Supporting Material

Enhanced Active Cross Bridges during Diastole: Molecular Pathogenesis of Tropomyosin's HCM Mutations

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SUPPORTING MATERIALS

1. Detailed methods

1.1. Muscle preparation

Hearts from freshly killed cows were collected from a slaughterhouse and prepared as described (1). We used a strip (length 20-40 mm, diameter 2-4 mm) of a trabecular muscle, which is thin and slender, and freely suspended in the right ventricle, and thus suitable for mechanical experiments. The strip was dissected to a small bundle (length 2-3 mm, diameter 90-110 μm) and attached to the experimental apparatus by small droplets of nail polish in the storage solution. The preparation was then soaked in the relaxing solution, which contains 40 mM BDM. All steps were performed at 0°C. The preparation was stretched until its slack was removed, which can be seen by a rise of small passive tension. This method establishes the sarcomere length of 2.1 μm as determined by confocal microscopy (1). At this time, the actual length (about 2 mm) and the diameter (90-110 μm) of the preparation were determined using an ocular micrometer. The conditions of the preparation were examined before the experiment was carried out, and if any damage was noticed, the preparation was discarded. At the beginning and the end of each experiment, the tension of the preparation was tested with the standard activating solution (5S8P, Table S2); when the final tension was <90% of the starting tension, the data was not used.

1.2 Thin-filament extraction and reconstitution

A fiber 90-110 μm in diameter was dissected from a skinned bundle on the day of an experiment, and two ends of the preparation were attached to a length driver and a tension transducer using nail polish. The fiber was then bathed at 0°C in the relaxing solution (Table S2). The volume of the muscle chamber was 80 μl (the small volume being advantageous because the quantity of protein used for reconstitution was limited). The fiber was further skinned in relaxing solution with 1% Triton-X100 for 20 min at 25°C. The length was then adjusted as described, and measured (L_0, about 2mm). The preparation was activated briefly to measure initial control tension at 25°C with the standard activating solution (5S8P, Table S2) and relaxed immediately. Brief activation was necessary to prevent fiber deterioration, which causes undesirable results in the subsequent steps. Reconstitution was performed by first extracting the thin filament in extraction solution (Table S2) with ~0.3 mg/ml gelsolin at 2°C. Generally, 60–120 min of extraction time was needed, depending on the strength of gelsolin and the diameter of the fiber. The degree of extraction was judged by a brief activation of tension at 25°C (5S8P, Table S2). When the tension was less than 10% of the control tension, the extraction was stopped by washing out gelsolin with the relaxing solution; overtreatment of fibers with gelsolin is not good for subsequent reconstitution, because a short segment of the thin filament remaining at the Z-line is essential to nucleate the growth of the actin filament.

The thin filament extracted fiber was then subjected to actin filament reconstitution from G-actin under the polymerizing condition in freshly made actin reconstitution solution (Table S2) with 1 mg/ml G-actin. The duration of reconstitution was limited to 7 min each time and performed at 0°C. The reconstitution procedure was repeated 5 times. The tension of this actin-filament reconstituted fiber without Tm or Tn was measured in 5S8P (Table S2) and defined as T_a. Actin reconstitution usually recovered 50–70% of initial tension. When tension recovery was

less than 50%, the treatment was repeated 2–4 additional times until the tension reached 50–70% of initial tension. The reconstitution was carried out in the presence of 40 mM BDM, because the reconstituted actin filament is spontaneously active owing to the lack of regulatory proteins.

The actin-filament reconstituted fiber was further reconstituted with regulatory proteins in a relaxing solution containing both Tm and Tn. This reconstitution was carried out overnight (15–18 h) at 0°C, because one Tm molecule binds to 7 actin monomers and neighboring Tm molecules are involved in the head-to-tail interaction. Thus, the realignment of misaligned Tm molecules by spontaneous unbinding/rebinding takes some time. Once regulatory proteins were reconstituted, the fiber became fully Ca^{2+} sensitive; at this point the active tension was tested. This fully reconstituted tension is 1.3–2x larger than T_a (actin-filament reconstituted tension). The fully reconstituted tension is about 95–105% of the initial control tension.

