Lys184 deletion in troponin I impairs relaxation kinetics and induces hypercontractility in murine cardiac myofibrils

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Aims To understand the functional consequences of the Lys184 deletion in murine cardiac troponin I (mcTnI_{D K184}), we have studied the primary effects of this mutation linked to familial hypertrophic cardiomyopathy (FHC) at the sarcomeric level.

Methods and results Ca\textsuperscript{2+} sensitivity and kinetics of force development and relaxation were investigated in cardiac myofibrils from transgenic mice expressing mcTnI_{D K184}, as a model which co-segregates with FHC. Ca\textsuperscript{2+}-dependent conformational changes (switch-on/off) of the fluorescence-labelled human troponin complex, containing either wild-type hcTnI or mutant hcTnI_{D K183}, were investigated in myofibrils prepared from the guinea pig left ventricle. Ca\textsuperscript{2+} sensitivity and maximum Ca\textsuperscript{2+}-activated and passive forces were significantly enhanced and cooperativity was reduced in mutant myofibrils. At partial Ca\textsuperscript{2+} activation, mutant but not wild-type myofibrils displayed spontaneous oscillatory contraction of sarcomeres. Both conformational switch-off rates of the incorporated troponin complex and the myofibrillar relaxation kinetics were slowed down by the mutation. Impaired relaxation kinetics and increased force at low [Ca\textsuperscript{2+}] were reversed by 2,3-butanedione monoxime (BDM), which traps cross-bridges in non-force-generating states.

Conclusion We conclude that these changes are not due to alterations of the intrinsic cross-bridge kinetics. The molecular mechanism of sarcomeric diastolic dysfunction in this FHC model is based on the impaired regulatory switch-off kinetics of cTnI, which induces incomplete inhibition of force-generating cross-bridges at low [Ca\textsuperscript{2+}] and thereby slows down relaxation of sarcomeres. Ca\textsuperscript{2+} sensitization and impairment of the relaxation of sarcomeres induced by this mutation may underlie the enhanced systolic function and diastolic dysfunction at the sarcomeric level.

KEYWORDS
Ca\textsuperscript{2+} sensitization; Cross-bridge kinetics; Diastolic dysfunction; Hypercontractility; Sarcomere dynamics

1. Introduction

Familial hypertrophic cardiomyopathy (FHC), a frequent hereditary disease of the myocardium, is associated with mutations in genes encoding sarcomeric proteins. The clinical course of the disease is extremely variable and may range from an asymptomatic to a malignant phenotype with sudden cardiac death.\textsuperscript{1} One of the most eminent functional disorders in affected individuals is diastolic dysfunction, which may have many underlying causes such as impaired inactivation of sarcomeres, altered Ca\textsuperscript{2+} sequestration at the myocyte level, and hypertrophy and fibrosis at the organ level.\textsuperscript{1,2} Thus, in order to understand the mechanistic interpretation of the altered physiological readouts of the organ function, it is essential to characterize first the primary genotype–phenotype relation at the sarcomeric level, which is the smallest functional contractile unit of the cardiomyocyte.

About 5% of FHC patients have mutations in the TNNI3 gene,\textsuperscript{3} which codes for the inhibitory subunit (cTnI) of the regulatory troponin complex (cTn). cTnI is essential for inactivation of cardiac contraction and hence for diastolic function. The functional consequences of cTnI mutations at the level of the contractile machinery have been investigated by several \textit{in vitro} protocols such as determination of actomyosin ATPase activity in reconstituted systems or exchange of the mutant protein against the endogenous one in skinned fibres or myofibrils.\textsuperscript{4–7} In skinned fibres, most cTnI mutations increase Ca\textsuperscript{2+} sensitivity of steady-state contraction,\textsuperscript{4}
which, however, does not allow to draw conclusions regarding dynamic heart function. Especially, sarcomeric diastolic dysfunction, i.e. impairment of the relaxation kinetics, is difficult to be investigated in skinned fibres due to the limited Ca\(^{2+}\)-buffering capacity of available caged Ca\(^{2+}\) chelators. Therefore, we used myofibrils, which allows visual observation of each individual sarcomere behaviour, as a favourable model to study the kinetics of the Ca\(^{2+}\)-regulated contraction and relaxation induced by rapid, defined changes in [Ca\(^{2+}\)].\(^{8}-11\) This approach can enhance the mechanistic understanding of systolic-diastolic dynamics, because the rate-limiting sarcomeric processes significantly sustain myocardial stiffness during ejection and isovolumetric relaxation.\(^{8,12}\)

We have previously shown that several kinetic results, obtained with cardiac myofibrils isolated from transgenic mice carrying the mutation R146G in the inhibitory region of cTnI (mcTnI\(^{R146G}\)), differed from those obtained by exchange of the endogenous wild-type troponin in myofibrils for the mutant protein.\(^{8}\) Based on this study, we believe that cardiac myofibrils isolated from transgenic mice are superior to exchanged myofibrils. Therefore, we generated a transgenic mouse model for one of the clinically best characterized mutations\(^{3,13}\) in the C-terminal mobile domain (Md) of cTnI, a Lys184 deletion (murine sequence), and analysed its functional consequences on contractile properties in myofibrils (TG myofibrils) isolated from the hearts of these transgenic mice (TG-\(\text{K184}\) mice).

