C1

Calmodulin regulation of the sheep cardiac ryanodine receptor is complex, involving modifications to both gating and conductance

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It is generally accepted that Ca^{2+}-bound calmodulin (Ca^{2+}CaM) reduces the open probability (P_o) of the cardiac ryanodine receptor (RyR2) channel whereas non Ca^{2+}-bound CaM (apoCaM) either does not influence channel function or slightly reduces P_o (1-3). However, this impression of how CaM regulates RyR2 function is predominantly drawn from [^{3}H]ryanodine binding studies which can only provide a very approximate assessment of changes in RyR2 gating behaviour and cannot inform about changes in single-channel conductance. We have therefore investigated how Ca^{2+}CaM and apoCaM modulate the single-channel properties of RyR2. As previously described (1), sarcoplasmic reticulum (SR) membrane vesicles were isolated from sheep hearts obtained from an abattoir and were either incorporated into artificial membranes for single-channel experiments or were used for [^{3}H]ryanodine binding.

In the presence of 100 μM cytosolic free Ca^{2+}, CaM produced a concentration-dependent inhibition of [^{3}H]ryanodine binding to SR vesicles (maximum inhibition was 33.67 ± 4.34% of control, SEM, n = 8), consistent with previous work (1). In contrast, mean data from the single-channel experiments, indicated that, in the presence of 100 μM cytosolic free Ca^{2+}, CaM did not significantly alter P_o; P_o was 0.32±0.06 before and 0.34±0.09 after the cytosolic addition of 1 μM CaM (SEM, n=16). Closer inspection of the data, however, revealed that CaM produced two different effects on channel gating. CaM reduced P_o (from 0.379 ± 0.149 to 0.118 ± 0.09 (SEM, n = 6, P<0.05, Student's t test)) in 37% of the experiments but increased P_o (from 0.292 ± 0.056 to 0.508 ± 0.103 (SEM, n = 10, P<0.05, Student's t test)) in 63% of experiments. Under these experimental conditions, at a holding potential of 0 mV, CaM had no effect on single-channel current amplitude.

After binding Ca^{2+}CaM to RyR2 incorporated into bilayers by incubation with 1 μM CaM and 100 μM Ca^{2+}, we then reduced the cytosolic free [Ca^{2+}] to a sub-activating level (<10 nM Ca^{2+}) to convert Ca^{2+}CaM to apoCaM. At this free [Ca^{2+}], the P_o of the sheep RyR2, without pre-incubation with Ca^{2+}CaM, is zero. However, after pre-incubation with Ca^{2+}CaM, P_o was 0.059 ± 0.042 (SEM, n = 10). Importantly, the observed channel open events were shown to be of significantly higher current amplitude (5.163 ± 0.101 pA) than control channel events (4.555 ± 0.057 pA (SEM, n = 10, P<0.001, Student's t test). The data suggest that apoCaM can increase the P_o and conductance of RyR2 but only if it is first pre-bound to the channel as Ca^{2+}CaM. The results of this study indicate that CaM may bind tightly to RyR2 and influence channel function in a complex manner throughout the cycle of high and low cytosolic [Ca^{2+}] that occurs during cardiac excitation-contraction coupling.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C2

Contractility of adult cardiomyocytes expressing human cardiac troponin I-wt and -R145G

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We investigated the effects of amino acid exchanges in cardiac troponin I (cTnI), known to induce familial hypertrophic cardiomyopathy (FHC), on the contractility of adult cardiomyocytes isolated post mortem from rat left ventricles. The extent and rate of sarcomer shortening as well as its relaxation rate are analysed in response to $\beta_{1/2}$-, β_1- and β_2-adrenergic stimulation. Contractions were induced by bipolar external stimuli (0.4 ms, 40 V). The stimulation protocol was 0.5, 3.0, 1.0, 2.0 and 1.5 Hz with 20 stimuli per frequency interrupted by a 30 s stimulation pause. Adenovirus driven expression of human cTnI-wt in adult rat myocytes, monitored by the expression of GFP (green fluorescent protein), does not alter the parameters listed above in comparison to non-infected myocytes. The localisation of human cTnI in the sarcomeres is controlled with anti-human cTnI monoclonal antibodies, not recognizing rat cTnI.

As expected, the extent and rate of shortening as well as the relaxation rate of infected and non-infected cardiomyocytes are enhanced in response to isoproterenol (1 μM) alone and the β_2-adrenergic receptor (AR) blocker ICI 118,551 (50 nM) added in addition to isoproterenol. Similar effects, but less pronounced, are obtained upon incubation with isoproterenol and the β_1-AR blocker CGP-20712A (300 nM).

