Uncoupling of myofilament Ca\(^{2+}\) sensitivity from troponin I phosphorylation by mutations can be reversed by epigallocatechin-3-gallate

Maria Papadaki, Petr G. Vikhorev, Steven B. Marston, and Andrew E. Messer*

National Heart and Lung Institute, Imperial College London, London W12 0NN, UK

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Aims
Heart muscle contraction is regulated via the β-adrenergic response that leads to phosphorylation of Troponin I (TnI) at Ser22/23, which changes the Ca\(^{2+}\) sensitivity of the cardiac myofilament. Mutations in thin filament proteins that cause dilated cardiomyopathy (DCM) and some mutations that cause hypertrophic cardiomyopathy (HCM) abolish the relationship between TnI phosphorylation and Ca\(^{2+}\) sensitivity (uncoupling). Small molecule Ca\(^{2+}\) sensitizers and Ca\(^{2+}\) desensitizers that act upon troponin alter the Ca\(^{2+}\) sensitivity of the thin filament, but their relationship with TnI phosphorylation has never been studied before.

Methods and results
Quantitative in vitro motility assay showed that 30 μM EMD57033 and 100 μM Bepridil increase Ca\(^{2+}\) sensitivity of phosphorylated cardiac thin filaments by 3.1- and 2.8-fold, respectively. Additionally they uncoupled Ca\(^{2+}\) sensitivity from TnI phosphorylation, mimicking the effect of HCM mutations. Epigallocatechin-3-gallate (EGCG) decreased Ca\(^{2+}\) sensitivity of phosphorylated and unphosphorylated wild-type thin filaments equally (by 2.15 ± 0.45- and 2.80 ± 0.48-fold, respectively), retaining the coupling. Moreover, EGCG also reduced Ca\(^{2+}\) sensitivity of phosphorylated but not unphosphorylated thin filaments containing DCM and HCM-causing mutations; thus, the dependence of Ca\(^{2+}\) sensitivity upon TnI phosphorylation of uncoupled mutant thin filaments was restored in every case. In single mouse heart myofibrils, EGCG reduced Ca\(^{2+}\) sensitivity of force and k\(_{\text{ACT}}\) and also preserved coupling. Myofibrils from the ACTC E361G (DCM) mouse were uncoupled; EGCG reduced Ca\(^{2+}\) sensitivity more for phosphorylated than for unphosphorylated myofibrils, thus restoring coupling.

Conclusion
We conclude that it is possible to both mimic and reverse the pathological defects in troponin caused by cardiomyopathy mutations pharmacologically. Re-coupling by EGCG may be of potential therapeutic significance for treating cardiomyopathies.

Keywords
Ca\(^{2+}\) sensitizers • Epigallocatechin-3-gallate • Ca\(^{2+}\) regulation of contractility • Troponin phosphorylation • Cardiomyopathies

1. Introduction
Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are common inherited diseases. HCM is detected in 1 in 500 of the adult population, and the causative mutations are overwhelmingly in the proteins of the cardiac muscle contractile apparatus. At least 30% of cases of idiopathic DCM are of genetic origin, and DCM with no additional complications such as conduction disease is usually caused by mutations in the contractile apparatus. In many cases, the cardiomyopathies result from missense mutations in one of the proteins of the muscle thin filament (actin, tropomyosin, troponin I, C, or T). Investigations of the mechanisms that cause HCM and DCM have generally found that mutations in the muscle thin filament cause abnormalities in the Ca\(^{2+}\) regulatory system of the muscle.

HCM is closely associated with enhanced myofilament Ca\(^{2+}\) sensitivity, although the process by which chronically high Ca\(^{2+}\) sensitivity leads to the symptoms of HCM, hypertrophy, fibrosis, and arrhythmias, is uncertain. In DCM, the situation is more complex. It has been

* Corresponding author. Tel: +44 0207 594 2732, +44 07722 966655, Email: a.messer@imperial.ac.uk
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suggested that DCM-causing mutations are associated with a reduced Ca\(^{2+}\) sensitivity, but comprehensive surveys now show that DCM-causing mutations in thin filament proteins can increase or decrease Ca\(^{2+}\) sensitivity. However, the DCM phenotype is always linked to the absence of modulation of Ca\(^{2+}\) sensitivity by troponin I (TnI) phosphorylation (uncoupling), and this has been proposed to be causative of the DCM phenotype. In addition, the coupling between TnI phosphorylation and change of Ca\(^{2+}\) sensitivity seems to be lost due to HCM mutations in thin filament proteins when studied by in vitro motility assay (IVMA) techniques and by myofilibrar assays. But in permeabilized muscle, this phenomenon is not observed. Thus, in HCM, the relationship between TnI phosphorylation and Ca\(^{2+}\) sensitivity is not clear.

Uncoupling appears to be closely associated with mutations in thin filament proteins that cause cardiomyopathies. The decrease in Ca\(^{2+}\) sensitivity upon phosphorylation of TnI and the corresponding increase in the rate of Ca\(^{2+}\) dissociation from troponin C (TnC) is a key component of the lusitropic response to β1-adrenergic stimulation in the heart. The uncoupling reported in DCM and HCM is likely to impact on cardiac reserve pathologically, and indeed, most studies of mouse models with cardiomyopathy mutations in thin filament proteins report a blunted response to adrenergic stimulation in vivo (reviewed in Ref. 17).

A number of small molecules have been found that alter myofilament Ca\(^{2+}\) sensitivity by binding to troponin and act as either Ca\(^{2+}\) sensitizers or Ca\(^{2+}\) desensitizers. The effect of these reagents on Ca\(^{2+}\) sensitivity is well documented, but the effect of small molecules on the coupling between Ca\(^{2+}\) sensitivity and TnI phosphorylation has not previously been considered.