Using this model, we confirmed the mutation-induced Ca\(^{2+}\)-sensitization of contraction. Kinetic analysis revealed that the mutation has no effect on the maximum rate of force development, but impairs relaxation kinetics and increases passive force. The Lys184 deletion did not alter switch-off kinetics of the isolated cTn, but it slows down the switch-off when cTn was incorporated into myofibrils. These results are interpreted according to structural models, which suggest that the Md is essential for returning to a relaxed state.\(^{14}\) We show for the first time that the Lys184 deletion affects the inter-sarcomeric dynamics, because TG myofibrils, but not wild-type myofibrils (nTG myofibrils), exhibit spontaneous oscillatory contractions (SPOCs) of sarcomeres. In conclusion, we report here that the Lys184 deletion leads to sarcomeric diastolic dysfunction.

2. Methods

2.1 Transgenic mice

We generated two transgenic mouse (C57BL/6) models (see Supplementary material online): TG-\(\text{K184}\) mice expressing mcTnI\(^{\text{K184}}\) and TG-flag mice expressing wild-type mcTnI with an N-terminal Flag-tag (mcTnI\(^{\text{WT-flag}}\)). We compared TG-\(\text{K184}\) mice with their nTG littermates to reduce transgene-independent inter-individual variability.

All animal investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US NIH (Publication No.85-23, revised 1996).

2.2 Troponin exchange in skinned fibres

Murine cTn (mcTn) containing either mcTnI\(^{\text{K184}}\) or mcTnI\(^{\text{WT}}\) was reconstituted from recombinant mcTn-subunits (see Supplementary material online) and used to replace the endogenous mcTn in skinned fibres isolated from TG-\(\text{K184}\), nTG- or TG-flag mice following an exchange protocol previously described.\(^{15}\)

2.3 Mechanical experiments with skinned fibres and myofibrils

Mice were sacrificed by cervical dislocation and the beating hearts quickly excised. Skinned fibres were dissected from left-ventricular papillary muscles, mounted and subjected to a successive Ca\(^{2+}\)-activation/relaxation protocol as described in Refs.\(^{15,16}\).

Myofibrils (sarcomere length, SL \(= 1.8\)–2.0 \(\mu\)m; diameter = 1.2–4.7 \(\mu\)m) for micro-mechanical experiments were prepared from papillary muscles as described in Ref.\(^{10}\). The experimental setup was previously described.\(^{9,17}\) The protocol for myofibrillar isometric force measurement and the assignation of steady-state and kinetic activation/relaxation parameters are shown in Figures 1B and C. Slack sarcomere lengths (S\(_{\text{Lo}}\)) of isometrically mounted myofibrils were measured in relaxing buffer (pCa 7.50). Experiments with fibres and myofibrils were performed at 10°C.

2.4 Troponin switch kinetics

Prior to reconstitution of the human cTn (hcTn) from recombinant hcTn-subunits, hcTnC was fluorescence-labelled at Cys84 with N-(2-(iodoacetoxy)ethyl)-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole (IANDB) as indicated in Ref.\(^{17}\). The IANDB-labelled hcTn was incorporated into left ventricular myofibrils.\(^{17}\)

The Ca\(^{2+}\)-dependent fluorescence change of myofibril-incorporated or isolated IANDB-labelled hcTn, containing either mcTnI\(^{\text{WT}}\) or mcTnI\(^{\text{K183}}\), was measured using a Stopped-Flow apparatus (SFM400/5 BioLogic, Claix, France) and the troponin switch-on/off kinetics were determined as previously published.\(^{11,17}\) Experiments were performed at 10°C.

Because the Stopped-Flow experiments require large quantities of myofibrils, they were prepared from guinea pig instead of murine ventricles.

2.5 Data analysis and modelling

The equations used for data analysis and modelling are explained in Supplementary material online. Analysis of myofibrillar force transients was explained previously.\(^{5-10}\) We used 1 and 4 pairs of nTG- and TG-\(\text{K184}\) mice aged 3 and 6–7 months, respectively, and 1 pair of 3 months aged nTG- and TG-flag mice. All data are presented as mean \(\pm\) SEM; \(n\) corresponds to the number of myofibrils/fibres analysed. Statistical significant differences were determined by two-tailed unpaired Student’s \(t\)-tests: *\((P < 0.05)\), **\((P < 0.01)\), ***\((P < 0.001)\).