The amino acid exchange R145G in the inhibitory region of cTnI reduced the rate of shortening (1.56 ± 0.35 to 0.52 ± 0.13 μm/s, n = 10, 2 Hz) and relaxation (0.94 ± 0.30 to 0.23 ± 0.08 μm/s, n = 10, 2 Hz) significantly ($P \leq 0.0007$, unpaired Student's t test) in non-AR stimulated ventricle cells. Upon stimulation via $\beta_{1/2}$-, β_1- and β_2-AR both parameters were enhanced as expected. However, in comparison to cells with TnI-wt rates of shortening and relaxation were reduced upon stimulation of $\beta_{1/2}$- (1.69 ± 0.22 to 1.14 ± 0.22 μm/s for shortening rate and 2.17 ± 0.59 to 1.29 ± 0.44 μm/s for relaxation rate, n = 10, 2 Hz, $P \leq 0.0074$) and β_2-AR (1.89 ± 0.48 to 1.23 ± 0.23 μm/s for shortening rate and 1.56 ± 0.45 to 0.94 ± 0.34 μm/s for relaxation rate, n = 10, 2 Hz, $P \leq 0.0027$), but not in ventricle cells stimulated via β_1-AR (2.18 ± 0.65 to 2.25 ± 0.27 μm/s for shortening rate and 2.10 ± 0.82 to 1.45 ± 0.65 μm/s for relaxation rate, n = 10, 2 Hz, $P \leq 0.1138$). Changes in the extent of shortening due to the mutation were only observed in cardiomyocytes which were not stimulated via β-AR depending on the stimulation frequency.

The fact that there are no differences between non-infected and hcTnI-wt infected cardiomyocytes but between hcTnI-wt and hcTnI-R145G infected cardiomyocytes depending either on the stimulation frequency or on the method of receptor stimulation is a further indicator that the subunit is incorporated into the rat troponin complex at the right position.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C3

Effects of $[K^+]_o$ and osmolarity on the 'warm-up phenomenon' in myotonic muscle

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Mutations in the chloride channel (ClC1) are causative for Thomsen's and Becker's myotonia which are characterized by prolonged muscle contraction and slowed relaxation due to involuntary electrical after-activity. Repetitive contractions reduce the myotonic stiffness by unknown mechanisms. The objective of this study was to investigate changes of microenvironmental conditions on this so called warm-up phenomenon. Force registrations were performed on mouse gastrocnemius muscle post mortem. Experimental myotonia was induced by Cl^--free solution (n=30), blocking ClC1 with 9-antracene carboxylic acid (n=31) or the use of myotonic mice (adr-mouse, n=35, control=31). Elevated osmolarity promoted the warm-up phenomenon. This effect was partially antagonized by bumetanide, an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC). Therefore, the antimyotonic effects of increased osmolarity may be explained by electrical stabilisation via NKCC. $[K^+]_o$ increase prevented the relaxation deficit in a concentration- and time-dependent manner. At 7 mM $[K^+]_o$ the relaxation time reached control levels. We conclude that a shift of the K^+ gradient reduces myotonic activity and contributes to the warm-up phenomenon. Potential mechanisms as inactivation of voltage gated Na^+-channels and increased K^+-conductance via BK^+-channels are discussed.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C4

Distribution of Na^+-K^+ pumps in skeletal muscle and its significance for maintenance of T-tubular K^+ homeostasis

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Contracting muscles lose K^+. This leads to increased $[K^+]_o$, which may interfere with fibre excitability. In the T-tubules, the build-up of K^+ is exaggerated due to a large ratio between membrane area and volume of the T-tubular lumen (Clausen, 2003). The build-up of $[K^+]_o$ is counteracted by the activity of the Na^+-K^+ pumps. In rat muscle, only the α_2 isoform of the Na^+-K^+ pump, which is ouabain sensitive, is present in the T-tubules. To quantify T-tubular Na^+-K^+ pumps, their content of ^{3}H-ouabain-binding sites was determined using a ^{3}H-ouabain-binding assay (Clausen & Hansen, 1977) combined with mechanical skinning of muscle fibres, which removes the sarcolemma (Lamb & Stephenson, 1990).

Extensor digitorum longus muscles isolated from rats post mortem were incubated at 30°C to saturation in buffer with 10^{-6} M ^{3}H-ouabain before being washed at 0°C and transferred to paraffin oil. Then fibre segments were isolated, mechanically skinned and taken for determination of ^{3}H-ouabain binding. Similarly, intact fibres were isolated. Fibre volume was determined from fibre length and diameter while still under paraffin oil. To express the results relative to cell solids, the relation between fibre volume and volume of cell solids in fibres while still under paraffin oil was calculated from similar experiments where muscles were washed at 0°C in buffer containing ^3H_2O, making it possible to determine the water content of muscles and fibre segments.

In intact fibres, the content of ^{3}H-ouabain-binding sites was 2150±271 pmol (cm cell solids)$^{-3}$ (mean ± SEM, n=10). Skinning of fibres reduced their content of ^{3}H-ouabain-binding sites to 1123±119 pmol (cm muscle)$^{-3}$, indicating that 53% of the total muscle content of ^{3}H-ouabain-binding sites was located in the T-tubules. In whole muscle, the content of ouabain-binding sites was 399±21 pmol (g wet wt)$^{-1}$ corresponding to a content of α_2 Na^+-K^+ pumps of 399 pmol (g wet wt)$^{-1}$. If 53% of these Na^+-K^+ pumps are situated in the T-tubules, this corresponds to 207 pmol (g muscle)$^{-1}$, which gives a maximum capacity for active re-uptake of T-tubular K^+ of 3300 nmol (g wet wt)$^{-1}$ min^{-1} (Clausen, 2003). Based on a T-tubular volume of 0.014 ml (g wet wt)$^{-1}$ (Launikonis & Stephenson, 2001) this allows the T-tubular Na^+-K^+ pumps to clear T-tubular K^+ at a maximal rate of ~4 mM s^{-1}, indicating that at end of exercise, the recover of T-tubular K^+ can be completed within seconds.

