Effects of troponin C isoform on the action of the cardiotonic agent EMD 57033

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The effects of the cardiotonic potentiator EMD 57033 on different TnC (troponin C) isoforms were investigated. Endogenous skeletal TnC was extracted from glycerinated, permeabilized rabbit psoas fibres and replaced with either purified native rabbit psoas TnC (fast TnC) or human recombinant cTnC (cardiac TnC) (3 mg/ml in relaxing solution for 30 min). In both conditions, 10 μ M EMD 57033 increased maximal calcium-activated force ($P_{\max}$) and gave a leftward shift in the pCa–tension curve. With cTnC, the increase in $P_{\max}$ was much greater (228 %) compared with the effect seen for fast TnC (137 %), which was the same as that in unextracted control fibres. When the whole troponin was replaced rather than just TnC, the effects of EMD 57033 on fibres replaced with cTn were the same as with the cTnC subunit alone,

except that the force at low Ca^{2+} concentrations was not increased as much. If TnC was only partially extracted, it was found that the degree of extraction did not influence the effect of EMD 57033, except when force was decreased to below 10 % of the pre-extraction $P_{\max}$. Dynamic stiffness was not altered by EMD 57033 in any of the preparations. The rate of tension recovery following a release–restretch method (k_{tr}) was decreased by EMD 57033. We conclude that EMD 57033 acts by a rate-modulating effect, and that the quantitative response of this effect is dependent on the TnC isoform present.

Key words: cardiac potentiator, EMD 57033, muscle contraction, single fibre, skeletal muscle, troponin.

INTRODUCTION

Current pharmacological treatments for progressive heart failure act by increasing the intracellular concentrations of free calcium (see [1] for a review). This can, however, have the side-effect of causing spontaneous calcium release from the sarcoplasmic reticulum, leading to arrhythmias. An alternative intervention is to increase the sensitivity of contractile proteins to calcium (positive inotropy) without altering intracellular free calcium levels. EMD 57033 is a drug that acts in such a fashion.

EMD 57033 is the positive optical isomer of the racemate EMD 53998. The negative isomer, EMD 57439, acts as a phosphodiesterase III inhibitor. EMD 57033, however, has almost no phosphodiesterase III inhibitory activity, but instead acts as a pure sensitizer [2,3]. It has been shown to increase maximal calcium activated force in a wide variety of tissues [1–3,5–7]. In skinned fibre systems, where the surface membrane has been chemically permeabilized, the sigmoidal pCa–tension relationship in the presence of EMD 57033 shows a leftward shift, indicating greater force for a smaller activating concentration of calcium [2,3]. As yet, however, the exact mechanism of action of EMD 57033 is unclear.

One mechanism of action would be to increase the apparent calcium sensitivity by increasing the calcium affinity of TnC (troponin C), thus increasing the number of cross-bridges that can be formed (recruitment) [4]. However, it has been shown that EMD 57033 has no effect on calcium binding to TnC [5]. Alternatively, it could have a direct effect on the cross-bridges themselves, either by altering the number of cross-bridges (recruitment) or the force for each cross-bridge, or by increasing the amount of time that each cross-bridge spends in a force-producing state (rate modulation). Rate modulation can occur by slowing the rate at which cross-bridges return to the non-force-producing states

(i.e. relaxation) or by increasing the rate at which they enter the force-producing states (activation) or by a combination of both [4].

Wang et al. [6] have shown, through NMR studies, that EMD 57033 binds to the C-terminus of TnC in the region of the high-affinity Ca^{2+} – Mg^{2+} sites. We have previously shown that EMD 57033 has a greater effect on force and activation rate in guinea-pig cardiac trabeculae compared with frog skeletal muscle [7]. To determine whether this is due to the difference between cardiac and skeletal isoforms of TnC, we have extracted native TnC [sTnC (skeletal TnC)] from glycerinated rabbit psoas fibres and replaced it with either wild-type fsTnC (fast skeletal TnC) or with hcTnC (human cardiac TnC). We have studied the effects of EMD 57033 on the pCa–tension relationship, on fibre dynamic stiffness and on the rate of activation, following a rapid release–restretch method (k_{tr}) in skeletal muscle fibres with a reconstituted regulatory system.