EMDS7033 and Bepridil are well-established Ca\(^{2+}\) sensitizers acting directly upon TnC. Epigallocatechin-3-gallate (EGCG), the principal polyphenol isolated from green tea, is reported to be a Ca\(^{2+}\) desensitizer that also acts via a binding site on TnC. Structural studies indicate that the regulatory Ca\(^{2+}\) binding site (Site II in the N-terminal lobe of TnC) is closely coupled both to the binding of the Tn switch peptide (147–163), critical for neutralizing the inhibitory action of TnI, and the cardiac-specific N-terminal peptide of TnI (1–30) that contains the phosphorylatable serines 22 and 23. We have proposed that DCM-causing mutations in thin filament proteins uncouple phosphorylation from the change in Ca\(^{2+}\) sensitivity by disrupting this coupled allosteric system. It is likely that Ca\(^{2+}\) sensitizers and desensitizers binding to TnC would also have an effect on the coupling between Ca\(^{2+}\) sensitivity and TnI phosphorylation by modulating the coupled system.

We have therefore investigated how EMDS7033, Bepridil, and EGCG affect the Ca\(^{2+}\) regulation and its modulation by phosphorylation in native human heart thin filaments and how these reagents interact with mutations in thin filament proteins associated with HCM or DCM. Using IVMA and single myofilibrar contractility, we have confirmed the Ca\(^{2+}\)-sensitizing effects of EMDS7033 and Bepridil and demonstrated that they also uncouple Ca\(^{2+}\) sensitivity from the TnI phosphorylation level, thus mimicking the effects of HCM-causing mutations. In contrast, EGCG decreases Ca\(^{2+}\) sensitivity in native thin filaments while retaining the modulation of Ca\(^{2+}\) sensitivity by TnI phosphorylation. Moreover, EGCG has the unique ability to restore the coupling to uncoupled HCM and DCM mutant thin filaments and myofibrils, thus antagonizing the disease-causing defect. This property of EGCG may be of therapeutic significance for treating some cardiomyopathies.

2. Methods

2.1 Sources of contractile proteins

Donor heart tissue, used as control, and end-stage failing heart tissue from explanted hearts were obtained from the Sydney Tissue Bank Sydney, Australia. Ethical approval for collection and use of tissue samples was obtained from the St Vincent’s Hospital, Sydney and Brompton, Harfield and NHLI Research Ethics Committees.

Troponin was isolated from 100 mg of human heart muscle using an anti-cardiac TnI mAb affinity column as described by Messer. Troponin containing the TNNC1 G159D mutation was purified from explanted heart samples as previously described. Recombinant TNNT2 K280N and TNNI3 K36Q was introduced into donor heart troponin by exchange as described. Wild-type α-tropomyosin (Tpm1.1) and the mutants E40K, E54K, and E180G were expressed in a baculovirus/SF9 system with a protocol based on that of Akkari et al. Native, E361G, and E99K mutant mouse cardiac acts were extracted from transgenic mouse hearts as described by Song et al.

2.2 Manipulation and measurement of TnI phosphorylation level

Troponin isolated from human heart samples has a high level of phosphorylation, which was reduced by treatment with shrimp AP (Sigma P9088). Recombinant TnI was phosphorylated by treatment with protein kinase A (PKA) catalytic subunit (Sigma, P2645–400) as previously described. To dephosphorylate mouse heart troponin, mice were treated with propranolol as described. TnI phosphorylation levels in myofilibrils and isolated troponin were measured by Phosphopeptide affinity SDS–PAGE as described; results are shown in Supplementary material online, SC.

2.3 Quantitative IVMA

Thin filaments were reconstituted with 10 nM rabbit skeletal or mouse cardiac muscle α-actin (labelled with TRITC, phalloidin), tropomyosin (40–60 nM), and troponin (60 nM) to study Ca\(^{2+}\) regulation of filament motility by the quantitative IVMA. Thin filament movement over a bed of immobilized rabbit fast skeletal muscle heavy meromyosin (100 μg/mL) was compared in paired motility chambers in which troponin varied by a single factor (mutation, phosphorylation state, or treatment with drug). Filament movement was recorded and analysed as previously described, yielding two parameters, the fraction of filaments moving, and the speed of moving filaments.

2.4 Contraction of isolated myofibrils

Contraction and relaxation of isolated mouse myofibrils were initiated using a fast-solution change system and sensitive force transducer system recently described. Further details are in Supplementary material online, SA.

3. Results

3.1 Relationship between phosphorylation and Ca\(^{2+}\) sensitivity

We measured the Ca\(^{2+}\) dependence of thin filaments containing human heart troponin by the IVMA. The assay yields two parameters, fraction of filaments motile and sliding speed of the motile thin filaments. Both parameters are Ca\(^{2+}\)-dependent, and Ca\(^{2+}\) activation curves were plotted (Figure 1). 3.9 μM Ca\(^{2+}\) increased the fraction of filaments motile from 0.03 to 0.83 and the sliding speed from 1.5 to 3.0 μm/s. The EC\(_{50}\) and Hill co-efficient (n\(_H\)) determined by fitting the Hill equation to the data were similar for both parameters under
all conditions; therefore, in this manuscript we have only shown the fraction motile parameter. Full data for both parameters are shown in Supplementary material online, SB.

Human heart troponin has a high level of TnI phosphorylation in the 1.4–1.8 mol Pi/mol range. This is reduced to 0.3 mol Pi/mol by treatment with phosphatase, see Supplementary material online, SC. The Ca$^{2+}$ sensitivity of unphosphorylated thin filaments is 1.88 ± 0.10 ($P < 0.0001, n = 16$) times greater than the native phosphorylated thin filaments as reported previously. $n_H$ was significantly reduced from 2.11 ± 0.18 for phosphorylated to 1.72 ± 0.11 for unphosphorylated thin filaments, $P = 0.017, n = 16$. The maximum sliding speed was not significantly affected by phosphorylation level (Table 1, Figure 1A).