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C5

A quantitative analysis of electrochemical equilibria in the sarcoplasmic reticulum of striated muscle

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A number of previous reports suggested that skeletal muscle sarcoplasmic reticular (SR) membranes are permeable to ions such as K^+ and Cl^- (1), but do not contain mechanisms for their active transport. This is compatible with their passive distribution in a Donnan equilibrium across the SR membrane in quiescent muscle.

A theoretical study using a multi-compartment Gibbs-Donnan analysis and charge-difference modelling (2) was used to explore the determinants of SR luminal $[K^+]$ and $[Cl^-]$, SR membrane

potential and SR volume in a system of this kind. It demonstrated that the principal parameters that influenced these key SR variables were the net charge carried by normally membrane-impermeant ions within the SR (such as Ca^{2+} and calsequestrin) and the *extracellular* membrane-permeant ion concentrations and osmolarity. Strikingly, SR concentrations of passively distributed ions were not influenced by sarcoplasmic ion concentrations or by the surface membrane potential.

These findings facilitate an understanding of the charge balancing that must occur following changes in SR Ca^{2+} content. In particular, they demonstrate (Fig. 1) that if the SR membrane potential in resting muscle is to be close to zero, as observed experimentally (3), then the net charge carried by membrane-impermeant ions (X) within the SR must be similar to that of the membrane-impermeant ions within the sarcoplasm and therefore negative. Figure 1 further provides a possible explanation for the observed SR volume increase following significant Ca^{2+} release (4): such loss of Ca^{2+}, which is normally membrane-impermeant, would tend to make the mean charge per osmole of membrane-impermeant ions within the SR ($z_{X(SR)}$) more negative. Conversely, Ca^{2+} overload of the SR might increase $z_{X(SR)}$ sufficiently to render the SR membrane potential significantly positive, contributing to the observed increases in Ca^{2+} release (5). This novel application of charge difference modelling thus provides a simple physiological basis for a number of key, yet hitherto unexplained, observations concerning SR volume and membrane potential.

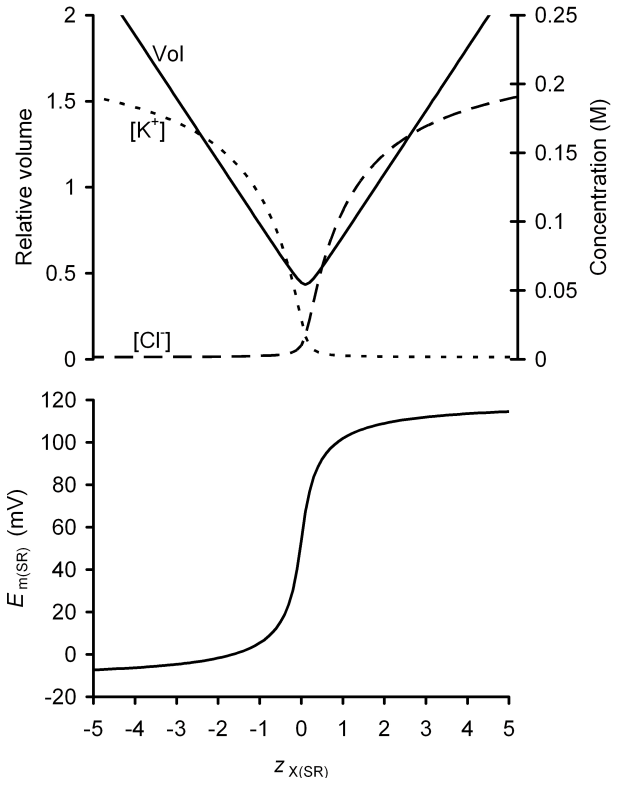

Figure 1. The predicted influence of the mean charge per osmole of intra-SR membrane-impermeant ions ($z_{X(SR)}$) upon SR volume, SR membrane potential ($E_{m(SR)}$) and intra-SR ion concentrations, calculated using Gibbs-Donnan relationships. Note that the SR membrane is normally impermeable to Ca^{2+} at rest, and total SR Ca^{2+} content (buffered + free) is high. Thus $z_{X(SR)}$ is likely to be profoundly influenced by the intra-SR Ca^{2+} content.

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C6

Dependence of store-operated Ca^{2+} flux on the electrochemical gradient in mammalian skeletal muscle