The degree of reconstitution of each protein was determined using SDS gels, as described in our previous publications (1-5).

2. Data for lumped and individual averages for Hill factor and pCa_{50}.

Because the pCa-tension curve rises steeply and saturates, if many experimental curves are averaged and fitted to Eq. 1, it is a natural mathematical consequence that the Hill coefficient (n_H: proportionate to the slope of the curve at the midpoint) is lower (Table S1, (B)) than for those that are individually fitted (Table S1, (A)). This means that values obtained from such methods do not represent the average property of the individual data; hence it is not a good way to analyze the data. For this reason, we fitted individual pCa-tension data to Eq. 1, and averaged the fitted parameters as described in section 2.3. As seen in Table S1, this effect is limited to the Hill coefficient. The effect is not pronounced for the pCa_{50} value, and similar results are obtained from either method.

Figure S1. Diagram of Tropomyosin and its mutations

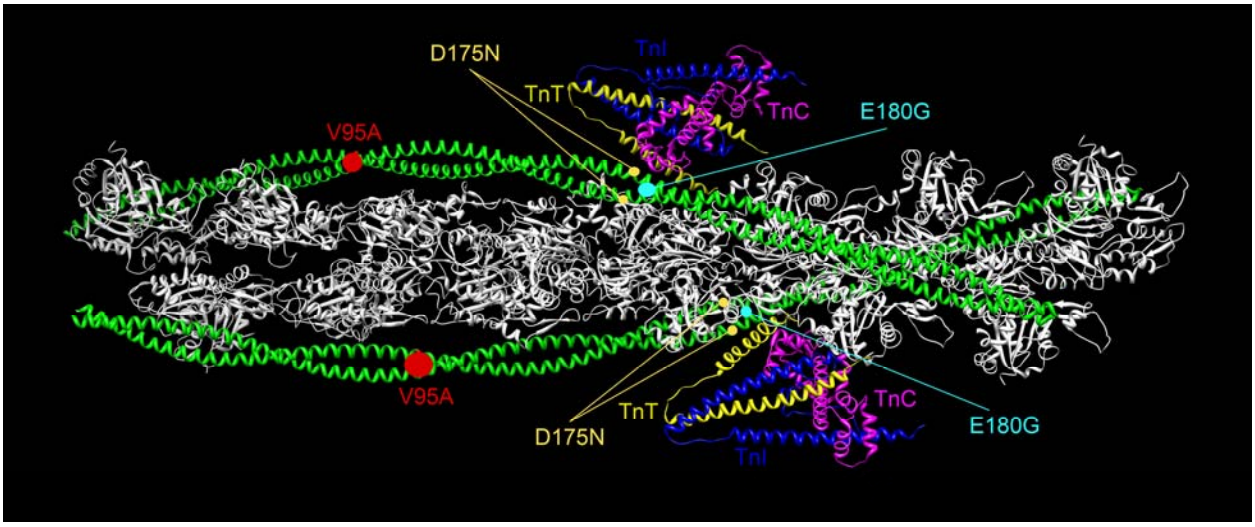

Figure S1. Diagram of Tropomyosin and its mutations. Two Tm α-helical chains (green), which wrap around each other to form a coiled-coil double helix, overlay the myosin binding sites on actin (white). One single Tm molecule (a dimmer) spans 7 actin subunits. Troponin T (yellow), troponin C (magenta) and troponin I (blue) in the absence of Ca^{2+} are also shown in the diagram. This diagram was adopted from Wu et al 2008 (PDB ID: 2w4u) with modifications.

