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.

Once completed, the report should be submitted electronically to the User Office via the User Portal:

https://wwws.esrf.fr/misapps/SMISWebClient/protected/welcome.do

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-2791
Beamline:	**Date of experiment:** from: 11 April 2018 to: 17 April 2018	**Date of report:** 2nd March 2020 *Received at ESRF:*
Shifts:	**Local contact(s):** Theyencheri Narayanan	

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Report:

Introduction: The aim of this project is to investigate the molecular basis of heart regulation. In previous visits we showed that in the heart as in the skeletal muscle (1) 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-sensing in the thick filament (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 (3, 4). 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 intracellular [Ca^{2+}] and the sensitivity of the thin filament to calcium (5,6), parameters that are under the control of several regulatory mechanisms like the increase in sarcomere length (SL) (Length Dependent Activation, which is the cellular basis of the Starling Law of the heart (7)) and the phosphorylation of contractile, regulatory, and cytoskeletal proteins (8,9). Previous work on demembranated preparations suggested that 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 alter the regulatory state of the thick filament, switching motors ON (10). This visit and the two previous ones (LS-2650 and LS-2719) were aimed at investigating the integration of thin and thick filament regulation by defining how the regulatory state of the thick filament in an electrically paced intact trabecula changes in diastole in relation to inotropic interventions able to potentiate the systolic force by a factor of 2. The interventions used were (i) the increase in sarcomere length (SL) from 1.95 to 2.22 μm with an extracellular [Ca^{+2}] of 1 mM, (ii) the increase in exracellular [Ca^{+2}] at constant SL, (iii) the addition to the perfusion solution of 10^{-7} M isoproterenol (ISO), a β-adrenergic agent which increases the degree of phosphorylation of MyBP-C. With LS-2791 we completed the investigation, demonstrating that all these inotropic interventions do not affect any of the myosin-based reflections related to the OFF state of the thick filament in diastole. The results clarify contradictory findings from previous X-ray diffraction experiments on intact trabeculae (11,12) and demonstrate the limits of using demembranated preparations, in which 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. The work, published in *J Gen Physiol* (13), solidifies the idea that the recruitment of myosin motors from their OFF state occurs with an energetically well suited mechanism downstream with respect to thin filament activation. In experimental visit LS-2791, in agreement with the beamline responsible, we dedicated 6 shifts to collect 2D patterns from single fibres from frog skeletal muscle to increase the statistical significance of the effect of the small molecule inhibitor blebbistatin on the resting and active X-ray reflections related to the thick filament regulatory state (see report LS-2721).

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 at the peak of the twitch either in control or following the addition of ISO 10^{-7} M to the perfusion solution. 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 (5-10 ms time windows) at the same trabecula lengths as those set for the 31 m frames.

Results. Addition of 10^{-7} M ISO to the physiological solution ([Ca^{2+}] 1 mM), which almost doubles the peak force of the systole at SL ~2.1 µm, does not affect, in diastole, the intensities and spacing of all the meridional myosin-based reflections (M3 originating from the axial repeat of the myosin motors, M6 mainly originating from an axial backbone periodicity, M2, M4, M5 forbidden reflections due to an axial perturbation induced by the MyBP-C) and the intensity of the ML1 layer line, originating from the three stranded helical symmetry of myosin motors on the surface of the thick filament. Instead, the intensities of both the so-called M1/C1 (contributed by the MyBP-C) and T1 (from the regulatory protein troponin on the thin filament) meridional reflections decrease, in diastole in the presence of ISO, by 20% by ISO, likely as a consequence of the different degree of phosphorylation of the two proteins.

Conclusions: Inotropic protocols that double the twitch peak by modulating the [Ca^{2+}]-dependent thin filament activation (the increase in SL (LS-2650) and the addition of ISO to the solution (LS-2719 and this report)), produce a corresponding increase in the fraction of switched ON motors but do not affect the OFF state of the thick filament in the diastole. Theresults of this work, published in *J Gen Physiol* (13), solidify the new concept that mechanosensing-dependent thick filament regulation is a energetically well suited downstream mechanism that rapidly adapts the fraction of switched ON motors to the loading conditions during the contraction. The novelty of this work gained a Commentary in *J Gen Physiol*: T Irving & R. Craig, 2019. DOI.org/10.1085/jgp.201812307.

References. 1. Linari *et al*. 2015, *Nature* **528**:276-9 ; **2.** Reconditi *et al*. 2017, *PNAS* **114**:3240-5; **3.** Woodhead *et al*. 2005, *Nature* **436**:1195-9; **4.** Stewart *et al*. PNAS **107**:430-5; **5.** Allen and Kentish 1985, *J Mol Cell Cardiol* **17**:821-40; **6.** ter Keurs 2012, *Am J Physiol Heart Circ Physiol* **302**:H38-50; 7. de Tombe *et al*, *J Mol Cell Cardiol*, **48**:851-858, 2010; **8**. Kumar et al. J Biol Chem 290:29241–49, 2015; **9**. Hidalgo & Granzier. Trends Cardiovasc Med 23:165–71, 2015; **10.** Colson et al. 20012, *J Mol Cell Cardiol*:53, 609-613. **11.** Farman *et al*. 2011, *Am J Physiol Heart Circ Physiol* 300:H2155-60; **12.** Ait-Mou *et al*. 2016, *PNAS* 113:2306-11.**13**. Caremani et al. 2019, J Gen Physiol **151**:53-65.