We found that EMD 57033 acts by rate modulation with either isoform of TnC present, but the effect on force is greater with the cardiac isoform of TnC compared with the skeletal isoform. We suggest a mechanism whereby EMD 57033 affects the interaction between TnI and TnC.

EXPERIMENTAL

Tissue

Female New Zealand White rabbits (~ 1.5 kg) were killed by lethal injection of barbiturate, in accordance with Home Office regulations. The psoas muscle was divided into strips approx. 3 mm in diameter and 3 cm in length and attached to plastic sticks. These strips were stored at -20°C in a 50:50 (v/v) glycerol/relaxing solution (pH 7.0) for up to 4 weeks.

Abbreviations used: TnC, troponin C; cTnC, cardiac TnC; fsTnC, fast skeletal TnC; hcTnC, human cTnC; sTnC, skeletal TnC; TnI, troponin I; TnT, troponin T.

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Purification of proteins

A pET11c construct encoding hcTnC was obtained as a gift from Professor I. P. Trayer (School of Biochemistry, University of Birmingham, U.K.); using this, TnC was overexpressed and purified as previously described [8].

Rabbit wild-type TnC was purified from rabbit muscle following the method described by Potter [9].

Whole troponin complexes were formed using the method described by Robinson et al. [10]. The subunits were mixed in a ratio of 1.5 TnC/1 TnI (troponin I)/1 TnT (troponin T) in 6 M urea, 1 M KCl, 10 mM imidazole, 50 μ M CaCl₂, 1 mM dithiothreitol and 0.01 % sodium azide (pH 7.0), and the concentrations of first urea and then the KCl were decreased to 0 and 200 mM respectively using a stepwise dialysis method. The mixtures were centrifuged (12 300 g, 5 min) to remove insoluble material, and intact troponin was purified by gel filtration using a Sepharose 200 column. Complexes were then dialysed in exchange buffer. The final proportions of individual subunits were measured by scanning densitometry and found to be 1.00:0.95:1.12 (TnT/TnI/TnC; $n = 3$).

Solutions

Solution compositions were calculated by a computer program using the affinity constants of Smith and Martell [11]. All relaxing solutions contained a final concentration (in mM) of 1 free Mg²⁺, 5 MgATP, 10 creatine phosphate, 7 EGTA and 10 imidazole. Ionic strength was made up to 0.18 mM using potassium propionate and pH was adjusted to 7.0 using potassium hydroxide (all calculated at 20 °C). Activating solutions were made by adding CaCl₂ to give final pCa values from 6.2 to 5.0. The relaxing solution was the same with no added calcium. All solutions contained 15 units/ml phosphocreatine phosphokinase and an EDTA-free protein kinase inhibitor tablet (Roche Diagnostics, Lewes, East Sussex, U.K.). EMD 57033 was a gift from Dr P. Schilling (E. Merck Pharmaceuticals, Darmstadt, Germany). It was prepared as a 10 mM stock solution in DMSO and was diluted in relaxing solution to obtain a final EMD 57033 concentration of 10 or 30 μ M, and a DMSO concentration of 0.1 or 0.3 % (v/v). Control solutions were prepared with an equivalent volume of DMSO, which had no effect on the fibres.

Methods

Small bundles of glycerinated psoas fibres [$\sim 100 \mu$ m in width and 4 mm in length (L_0)] were dissected out and attached by cyanoacrylate glue to steel wire hooks. One hook was attached to an Akers 801 piezoelectric force transducer (Sensonor, Horten, Norway) and the other to a moving coil stretcher motor to allow rapid length changes. These fibre bundles were then permeabilized in relaxing solution containing 1 % (v/v) Triton X-100 in relaxing solution for 2 min. Sarcomere length was measured by monitoring the diffraction pattern resulting from the incident beam from a small diode laser (8 mW maximum output; Model PM08G01, Power Technology, U.S.A.). Initial sarcomere length was set to 2.5 μ m. All experiments were conducted at a room temperature of 20 °C.