3. Results

3.1 Transgenic protein expression levels, viability and heart weights

TG-\(\text{K184}\) mice were viable with normal life spans and no significant differences in body weights. Surprisingly, the mutant hearts did not exhibit hypertrophy. Rather the heart-to-body weight ratio was decreased: 3.55 \(\pm\) 0.09, \(n = 11\) for TG-\(\text{K184}\) mice; 4.28 \(\pm\) 0.09, \(n = 15\) for nTG mice; \(P < 0.001\); 6 months old.

In the cardiac myofibrils isolated from TG-\(\text{K184}\)- and TG-flag mice, \(\geq 90\%\) of the mcTnI\(^{\text{WT}}\) protein was replaced by the transgenic mcTnI\(^{\text{K184}}\) and mcTnI\(^{\text{WT-flag}}\) proteins, respectively (Figure 1A), but the total mcTnI protein content was preserved (for the estimation of heart weights and protein expression levels, see Supplementary material online).

3.2 Ca\(^{2+}\)-activated myofibrillar force

TG myofibrils developed significantly higher maximum force (\(F_{\text{max}}\)) per cross-sectional area (CSA) compared with nTG
Figure 1 (A) Transgenic mcTnl expression levels (% of total mcTnl protein) in myofibrils prepared from ventricles of TG-DK184-, nTG- and TG-flag mice (see Supplementary material online). (B) Representative full force transients of isolated nTG myofibrils. [Ca\textsuperscript{2+}] is rapidly (10–20 ms) changed from pCa 7.50 to 4.53 and back to 7.50 to induce contraction and relaxation, respectively. Ca\textsuperscript{2+}-induced force (\(F_{\text{ACT}}\)) develops mono-exponentially (\(k_{\text{ACT}}\)). Force redevelopment (\(k_{\text{TR}}\)) is induced mechanically by slackening–restretching the myofibril during Ca\textsuperscript{2+} activation. Passive force (\(F_{\text{pass}}\)) is determined prior to activation by transiently slackening the myofibril from the sarcomere length of 15% above SL\textsubscript{0}. (C) The best fit (black) superimposed on the force transient (grey trace) during relaxation is a biphasic, linear-exponential function\textsuperscript{10} yielding \(k_{\text{LIN}}, \ell_{\text{LIN}}\) and \(k_{\text{REL}}\). (D) nTG myofibrils mounted isometrically.

Figure 2 (A) Passive force (left), Ca\textsuperscript{2+}-induced force amplitude (middle), and total active force (right), normalized to cross-sectional area (CSA), developed by myofibrils of 6–7 months old TG-DK184 mice (grey-filled bars) and nTG mice (open bars); \(F_{\text{max}} = F_{\text{ACT}} + F_{\text{pass}}\). (B) Force–pCa relations for TG myofibrils (closed circles) and nTG myofibrils (open circles). The curves represent Hill-functions (equation (1), see Supplementary material online) fitted to force data normalized to \(F_{\text{max}}\) at pCa 4.53. (C) Data of B are linearized (see Supplementary material online) and plotted as function of pCa. Zero-crossing values correspond to pCa\textsubscript{0.5} at half-maximum force. The biphasic relation of data from nTG myofibrils indicates that the degree of cooperativity above pCa\textsubscript{0.5} (\(n_{H1} = 3.77 \pm 0.28\)) is significantly higher than below pCa\textsubscript{0.5} (\(n_{H2} = 1.50 \pm 0.35\)). Encircled points indicate data at which Ca\textsuperscript{2+}-induced spontaneous oscillatory contractions (Ca-SPOCs) were detected by video-microscopy (see Supplementary material, movies). Numbers next to each data point give Ca-SPOC events detected per number of videos investigated.
myofibrils or TG-flag myofibrils, irrespective of the age of the mice (Table 1). This is due to enhancements of both passive force ($F_{\text{pass}}$) and Ca$^{2+}$-induced force amplitude ($F_{\text{ACT}}$) (Figure 2A).

The force–pCa relation of TG myofibrils was shifted to the left (Figure 2B), indicating an increase in Ca$^{2+}$ sensitivity of force development relative to nTG myofibrils (Table 1). The Hill-coefficient, reflecting the overall cooperativity of the force–pCa relation, was significantly reduced (Table 1). This loss in cooperativity occurs specifically at lower Ca$^{2+}$-activated force levels (Figure 2C). Interestingly, at the same sub-half-maximal force levels SPOC of sarcomeres was observed (see Supplementary material online, movies). The occurrence of Ca$^{2+}$-induced SPOC (Ca-SPOC) is indicated in Figure 2C by the encircled data points. Ca-SPOC was never observed in nTG myofibrils at any partial [Ca$^{2+}$].

### 3.3 Force–pCa relations in skinned papillary muscle fibres

Skinned fibres isolated from TG-ΔK184 mice exhibited similar features in the force–pCa relation like myofibrils when compared with either nTG- or TG-flag fibres (Figure 3A and B; Table 2). There were no significant differences between force–pCa relations of nTG- and TG-flag fibres (Figure 3A).