We have investigated thin filaments incorporating six DCM-causing mutations that have been shown to abolish this relationship (uncouple) in previous studies and three HCM mutations that uncouple in vitro (Figure 1B).

Figure 1 Relationship between EC$_{50}$ and Tn phosphorylation in native human thin filaments and thin filaments containing HCM-causing mutation TPM1 E180G. Sliding speed and fraction of filaments motile, measured in the same experiment by IVMA, are plotted against [Ca$^{2+}$] for representative experiments. Raw data from one experiment are shown here; the mean values of EC$_{50}$ from replicate experiments are shown in Table 2. Solid lines and points, phosphorylated troponin (P); dotted lines and open points, unphosphorylated troponin (unP). Error bars represent SEM of four measurements of motility in the same motility chamber. Blue, native thin filaments; purple, HCM-causing mutation TPM1 E180G present. (A) Native thin filaments: phosphorylation increased EC$_{50}$ (decreased Ca$^{2+}$ sensitivity) but had no effect on the maximum sliding speed or fraction of filaments motile at saturating Ca$^{2+}$. (B) Thin filaments containing TPM1 E180G HCM-causing mutation. The relationship of Ca$^{2+}$ sensitivity to TnI phosphorylation is uncoupled.
### Table 1: Effect of EMD57033, Bepridil, and EGCG on Ca$^{2+}$ regulation of motility

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC$_{50}$</th>
<th>n$_{4i}$</th>
<th>$\Delta V_{\text{max}}$</th>
<th>$\text{EC}_{50}$, TPM1 E54K DCM mutation</th>
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<tbody>
<tr>
<td>Native</td>
<td>0.16±0.03</td>
<td>2.08±0.14</td>
<td>1.40±0.11</td>
<td>0.12±0.02</td>
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<tr>
<td>P</td>
<td>0.091±0.014</td>
<td>1.51±0.25</td>
<td>1.30±0.04</td>
<td>0.11±0.008</td>
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<tr>
<td>+EMD57033</td>
<td>0.057±0.010</td>
<td>1.26±0.07</td>
<td>1.55±0.08</td>
<td>0.041±0.002</td>
</tr>
<tr>
<td>P</td>
<td>0.053±0.010</td>
<td>1.34±0.14</td>
<td>1.54±0.08</td>
<td>0.041±0.002</td>
</tr>
<tr>
<td>Native</td>
<td>0.13±0.007</td>
<td>2.89±0.48</td>
<td>2.09±0.14</td>
<td>0.13±0.014</td>
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<tr>
<td>unP</td>
<td>0.072±0.002(6)</td>
<td>1.96±0.08</td>
<td>2.13±0.20</td>
<td>0.13±0.012</td>
</tr>
<tr>
<td>+Bepridil</td>
<td>0.045±0.001(8)**</td>
<td>1.94±0.08</td>
<td>2.40±0.15 (8)**</td>
<td>0.060±0.009</td>
</tr>
<tr>
<td>P</td>
<td>0.045±0.001(6)**</td>
<td>2.03±0.13</td>
<td>2.24±0.16</td>
<td>0.058±0.006</td>
</tr>
<tr>
<td>Native</td>
<td>0.14±0.03</td>
<td>1.61±0.19</td>
<td>1.91±0.18</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>unP</td>
<td>0.059±0.011(6)**</td>
<td>1.50±0.21</td>
<td>1.92±0.22</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>+EGCG</td>
<td>0.26±0.02(7)**</td>
<td>1.96±0.24</td>
<td>1.38±0.12 (7)**</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>P</td>
<td>0.15±0.02(6)**</td>
<td>1.79±0.30</td>
<td>1.52±0.13 (6)*</td>
<td>0.071±0.005</td>
</tr>
</tbody>
</table>

Paired t-test was used for EC$_{50}$, n$_{4i}$, and $\Delta V_{\text{max}}$, (the change in velocity due to Ca$^{2+}$). EC$_{50}$ values rounded to two significant figures. The number of experiments is given in brackets.

*P < 0.05, **P < 0.01, for presence and absence of reagent.

†P < 0.05, ††P < 0.01, unphosphorylated compared with phosphorylated.

#### 3.2 EMD57033 and Bepridil increase Ca$^{2+}$ sensitivity and uncouple TnI phosphorylation from changes in Ca$^{2+}$ sensitivity

EMD57033 and Bepridil substantially increased the Ca$^{2+}$ sensitivity (Figure 2A and D) and slightly increased the maximum sliding speed of native human thin filaments as expected.

The effect of EMD57033 on EC$_{50}$ for Ca$^{2+}$ activation of motility was biphasic with a maximum at 30 µM and an EC$_{50}$ in the range of 15–20 µM (Figure 2C). Interestingly, we observed that 30 µM EMD57033 increased the Ca$^{2+}$ sensitivity of thin filaments containing phosphorylated troponin more than unphosphorylated TnI (mean ratio EC$_{50}$ P/EC$_{50}$ unP + EMD57033 = 1.83±0.36, P = 0.07, n = 4 in unphosphorylated, Table 1, Figure 2A, see Supplementary material online, SB). Consequently, in the presence of EMD57033, Ca$^{2+}$ sensitivity of thin filaments was the same, independent of the level of phosphorylation (EC$_{50}$ P + EMD57033/EC$_{50}$ unP + EMD57033 = 1.08±0.03, P = 0.06, n = 4). EMD57033 increased sliding speed slightly at saturating Ca$^{2+}$ concentration in phosphorylated thin filaments and in unphosphorylated thin filaments by 18.4±4.1% (P = 0.02, n = 4), $n_{4i}$ was slightly decreased in phosphorylated but not in unphosphorylated thin filaments. EMD57033 also increased the Ca$^{2+}$ sensitivity of thin filaments containing the DCM-causing mutations, TPM1 E40K and E54K. In both these cases, Ca$^{2+}$ sensitivity was independent of troponin phosphorylation (uncoupled) due to the mutation (Table 1, Figure 2B).