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In most eukaryotic cells, depletion of the internal Ca^{2+} store activates store-operated calcium entry (SOCE). We used shifted excitation and emission ratioing of fluorescence (Launikonis et al. 2005) to image mag-indo-1 trapped in the t-system of mechanically skinned rat skeletal muscle fibres, simultaneously with rhod-2 in the cytoplasm, to measure for the first time SOCE during intracellular Ca^{2+} release. Ca^{2+} release was induced pharmacologically, to avoid activation of voltage-dependent contributions to Ca^{2+} flux. Spatially and temporally resolved images of t-system $[Ca^{2+}]$ ($[Ca^{2+}]_{t\text{-}sys}$) allowed estimation of Ca^{2+} flux from the rate of change of $[Ca^{2+}]_{t\text{-}sys}$. An initial uptake of Ca^{2+} by the t-system was observed during the release of Ca^{2+} from sarcoplasmic reticulum (SR) at rates approaching 1 mM s^{-1} (relative to t-system volume; n = 8 fibres), depending upon cytoplasmic buffering conditions. Activation of SOCE occurred prior to the peak of the cytoplasmic Ca^{2+} transient, as indicated by an abrupt decay in $[Ca^{2+}]_{t\text{-}sys}$. Cytoplasmic Ca^{2+}-buffering conditions did not affect SOCE activation or Ca^{2+} entry through the t-system membrane. Once SOCE was activated, Ca^{2+} entry flux was exponentially dependent (rate constant, 0.9 s^{-1}) on the concentration gradient, essentially $[Ca^{2+}]_{t\text{-}sys}$, as well as the transmembrane potential (n = 16 fibres). $[Ca^{2+}]_{t\text{-}sys}$ could only recover upon a net uptake of Ca^{2+} by the SR in a low $[Mg^{2+}]_{cyto}$ solution (n = 4 fibres; see $[Ca^{2+}]_{SR}$ measurements under similar conditions in Launikonis et al. 2006), indicating SOCE termination required SR refilling. Depletion without recovery of $[Ca^{2+}]_{SR}$ in caffeine allowed activation but not termination of SOCE (n = 12 fibres). The concomitant change in sign of the t-system Ca^{2+} flux with depletion and recovery of $[Ca^{2+}]_{SR}$ is diagnostic of SOCE and validates the measurements. Ca^{2+} entry flux was 13.3 μM s^{-1} during SOCE in the polarized cell at physiological $[Ca^{2+}]_{t\text{-}sys}$. This flux is consistent with SR refilling rates mediated by SOCE in experiments with intact EDL fibres of rat (Kurebayashi & Ogawa, 2001). The experiments also showed that activation

of SOCE was regulated by a local signal, did not require full depletion and was not clearly graded with depletion. Termination of SOCE required a net increase in $[Ca^{2+}]_{SR}$.

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PC1

The control of sequential cross-bridge operation

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In a communication to the European Muscle Congress (2006) meeting Elliott & Worthington [1] set out the very strong experimental evidence from biochemistry and physiology suggesting that cross-bridges act sequentially (rather than simultaneously) along a given actin filament during the contraction of striated muscle. Here we suggest how sequential operation is initiated and controlled.

In glycerinated muscle (psoas from rabbits killed by approved procedures) Coomber et al. [2,3] used Donnan potential measurements with KCl-filled microelectrodes to show that the electric charge on the actin-myosin matrix undergoes a sharp switch-like transition at pCa 50 = 6.2. The potentials are about 2 mV less negative at the lower pCa ($P<0.001$; Z test for unmatched samples) (e.g. Fig. 2 in [2]). Although these were muscles in rigor, since in the presence of ATP the muscle contracts and breaks the microelectrode, there is no reason to suppose a priori that a similar switch does not occur in contracting muscle.

The charge decrease within the matrix decreases the depth of the potential wells between the protein filaments (Fig. 8 of [4] illustrates these potential wells for the parallel case of collagen fibrils in corneal stroma). The decrease may suffice to activate the ATP-ase on the cross-bridges, in analogy with the threshold potential in nerve conduction. Following that analogy, the effect would start at the A-I junction, where edge effects will in any case cause the depth to be less, and then proceed along the train of myosin heads around a given actin filament, each event initiating the following one. Thus sequential operation would occur.

Coomber [5] (Ch 2) found that in muscles stretched beyond overlap (sarcomere length = 4.2 μm) the calcium dependence was still apparent, and was also seen in the gap filaments between the A- and I-band ends; further stretching (to sarcomere length > 4.3 μm) abolished the dependence. These experiments suggest very strongly that the calcium dependence is controlled by the titin component of the sarcomere, and is lost when titin filaments break.

There is now renewed interest in the electric charge of those proteins in muscle within the structural system; here we suggest how changes in these charges may control the calcium activation process.

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PC2

Effect of repetitive contraction on the dynamics of intracellular calcium handling in dystrophin-deficient mouse myotubes

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Dystrophin and its associated protein and glycoprotein complex is widely thought to confer integrity on the muscle fibre membrane during repeated cycles of contraction and relaxation. In the present study dystrophin-deficient *mdx* myotubes were subjected to repetitive electrical stimulation of variable frequencies (0.3–3 Hz) to test their ability to respond to this mechanical challenge. Calcium (Ca^{2+}) handling associated with these contractions in the *mdx* myotubes was compared with that in control (B10) myotubes by use of the calcium-sensitive fluorescent dye Indo-1 AM (4 μM). Each myotube that responded to electrical stimulation showed no change in it's individual Ca^{2+} handling ability when stimulated to contract at 0.5 Hz for an hour or more. However, relative to control myotubes (n = 53), contractile *mdx* myotubes (n = 74) exhibited a significant (p = 0.004) elevation in resting levels of intracellular Ca^{2+} (1.03 ± 0.17 ratio units in *mdx* compared with 0.90 ± 0.27 in B10; mean ± SEM, significance assessed by two sample t test), but a significant decrease in amplitude of the electrically induced Ca^{2+} transient (0.38 ± 0.14 ratio units in *mdx* compared with 0.52 ± 0.36 in B10 myotubes, p=<0.0001). However, these differences were not associated with any alteration in the distribution of the sarcoplasmic/endoplasmic reticulum ATPases or ryanodine receptors as assessed by immunostaining and imaging with a Leica TCS4D confocal laser scanning microscope. Overall these findings confirm that Ca^{2+} levels are elevated in dystrophin-deficient myotubes, but do not support the hypothesis that repetitive cycles of contraction and relaxation directly compromise sarcolemmal integrity and lead to aberrant Ca^{2+} dynamics. Analyses of induced Ca^{2+} transients over extended periods of rapid repetitive electrical stimulation revealed stable resting and peak Ca^{2+} concentrations in both *mdx* and control (B10) myotubes.