To exclude that the mutation causes adaptive effects in the myofibrils which may affect the force–pCa relation, the endogenous mcTn in the skinned fibres of TG-ΔK184- and nTG mice was replaced by recombinantly expressed murine wild-type troponin complex (mcTn$^{\text{WT}}$). The incorporation of mcTn$^{\text{WT}}$ into TG- and nTG fibres completely abolished the differences in force–pCa relations (Figure 3C; Table 2) suggesting that Ca$^{2+}$ sensitization is solely mediated by the mutation. This is supported by the finding that replacement of the endogenous mcTn from skinned nTG fibres by mutant mcTn caused Ca$^{2+}$ sensitization and loss in cooperativity, while replacement by mcTn$^{\text{WT}}$ had no significant effect (Figure 3D; Table 2).

Since endogenous mcTnI and exogeneous mcTnI$^{\text{WT}}$ or mcTnI$^{\text{WT}}$ cannot be separated by SDS-PAGE, we used skinned fibres isolated from TG-flag mice to estimate the efficiency of exchange. ~80% of endogenous mcTn$^{\text{WT}}$-flag protein was replaced by the recombinant mcTn$^{\text{WT}}$ or mcTnI$^{\text{WT}}$ protein (Figure 3E).

### 3.4 Myofibrillar Ca$^{2+}$-induced kinetics of force development

Force transients induced by a sudden increase in [Ca$^{2+}$] from pCa 7.50 to pCa 4.53 (saturating [Ca$^{2+}$]) (Figure 4A) were fitted by a mono-exponential function yielding the kinetic rate constant $k_{\text{ACT}}$, which was similar for TG- and nTG- or TG-flag myofibrils (Table 1). All these myofibrils displayed similar rate constants of mechanically induced force redevlopment, $k_{\text{TR}}$ (Table 1).

At partial Ca$^{2+}$ activation, in TG myofibrils, $k_{\text{ACT}}$ is higher than in nTG myofibrils (Figure 4B). This effect relates to the increased Ca$^{2+}$ sensitivity of contraction in TG myofibrils as shown by plotting $k_{\text{ACT}}$ against the relative force, $F_r$ (Figure 4C). Fitting the $k_{\text{ACT}}$–force data to equation (2) (see Supplementary material online) yielded similar curves for both TG myofibrils and nTG myofibrils (Figure 4C) and the extrapolation of the curves to $F_r \to 0$ gives an estimation of $g_{\text{app}}$.8

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**Table 1** Steady-state and kinetic parameters of cardiac myofibrils isolated from TG-ΔK184-, TG-flag- and nTG mice (passive force was measured at SL = 1.15 × SL0)

<table>
<thead>
<tr>
<th>Age of mice:</th>
<th>3 months</th>
<th>6-7 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nTG mice</td>
<td>TG-flag mice</td>
</tr>
<tr>
<td>SL0 (μm) at pCa 7.50</td>
<td>2.03 ± 0.03</td>
<td>1.99 ± 0.02</td>
</tr>
<tr>
<td>$F_{\text{max}}$ (nN/μm$^2$) at pCa 4.53</td>
<td>58.1 ± 4.4</td>
<td>59.7 ± 9.3</td>
</tr>
<tr>
<td>$F_{\text{pass}}$ (nN/μm$^2$) at pCa 7.50</td>
<td>3.3 ± 0.4</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>$pC_{\text{A0}}$</td>
<td>5.49 ± 0.03</td>
<td>n.a.</td>
</tr>
<tr>
<td>$n_{\text{h}}$</td>
<td>2.67 ± 0.16</td>
<td>n.a.</td>
</tr>
<tr>
<td>$k_{\text{ACT}}$ (s$^{-1}$) at pCa 4.53</td>
<td>5.57 ± 0.18</td>
<td>5.63 ± 0.46</td>
</tr>
<tr>
<td>$k_{\text{TR}}$ (s$^{-1}$) at pCa 4.53</td>
<td>5.69 ± 0.19</td>
<td>6.10 ± 0.45</td>
</tr>
<tr>
<td>$k_{\text{LH}}$ (s$^{-1}$)</td>
<td>2.17 ± 0.09</td>
<td>2.22 ± 0.20</td>
</tr>
<tr>
<td>$t_{\text{LH}}$ (ms)</td>
<td>34.6 ± 2.2</td>
<td>31.9 ± 2.1</td>
</tr>
<tr>
<td>$k_{\text{REL}}$ (s$^{-1}$)</td>
<td>33.6 ± 2.3</td>
<td>36.2 ± 2.8</td>
</tr>
<tr>
<td>$t_{\text{REL}}$ (ms)</td>
<td>63.0 ± 3.7</td>
<td>58.5 ± 4.0</td>
</tr>
</tbody>
</table>

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8 $g_{\text{app}}$ is the apparent compliance of the sarcomere.
Table 2  Functional parameters of skinned fibres from the papillary muscles of 6–7 months old TG-ΔK184- and nTG mice before and after exchange with mcTn containing either mcTn\textsuperscript{ΔK184} or mcTn\textsuperscript{WT}.