The bathing solution was changed by means of a rotating bath mechanism [13], and for the pCa curves, fibres were cycled between relaxing solution and pCa solution of increasing Ca²⁺ concentration. Solutions of pCa 5.0 were used for maximal calcium activation, since decreasing the pCa to 4.0 caused no further increase in force. For the dynamic stiffness measurements, fibres were activated in pCa 5.0 solution until force had reached a plateau. A stepwise length change of +1 % of L_0 (~ 4 nm) was applied and the initial change in force was recorded to give the

dynamic stiffness. The length change step was complete within 0.5 ms and held for 5 ms.

To measure k_{tr} , the rate of recovery of tension following a rapid release–restretch method, fibres were rapidly shortened (the step was completed within 0.5 ms) and held at a slack length for 100 ms. After this, they were rapidly returned to L_0 . The resulting force recovery was recorded on a Tektronics TDS 210 digital oscilloscope and captured using Tektronics Wavestar software. It was fitted using a monoexponential function.

Extraction and replacement methods

TnC subunit only

To extract TnC, the fibres were bathed in a rigor solution comprising of 5 mM EDTA, 10 mM Hepes and 500 μ M trifluoperazine (pH 7.0) for 30 min at 20 °C and were subsequently washed twice in relaxing solution. The fibres were then tested in pCa 5.0 solution. Post-extraction force was typically < 5 % of pre-extraction maximum force at pCa 5.0. Extracted fibres were then bathed in either 3 mg/ml cTnC or 3 mg/ml fsTnC in an exchange buffer solution of (in mM) 10 imidazole, 170 NaCl, 5 MgCl₂, 5 EGTA and 5 dithiothreitol (pH 6.8) for 30 min at 20 °C. Restored force was then measured in pCa 5.0 solution. Replacement with fsTnC returned the force to pre-extraction levels, whereas replacement with cTnC restored approx. 60 % of pre-extraction force. Incubation for longer or at higher temperatures did not increase this value. Dummy replacement experiments followed the same method, but used an exchange buffer without added protein. Dummy exchange fibres were not different from control (results not shown).

Whole troponin

Whole troponin replacement was conducted using the method described by Brenner et al. [12]. Fibres were first brought to rigor by washes in pre-rigor solution [(in mM) 10 imidazole, 2.5 EGTA and 15 EDTA (pH 7.0)] followed by rigor solution [(in mM) 10 imidazole, 2.5 EGTA and 2.5 EDTA (pH 7.0)] to ensure complete removal of ATP. The fibres were then incubated in 2.14 mg/ml recombinant human whole troponin solution for 2 h at 20 °C. Following this, the fibres were washed with exchange buffer and relaxing solution to remove the excess protein.

Protein content analysis

At the end of each experiment, fibres were placed in Laemmli buffer [in % (v/v) 47.5 distilled water, 12.5 Tris/HCl (made to 0.5 M, pH 6.8), 10 glycerol, 5 2-mercaptoethanol, 20 SDS (made to 10 % w/v) and 5 Bromophenol Blue (made to 1 % w/v)] and stored at –20 °C. They were analysed using a SDS/PAGE (15 % polyacrylamide) gel, against known concentrations of standard fsTnC and hcTnC. Protein content was ascertained using densitometry scans of silver-stained gels.

Statistical analysis

Data were analysed using SigmaPlot. Individual traces were fitted to the relevant curve fitting function (see below) and then the parameters were averaged to obtain the mean and S.E.M. Student's t test was used to determine the significant difference. The pCa–tension curves were fitted to the function $y = P_{\max} \times (c)^h / 1 + (c)^h$, where $c = [\text{Ca}^{2+}]_y / [\text{Ca}^{2+}]_{50}$, where $[\text{Ca}^{2+}]_y$ is the $[\text{Ca}^{2+}]$ that gives the force y and $[\text{Ca}^{2+}]_{50}$ is the $[\text{Ca}^{2+}]$ that gives 50 % $P_{\max}$. Data from the k_{tr} experiments were fitted with a single exponential function $y = \exp^{(kx)} + C$, where C is a constant and k the rate constant.

Figure 1 Relationship between calcium concentration (expressed as pCa) and tension in glycerinated rabbit psoas fibres both for control (○, $n = 7$) and for 10 μM EMD 57033 (●, $n = 5$)

(a) Shows tension normalized to maximal tension in control fibres (○), and (b) shows both curves normalized to maximal tension. In all Figures, error bars denote S.E.M. All the experiments were performed at room temperature.

