ESRF	**Experiment title:** **Molecular bases of regulation of cardiac muscle contractility**	**Experiment number**: LS-2990
Beamline: ID02	**Date of experiment**: from: 22/06/2021 to:28/06/2021	**Date of report**: Updated 4th March 2022
Shifts: **15**	**Local contact(s)**: Peter Boesecke	*Received at ESRF:*

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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 in 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, which is the cellular basis of the Starling Law of the heart (5)) 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 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, as expected from an energetically well suited downstream mechanism as thick filament mechanosensing, which adapts the recruitment of myosin motors to the load (10). The results prove the unique effectiveness of intact trabeculae approach in structural investigations on thick filament regulation and related myopathies and suggest that in skinned preparations the membrane permeabilisation likely affects the intramolecular (head-head and head-tail) and the intermolecular (Myosin-MyBP-C-titin) interactions that keep the myosin motors in the OFF state. To further understand the mechanism underlying the thick filament regulation, we investigated in intact trabeculae the effects on the thick filament of the small molecule Omecamtiv Mecarbil (OM) that binds specifically to myosin and is known to alter the state of the thick

filament in demembranated preparations in the absence of calcium (11). We found that 1 μM OM affects the OFF state of the thick filament in diastole, switching ON ~ 20% of motors (Report LS-2867). OM is a putative positive inotropic tool for treatment of systolic heart dysfunction (12,13), currently in phase-three clinical trial (14). OM binds to the catalytic domain of both α cardiac myosin (the main isoform in the mouse and rat heart and in the atrium of large mammals and human), β cardiac myosin (the main isoform in the ventricle of large mammals and human) and the slow skeletal isoform (15), increasing the affinity for actin attachment, and thus causing, in skinned myocytes, a leftward shift in the relation between force and Ca^{2+} concentration (15, 16). However the maximum force developed at saturating Ca^{2+} is reduced to ½ that of control because myosin motors that bind OM are unable to undergo the force generating stroke (16,17). The different effects that OM and ISO have on the regulatory state of the thick filament in diastole represent a significant step toward the understanding of thick filament mechano-sensing, the role of accessory proteins and their phosphorilation and the modulatory effect of small molecules. In the subsequent visit (LS-2944) we investigated in intact trabeculae the structural basis of the inotropic action of OM, by recording how it influences the transition to active state of the thick filament in systole at different levels of peak force (T_p). It has been found that in the presence of 1μM OM the changes of the X-ray signals that mark the load-dependent switching ON of the thick filament in systole were anticipated by 15-20 kPa with respect to the control (18) indicating that OM inotropic action is explained by the changes induced on the thick filament in diastole that add to those induced by thick filament stress during the systole. Due to COVID-19 restrictions a reduced team and only part of the allocated shifts have been allowed and the statistics required has been attained during LS-2990, which was mainly dedicated to clarify the structural basis of inotropic action of ISO.

Methods. Intact trabeculae or papillary muscles, dissected from the right ventricle of the rat, are mounted in a thermoregulated trough perfused with oxygenated solution (1.2 ml/min, 27°C) and attached, via titanium double hooks, to the lever arms of a strain gauge force transducer and a loudspeaker motor carried on the moveable stage of a microscope. The length of the sample is adjusted to have an initial SL of ~2.1 μm. A pair of mylar windows is positioned close to the sample, about 1 mm apart, to minimize the X-ray path in the solution. The trough is sealed to prevent solution leakage and the sample is vertically mounted in the beam path. The sample is electrically stimulated to produce twitches. 2D X-ray patterns are collected either at the peak of isometric twitch or under different afterload both in control solution and in solution with ISO 1 μM. A FReLoN CCD detector is placed at 31 m from the preparation to collect the first orders of the sarcomeric reflections with 2 ms time windows and set the initial SL to 2.2 μm. The detector is then moved to 1.6 m to collect up to the 6th order of the myosin-based meridional reflections (2-5 ms time windows).

Results. In the presence of 1 μM ISO, which potentiates the peak force of the isometric twitch and the velocity of shortening in afterloaded contractions, the relations between the X-ray signals marking the degree of thick filament activation and force superimpose on those in control covering a range of thick filament activation-force points extended to higher values.

Conclusions. The results indicate that the ISO-dependent enhancement of the degree of phosphorylation of accessory proteins modulates the mechanical performance of the cardiac systole via the same mechano-sensing mechanism as in control, by increasing the gain of the positive feedback between stress and thick filament activation.

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