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Report:

Introduction: The aim of the project is to investigate the molecular bases of heart regulation. Using X-ray diffraction on electrically paced intact trabeculae from the rat ventricle at ID02, we have shown that in the heart as in the skeletal muscle a dual filament mechanism of regulation of contraction operates: the Ca^{2+}-dependent thin filament activation, making the actin sites available for binding of the myosin motors, and the mechano-sensitivity of the thick filament (1,2), acting as a downstream mechanism that adapts to the load the recruitment of the myosin motors from their OFF state, in which they lie on the surface of the thick filament unable to split ATP and bind actin. In a heartbeat, unlike during skeletal muscle tetanic contraction, the rise of internal $[Ca^{2+}]$ is transient and may not reach the level for full thin filament activation, thus the mechanical response depends on both the internal $[Ca^{2+}]$ and the sensitivity of the thin filament to calcium (3,4), parameters that are under the control of several regulatory mechanisms among which the increase in sarcomere length (SL) (Length Dependent Activation, the cellular basis of the Starling Law of the heart (5), that relates the systolic performance to the degree of ventricular filling in diastole, and the phosphorylation of contractile, regulatory, and cytoskeletal proteins (6-8). Previous work on demembranated preparations suggested that the increase of SL and degree of phosphorylation of the Myosin Binding Protein-C (MyBP-C), an accessory protein that lies on the thick filament and can bind the thin filament with its N-terminus, can by themselves alter the regulatory state of the thick filament, switching motors ON at low Ca^{2+} (9). In contrast, our recent X-ray diffraction experiments on intact trabeculae from the rat heart have demonstrated that inotropic interventions able to double the systolic force like increase in SL from 1.95 to 2.22 μm or addition of isoprenaline (ISO) 10^{-7} M to the bathing solution (which increases the degree of phosphorylation of MyBP-C) do not affect any of the myosin based reflections related to the OFF state of the thick filament in diastole (10), as expected from an energetically well suited downstream mechanism as thick filament mechanosensing, which adapts the recruitment of myosin motors to the load during contraction (1). This idea is solidified by the recent finding in skeletal muscle that, at physiological sarcomere length, upon activation titin in the A band increases its stiffness by 100 time, in this way becoming able to control the orientation of myosin motors in relation to the load (11). However, the classical view of the Starling has been recently reaffirmed in demembranated bundles dissected from the papillary muscle of Yucatan mini-pig showing that, at 22°C and without recovering the native lattice dimension thick filament is activated at low Ca^{2+} by SL increase from 2 to 2.2 μm (12). Considering the lack in that experiment of the requirements for preserving the OFF state of myosin motors in demembranated fibres (13, 14) and that the porcine ventricle, but not the rat ventricle, has the same myosin isoform (β-cardiac myosin) as the human ventricle, we found compelling to solve the contradiction by applying our mechanical and X-ray diffraction methods to define thick filament activation on intact preparations from the minipig heart. The fundamental step pursued during LS 3208, was to define a suitable intact preparation from minipig heart, in particular if the thick filament is activated by lowering temperature, as reported in both intact skeletal and heart muscle (14, 15).

Methods. One heart from 2-years old male minipig is harvested, rinsed, and brought in cold cardioplegia solution to the ESRF. The suitable intact preparation has been identified in the trabecula from the left ventricle (a pillar like multicellular preparation of 4 mm length and 1 mm diameter). Trabeculae are dissected from the internal wall of the left-ventricle and mounted vertically in a thermo-regulated trough between the lever arms of the force and length transducers via T-shaped aluminum clips, continuously perfused with physiological solution bubbled with oxycarb (95% O2, 5% CO2) and electrically stimulated to produce twitches at 0.5 Hz by means of platinum electrodes (SL 2.1 μm; temp. 12-37°C). 5 ms 2D diffraction patterns are recorded with the EIGER2-4M detector (2068x2162 pixels, active area 155x162 mm^2) at the different temperature. To mitigate radiation damage from the high photon flux (10^{13} photons/s) at the ESRF-EBS, the trabecula is shifted axially by 200 μm between exposures. Fast shutters are used to limit the exposure to the acquisition time. 4.8 m camera length allows the spatial resolution adequate to resolve the fine structure of the reflections marking the state of the thick filament, up to the M6. 31 m camera length is used to record the SL.

Results. Lowering temperature from 37 to 12°C, the analysis of X-ray data so far shows: (i) decrease of the intensity ratio of the 1,1 over the 1,0 equatorial reflections; (ii) decrease of the intensity (to 1/3) and the spacing (by 0.35%) of the M3 reflection; (iii) increase of the spacing of the M6 reflection by 0.38% and (iv) decrease of the intensity of the first myosin layer by 5-fold. All these changes indicate, in agreement with previous findings on intact skeletal and cardiac muscle, that cooling produces a progressive disruption of the OFF state of the thick filament, with motor moving away from the ordered helical tracks on the surface of the thick filament.

Conclusions. The intact trabecula from the porcine ventricle is a suitable preparation to investigate the structural dynamics of thick filament activation with the same β-cardiac myosin isoform as in the human ventricle.

References. 1. Reconditi *et al. PNAS* **114**:3240-5, 2017; **2.** Piazzesi *et al. Front Physiol* **9**:736-743, 2018; **3.** Allen and Kentish, *J Mol Cell Cardiol* **17**:821-40, 1985; **4.** ter Keurs, *Am J Physiol Heart Circ Physiol* **302**:H38-50, 2012; **5.** de Tombe *et al. J Mol Cell Cardiol*, **48**:851-858, 2010; **6**. Herron *et al. Circ. Res* **89**:1184-1190, 2001; **7**. Kumar *et al. J Biol Chem* **290**:29241–9, 2015; **8**. Hidalgo & Granzier. *Trends Cardiovasc Med* **23**:165–71, 2015; **9.** Colson *et al. J Mol Cell Cardiol* **53**: 609-613, 2012; **10.** Caremani *et al. J Gen Physiol* **151**:53-65, 2019; **11.** Squarci *et al. PNAS* 120: e2219346120, 2023; **12.** Ma *et al. Circ Res* **129**:617, 2021; **13.** Caremani *et al. J Gen Physiol* **153**:e202012713, 2021; **14.** Ovejero *et al. J Gen Physiol* **154**:e202113029, 2022; **15.** Caremani *et al. J Gen Physiol* **151**:1272-1286, 2019.