RESULTS

Effect of EMD 57033 with skeletal isoforms

Figure 1(a) shows the pCa–tension curves for un-extracted psoas fibres (with native TnC), both control (open symbols) and with 10 μM EMD 57033 (filled symbols). Figure 1(b) shows the same curves normalized to $P_{\max}$. It can be seen that 10 μM EMD 57033 significantly increases maximal calcium-activated force to $140.7 \pm 5.5\%$ of control values (means $\pm$ S.E.M., $n = 5$, $P < 0.001$). In the normalized traces (Figure 1b), it is clear that the pCa–tension curves are shifted leftwards by 10 μM EMD 57033, with a significant increase in the pCa value for half-maximal force (pCa_{50}) from a control value of 5.61 ± 0.03 (means $\pm$ S.E.M., $n = 7$) to 5.73 ± 0.02 in the presence of 10 μM EMD 57033 (means $\pm$ S.E.M., $n = 5$, $P < 0.01$). This shows an increase in calcium sensitivity. The slope of the curve, as measured by the Hill coefficient (h), is not significantly altered [2.16 ± 0.33 in control (means $\pm$ S.E.M., $n = 7$); 2.06 ± 0.37 with 10 μM EMD 57033 (means $\pm$ S.E.M., $n = 5$)]. Thus, using this index, co-operativity of the fibre is not affected by 10 μM EMD 57033. Fibres were also tested with 30 μM EMD 57033, but this gave no significant increase over 10 μM EMD 57033 (results not shown).

Figure 2(a) shows the effect of replacement with fsTnC and Figure 2(b) shows the same curves normalized to $P_{\max}$. The effects of EMD 57033 on $P_{\max}$, pCa_{50} and h are not significantly changed in the fsTnC-replaced fibres compared with the control. This

Figure 2 Relationship between calcium concentration (expressed as pCa) and tension in glycerinated rabbit psoas fibres with native TnC extracted and replaced by fast skeletal wild-type TnC, control (□, $n = 6$) and with 10 μM EMD 57033 (●, $n = 6$)

The native control curve is shown by the open circles and dashed lines ($n = 7$). (a) Tension normalized to maximal tension in control fibres (○), and (b) all curves normalized to maximal tension.

shows that the EDTA extraction/TnC replacement method has no effect on the action of 10 μM EMD 57033.

Effect of EMD 57033 with cTnC replacement

Figure 3(a) shows the pCa–tension curves that result when the native rabbit sTnC (open circles) is replaced with recombinant hcTnC (open triangles), and the effect of 10 μM EMD 57033 on the replaced fibres (filled triangles). In Figure 3(a), fibres without 10 μM EMD 57033 are normalized to 100% tension in pCa 5.0 solution. As with the fibres with sTnC, it was found that force was not further increased by decreasing the pCa to 4.0 (results not shown). In Figure 3(b), all three curves are normalized to maximal tension and to zero at pCa 6.2. It is clear that replacement with cTnC causes a decrease in co-operativity, as shown by a decrease of h from 2.16 ± 0.33 (means $\pm$ S.E.M., $n = 7$) in control fibres to $h 0.93 \pm 0.21$ (means $\pm$ S.E.M., $n = 9$) in cTnC-replaced fibres ($P < 0.01$), as deduced from Figure 3(a). However, there is no change in pCa_{50} (cTnC replaced; 5.59 ± 0.08 , means $\pm$ S.E.M., $n = 9$). In the cTnC-replaced fibres, 10 μM EMD 57033 significantly increased $P_{\max}$ compared with control fibres (228.4 ± 18.9 , $P < 0.001$), significantly increased pCa_{50} (5.73 ± 0.07 , means $\pm$ S.E.M., $n = 9$, $P < 0.001$) and did not alter the h value (0.93 ± 0.22 , means $\pm$ S.E.M., $n = 7$).

Figure 3 Relationship between calcium concentration (expressed as pCa) and tension in glycerinated rabbit psoas fibres with native TnC extracted and replaced by recombinant hcTnC, control (Δ , $n = 9$) and with 10 μ M EMD 57033 ($\blacktriangle$, $n = 7$)

The native control curve is shown by the open circles and dashed lines ($n = 7$). (a) Tension normalized to maximal tension in control fibres ($\circ$), and (b) all curves normalized to maximal tension.