<table>
<thead>
<tr>
<th>Genotype of mice:</th>
<th>nTG</th>
<th>TG-ΔK184</th>
<th>nTG</th>
<th>TG-ΔK184</th>
<th>nTG</th>
<th>nTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exchange of cTn containing:</td>
<td>–</td>
<td>–</td>
<td>mcTn\textsuperscript{WT}</td>
<td>mcTn\textsuperscript{WT}</td>
<td>mcTn\textsuperscript{WT}</td>
<td>mcTn\textsuperscript{ΔK184}</td>
</tr>
<tr>
<td>(pC_{a0})</td>
<td>5.60 ± 0.02</td>
<td>5.79 ± 0.02</td>
<td>5.52 ± 0.04</td>
<td>5.56 ± 0.02</td>
<td>5.52 ± 0.04</td>
<td>5.65 ± 0.03</td>
</tr>
<tr>
<td>(n_H)</td>
<td>4.27 ± 0.15</td>
<td>2.77 ± 0.07</td>
<td>2.30 ± 0.16</td>
<td>2.18 ± 0.23</td>
<td>2.30 ± 0.18</td>
<td>1.83 ± 0.13</td>
</tr>
<tr>
<td>(F_{\text{max}}) (normalized)</td>
<td></td>
<td></td>
<td>0.73 ± 0.02</td>
<td>0.66 ± 0.04</td>
<td>0.73 ± 0.02</td>
<td>0.74 ± 0.07</td>
</tr>
</tbody>
</table>

Figure 3  Force–pCa relations of skinned fibres from papillary muscles of 6–7 months old TG-ΔK184- and nTG- or TG-flag mice. (A) Force–pCa relations from TG fibres (mcTn\textsuperscript{WT}) (closed circles), nTG fibres (mcTn\textsuperscript{WT}) (open circles) and TG-flag fibres (mcTn\textsuperscript{WT-flag}) (open squares). (B) Force data of A from TG fibres (closed circles) and nTG fibres (open circles) are linearized as in Figure 2C. The linearized relation for nTG fibres is biphasic: above \(pC_{a0}\), \(n_H = 5.51 ± 0.45\); below \(pC_{a0}\), \(n_H = 3.02 ± 0.47\). (C) Endogenous troponin-complex of either nTG fibres (open triangles) or TG fibres (closed triangles) was exchanged for recombinant mcTn\textsuperscript{WT}. (D) Endogenous troponin of nTG fibres was exchanged for recombinant mcTn containing either mcTn\textsuperscript{I} \(\text{D}_{K184}\) (open nablas) or mcTn\textsuperscript{ΔK184} (closed nablas). (E) Western blot showing the replacement of endogenous mcTn\textsuperscript{WT-flag} by the recombinant mcTn containing either mcTn\textsuperscript{ΔK184} or mcTn\textsuperscript{WT}.
indicates that in adult TG-DK184 mice there is no shift of the $\alpha$-MHC to the $\beta$-MHC isoform, which would result in a slower cross-bridge cycling.

3.5 Myofibrillar relaxation kinetics

Both, TG- and nTG myofibrils exhibit a biphasic force decay consisting of an initial, slow phase with the rate constant $k_{\text{LIN}}$ and a duration $t_{\text{LIN}}$, followed by a rapid, exponential phase with the rate constant $k_{\text{REL}}$. We have shown previously that the sarcomeres remain isometric during the slow phase, whereas the second phase initiates from sequential sarcomere dynamics which enable the myofibril to relax faster than it contracts ($k_{\text{REL}} > k_{\text{ACT}}$).

Due to a significant increase in the duration $t_{\text{LIN}}$ and a significant decrease in the rate constant $k_{\text{REL}}$, the overall relaxation process is delayed and slower in TG myofibrils than in nTG myofibrils (Figures 5A and B; Table 1). In contrast, there was no significant difference in the rate constant $k_{\text{LIN}}$ (Figure 5B-left; Table 1). The overall relaxation kinetics can be expressed by the relaxation time constant, $\tau_{\text{REL}}$ (equation (3), see Supplementary material online). For 3 and 6–7 months old mice, the mutation significantly increased $t_{\text{REL}}$ by 46% and 30%, respectively ($P < 0.01$; Table 1). There were no significant differences between the relaxation parameters of nTG- and TG-flag myofibrils (Table 1).