Figure S2. Tension-Pi and tension-ATP plots

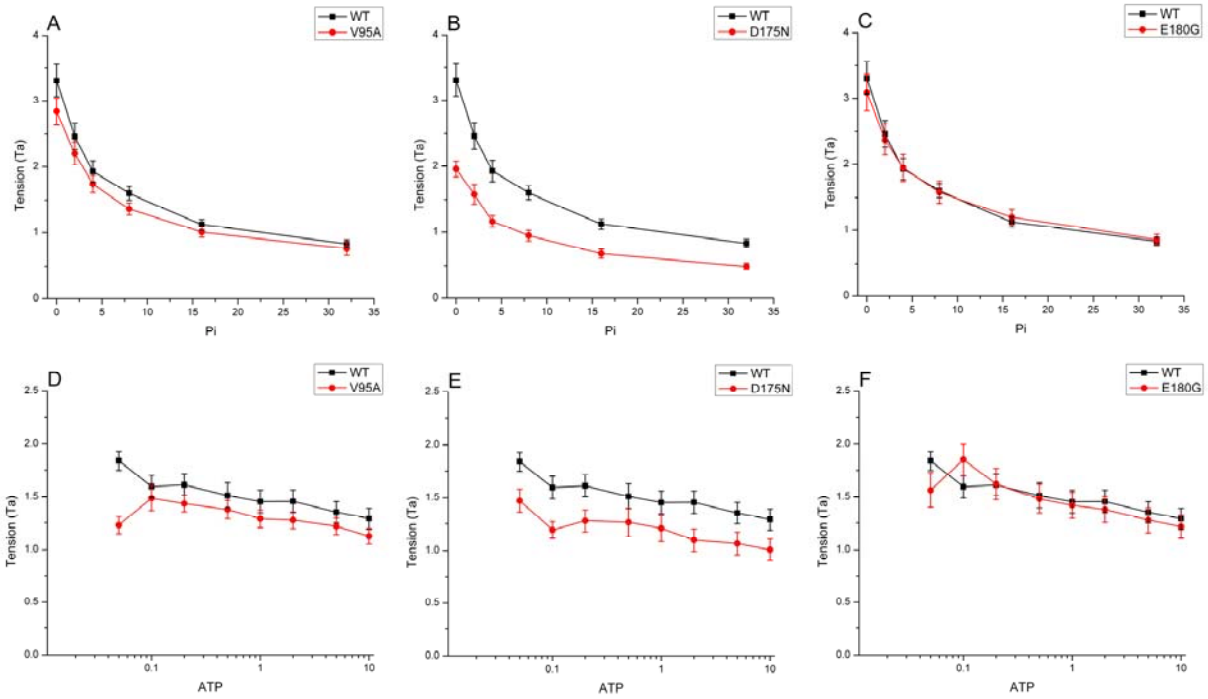

Figure S2. Tension-Pi and tension-ATP plots. Tension of reconstituted myocardium with mutants (red) and WT (black) Tm. The mean±SE are shown. A-C: Tension is plotted against [Pi]. D-F: Tension is plotted against [MgATP].

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2. Fujita, H., X. Lu, M. Suzuki, S. Ishiwata, and M. Kawai. 2004. The effect of tropomyosin on force and elementary steps of the cross-bridge cycle in bovine myocardium. J. Physiol. 556:637-649.
3. Kawai, M., X. Lu, S. E. Hitchcock-DeGregori, K. J. Stanton, and M. W.Wandling. 2009. Tropomyosin Period 3 Is Essential for Enhancement of Isometric Tension in Thin Filament-Reconstituted BovineMyocardium. J. Biophys. 2009: Online paper.
4. Lu, X., M. K. Bryant, K. E. Bryan, P. A. Rubenstein, and M. Kawai. 2005. Role of the N-terminal negative charge of actin in cross-bridge kinetics and force generation in reconstituted bovine myocardium. J. Physiol. 564:65-82.
5. Lu, X., L. S. Tobacman, and M. Kawai. 2003. Effects of tropomyosin internal deletion Δ23Tm on isometric tension and the cross-bridge kinetics in bovine myocardium. J. Physiol. 553:457-471.