Effect of EMD 57033 with cTnC replacement

Figure 4(a) shows the pCa–tension curve of psoas fibres after replacement with human recombinant whole cTnC (open triangles) and the effect of 10 μ M EMD 57033 (filled triangles). The native pCa–tension curve is shown by open circles and dashed line. Figure 4(b) shows the same results normalized. As for replacement with cTnC alone, h is significantly decreased by the cardiac isoform compared with the pre-replacement fibres (h 0.67 ± 0.18 , means $\pm$ S.E.M., $n = 3$, $P < 0.05$) and the pCa₅₀ is unchanged (5.62 ± 0.10 , means $\pm$ S.E.M., $n = 3$). The effect of 10 μ M EMD 57033 in cTnC-replaced fibres is similar to that in cTnC-replaced fibres, except that the increase in $P_{\max}$ is not as extreme (146.2 ± 10.8 %, means $\pm$ S.E.M., $n = 4$). The pCa₅₀ value is significantly increased (5.73 ± 0.85 , means $\pm$ S.E.M., $n = 4$, $P < 0.001$) but the h value is not significantly changed (0.83 ± 0.33 , means $\pm$ S.E.M., $n = 4$).

Effect of EMD 57033 on replaced fibres (pCa 6.2)

Figure 5 shows a bar chart of normalized pCa–tension data for native fibres and fibres replaced with cTnC or whole cTnC (open bars), and the effects of 10 μ M EMD 57033 (grey bars). It clearly shows one of the more striking effects of EMD 57033 on psoas fibres replaced with cardiac isoforms of regulatory proteins, the

Figure 4 Relationship between calcium concentration (expressed as pCa) and tension in glycerinated rabbit psoas fibres with whole troponin replaced by recombinant hcTnC, control (Δ , $n = 3$) and with 10 μ M EMD 57033 ($\blacktriangle$, $n = 4$)

The native control curve is shown by the open circles and dashed lines ($n = 7$). (a) Tension normalized to maximal tension in control fibres ($\circ$), and (b) all curves normalized to maximal tension.

Figure 5 Force measurements at pCa 6.2 in native and replaced fibres

Open bars denote the absence of drug. Grey bars denote the presence of 10 μ M EMD 57033. Native psoas fibres, $n = 7$. Whole hcTnC replacement, $n = 3$. hcTnC replacement, $n = 9$.

large increase at low calcium. Whereas in native fibres, the tension at pCa 6.2 + 10 μ M EMD 57033 is 4.8 ± 1.6 % of $P_{\max}$ (means $\pm$ S.E.M., $n = 5$), it increases to 37.4 ± 9.2 % of $P_{\max}$

Figure 6 Effect of 10 μ M EMD 57033 on force, varying with partial extraction of TnC

Each bin represents one fibre, extracted in EDTA solution for a varying time to functionally remove some TnC. Open bars, the post-extraction force at pCa 6.2. Filled bars, the post-extraction force at pCa 6.2 in the presence of 10 μ M EMD 57033. Grey-shaded bars, the post-extraction force at pCa 5.0 in the presence of 10 μ M EMD 57033.

(means $\pm$ S.E.M., $n = 8$, $P < 0.002$) with normalized cTnC replacement and $30.0 \pm 5.2\%$ of $P_{\max}$ (means $\pm$ S.E.M., $n = 5$, $P < 0.01$) with normalized whole cTn replacement. In the non-normalized traces, these values are $68.6 \pm 11.9\%$ (means $\pm$ S.E.M., $n = 8$) for cTnC alone and $54.7 \pm 13.5\%$ (means $\pm$ S.E.M., $n = 5$) for cTn.