A similar pattern of results was observed with Bepridil. The dose–response curve was cooperative with a calculated EC$_{50}$ of 52 µM (Figure 2F). At 100 µM, which is the saturated concentration, the increase in Ca$^{2+}$ sensitivity of phosphorylated donor thin filaments (measured by the mean ratio EC$_{50}$ P/EC$_{50}$ P + Bepridil) was 2.8±0.14-fold for fraction motile (P < 0.0001, n = 6) and 3.09±0.59-fold for sliding speed (P = 0.016, n = 6), see Supplementary material online, SB.

Bepridil increased the Ca$^{2+}$ sensitivity of thin filaments and also uncoupled Ca$^{2+}$ sensitivity from TnI phosphorylation, as shown by the EC$_{50}$ ratios of phosphorylated to dephosphorylated troponin (EC$_{50}$ P/EC$_{50}$ unP = 1.73±0.03, P < 0.0001; EMD57033 P + Bepridil/EC$_{50}$ unP + Bepridil = 1.03±0.01, P = 0.04, n = 6) (Figure 2D, see Supplementary material online, SB). Bepridil increased sliding speed at saturating Ca$^{2+}$ concentrations significantly in phosphorylated thin filaments (14.8±3.2%, P = 0.006, n = 6) but did not have a significant effect in unphosphorylated thin filaments (7.2±4.6%, P = 0.18, n = 6); it had no significant effect on $n_{4i}$.

Bepridil also reversibly increased the Ca$^{2+}$ sensitivity of both phosphorylated and unphosphorylated thin filaments containing the DCM-causing TPM1 E54K mutation, and these mutant thin filaments remained uncoupled (Table 1, Figure 2E, see Supplementary material online, SE).

#### 3.3 EGCG decreases Ca$^{2+}$ sensitivity while retaining coupling

EGCG decreased Ca$^{2+}$ sensitivity of human cardiac thin filaments measured by IVMA and at saturating concentrations (100 µM) reduced the maximum sliding speed up to 20% but had no significant effect on $n_{4i}$ (Table 1, Figure 3, see Supplementary material online, SB). Titration of EGCG at 0.074 µM Ca$^{2+}$ yielded a cooperative dose–response curve with a calculated EC$_{50}$ of 70±7 µM for phosphorylated and 60±1 for unphosphorylated (Figure 3C). Unlike EMD57033 and Bepridil, 100 µM EGCG had a similar effect on the EC$_{50}$ of thin filaments with phosphorylated and unphosphorylated troponin, (mean ratio EC$_{50}$ +
EGCG/EC50 control = 2.15 ± 0.45-fold for phosphorylated and 2.80 ± 0.48-fold for unphosphorylated thin filaments, see Supplementary material online, SB). Therefore, the coupling of TnI phosphorylation level to changes in Ca2+ sensitivity was retained (EC50 P/EC50 unP = 2.24 ± 0.10, P < 0.0001, n = 6; EC50 P + EGCG/EC50 unP + EGCG = 1.73 ± 0.16, P = 0.006, n = 6) (Table 1, Figure 3A, see Supplementary material online, SB).

### 3.4 EGCG restores coupling to HCM and DCM mutant thin filaments and myofibrils

EGCG also affected Ca2+ sensitivity in thin filaments containing mutations associated with HCM or DCM in a phosphorylation-dependent way. This was tested with five DCM-causing mutations and three HCM-causing mutations. EGCG decreased the Ca2+ sensitivity of phosphorylated mutant thin filaments in a similar way to wild-type troponin (ratio EC50 with/without EGCG was 1.36–2.85). However, with unphosphorylated troponin, EGCG tended to have no effect or, in the case of the three tropomyosin mutations, increased Ca2+ sensitivity (Table 2). As a result, the dependence of Ca2+ sensitivity on TnI phosphorylation level was restored. For example, thin filaments containing the DCM-causing TPM1 E54K mutation were uncoupled with EC50 of 0.106 ± 0.013 μM for phosphorylated and 0.114 ± 0.019 μM for unphosphorylated giving a ratio of 0.95 ± 0.03 compared with a ratio of 2.24 ± 0.10 for wild-type thin filaments (Table 1). In the presence of 100 μM EGCG, the EC50 was 0.23 ± 0.03 μM for phosphorylated and 0.071 ± 0.005 μM for unphosphorylated giving a ratio of 3.17 ± 0.37 (P = 0.004, n = 5) (Table 1, Figure 3B). A dose–response assay for recoupling yielded an EC50 of 58.8 ± 13.3 μM (Figure 3D).

EGCG was able to reversibly restore coupling of all the thin filament HCM and DCM-causing mutations in this study (Table 2, Figures 4 and 5, see Supplementary material online, SF).

### 3.5 The effect of EGCG on contraction of myofibrils

We studied the effect of EGCG on mouse myofibril contractility in basally phosphorylated and dephosphorylated states, obtained...
by treating mice with propranolol prior to sacrifice (Table 3, Figure 6). We found that 10 μM EGCG decreased Ca^{2+} sensitivity of isometric force for both phosphorylated and unphosphorylated mouse myofibrils equally (EC_{50} P/EC_{50} unP + EGCG = 0.50 ± 0.06, P < 0.001, n = 9, EC_{50} unP/EC_{50} unP + EGCG = 0.45 ± 0.07, P < 0.00, n = 5). Consequently, the effect of phosphorylation on Ca^{2+} sensitivity in wild-type myofibrils was preserved in the presence of EGCG (EC_{50} P + EGCG/EC_{50} unP + EGCG = 1.64 ± 0.24, P < 0.001, n = 5).