Supplemental Tables

Table S1. The method of averaging the Hill factor makes a difference in results.

	(A) Parameters individually fitted to Eq. 1 were averaged		(B) Averaged data were fitted to Eq. 1	
	Hill Factor	pCa_{50}	Hill Factor	pCa_{50}
WT	2.79±0.26 (N=15)	5.85±0.03 (N=15)	1.80±0.24	5.95±0.04
V95A	1.70±0.12 (N=13)	6.20±0.06 (N=13)	1.59±0.29	6.31±0.07
D175N	1.87±0.09 (N=7)	5.88±0.05 (N=11)	1.66±0.15	5.80±0.06
E180G	1.75±0.14 (N=7)	6.49±0.02 (N=11)	1.70±0.30	6.48±0.03

Table S1. The method of averaging the Hill factor makes a difference in results. This Table compares the results of averaging the data in two different ways. In (A), individually fitted parameters were averaged with ± indicating S.E.M. In (B), averaged data were fitted to Eq. 1, with ± representing the 95% confidence limits of the fitted parameters. To plot theoretical curves of Fig. 1, we used Eq. 1 with parameters listed under (B) of this Table.

Table S2: Solutions

Solutions	$K_2CaEGTA$ (mM)	K_2EGTA (mM)	Na_2MgATP (mM)	Na_2 K_2ATP (mM)	$MgAc_2$ (mM)	Na_2CP (mM)	$K_{1.5}Pi$ (mM)	NaAc (mM)	KAc (mM)	NaN_3 (mM)	MOPS (mM)	BDM (mM)	Creatine Kinase (U/ml)
Na Skinning	—	10	2	5	—	—	—	122	—	—	10	30	—
K Skinning	—	10	2	5	—	—	—	—	122	—	10	30	—
Storage*	—	10	2	5	—	—	—	—	122	—	10	30	—
Extraction**	2.0	—	—	2.2	—	—	—	—	—	—	20	40	—
Actin Reconstitution †	—	4	4	—	—	—	20	—	—	—	—	40	—
Tm and Tn Reconstitution ††	—	6	2.2	5	—	—	8	41	74.5	—	10	40	—
Relaxing	—	6	2.2	5	—	—	8	41	74.5	—	10	40	—
Rigor	—	—	—	—	—	—	8	55	122	—	10	—	—
5S0P	6	—	5.83	1.36	—	15	—	0.6	91.7	10	10	—	320
5S32P	6	—	5.7	1.36	—	15	32	0.9	17.3	10	10	—	320
0S8P	6	—	—	—	0.85	15	8	15	87.6	10	10	—	320
5S8P, Standard activating solution	6	—	5.8	1.36	—	15	8	0.7	73.1	10	10	—	320
00D	6	—	2.83	0.07	—	15	8	9.2	82.7	10	10	—	320
0D‡	6	—	2.75	0.178	—	—	8	39.1	97.3	10	10	—	—
3D‡‡	6	—	2.86	—	2.81	—	8	21.5	62.3	10	10	—	—

*Includes 50% (v/v) glycerol. ** Includes 121 mM KC1, 4.25 mM $MgCl_2$, 2 mM leupeptin, 2 mM DFP and 0.3 mg/ml gelsolin. †Includes 1 mg/ml G-actin, 80 mM KI and 8 mM KC1. ††Includes 0.6 mg/ml Tm (WT or mutant) and 0.6 mg/ml bovine Tn. ‡Includes 0.2 mM P^1, P^5-di(adenosine-5') pentaphosphate, pentalithium salt (A_2P_5). ‡‡ Includes 0.2 mM A_2P_5 and 11.84 mM NaADP. All the skinning solutions include 2 mM DTT. The pH of all solutions was adjusted to 7.00. Abbreviations: Prop, propionate; CP, creatine phosphate; CK, creatine kinase; DTT, dithiothreitol; BDM, 2, 3-butanedione 2-monoxime; and DFP, diisopropyl fluorophosphate.

