EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

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

Deadlines for submission of Experimental Reports

Experimental reports must be submitted within the period of 3 months after the end of the experiment.

Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report"),*
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

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.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number 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.

ESRF	**Experiment title:** **Molecular bases of regulation of cardiac muscle contractility**	**Experiment number**: LS-3081
Beamline: ID02	**Date of experiment**: from: 15/04/2022 to: 19/04/2022	**Date of report**: Updated 13th September 2022
Shifts: **12**	**Local contact(s)**: Theyencheri Narayanan	*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 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, 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 phosphorylation and the modulatory effect of small molecules. In the subsequent visit (LS-2944) we investigated, in intact trabeculae and papillary muscles, 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 visit. After completing the experiments with the OM activator, the remaining of the LS-2990 beam time has been dedicated to clarify the structural bases of inotropic action of ISO. LS-3081 visit has been dedicated to implement statistics to achieve the S:N of the diffraction signals that mark the degree of thick filament activation and complete the experiments aimed at understanding the inotropic action of ISO.

Methods. Intact trabeculae or papillary muscles, dissected from the right ventricle of the rat, were 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 was adjusted to have an initial SL of ~2.1 μm. A pair of mylar windows was positioned close to the sample, about 1 mm apart, to minimize the X-ray path in the solution. The trough was sealed to prevent solution leakage and the sample was vertically mounted in the beam path. The sample was electrically stimulated to produce twitches. 2D X-ray patterns were collected either at the peak of isometric twitch or under different afterload both in control solution and in solution with 1 μM ISO. A FReLoN CCD detector was 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 was 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 power output in afterloaded contractions, the relation between the intensity of ML1 reflection, marking the degree of thick filament activation, and force is shifted to the left with respect to the relation in control indicating a larger stress-sensitivity of thick filament.

Conclusions. The results confirm that the ISO-dependent enhancement of the degree of phosphorylation of accessory proteins modulates the mechanical performance of the cardiac systole by increasing the gain of the positive feedback between stress and thick filament activation, with a larger gain at lower stresses.

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**. Kampourakis *et al*. *J Physiol* **596**:31-46, 2018. **12**. Malik *et al. Science* **331**:1439-1443, 2011. **13**. Morgan *et al. ACS medicinal chemistry letters* **1**:472-477, 2010. **14.** Kaplinsky and Mallarkey D*rugs in context* **7**:212518, 2018. **15.** Nagy *et al. Br J Pharmacol* **172**:4506-4518, 2015. **16**. Governali *et al*. *Nat Commun* **11**:3405, 2020. **17.** Woody *et al. Nat comm* **9**:3838, 2018. **18.** Lombardi *et al*. *Biophys J* **121**:p36a, 2022.