ESRF	**Experiment title:** **Molecular bases of regulation of cardiac muscle contractility**	**Experiment number**: LS-2944
Beamline: ID02	**Date of experiment**: from: 03/02/2021 to:09/02/2021	**Date of report**:
Shifts: **15**	**Local contact(s)**: Peter Boesecke; Narayanan Theyencheri	*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 text-book 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 bind actin and split ATP. 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 interactions (head-head and head-tail) and the intermolecular interactions (Myosin-MyBP-C-titin) 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 demebranated 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 LS2867). 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). In 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).

Methods. The intact heart trabecula, dissected from the right ventricle of the rat, is 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. SL is measured with a 40x dry objective and a 25x eyepiece. The length of the trabecula is adjusted to have an initial SL of ~2.1 μm (L_0 length). A pair of mylar windows is positioned close to the trabecula, about 1 mm apart, to minimize the X-ray path in the solution. The trough is sealed to prevent solution leakage and the trabecula is vertically mounted in the beam path. Trabeculae are electrically stimulated at 0.5 Hz to produce twitches. 2D X-ray patterns are collected during diastole and at the peak of the twitch both in fixed-end and in sarcomere length clamp conditions (18) either in control solution or in solution with 1μM OM. This OM concentration is used as it is known to potentiate the steady force attained by a skinned preparation at partial Ca^{2+} activation (pCa ~6.5, which is presumably the intracellular [Ca^{2+}] attained during the systole by an electrically paced trabecula, see also (15)). A FReLoN CCD detector is placed at 31 m from the preparation to collect the first orders of the sarcomeric reflections with 1.6 ms time windows. 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) at the same trabecula lengths as those set at 31 m. Given the long time taken by OM equilibration into the trabecula (45min), the absence of full recovery after OM washout and to avoid confounding effect of radiation damage, the data in control and in OM were collected from different trabeculae. The parameter that revealed the most effective for the normalisation of the intensity of the reflections for the different mass of individual trabeculae was the sum of the intensities of the low angle equatorials (I1,0+ I1,1).

Results. In diastole, addition of 1 μM OM to the physiological solution ([Ca^{2+}] 1 mM) reduces by 30-40% the intensity of the ML1 layer line, originating from the three-stranded helical symmetry of myosin motors on the surface of the thick filament, and increases by 0.3% the spacing of M6, marking the extension of the thick filament. In the presence of OM, the systolic force (T_p) at a given SL reduces to ½ the value in control. The changes of the X-ray marking the regulatory state of the thick filament as a function of T_p are anticipated by 15-20 kPa in the presence of OM.

Conclusions.

The results indicate that partial activation of the thick filament and the structural changes induced by 1 μM OM on the thick filament in diastole add to those induced by the mechanosensing that accompany the rise of force, i.e. in the presence of both Ca^{2+} and stress on the thick filament.

Due to the COVID-19 restrictions, only a reduced team of three people has been allowed on site for the planned experiments, therefore no more than 2/3 of the 15 allocated shifts has been exploited. The statistics required for a more detailed analysis of the data has been integrated with the results obtained in the subsequent visit (LS-2990 and associated report)

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