To study the contribution of cross-bridges to the deceleration of the relaxation in TG myofibrils, the effect of 2,3-butanedione-monoxime (BDM) on the relaxation kinetics was investigated. BDM, an uncompetitive inhibitor of the myosin ATPase activity, traps cross-bridges in pre-powerstroke non-force generating states. BDM accelerated the relaxation kinetics of both TG- and nTG myofibrils. Increasing [BDM] in the relaxing buffer diminishes the difference of the relaxation time between TG- and nTG myofibrils (Figure 5C) and at 10 and 15 mM BDM no significant differences were observed ($P > 0.05$, $n = 7$). In the absence of Ca$^{2+}$, myofibrils were stretched by 15% of their slack sarcomere length. This imposed mechanical perturbation induced a larger increment in passive force in TG myofibrils compared with nTG- or TG-flag myofibrils.
The enhancement was diminished by BDM in TG myofibrils but not in nTG myofibrils (Figure 5D). Hence, in the presence of 15 mM BDM, there was no significant difference in the passive force ($P > 0.05$, $n = 7$).

3.6 Ca$^{2+}$-controlled switch-kinetics of isolated and myofibril-incorporated troponin

To investigate whether altered force kinetics of TG myofibrils could be explained by the effects of the mutation on the switch-on/off kinetics of troponin, recombinantly expressed hcTn, containing either hcTnIWT or hcTnID$^\text{DK183}$, was incorporated into myofibrils. Conformational changes of the incorporated hcTn, sensed by IANBD-labelled hcTnC, were measured by mixing the myofibrils with Ca$^{2+}$ (switch-on kinetics) (Figure 6A) or the Ca$^{2+}$-chelator BAPTA (switch-off kinetics) (Figure 6B) as previously described. While the mutation did not significantly alter the observed rate constant of Ca$^{2+}$-induced switch-on kinetics ($k_{\text{obs},+\text{Ca}}$) (Figure 6C), it significantly decreased the observed rate constant of switch-off kinetics ($k_{\text{obs},-\text{Ca}}$) induced by Ca$^{2+}$-removal (Figure 6D). Thus, in line with the kinetics of force decay during relaxation (Table 1), the mutation only affected the inactivation (switch-off) kinetics. Both $k_{\text{obs},+\text{Ca}}$ and $k_{\text{obs},-\text{Ca}}$ are too fast to directly rate-limit the intrinsic cross-bridges cycling as explained previously. Interestingly, neither switch-on kinetics (Figure 6E) nor switch-off kinetics (Figure 6F) of isolated hcTn was significantly affected by the mutation. Thus, hcTnID$^\text{DK183}$ impairs the switch-off kinetics only when incorporated into the sarcomeric environment.

4. Discussion

One of the prominent clinical characteristics of FHC-patients carrying mutations in hcTnI is diastolic dysfunction. However, the underlying cause is not fully understood. The major finding of our study using isolated myofibrils from transgenic murine hearts carrying a Lys184 deletion in the C-terminal mobile domain of cTnI is that this mutation leads to a decrease in the switch-off kinetics of troponin, a
deceleration of myofibrillar force relaxation kinetics and an increase in their passive force due to residual activated cross-bridges in the absence of Ca$^{2+}$. The mutation further enhances the Ca$^{2+}$-sensitivity of both myofibrils and multicellular skinned fibres. Troponin exchange studies showed that this is due to the mutation rather than to secondary mutation-induced effects. Interestingly, we found that the Lys184 deletion also induces SPOCs at partial Ca$^{2+}$ sensitization and loss in cooperativity observed in TG myofibrils and nTG myofibrils implies that the intrinsic cross-bridge kinetics, which depend on the MHC isoform, were not affected by the mutation. Consistently, there was no shift in the MHC isoform. Therefore, intrinsic kinetic properties of cross-bridges are not responsible for the observed Ca$^{2+}$ sensitization and reduced cooperativity in TG myofibrils.

**4.1 Ca$^{2+}$ sensitization is not due to the alteration in intrinsic turnover kinetics of cross-bridges**

In agreement with previous studies,$^4,5$ we found an increase in Ca$^{2+}$-sensitivity of both TG myofibrils and skinned TG fibres. The effect was also mimicked in skinned fibres from nTG mice with incorporated mcTnI$^{K184}$ and was lost when the endogenous mutant cTn from skinned TG fibres was exchanged for wild-type troponin. The troponin exchange studies suggest that the Ca$^{2+}$-regulated actin filament accessibility for steady cross-bridge cycling is altered due to loss of cooperative inhibition.

\[ k_\text{ACT} = f_\text{app} + g_\text{app} \]

\[ k_\text{ACT} \approx g_\text{app} \] at very low [Ca$^{2+}$], i.e. when myofibrils relax. Both, $g_\text{app}$ and $k_\text{LIN}$ ($k_\text{LIN}$ giving an independent estimation for $g_\text{app}$) were similar for TG- and nTG myofibrils, suggesting that the intrinsic rates of cross-bridge detachment ($g_\text{app}$)
are unaffected by the mutation. Thus, the impairment of myofibrillar relaxation is not due to the alteration of the intrinsic cross-bridge kinetic properties.

We note that, despite of unaltered maximum $k_{\text{act}}$, maximal force is increased in TG myofibrils, but the molecular mechanism remains to be solved. However, a significant enhanced maximum force has not been previously detected in skinned fibres containing the Lys183 deletion\(^6\) or other C-terminal cTnI mutations.\(^5\) This may be due to non-homogeneity of myofibrils in the analyzed skinned fibres.