As previously shown, in myofibrils from ACTC E361G DCM mice, the Ca^{2+} sensitivity of force is uncoupled (Table 3, Figure 6). EGCG decreased Ca^{2+} sensitivity in myofibrils from ACTC E361G mice; however, this effect was greater in phosphorylated myofibrils than unphosphorylated myofibrils. Thus, EGCG restored the phosphorylation-dependent shift in Ca^{2+} sensitivity for ACTC E361G myofibrils to the same level as in wild-type myofibrils (EC_{50} P + EGCG/EC_{50} unP + EGCG = 1.62 ± 0.20, P < 0.01, n = 5 compared with 1.82 ± 0.24 for wild-type in the absence of EGCG). n_{H} values were similar to those found in wild-type

**Figure 3** Effect of EGCG on Ca^{2+} control of motility. (A and B) Fraction of filaments motile, measured by IVMA, is plotted against [Ca^{2+}], details as in Figure 1. Raw data from one experiment are shown here; the mean values of EC_{50} from replicate experiments are shown in Tables 1 and 2. (A) Effect of EGCG on Ca^{2+} regulation of native thin filaments. Blue, native thin filaments; green, the presence of 100 μM EGCG. (B) Effect of EGCG on Ca^{2+} regulation of thin filaments containing TPM1 E54K DCM-causing mutation. Red, E54K-containing thin filaments; green, the presence of 100 μM EGCG. (C and D) Dose–response curves, the fraction motile was measured at a constant [Ca^{2+}] with increasing concentrations of EGCG for a representative experiment. (C) Effect of EGCG on donor thin filaments at 0.074 μM Ca^{2+}. The inset plots the change in fraction motility with increasing EGCG concentration, and the curve represents the fit of the pooled data to the Hill equation. Values of parameters obtained are shown. (D) Effect of EGCG on thin filaments containing TPM1 E180G tropomyosin at 0.037 μM Ca^{2+}. Initially, motility is the same (see Figure 1); the addition of EGCG reduced motility of phosphorylated and increased motility of unphosphorylated thin filaments, indicating recoupling or the restoration of the phosphorylation-dependent Ca^{2+} sensitivity difference (see Figure 3B). Details as in Figure 2F.
Table 2 Effect of EGCG and TnI phosphorylation on the Ca\textsuperscript{2+} sensitivity of thin filaments containing HCM and DCM mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EC\textsubscript{50} of thin filaments containing phosphorylated troponin μM ± SEM</th>
<th>Ratio EC\textsubscript{50} P/unP ± SEM</th>
<th>Ratio EC\textsubscript{50} P + EGCG/EGCG ± SEM</th>
<th>Ratio EC\textsubscript{50} P + EGCG/unP ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.14 ± 0.03 (7)</td>
<td>2.24 ± 0.10 (6)††</td>
<td>1.73 ± 0.16 (6)††</td>
<td>2.15 ± 0.45 (7)</td>
</tr>
<tr>
<td>DCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPM1 E54K</td>
<td>0.11 ± 0.013 (5)</td>
<td>0.95 ± 0.03 (5)</td>
<td>0.071 ± 0.005 (5)††</td>
<td>3.17 ± 0.37 (5)††</td>
</tr>
<tr>
<td>TPM1 E40K</td>
<td>0.18 ± 0.03 (8)</td>
<td>1.02 ± 0.007 (8)</td>
<td>0.052 ± 0.01 (5)†</td>
<td>4.63 ± 0.96 (5)††</td>
</tr>
<tr>
<td>TNNC1 G159D</td>
<td>0.092 ± 0.004 (5)</td>
<td>0.97 ± 0.03 (5)</td>
<td>0.088 ± 0.005 (5)††</td>
<td>2.25 ± 0.41 (5)††</td>
</tr>
<tr>
<td>TNNJ3 K36Q</td>
<td>0.18 ± 0.03 (10)</td>
<td>1.02 ± 0.05 (10)</td>
<td>0.10 ± 0.009 (5)††</td>
<td>1.97 ± 0.12 (5)††</td>
</tr>
<tr>
<td>ACTC E361G</td>
<td>0.087 ± 0.002 (5)</td>
<td>1.08 ± 0.02 (5)</td>
<td>0.09 ± 0.002 (5)††</td>
<td>2.41 ± 0.28 (5)††</td>
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<td>HCM</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TPM1 E180G</td>
<td>0.086 ± 0.013 (3)</td>
<td>0.98 ± 0.02 (3)</td>
<td>0.048 ± 0.003 (5)††</td>
<td>2.41 ± 0.32 (5)††</td>
</tr>
<tr>
<td>TNNT2 K280N</td>
<td>0.11 ± 0.007 (5)</td>
<td>1.08 ± 0.04 (5)</td>
<td>0.10 ± 0.004 (5)†</td>
<td>1.96 ± 0.24 (5)††</td>
</tr>
<tr>
<td>ACTC E99K</td>
<td>0.074 ± 0.005 (5)</td>
<td>0.99 ± 0.02 (5)</td>
<td>0.10 ± 0.007 (5)††</td>
<td>2.03 ± 0.21 (5)††</td>
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</table>

Ratios: single value t-test compared with 1. ANOVA analysis of this data set is shown in Supplementary material online. SE. EC\textsubscript{50} values rounded to two significant figures. The number of experiments is given in brackets.