Table S3: Measured parameters of WT and mutant Tm

Parameter (unit)	WT	V95A	D175N	E180G
T_{LC} (T_a)	0.122±0.025 (n=25)	0.393±0.04**** (n=12)	0.287±0.044*** (n=8)	0.425±0.052**** (n=21)
T_{HC} (T_a)	1.59±0.06 (n=11)	1.49±0.09 (n=12)	1.45±0.07 (n=9)	1.87±0.06**** (n=10)
T_{act} (T_a)	1.52±0.09 (n=11)	1.10±0.07** (n=12)	1.12±0.08** (n=8)	1.48±0.11 (n=10)
Y_{LC} (T_a)	11.87±1.21 (n=9)	28.91±2.53**** (n=13)	25.99±3.30**** (n=8)	27.06±1.61**** (n=10)
Y_{HC} (T_a)	57.04±4.88 (n=9)	69.15±4.61 (n=13)	57.14±4.56 (n=8)	76.09±4.10** (n=10)
Y_{act} (T_a)	45.17±4.08 (n=9)	40.24±3.45 (n=13)	31.15±2.63** (n=8)	49.03±4.28 (n=10)
C_{LC} (MPa)	0.131	0.297	0.263	0.280
C_{HC} (MPa)	0.782	0.759	0.812	0.913
C_{act} (MPa)	0.651	0.462	0.549	0.633
B_{LC} (MPa)	0.044	0.074	0.083	0.083
B_{HC} (MPa)	0.118	0.102	0.151	0.163
B_{act} (MPa)	0.074	0.028	0.069	0.079
pCa_{50}	5.85±0.03 (n=15)	6.20±0.06**** (n=13)	5.88±0.05 (n=7)	6.49±0.02**** (n=11)
Hill Factor (Cooperativity)	2.79±0.26 (n=15)	1.70±0.12*** (n=13)	1.87±0.09* (n=7)	1.75±0.14*** (n=11)
K_0 (mM^{-1})	24.52±2.71 (n=8)	14.84±0.85** (n=7)	19.53±3.05 (n=5)	33.23±1.25* (n=5)
K_1 (mM^{-1})	1.76±0.35 (n=9)	0.86±0.16* (n=9)	0.91±0.13 (n=9)	1.84±0.32 (n=9)
K_2	1.37±0.13 (n=10)	0.93±0.06* (n=9)	1.69±0.16 (n=9)	1.49±0.07 (n=9)
K_4	0.96±0.19 (n=10)	1.17±0.18 (n=10)	1.11±0.24 (n=10)	0.77±0.09 (n=9)
K_5 (mM^{-1})	0.58±0.13 (n=9)	0.22±0.04* (n=10)	0.26±0.04* (n=10)	0.28±0.08 (n=9)
k_2 (s^{-1})	25.57±1.87 (n=10)	28.00±1.80 (n=9)	33.03±2.27* (n=9)	26.14±1.49 (n=9)
k_{-2} (s^{-1})	19.36±1.18 (n=10)	30.32±1.17**** (n=9)	20.28±1.58 (n=9)	17.66±0.97 (n=9)
k_4 (s^{-1})	5.93±0.65 (n=10)	8.32±0.62* (n=10)	7.08±0.59 (n=10)	5.81±0.70 (n=9)
k_{-4} (s^{-1})	7.42±1.03 (n=10)	8.19±1.14 (n=10)	8.05±1.23 (n=10)	7.69±0.79 (n=9)

Note: *: p<0.05, **: p<0.01, ***: p<0.005, and ****: p<0.001 compared to WT. T_a: Tension of actin-filament reconstituted myocardium at standard activating conditions (5S8P, Table S2). T_a=3.88±0.64 KPa (n=51)