### 4.2 mcTnI\(^{K184}\) mutation reduces cooperativity and induces spontaneous oscillatory contractions

A high slope of the force–$pCa$ relation is an indicator for a strong cooperative behaviour. Basically, two sources of cooperativity have been proposed: (i) attachment of strongly bound cross-bridges within a regulatory troponin-tropomyosin unit turns-on the unit favouring further cross-bridge attachment and (ii) already turned-on units facilitate further turning-on of neighbouring units.

Other studies indicated that mutations which alter charges in cTnI could cause a loss of the cooperative behaviour,\(^7,22\) as we observed in TG myofibrils, which specifically occurs in the lower half of the force–$pCa$ relation.

Simulation of force–$pCa$ relations, using sophisticated models which involve several types of cooperativity,\(^23\) showed that strong neighbour interactions of troponin-tropomyosin produce asymmetric relations. These were clearly observed for nTG myofibrils, presenting a higher slope at low $Ca^{2+}$ activation levels, but not for TG myofibrils. Specific abolishment of the higher cooperativity at lower $[Ca^{2+}]$ in TG myofibrils therefore most likely indicates that the Lys184 deletion impairs the communication between neighbouring units along the thin filament.

In the same range of $Ca^{2+}$ activation where cooperativity was lost, TG myofibrils exhibited the striking feature of $Ca^{2+}$-induced SPOC, which did not occur in nTG myofibrils. Ca-SPOC had been first described for cardiac tissues\(^24\) and afterwards for myofibrils\(^25\) containing predominantly the slow $\beta$-MHC isoform. Recently, SPOC had been investigated in myocardium of various species.\(^26\) SPOC was found to be less pronounced in rat myocardium containing a mixture of $\alpha$-MHC and $\beta$-MHC. To our knowledge there is no study describing Ca-SPOC in the murine myocardium, which might be due to the fact that it contains only the fast $\alpha$-MHC isoform. Thus, it may be plausible that we do not find Ca-SPOC in nTG myofibrils.

Since MHC isoform composition was not changed in TG myofibrils, we propose that Ca-SPOC might arise from attenuated cooperative responses within the thin filaments. This is consistent with the interpretation described in Ref.\(^27\) that a diminished cooperative behaviour of partial $Ca^{2+}$-activated cross-bridges along the thin filament could favour the occurrence of Ca-SPOC.

### 4.3 mcTnI\(^{K184}\) mutation impairs relaxation kinetics of sarcomeres

It is known from studies on reconstituted thin filaments that several cTnI mutations weaken the ability of cTn to fully inhibit actin–myosin ATPase and that this incomplete inhibition is associated with weakening of cTnI–actin interactions.\(^7,22\) Analogously, we showed here that the Lys183 deletion selectively impairs the switch-off kinetics in the sarcomere without affecting the switch-on kinetics and, thus, destabilizing the off-state of the $Ca^{2+}$-regulated actin filament. This effect does not occur in the isolated cTn-complex, i.e. without actin. Therefore, the presence of actin filaments, i.e. an organized sarcomeric environment, is a prerequisite for the effect of the hTnI\(^{K184}\) mutation on the tropinin switch-off kinetics, suggesting that the C-terminus of cTnI has a functional importance in the rate modulating process of the actin–myosin interaction.

This interpretation is supported by previous structural studies: within the highly flexible C-terminal mobile domain (Md) of cTnI, Lys184 is located in a fragile $\beta$-bulge motif of an antiparallel $\beta$-sheet.\(^14\) During diastole, when $[Ca^{2+}]$ is very low, the Md and the inhibitory region (Ir) of cTnI interact strongly with actin and shield the ‘secondary’ and ‘primary’ myosin-binding sites, respectively, on the actin filaments preventing actin–myosin interaction.\(^14\) Deletion of the positively charged Lys184 breaks one of the salt-bridges involved in Md–actin interaction and destabilizes some hydrogen bonds,\(^14\) probably shifting the $\beta$-bulge motif into a more rigid $\beta$-turn (Wakabayashi, personal communications). On the other hand, the $\beta$-sheet of the Md, containing Lys184, does not interact with cTnC\(^14\) as, for example, Arg146 in the Ir does. Therefore, the Ca$^{2+}$-induced conformational change (switch-on) of cTnI during systole is not affected by the Lys184 deletion, which is in agreement with our and previous interpretations from functional measurements.\(^4\)

Considering the proposed mechanism in Ref.\(^28\) by which the initially intrinsically unfolded Md at high $[Ca^{2+}]$ nucleates by binding to actin (flycatching hypothesis) upon Ca$^{2+}$ removal, Lys184 deletion could interfere with the efficient flycatching activity. As a consequence, a possible scenario is that the kinetics of the complete transfer of Ir to actin (switch-off) can be indirectly disturbed by the mutation in Md, but not the reverse transition towards cTnC (switch-on) upon Ca$^{2+}$-binding to cTnC.