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PC3

Patch-clamp characterization of excitable properties of enzymatically isolated adult skeletal muscle fibres from the honeybee *Apis mellifera*

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Electrical properties of adult honeybee skeletal muscle fibres enzymatically isolated from the metathoracic leg tibia were

explored with the whole-cell patch-clamp technique. In a physiological-like extracellular solution, pressure perfusion (2 psi) with L-glutamate, an excitatory neurotransmitter at the neuromuscular synapse of invertebrates, induced fast activation of an inward current in fibres voltage clamped at a resting membrane potential of -80 mV. During the course of a 3 s application of L-glutamate (1 mM), half of the fibres responded with an initial peak showing fast inactivation, followed by a sustained component, while the other half of the fibres apparently lacked the initial peak. On average, the mean relative amplitude of the sustained component was 49 ± 8% of the initial peak one (n=23, mean ± S.E.M.). Responses to voltage ramps indicated that the sustained current component had a reversal potential around 0 mV. In another set of experiments, single action potentials or trains of action potentials overshooting to ~ +30 mV, were recorded in current-clamp mode in response to current steps from a membrane potential adjusted to -80 mV with slight basal current injection (on average, the resting potential of fibres before adjustment was -47 ± 5 mV, n=9). While the voltage-dependant sodium channel blocker tetrodotoxin (TTX, 1 μM) did not affect action potential responses, cadmium and lanthanum applied together (0.5 and 0.3 mM, respectively) induced disappearance of this all-or-none response. In voltage-clamp, the inward and outward currents underlying this action potential activity were recorded in response to controlled membrane depolarizations from a resting potential of -80 mV, in a physiological-like extracellular solution. In an extracellular solution devoid of sodium but containing 2 mM calcium and the potassium channel blockers tetraethylammonium and 4-aminopyridine, a current with an L-type calcium channel profile was recorded. This current was not affected by TTX, but was completely blocked by Cd^{2+}-La^{3+}. The activation threshold potential for this calcium current was ~ -40 mV and its mean maximal amplitude, reached at ~0 mV, was -8.5 ±1.9 A/F (n=13). Individual I-V curves were fitted with equation $I(V)=G_{max}(V-V_{rev})/(\{1+\exp[(V_{0.5}-V)/k]\})$ and mean values for G_{max}, V_{rev}, $V_{0.5}$ and k were 217 ± 35 S/F, +39.8 ± 4.1 mV, -13.7 ± 1.8 mV and 5.4 ± 0.5 mV, respectively (n=13). This original cell preparation appears particularly suitable for patch-clamp studies and future investigations will allow a complete characterization of the pharmacological sensitivity of its membrane ionic currents as well as its calcium release apparatus properties, in order to explore excitation-contraction coupling in this insect model.

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PC4

Effect of high $[K^+]_o$ and Na^+-K^+ pump stimulation on contractile endurance during excitation in rat skeletal muscle

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During contractile activity, muscle cells often undergo a net loss of K^+, leading to increased interstitial concentration of K^+, depolarization and loss of excitability. For almost 70 years, it has repeatedly been proposed that this sequence of events might be an important cause of fatigue (Sejersted & Sjøgaard, 2000). Objections to this idea are that during work, interstitial K^+ may not increase sufficiently to elicit inhibition of contractions. Moreover, recent studies on intact mouse muscles failed to detect any inhibitory effect of exposure to 10 mM K^+ (Zhang et al. 2006). We have therefore examined this hypothesis by measuring force development in intact soleus muscles. Muscles prepared post mortem from 4-week-old Wistar rats were incubated in oxygenated Krebs-Ringer bicarbonate buffer (30°C) containing K^+ in the physiological range (4-10 mM). Immediate increase from 4 to 10 mM K^+ caused no significant change in initial force or subsequent force decline seen during continuous stimulation with 0.2 ms 10 V pulses at 60 Hz at 30°C. However, following 20 min of exposure to 10 mM K^+, the rate of force decline increased by 618% in comparison to the values recorded at 4 mM K^+ (n = 14 vs 8, $P < 0.001$, unpaired t test). Twenty minutes of exposure to 8 mM K^+ increased the rate of force decline by 135% (n = 8 vs 8, $P < 0.001$). Following return to 4 mM K^+, the inhibitory effect of elevated K^+ was completely reversible. Since a rise in interstitial K^+ can be counteracted by stimulating the Na^+-K^+ pumps, we examined agents known to exert such an action. The beta2-agonist salbutamol, adrenaline, rat calcitonin gene related peptide (rCGRP) and insulin, when added to the K^+-enriched buffers, all induced a highly significant or complete restoration of contractile endurance to the level obtained at 4 mM K^+. When the Na^+-K^+ pumps were activated by intermittent stimulation (1 s 60 Hz trains of 0.2 ms 10 V pulses every 2 min for 20 min before the fatiguing stimulation), contractile endurance was improved by 151% (n = 6 vs 8, $P < 0.001$).