Effect of EMD 57033 with partial extraction

It is possible that a large percentage increase in tension seen in replaced fibres with EMD 57033 is an artifact of either the lower initial force or of EMD 57033, compensating for a loss of co-operativity due to a partial troponin system. To test for this, psoas fibres were extracted in EDTA solution for 2–20 min to produce fibres in which maximal force was functionally decreased by varying amounts, due to the loss of varying amounts of TnC. There was no subsequent troponin replacement. The effect of 10 μ M EMD 57033 at pCa 6.2 and pCa 5.0 was tested in these fibres. Figure 6 shows a plot of the force decrease (post-extraction force expressed as a percentage of pre-extraction force) versus the resultant force in pCa 6.2 (open bars) and the effects of 10 μ M EMD 57033 at pCa 6.2 (dark shaded bars) and pCa 5.0 (light shaded bars). As can be seen, the effect of EMD 57033 was similar at all levels of post-extraction force until $< 10\%$ of pre-extraction force. Since the average force for replaced fibres was approx. 60% of pre-extraction force, it appears that the increased effect of 10 μ M EMD 57033 in cardiac protein replaced fibres is due to the protein isoform and not an artifact of the replacement procedure.

Effect of EMD 57033 on stiffness

Fibre stiffness gives an indication of the number of cross-bridges formed in a contracting fibre. If force increases are due to an increased number of active cross-bridges, then the ratio of stiffness to force will be unchanged. If, however, $P_{\max}$ increases because the force for each cross-bridge changes, then force will be proportionally larger than stiffness and thus the ratio of stiffness/force will be decreased. In these experiments, 30 μ M EMD 57033 was used to ensure a saturating concentration of the drug. Figure 7 shows a plot of tension versus stiffness (normalized to the highest values within each fibre) caused by a rapid, transient stretch of 1% fibre length. Circles show prerenplacement native psoas fibres.

Figure 7 Stiffness response to a rapid 1% L_0 stretch plotted against the initial force (steady-state maximal calcium-activated force at pCa 5.0, data normalized to the results at pCa 5.0 in the unreplaced state in each fibre)

Open symbols denote control fibres and filled symbols denote the presence of 30 μ M EMD 57033. Circles, data from native rabbit psoas fibres; triangles, data from rabbit psoas fibres replaced with hcTnC. Each point represents one experiment.

Triangles show fibres replaced with cTnC. Open symbols show the control results and filled symbols show the effects of 30 μ M EMD 57033. At higher forces, stiffness in the filaments will be a contributing factor. To account for this, the data were fitted with the function $k_{\text{tot}} = k_b \times k_f / (k_b + k_f)$, where k_{tot} is the total stiffness, k_f is the stiffness in the filaments (calculated to be 0.5 when the tension is 1) and k_b is the stiffness in the cross-bridges. The continuous line shows the fit for the combined control data (replaced and non-replaced fibres), and the dashed line shows the fit for the combined EMD 57033 data. It can be seen that the stiffness/force relationship is unaltered by EMD 57033 with both skeletal and cardiac isoforms of TnC present, and suggests that the force for each cross-bridge is unaltered.

Effect of EMD 57033 on k_{tr}

The rate of force redevelopment (k_{tr}) after a slack–restretch method was measured in psoas fibres containing native TnC and replaced with cTnC. Figure 8(a) is a trace from one fibre (220404#3) showing the effects of 30 μ M EMD 57033 on k_{tr} . Figure 8(b) shows the mean rate constant data from all fibres under all conditions. In native fibres, 30 μ M EMD 57033 caused a significant ($P < 0.05$) decrease in the rate of activation from $20.0 \pm 3.0 \text{ s}^{-1}$ in native fibres (means $\pm$ S.E.M., $n = 12$) to $12.5 \pm 1.8 \text{ s}^{-1}$ in native fibres + 30 μ M EMD 57033 (means $\pm$ S.E.M., $n = 9$). In fibres replaced with cTnC, EMD 57033 again caused a significant decrease ($P < 0.001$) in k_{tr} from $22.0 \pm 0.7 \text{ s}^{-1}$ in cTnC-replaced fibres (means $\pm$ S.E.M., $n = 9$) to $14.0 \pm 3.0 \text{ s}^{-1}$ in cTnC-replaced fibres + 30 μ M EMD 57033 (means $\pm$ S.E.M., $n = 9$).