*P < 0.05, **P < 0.01, for the presence and absence of EGCG using paired t-test.

†P < 0.05, ††P < 0.01, unphosphorylated compared with phosphorylated using paired t-test.
myofibrils (Table 3). The duration of the initial, nearly isotonic, phase of relaxation, \( t_{\text{LIN}} \), in phosphorylated and unphosphorylated \( \text{ACTC} \; E361G \) myofibrils was restored to wild-type values (Table 3). EGCG decreased \( k_{\text{ACT}} \) in unphosphorylated wild-type myofibrils (decreased by 23%, \( P < 0.05, n = 10 \)) as well as in phosphorylated and unphosphorylated \( \text{ACTC} \; E361G \) (decreased by 21%, \( P < 0.001, n = 11 \)).

4. Discussion

In previous studies, it was found that mutations in proteins of the cardiac muscle thin filament that are associated with inherited cardiomyopathies (HCM and DCM) alter myofibrillar \( \text{Ca}^{2+} \) sensitivity. They also cause the modulation of myofilament \( \text{Ca}^{2+} \) sensitivity to become independent of the PKA-dependent phosphorylation of TnI. We have named this phenomenon uncoupling. Moreover, this uncoupling effect may be sufficient to generate the disease phenotype of familial DCM.9,17 We have investigated whether small molecules might be able to mimic or reverse the molecular effects of mutations.

The \( \text{Ca}^{2+} \) sensitizers EMD57033 and Bepridil, known to bind to TnC, induce uncoupling in wild-type thin filaments, thus mimicking the effects of HCM mutations. The \( \text{Ca}^{2+} \)-desensitizer EGCG has an opposite effect. It preserves coupling in wild-type troponin and restores coupling to thin filaments with HCM- and DCM-causing mutations in TnI, TnC, TnT, tropomyosin, and actin, thus antagonizing the effect of the HCM or DCM mutation. These findings suggest the potential of EGCG for treating the symptoms of inherited cardiomyopathies.

4.1 \text{Ca}^{2+} \) sensitizers mimic the effects of HCM-causing mutations in thin filaments

Both EMD57033 and Bepridil increase \( \text{Ca}^{2+} \) sensitivity of phosphorylated thin filaments by 2.8- and 2.9-fold, measured by quantitative
Table 3  Effect of phosphorylation, ACTC E361G mutation, and EGCG on Ca²⁺ regulation of myofibril contraction

<table>
<thead>
<tr>
<th></th>
<th>F_{max} (kN/m²)</th>
<th>EC_{50} (µM)</th>
<th>n_M</th>
<th>k_{ACT} (s⁻¹)</th>
<th>t_{LIN} (ms)</th>
<th>k_{REL} (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT⁺</td>
<td>100.9 ± 6.3 (14)</td>
<td>0.93 ± 0.06 (11)</td>
<td>10.43 ± 1.84 (10)</td>
<td>4.16 ± 0.43 (11)</td>
<td>50.8 ± 3.5 (12)</td>
<td>35.0 ± 4.0 (10)</td>
</tr>
<tr>
<td>unP</td>
<td>87.1 ± 6.0 (16)</td>
<td>0.51 ± 0.06 (14) †††</td>
<td>4.74 ± 0.66 (13) †††</td>
<td>4.50 ± 0.24 (15)</td>
<td>67.0 ± 4.2 (16) †</td>
<td>23.2 ± 2.8 (11) †††</td>
</tr>
<tr>
<td>WT + EGCG</td>
<td>92.5 ± 5.0 (15)</td>
<td>1.85 ± 0.17 (9) †***</td>
<td>4.30 ± 0.85 (8) †**</td>
<td>4.44 ± 0.24 (15)</td>
<td>46.7 ± 3.9 (12)</td>
<td>41.0 ± 4.0 (12)</td>
</tr>
<tr>
<td>E361G⁺</td>
<td>92.6 ± 8.9 (8)</td>
<td>1.13 ± 0.13 (5) ††††</td>
<td>5.08 ± 1.24 (5) ††††</td>
<td>3.47 ± 0.55 (6) ††††</td>
<td>88.9 ± 9.9 (7) ††††</td>
<td>22.3 ± 1.2 (7) ††††</td>
</tr>
<tr>
<td>E361G + EGCG</td>
<td>93.5 ± 8.9 (11)</td>
<td>0.38 ± 0.05 (12) †††</td>
<td>5.39 ± 1.26 (9) †††</td>
<td>4.51 ± 0.32 (13) †††</td>
<td>75.4 ± 6.5 (10) †††</td>
<td>21.6 ± 2.8 (10) †††</td>
</tr>
<tr>
<td>unP</td>
<td>89.3 ± 8.6 (10)</td>
<td>0.43 ± 0.06 (10) †††</td>
<td>4.48 ± 0.70 (8) †††</td>
<td>4.12 ± 0.32 (11) †††</td>
<td>70.8 ± 9.7 (11) ††</td>
<td>21.6 ± 2.1 (10)</td>
</tr>
<tr>
<td>E361G + EGCG</td>
<td>89.1 ± 4.5 (12)</td>
<td>2.07 ± 0.20 (5) †††† †††</td>
<td>7.18 ± 1.54 (5) ††††</td>
<td>3.43 ± 0.20 (13) †††</td>
<td>49.7 ± 3.3 (12) ††</td>
<td>31.0 ± 2.6 (12) ††</td>
</tr>
<tr>
<td>unP</td>
<td>96.3 ± 11.1 (11)</td>
<td>1.28 ± 0.10 (8) †††† †††</td>
<td>4.14 ± 0.42 (7) †††</td>
<td>3.25 ± 0.26 (11) †††</td>
<td>63.0 ± 4.7 (12) ††</td>
<td>31.0 ± 2.0 (12) ††††</td>
</tr>
</tbody>
</table>

The number of experiments is given in brackets. Statistical analysis carried out by un-paired t-test (equal variance).

