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Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

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Reports supporting requests for additional beam time

Reports can be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

	Experiment title: Molecular bases of regulation of cardiac muscle contractility	**Experiment number**: LS-2867
Beamline:	**Date of experiment**: from: 05 December 2018 to: 10 December 2018	**Date of report**: 2nd March 2020
Shifts:	**Local contact(s)**: Theyencheri Narayanan	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Vincenzo Lombardi*, University of Florence, Italy
Gabriella Piazzesi*, University of Florence, Italy
Marco Linari*, University of Florence, Italy
Marco Caremani*, University of Florence, Italy
Massimo Reconditi*, University of Florence, Italy
Matteo Marcello*, University of Florence, Italy
Ilaria Morotti*, University of Florence, Italy

Report:

Introduction: The aim of this project is to investigate the molecular basis 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 canonical 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. Omecamtiv Mecarbil (OM) is a putative positive inotropic tool for treatment of systolic heart dysfunction

(11,12), currently in phase-three clinical trial (13). 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 (14), increasing the affinity for actin attachment, and thus causing, in skinned myocytes, a leftward shift in the relation between force and Ca^{2+} concentration (14, and our preliminary experiments). 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 (15). In LS-2867 we investigated the structural basis of the inotropic action of OM, whether it influences the regulatory state of the thick filament in diastole.

Methods. The 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 a the peak of the twitch both in fixed and in sarcomere length clamp conditions (16) either in control solution or in solution with 1μM OM. This OM concentration is used as it is know 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 (14)). 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 (5-20 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 ($I_{1,0}$+ $I_{1,1}$).

Results. 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, the intensity of the meridional myosin-based reflections (M1, also contributed by the MyBP-C, M3 originating from the axial repeat of the myosin motors, M6 from the backbone periodicity) and the intensity of the meridional T1 reflection from the axial repeat of regulatory protein troponin on the thin filament. Moreover the spacing of M6, marking the extension of the thick filament, is increased by 0.3% by OM in diastole.

Conclusions: The results indicate that 1 μM OM affects the OFF state of the thick filament in diastole, indicating that ~20% of motors are switched ON in the absence of both Ca^{2+} activation of the thin filament and stress on the thick filament.

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**. Malik *et al. Science* **331**:1439-1443, 2011. **12**. Morgan *et al. ACS medicinal chemistry letters* **1**:472-477, 2010. **13.** Kaplinsky & Mallarkey D*rugs in context* **7**:212518, 2018. **14.** Nagy *et al, Br J Pharmacol* **172**:4506-4518, 2015. **15**. Woody *et al. Nat comm* **9**.3838, 2018. **16.** Caremani *et al. PNAS* **113***:*3675-3680, 2016.