In conclusion, exposure of muscles to elevated $[K^+]_o$ for sufficient time (20 min) to allow access of K^+ to the interstitial water space, leads to marked but reversible inhibition of contractile performance. This effect shows progressive increase with the K^+ concentration. Stimulation of the Na^+-K^+ pumps counteracts or alleviates this inhibition, indicating that muscle fatiguability depends on the balance between excitation-induced rise in $[K^+]_o$ and clearance of this K^+ via the Na^+-K^+ pumps.

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PC5

The role of α-adrenergic receptors on the spasmolytic effect of *petroselinum crispum* (Parsley) seed alcoholic extract on adult male rat ileum

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Certain medical herbs have been used instead of chemical medicines because they have fewer side effects. *Petroselinum crispum* (Parsley) is a herb belong ing to the umbelliferae family. As a medicinal plant, parsley has traditionally been used as an antispasmodic (2), carminative, diuretic (1), emmenagogue and stomachic. The plant has also been used as a remedy for asthma,

conjunctivitis, dropsy, fever and jaundice. The essential oil of parsley seed has been reported to stimulate hepatic regeneration (4). This work aims to provide the scientific evidence that would confirm or reject the claimed spasmolytic role of parsley.
The extract was prepared from parsley seeds with 80% ethanol. The ethanol was then removed by evaporation and different amounts of the residue were dissolved in Tyrode solution to give various concentrations and tested on the ileum of adult male Wistar rats (225±25g). Post mortem, 2cm of ileum were removed and placed in an organ bath containing Tyrode solution (37°C, pH=7.4), bubbled with air. An isotonic transducer and Harvard universal oscillograph were used for recording contractions of the ileum after administration of 60 mM KCl (non-receptor stimulation for opening Ca^{2+} channels) (3), to the bath solution. Various concentrations of parsley seed extract (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg/ml) were added to the bath.
Statistical comparisons performed using Student's paired t test and one-way analysis of variance (ANOVA), show that various concentrations of *Petroselinum* extract significantly (n=7, P<0.001) decreased the KCl-induced contraction. Laboratory experiments were performed to investigate the mechanism of relaxation of parsley seed extract on smooth muscle with an α-adrenergic receptor antagonist (propranolol, 1 μM). In addition block of α-adrenoceptors by propranolol did not have a significant effect on the relaxation response of parsley.
Since the present study showed that propranolol had no influence on the relaxation effect of ileum smooth muscle, it therefore seems that the relaxation effect of parsley seed extract was not performed by α-adrenergic receptors.

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PC6

The role of β-adrenergic receptors on the spasmolytic effect of the hydroalcoholic extract of *Ruta chalepensis* leaf on the male rat ileum

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Rue (*Ruta chalepensis*) belongs to the *Rutaceae* family. It grows in the north of Boshehr(south Iran). The plant is rich in coumarins, it has anti-fertility effects and some quinoline alkaloid mutagenic actions as well as curare-like ganglionic–blocking effects and spasmolytic activities (1). The aerial part of *Ruta chalepensis* is used for delaying the onset of seizures; a dose-dependent suppression in the tonic phase induced by pentylenetetrazole (PTZ) has been reported (2). Its extract increases blood levels of nitrite, an indicator of nitric oxide production (3). This study investigated the effect of a hydroalcoholic extract of Rue leaves (RLHE) on the contraction of rat ileum induced by 60 mM KCl (4).
Rue leaves (10 g) were macerated in 70% ethanol. The mixture was filtered and the solvent was evaporated. Ileum segments were removed post mortem from adult male Wistar rats (225±25 g) and placed under 1 g initial tension in an organ bath containing Tyrode solution (pH=7.4, 37[ı ı]C). Contractions were recorded using an isotonic transducer and oscillograph.
Statistical comparisons performed using Student's paired t test and one-way analysis of variance (ANOVA), show that various concentrations of *Ruta chalepensis* extract (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07 mg/ml) significantly (n=7, P<0.001) decreased the KCl-induced contraction. Laboratory experiments were performed to investigate the mechanism of relaxation of the rue extract on smooth muscle with the β-adrenergic receptor antagonist propranolol (1 μM). Block of the β-adrenoceptors by propranolol did not significantly affect the relaxation response of rue.
Since the present study showed that propranolol had no influence on the relaxation effect of smooth muscle ileum, it seems therefore that the relaxation effect of rue leaf extract was not performed by β-adrenergic receptors.

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PC7

Effects of stimulation and cellular changes associated with fatigue on tubulo-sarcoplasmic reticular (T-SR) distance

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Excitation-contraction coupling in skeletal muscle probably involves specific allosteric interactions between transverse (T)-tubular dihydropyridine receptors and sarcoplasmic reticular (SR) ryanodine receptors (Franzini-Armstrong & Protasi, 1997). This coupling is therefore potentially vulnerable to anatomical disruption of T-SR membrane spacing. This study on *Rana temporaria* sartorius muscles examined the effect of fatiguing electrical stimulation on this T-SR distance and compares this with exposing resting fibres to a range of extracellular conditions that each replicate one of the major changes associated with muscular activity: membrane depolarisation, volume increase, acidification and intracellular lactate accumulation. Following each