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⁺P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001, EGCG treated compared with no EGCG.

†P < 0.05, ††P < 0.01, †††P < 0.001, unphosphorylated compared with phosphorylated.

§P < 0.05, §§P < 0.01, §§§P < 0.001, ACTC E361G compared with wild type.

Fig 6  EGCG restores the relationship between Ca²⁺ sensitivity of force production and TnI phosphorylation in wild-type and ACTC E361G mouse myofibrils. Phosphorylation level was reduced by propranolol treatment and measured by phosphate affinity SDS–PAGE. Basally phosphorylated wild-type was 1.02 ± 0.03 and ACTE E361G was 1.08 ± 0.01 mol Pi/mol TnI. For propranolol-treated (dephosphorylated) muscle, the phosphorylation level was 0.30 ± 0.04 in wild type and 0.34 ± 0.07 mol Pi/mol TnI for ACTE E361G. (A) Ca²⁺ activation curves for ACTC E361G myofibrils in the presence (green) and absence (red) of 10 µM EGCG. Solid lines and points, phosphorylated troponin (P); dotted lines and open points, unphosphorylated troponin (unP). The plot shows averaged isometric tension ± SEM from 11 to 14 myofibrils for experiments performed at SL = 2.17 µm. EGCG shifts the activation curve to the right and restores the difference between phosphorylated and unphosphorylated myofibrils. (B) Effects of the ACTC E361G mutation, phosphorylation, and EGCG on the mean EC_{50} (± SEM) for myofibril isometric contraction. Significant differences, calculated by t-test, between phosphorylated and unphosphorylated myofibrils are indicated: †††P < 0.01; ††††P < 0.001. Data from Table 3.

IVMA, in common with previous measurements. However, it has not been shown before that they increase Ca²⁺ sensitivity of unphosphorylated thin filaments considerably less than phosphorylated thin filaments. As a consequence, Ca²⁺ sensitivity becomes independent of the TnI phosphorylation level (uncoupled).

The effect of Ca²⁺ sensitisers is analogous to the effect of HCM-causing mutations in vitro (Table 2) where both an increase in the Ca²⁺ sensitivity and uncoupling of the Ca²⁺ sensitivity from the TnI phosphorylation are observed. Uncoupling due to HCM mutations was first reported in 2001 and several more thin filament mutations
have subsequently been demonstrated by in vitro assays,\textsuperscript{5,17} as the TPM1 E180G mutation, shown in Figure 1B, demonstrates.

### 4.2 Mechanistic considerations of Ca\textsuperscript{2+} sensitizers

Since both EMD57033 and Bepridil appear to modulate Ca\textsuperscript{2+} sensitivity with a minimal effect on the sliding speed in IVMA or maximum isometric force in muscle fibres,\textsuperscript{10,42} it is likely that they are acting on troponin rather than the cross-bridge cycling mechanism in our systems.

The Ca\textsuperscript{2+} sensitivity of cardiac troponin is modulated by the unique N-terminal peptide of TnI (1–30) that contains the PKA phosphorylation sites, Ser22 and 23. When unphosphorylated, the peptide interacts with the N-terminal domain of TnC, close to the regulatory Ca\textsuperscript{2+}-binding site and this interaction is lost or reduced when the two serines are phosphorylated.\textsuperscript{43,44} The change in Ca\textsuperscript{2+} sensitivity with TnI phosphorylation is a two- to three-fold reduction.

Bepridil is suggested to displace TnI 1–30 and/or the linker helix TnI 31–70.\textsuperscript{20,45} EMD57033 is believed to bind to the C-terminal domain of TnC and also displaces the TnI 31–70 peptide.\textsuperscript{18,19,46} Thus, both these compounds could act allosterically by interfering with the modulation of Ca\textsuperscript{2+} sensitivity due to Ser22/23 phosphorylation, resulting in uncoupling.

Interestingly, in experiments measuring isometric force in cardiac muscle myofibrils, EMD57033 increased Ca\textsuperscript{2+} sensitivity but only caused uncoupling at short sarcomere lengths. Thus, the uncoupling phenomenon may be graded rather than all-or-none. One possibility is that uncoupling may be related to Ca\textsuperscript{2+} sensitivity if the Ca\textsuperscript{2+} sensitizer shifts the conformational equilibrium so far towards the N-terminal bound conformation that it cannot be significantly influenced by phosphorylation of the N-terminal peptide of TnI (see Supplementary material online, SD). However, the Ca\textsuperscript{2+}-sensitizing property of these compounds does not necessarily involve the same molecular mechanism as uncoupling.

The actions of small molecules that uncouple highlight the crucial role of the allosteric coupling between the TnI 1–30 peptide and ligand-binding sites that could be remotely located. Likewise, it is notable that DCM-causing mutations that uncouple are distributed in all the proteins of the thin filament, also demonstrating long-range allosteric interactions.\textsuperscript{9,47} We have proposed that these mutations destabilize the unphosphorylated state of the 1–30 peptide.\textsuperscript{9}

### 4.3 The Ca\textsuperscript{2+} desensitizer EGCG restores coupling to thin filaments with HCM and DCM mutations

Our observation that EGCG reduces Ca\textsuperscript{2+} sensitivity two- to three-fold both in human thin filaments and in mouse myofibrils with only small effects on maximum sliding speed, isometric force or Hill coefficient, is in accord with previous measurements in skinned cardiac muscle fibres.\textsuperscript{21,23} Moreover, we have demonstrated that the phosphorylation-dependent shift in myofilament Ca\textsuperscript{2+} sensitivity is unaffected by EGCG in both isolated filaments and intact myofibrils, in contrast to the Ca\textsuperscript{2+} sensitizers (Figures 2, 3, and 6).