DISCUSSION

Model of muscle regulation

To discuss the action of EMD 57033, it is necessary to consider the mechanism of the regulatory proteins. This has been modelled as a three-state process [14]. In the 'B' state, tropomyosin sterically blocks myosin S1 binding to actin. TnI maintains this state in the absence of calcium by the binding of two regions, the inhibitory peptide and the switch peptide, to actin. In skeletal TnI, the inhibitory region comprises residues 96–115, and in cTnI, the residues 130–147. The switch peptide comprises residues 116–129 in skeletal TnI and residues 150–165 in cTnI [15,16]. When calcium binds to TnC, a hydrophobic pocket at the N-terminal

Figure 8 Effect of EMD 57033 on time for force redevelopment following a release–restretch method (k_{tr})

(a) An example trace from one fibre (220404#3). The slowing effect of 30 μ M EMD 57033 can clearly be seen in the lower trace. (b) A bar chart of the mean values for k_{tr} taken from fibres with native or cardiac-replaced TnC, and the effect of 10 μ M EMD 57033.

region of TnC opens up and this is capable of accepting the switch peptide of TnI. The inhibitory peptide binds to a cleft in the C-terminal of TnC and this removes the inhibition of tropomyosin to myosin [17–19]. The system can proceed to the 'C' state, whereby myosin is capable of weakly binding to actin. If ATP is present, the myosin heads can proceed to strongly bound states and the system enters the 'M' state, where the strongly bound cross-bridges increase the probability of adjacent actin–tropomyosin–tropomyosin units to shift from the B-state to the C-state. Thus both calcium and strongly bound cross-bridges regulate the contractile system.

Effect of troponin replacement with cardiac isoforms

Replacement of native TnC with cTnC caused a decrease in the co-operativity of the fibres, but did not significantly alter calcium sensitivity as measured by pCa_{50} . The fact that cTnC has only one Ca^{2+} -specific binding site is probably the main cause for decreased co-operativity [20], although it is possible that inefficient interactions between cTnC and inherent skeletal isoforms of TnT and TnI may be responsible [21]. To test this, whole cTn replacements were performed. The results are the same as with cTnC and suggest that the decreased co-operativity is not due to diminished communication between the native and replaced proteins, but due to an inherent property of the cTnC isoform. It is unlikely that the exchange methods are responsible for the changes in co-operativity, since fsTnC exchange does not alter the parameters of the pCa –tension relationship in any significant way, and the cardiac replacement experiments use two different methods for cTnC and cTn, yet still produce very similar results.

Effect of EMD 57033 on force, stiffness and kinetics

The predominant difference in the effect of EMD 57033 seen in the present study on native and replaced fibres is the great increase in force when cardiac isoforms of TnC are present, both at P_{max} and at low Ca^{2+} concentrations. In case this was an artifact caused by the fact that force is typically lower in the replaced fibres (and thus a small absolute response will give a proportionally larger percentage response), the effect of EMD 57033 on partially extracted fibres was studied. It can be seen that the apparent effect of EMD 57033 only increases when the force is below 10% P_{max} . Force in the replaced fibre was typically approx. 60% of the pre-replacement force and thus well above the level seen for this effect.

From the normalized traces, it can be seen that EMD 57033 causes a leftward shift in the pCa –tension curve in all conditions, indicating that the drug enables the fibre to generate more force for a given free $[Ca^{2+}]$. The fact that force increases even at P_{max} , where there is a saturating calcium concentration, implies that the effect of EMD 57033 must be acting on the cross-bridges and is not simply altering calcium occupancy of TnC. In this case, the force produced by a muscle fibre depends on three factors, the rate at which cross-bridges enter force-producing states (given by the apparent rate constant f_{app}), the rate at which they return to weakly bound or detached states (g_{app}) and the force/cross-bridge (n). The proportion of cross-bridges in strongly bound states is given by $f_{app}/(f_{app} + g_{app})$ and the force is proportional to this expression [22]. Increasing f_{app} or decreasing g_{app} will increase the proportion of force-producing states and hence increases the force. This can also occur by increasing n or a combination of all three. The stiffness measurements show that force/cross-bridge (n) is not altered.