The hypothesis is supported by considering both, our present and previous\(^4\) functional results. In Ref.\(^6\) the mutation R146G, expressed in TG mice, slowed down both relaxation kinetics and $k_{\text{act}}$ at high $[Ca^{2+}]$ implying that R146G in the Ir impaired both full inhibition of the force-generating actin-myosin interactions in the absence of $Ca^{2+}$ (as $\Delta K184$ does) and the release of this inhibition at high $[Ca^{2+}]$ (as $\Delta K184$ does not). This is because Ir competes with myosin for the ‘primary’ myosin-binding site on actin (at low $[Ca^{2+}]$) and binds to cTnC (at high $[Ca^{2+}]$), which is not the case for the $\beta$-sheet, containing Lys184, and the downstream located $\alpha$-helices of the Md.\(^14\)

Based on this, we propose that the Lys184 deletion impairs the rapidly tumbling Md upon Ca$^{2+}$ removal and, thus, restricts tropomyosin to adopt an optimal configuration for an efficient inhibition of the actin–myosin interaction, but it could also indirectly affect the inhibitory role of the Ir.

Thus, our findings are in line with the hypothesis\(^28\) that perturbations of folding of the Md primarily affect relaxation kinetics. Following a similar way of thinking and considering previous results in Ref.\(^8\) we assume that a perturbation of the folding-unfolding kinetics of the Ir (e.g. by the mutation R146G) would impair both relaxation and Ca$^{2+}$ activation kinetics.
Following this mechanism, destabilization of the switched-off state leads to an impaired inhibition of force-generating actin–myosin interactions as indicated by the increased passive force in TG myofibrils and its reduction by BDM close to the passive force level recorded for nTG myofibrils. The Lys184 deletion decreased the switch-off rate, preserved the switch-on rate and consequently increased the switch on–off equilibrium constant of tropomin. This enables cross-bridges to reattach to the actin filament even at very low [Ca\(^{2+}\)] during relaxation. The slow-down of relaxation is comparable with that caused by an incomplete Ca\(^{2+}\)-removal from cTnC, i.e. incomplete turning-off of the thin filament: it has been shown that myofibrillar relaxation kinetics are slowed down very sensitively by turning to a lower, residual Ca\(^{2+}\) activation instead of a complete relaxation.\(^8,29\)

When added during the relaxation process, BDM prevents the reattachment of cross-bridges by trapping them in non-force-generating states\(^19\) such that the effect of the mcTnI\(^{K184}\) mutation to incompletely inhibit force-generating actin–myosin interactions becomes diminished. As a consequence, the relaxation time constant, \(\tau_{REL}\), of TG myofibrils approaches that of nTG myofibrils. It had also been shown that BDM fastens the rate of cross-bridge detachment, presumably by increasing the affinity of nucleotide binding to myosin.\(^30\) Thus, targeting myosin motors with uncompetitive inhibitors, such as BDM, could accelerate the relaxation kinetics and therefore ameliorate the consequences of the incomplete inhibition of actin–myosin interaction, i.e. the slow-down in relaxation kinetics. This finding could suggest new hints concerning a potential therapeutic approach in the treatment of sarcomeric diastolic dysfunction induced by a less-functional inhibition of actin–myosin interaction in the absence of Ca\(^{2+}\).

A proteolytic cTnI truncation (hcTnI\(_{1–192}\))\(^{31}\) not related to FHC, exhibited features resembling those induced by mcTnI\(^{K184}\): impairment of the relaxation kinetics, Ca\(^{2+}\)-sensitization and loss in cooperativity, which probably occurred because Ca\(^{2+}\)-activation of force becomes more dependent, via the Ir, on Ca\(^{2+}\)-binding to cTnC, which is a relatively low cooperativ process.

Similar features were reported in a recent study using cardiomyocytes expressing the mutation R145G in human cardiac troponin I on the kinetics of force-generating actin–myosin interaction in the absence of Ca\(^{2+}\).\(^\text{1.20}\) \n
In conclusion, TG myofibrils generate higher forces at low and high [Ca\(^{2+}\)] and exhibit an increased Ca\(^{2+}\)-sensitivity of contraction, which is based on the loss of cooperative inhibition rather than on facilitated activation and accompanied by Ca-SPOC. The incomplete inhibition of actin–myosin interactions in the absence of Ca\(^{2+}\) in TG myofibrils and not an alteration of the intrinsic cross-bridge kinetic properties, or a shift of \(\alpha\)-MHC to the slow \(\beta\)-MHC, can explain the slow-down of the relaxation kinetics. Therefore, the impaired relaxation and elevated Ca\(^{2+}\)-independent residual force of the sarcomeres carrying mcTnI\(^{K184}\) could involve disturbances in ventricular relaxation, distensibility or filling of the non-hypertrophied murine transgenic heart with probable unfavourable consequences on the diastolic function.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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