treatment, muscles were immersed in isotonic fixative solution and processed for electron-microscopy. Mean distances between junctional SR and the T-system were estimated using the orthogonal intercept method to provide unbiased stereological estimators of diffusion distances between T and SR membranes (Jenson *et al.* 1979). Measurements from muscles fatigued by low frequency intermittent stimulation (80 sets of a 2s stimulus at intervals of 8s with impulses 20ms long at 20V and 25Hz) showed significant ($p << 0.05$) reversible increases in T-SR distance (Table 1). Exposure to isosmotic 15mM extracellular [K^+] to produce membrane depolarisation and cell swelling comparable to that following stimulation did not significantly affect T-SR distance although increasing extracellular [K^+] to 60mM reduced it. This latter finding persisted with Cl^- deprivation which prevents the volume changes normally accompanying increased [K^+] (Usher-Smith *et al.* 2006b), thereby excluding additional effects of isosmotic cell swelling. Acidification alone produced by NH_4Cl addition and withdrawal also decreased T-SR distance and a similar reduction occurred following exposure to extracellular Na-lactate where such acidification was accompanied by elevations of intracellular lactate (Usher-Smith *et al.* 2006a). Thus, under these conditions, lactate has no additional effect on T-SR distance. Overall, this study demonstrates significant *increases* in T-SR distances following stimulation, whilst showing that membrane depolarisation and acidification *decrease* T-SR distance and isosmotic volume changes and lactate appear to have no additional effect.

Table 1

Experimental condition	T-SR distance (nm) (means±SEM)	*n* (fibres, muscles)
Normal Ringer solution	15.97 ± 0.37	40, 4
Following stimulation	20.15 ± 0.60	30, 3
60 min after stimulation	15.28 ± 0.42	30, 3
15 mM KCl	16.50 ± 0.31	31, 3
60 mM KCl	9.80 ± 0.20	31, 3
60 mM K_2SO_4	9.13 ± 0.23	20, 2
40 mM NH_4Cl withdrawal	9.99 ± 0.21	31, 3
80 mM lactate	10.12 ± 0.46	30, 3

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PC8

γ_1-subunit deficiency alters phenylalkylamine action on DHP receptor inactivation in mouse skeletal muscle fibres

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Adult skeletal muscle fibres of mice lacking the DHP receptor γ_1-subunit exhibit changes of voltage dependence of inactivation of both Ca^{2+} conductance and sarcoplasmic reticulum Ca^{2+} release. The steady state inactivation curves were found to be shifted to more positive potentials indicating that γ_1 favours the inactivated state of the voltage sensor (Ursu et al. 2004). These effects of the γ_1-subunit resemble the effect reported for certain Ca^{2+} channel antagonists on excitation-contraction coupling (e.g. Erdmann & Lüttgau, 1989) raising the possibility of a common modulatory mechanism. We performed experiments in single voltage-clamped interosseus fibres and measured the voltage-activated Ca^{2+} release flux by analysing fura-2 fluorescence signals (Ursu et al. 2005) to determine whether wild type (γ_1+/+) and γ_1-null (γ_1-/-) muscles differ from each other with respect to their sensitivity to the phenylalkylamine L-type Ca^{2+} channel antagonist (-)D888. We studied the time course of recovery from inactivation of Ca^{2+} release flux in the presence of different concentration of (-)D888. Inactivation was caused by a depolarising voltage step to +10 mV lasting 60 s. Recovery was assessed at different holding potentials using 100 ms depolarising test pulses to +20 mV. (-)D888 caused a substantial concentration-dependent slowing of recovery and left shift of the voltage dependence of recovery both in γ_1-/- and γ_1+/+ fibres. In γ_1-/- fibres the voltages of half-maximum recovery ($V_{1/2}$) at quasi steady state (190 s after inactivation) in the presence of 0, 5 and 10 μM (-)D888 were -36, -49 and -55 mV, respectively. In γ_1+/+ fibres $V_{1/2}$ values were -47, -67 and -69 mV, respectively. In γ_1-/- muscle fibres, 5 μM (-)D888 reversed the effect of γ1-subunit deficiency underlining the Ca^{2+} antagonist-like modulation of voltage-controlled Ca^{2+} release by this auxiliary subunit. Moreover, in γ_1+/+ fibres the effect of (-)D888 on recovery was enhanced which may point to a positive allosteric interaction of the subunit with the phenylalkylamine binding site.

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PC9

Triadin modifies EC coupling in C2C12 myotubes

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Triadin is localized in the sarcoplasmic reticulum (SR) and is connected to the ryanodine receptor (RyR) and calsequestrin. It has different isoforms in skeletal muscles of vertebrates. So far 32, 49, 51 and 95 kDa isoforms were described in rat skeletal muscle which originate from the same gene. It was suggested earlier that Triadin can play a role in regulating calcium release from the SR and probably in the differentiation of myotubes.

The 95 kDa Triadin (TRISK95) was over-expressed in C2C12 cells and primary myotubes from mouse and the properties of

EC coupling were examined. Whole cell intracellular calcium concentration was measured using the acetoxymethylesther form of the fluorescent dye fura-2. While the KCl-induced calcium release from the SR was significantly smaller in Triadin over-expressing myotubes, caffeine-induced release was identical in the two cases. The former effects depended on the presence of external calcium concentration. Sparks and embers, the two forms of spontaneous calcium release events, were measured with confocal microscopy using fluo-3 on control and TRISK95 over-expressing cells. Triadin decreased the frequency, from the control 21.1±0.6 Hz (n=577) to 13.3±0.7 Hz (n=332), and the amplitude of sparks, but did not change their duration. In the case of embers the frequency decreased from the control 21.1±1.9 Hz (n=63) to 12.2±2.1 Hz (n=41) and while their duration also decreased, their amplitude was not affected.
We propose that Triadin over-expression inhibits RyRs and thus decreases calcium release from the SR.