The parameter k_{tr} measures the overall cycling rate of cross-bridges, in other words ($f_{app} + g_{app}$). End compliance in rabbit psoas fibres can cause k_{tr} measurements to be underestimated if sarcomere length is not controlled [23]. We do not measure sarcomere length during contraction, since it is difficult to maintain a viable diffraction pattern. However, all fibres are set to the same initial sarcomere length before contraction, and using cyanoacrylate glue to attach the fibre ends can minimize end compliance. Furthermore, since all experiments were conducted under the same conditions, it is still applicable to record the effects as normalized data. EMD 57033 slows k_{tr} and thus must be affecting the rate of transition between cross-bridge states. To increase the force, the predominant effect must be to decrease g_{app} . This is different from previous results from this laboratory, where no significant effect was seen on the rate of relaxation in either tissues when the laser photolysis of the caged calcium chelator diazo-2 was used [7,24]. It has been shown that EMD 57033 itself is photolabile and this may be a confounding factor in caged compound experiments [25]. However, we have shown that photolysis of EMD 57033 occurs much more readily when a UV flashlamp is used as the UV source [25] compared with a frequency-doubled ruby laser [7,26]. It is also possible that the rate of relaxation after photolysis of diazo-2 does not give a representation of g_{app} . The attachment and detachment transitions that occur during steady state force and constant pCa (at P_{max}) may well have different kinetics from those that occur when force and pCa are changing [27].

Effect of EMD 57033 in different TnC isoforms

It appears from these results that EMD 57033 acts by rate modulation when skeletal or cardiac isoforms of TnC are present, but the results also show that the effect on force is greater with the cardiac isoform. This is expected from the apparent rate constant

model, as the effect on force of altering g_{app} is larger at low calcium concentration [23]. Activation experiments performed by this laboratory using the caged calcium nitr-5 have found that EMD 57033 has more effect on the rate of activation in cardiac fibres compared with skeletal muscle [7,26]. This implies that, in cardiac muscle, EMD 57033 could be increasing f_{app} as well as slowing g_{app} compared with just slowing g_{app} in skeletal muscle, hence a greater effect on force. This observation is subject to the same caveats concerning caged-compound experiments and EMD 57033 as discussed above.

Recent evidence shows that EMD 57033 binds directly to the C-terminal of cTnC, in such a position that it competes with the binding of the N-terminal of TnI [6]. In skeletal muscle, this region comprises residues 1–40 and in cardiac muscle, it comprises residues 34–71. At the same time, the binding of EMD 57033 increases the affinity of TnC for the inhibitory peptide of TnI, possibly by stabilizing the open hydrophobic pocket on the C-terminal of TnC. The net effect is to draw the inhibitory region of TnI away from actin and towards TnC. This pushes the regulatory system closer to the C-state and allows weak-binding of cross-bridges to occur more easily. Dephosphorylation of TnI produces a similar effect, although EMD 57033 also increases P_{max} , whereas TnI dephosphorylation does not [28]. Barth et al. [29] found that EMD 53998, the racemic mixture of EMD 57033 and its negative enantiomer, decreased the inhibition caused by the cardiac inhibitory peptide cTnI_{137–147}. They surmised that EMD 53998 acted by weakening the attraction between cTnI_{137–147} and actin, but in fact the converse could be true that EMD 57033 increases the attraction between TnI_{137–147} and TnC. The effect would be the same, i.e. to shift the state of TnI from inhibitory to non-inhibitory. If EMD 57033 were maintaining the 'on' configuration of troponin (or the M-state of the regulated cross-bridge assembly), this could slow the rate of transition from strongly bound force-producing states back to non-force producing states, which might be seen as a slowing of g_{app} .

In the cardiac system, the hydrophobic cleft of the N-terminal TnC does not open fully until the TnI switch peptide has already bound, unlike the skeletal isoform where the hydrophobic cleft opens readily to accept the TnI switch peptide. This means that the skeletal system is more probable to enter the C-state than the cardiac. If EMD 57033 helps in stabilizing the opening of the hydrophobic cleft [6], then this effect will have greater importance in cardiac muscle compared with skeletal muscle, and this could explain why the apparent effect on force of EMD 57033 is larger in fibres with the cardiac isoform of TnC compared with fibres with the skeletal isoform of TnC.

It is apparent from our results that EMD 57033 must act by rate modulation since the force/stiffness ratio remains unaltered, and we suggest that it acts primarily by slowing g_{app} . Since this would slow relaxation, this diminishes its usefulness as a clinical intervention, but provides an interesting tool for examining the processes of muscle regulation, as the different effects on the TnC isoforms demonstrates. It may be possible that EMD 57033 will prove as a useful tool in determining the interactions of TnC and TnI that underlie muscle regulation.

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