EGCG was also able to decrease the Ca\textsuperscript{2+} sensitivity of phosphorylated thin filaments containing HCM- and DCM-causing mutations; however, it had different effects on phosphorylated and unphosphorylated thin filaments. With phosphorylated troponin, EGCG decreased Ca\textsuperscript{2+} sensitivity similarly to wild-type troponin, but EGCG either had no effect or increased Ca\textsuperscript{2+} sensitivity with unphosphorylated troponin (Table 2). This resulted in the restoration of the coupling of Ca\textsuperscript{2+} sensitivity change to TnI phosphorylation (Figures 3, 4, and 5).

Several studies have located EGCG binding in the C-terminal domain of TnC in the region of the hydrophobic cleft.\textsuperscript{21,22,46} Molecular dynamics (MD) simulation suggests that it can bind in several interchangeable orientations,\textsuperscript{46} and unlike EMD57033, EGCG can bind to the C-terminal domain of TnC even in the presence of TnI 34–71 helix.\textsuperscript{46} We suggest that it is possible that EGCG can re-stabilize the TnI–TnC interaction, restoring the Ca\textsuperscript{2+} response to phosphorylation found in DCM and HCM. MD calculations indicate that the uncoupling mutation cTnC G159D strengthens TnI 34–71 helix binding to the hydrophobic cleft while EGCG weakens this interaction,\textsuperscript{46} compatible with their opposite effects on coupling.

### 4.4 Effects of EGCG on myofibrillar contractility

EGCG decreased Ca\textsuperscript{2+} sensitivity in phosphorylated and unphosphorylated wild-type myofibrils by approximately two-fold but did not change the relaxation parameters \(t_{IN}\) and \(k_{DEL}\) in phosphorylated myofibrils (Table 3). The rate of force development \(k_{ACT}\), measured at high Ca\textsuperscript{2+}, was unchanged in myofibrils with phosphorylated TnI and slightly decreased \((P = 0.059)\) in unphosphorylated myofibrils. As \(k_{ACT}\) depends strongly on the Ca\textsuperscript{2+} sensitivity,\textsuperscript{10} we conclude that EGCG shifts the \([\text{Ca}^{2+}]_{1}\)–\(k_{ACT}\) relationship towards higher Ca\textsuperscript{2+} concentration in agreement with IVMA data (Table 1). Thus, unlike EMD57033, EGCG decreases cross-bridge activation kinetics.\textsuperscript{10}

EGCG reduced Ca\textsuperscript{2+} sensitivity and \(k_{ACT}\) in both phosphorylated and unphosphorylated ACTC E361G myofibrils. In addition, it restored the lost difference in Ca\textsuperscript{2+} sensitivity between phosphorylated and unphosphorylated myofibrils and also the difference in the relaxation parameter \(t_{IN}\) (Table 3). The observation that EGCG does not affect the EC\textsubscript{50} \(P/EC_{50}\) unP or \(t_{IN}\) in wild-type myofibrils but changes them in ACTC E361G suggests that EGCG can restore coupling in ACTE E361G myofibrils independently of its Ca\textsuperscript{2+}-desensitizing function.

Although uncoupling can be clearly demonstrated at the level of skinned muscle fibres or myocytes with thin filament mutations causing DCM,\textsuperscript{35,48,49} this does not appear to be true for HCM-causing mutations, despite being indistinguishable at the single filament level. A near-normal response of Ca\textsuperscript{2+} sensitivity to TnI phosphorylation has been reported for TNNC1 L29Q and TPM1 E180G in transgenic mice,\textsuperscript{50,51} and TNNI3 R145W and TNNT2 K280N in human heart myectomy samples.\textsuperscript{14} Despite this, both DCM- and HCM-linked mutations in transgenic mouse models are associated with an impaired response to \(\beta1\)-adrenergic stimulation and reduced cardiac reserve as would be expected if uncoupled (reviewed by Messer\textsuperscript{17}). The physiological manifestations of the uncoupling seen in unloaded filaments require further investigation.

### 4.5 Clinical relevance of re-coupling by EGCG

Ca\textsuperscript{2+} antagonists have been suggested as being potentially useful for treatment of HCM.\textsuperscript{52} EGCG represents a new class of Ca\textsuperscript{2+} antagonists with a very favourable functional profile. EGCG acts directly on the Ca\textsuperscript{2+} regulatory system of the thin filament that is also the main target of HCM-causing mutations in sarcomeric proteins.\textsuperscript{5} By binding to troponin, it decreases the enhanced Ca\textsuperscript{2+} sensitivity characteristic of HCM while also reversing the uncoupling effect we observed in
doses of EGCG have a positive inotropic effect in intact heart muscle, mutations pharmacologically. The coupling of Ca\(^{2+}\) in the contractile apparatus.\(^{54,55}\)

Although EGCG is too non-specific to be a viable drug for treating HCM, this study has demonstrated a significant proof of principle: it is possible to directly reverse the molecular mechanism of HCM-causing mutations pharmacologically. The coupling of Ca\(^{2+}\) sensitizers to cTnI phosphorylation has been demonstrated to be a labile property of troponin. HCM or DCM mutations and Ca\(^{2+}\) sensitizers can induce uncoupling, indicating that small perturbations can destabilize tropo-

It is very unusual to find a reagent that will give a gain of function by apparently restoring the native conformational state in the presence of mutations that destabilized this state. Our findings provide a starting point for investigating molecules related to EGCC that may be more efficacious and act specifically on troponin.\(^{56}\)

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### Conflict of interest

none declared.

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