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PC10

Frequency-dependent interactions between ion channels in the triad junction and the induction of post-tetanic potentiation

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In response to a regular spike-train, skeletal muscles will gradually increase their tension until a plateau is reached that matches the spike-train frequency. This is a relatively slow process taking in the order of 50-500 ms depending on muscle type and activation frequency (see e.g. Burke et al. 1976), and it is an indication that force production is a cumulative process that depends on systems with slower dynamics than the spikes themselves. In experiments (Abbate et al. 2001 and references therein) the actual position of the force-plateau matching a particular frequency can be shifted by tetanic bursts of high-frequency stimulation in a process known as post-tetanic potentiation (PTP). It is generally accepted that the force increase associated with PTP is related to phosphorylation of myosin light chains (reviewed in Abbate et al. 2001); however, the phosphorylation itself might be triggered by an increase of myoplasmic Ca^{2+} following tetanic stimulation of a muscle fibre (Decostre et al. 2000). A Ca^{2+} resource allocation based model of the excitation-contraction coupling has here been developed to investigate frequency-dependent changes in muscle force response. This model rests on the common assumption that Ca^{2+} resources are unevenly distributed throughout the muscle fibres (myoplasm, sarcoplasm and T-tubules). For simplicity it is also assumed that regulation and transport within and between these compartments follows simple first-order dynamics associated to protein actions either in the form of Ca^{2+} binding proteins (parvalbumin, calsequestrin and troponin C), or through the action of membrane bound Ca^{2+} channels (sarcoendoplasmatic reticulum Ca^{2+}-ATPase, ryanodine receptor, dihydropyridine receptor). Each of these systems contributes to the overall Ca^{2+} dynamics via time constants ultimately determining the rate of Ca^{2+} transport between compartments. The system is highly sensitive to motoneuronal spike arrival times (triggering release of Ca^{2+} from sarcoplasm) and therefore several frequency-dependent muscle force responses like PTP and the catch-like effect of skeletal muscle (Burke et al. 1976) are easily modelled. It is concluded that the small amount of additional extracellular Ca^{2+} that gains access to the myoplasm via delayed slow opening of the L-type DHPR Ca^{2+} channel (Shtifman et al. 2004 and references therein) might be sufficient to account for PTP in response to tetanic activation. This would probably involve retrograde coupling in which RyR channels modulate currents through the DHPR channel (see review by Dulhunty et al. 2002).

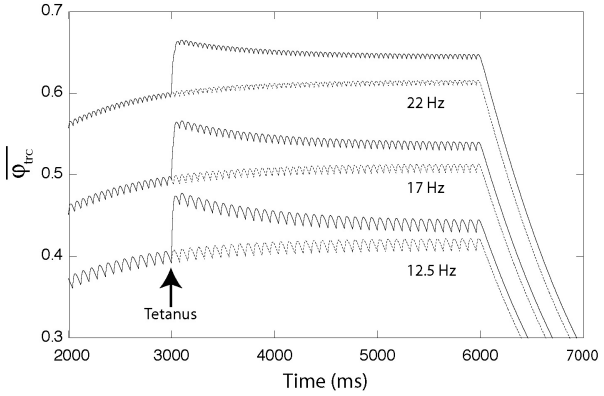

Figure 1. Fraction of Ca^{2+} bound to troponin C as a function of time during regular spiking activity (at 12.5, 17 and 22 Hz). Arrow marks time at which the total amount of Ca^{2+} within the muscle fibre is suddenly increased by 5%, here corresponding to cumulative additional extracellular Ca^{2+} entering the myoplasm during tetanus via slow L-type Ca^{2+} channels like the DHPR receptor.

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PC11

Rapid regional solution switching in subcompartments of the extracellular space around isolated adult ventricular myocyte

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Biophysical studies of isolated cardiac myocytes would benefit from devices that allow the rapid microfluidic manipulation of

the extracellular space around limited regions of a single cell. This abstract describes a method to partition the extracellular space around a single cardiac myocyte bridging a hydrophobic gap, produced with polymer soft-lithography. This system allows the two ends of a single cardiac cell to be independently superfused using an arrangement of concentric micropipettes. The inner pipette is used for delivery of the superfusate and the outer pipette for the removal of the extracellular solution around the cell ends. An additional concentric pipette system, loaded with a separate test solution (containing drugs, ions or permeabilisation reagents) can be inserted into a selected compartment for variable periods using a piezo-stepper, enabling digitally controlled rapid solution switching within the picolitre volume around either end of the cell. Planar gold electrodes were also integrated into the device, enabling electrical stimulation and recording of the evoked action potential. The device was mounted on an inverted microscope to allow sarcomere length and epifluorescence measurements. This system allows the demonstration of spatially restricted effects of the test solution by observing the response at one end of the cardiac cell in comparison with the untreated region. Initial measurements using this novel device include: (i) the regional permeabilization of the surface membrane of the cardiac cell and the subsequent perfusion of the intracellular space; (ii) the local application of caffeine thereby generating spatially confined intracellular Ca^{2+} transients; (iii) differential recording across the high resistance (~50MΩ) between the separate compartments to reveal the extracellular signal associated with an evoked action potential.

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