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Report:

Introduction. At the level of the sarcomere, the structural unit of the striated (skeletal and cardiac) muscles, contraction is due to the cyclical interactions of myosin motors extending from the thick filaments and the nearby actin containing thin filaments. The aim of this project is to investigate the structural bases of the regulation of the contraction, which involves proteins other than the contractile proteins myosin and actin. Previous X-ray diffraction experiments at ID02[1,2,3] demonstrated that, beyond the classical Ca^{2+}-dependent thin filament activation that makes the actin available for interaction with the myosin motors, a mechano-sensing mechanism in the thick filament recruits myosin motors from their resting (OFF) state as a function of the load of the contraction. This finding raised interest in describing in detail the molecular mechanism of thick filament regulation, and research has focused on two sarcomeric proteins, (*i*) the giant protein **titin** and (*ii*) the **myosin-binding protein C (MyBP-C)**. Titin runs from the midpoint of each thick filament to its tip in the A-band and then extends from the thick filament tip to the end of the sarcomere in the I-band. I-band titin in this way is a spring in parallel to the myosin motors and is responsible for passive tension in the resting muscle and likely provides the load to prevent development of large inhomogeneity in serially linked half-sarcomeres with different force capability[4] in the active muscle. However, due to its large extensibility at the physiological sarcomere length (SL) it looked inadequate to transmit external stress to the thick filament and account for its mechanosensitivity. MyBP-C is bound with its C-terminal to the backbone of the thick filament in the central one-third of each of its halves (C-zone) and with its N-terminal[5] to the thin filament. Both MyBP-C and titin establish periodic interactions with the myosin motors in the OFF state[6,7] suggesting a role in the OFF-ON transition of the motors. A detailed description of the dynamics of the interactions between myosin, titin and MyPB-C underlying thick filament regulation is of fundamental interest because mutations in the specific sites of these proteins are the principal causes of myopathies. We combine sarcomere-level mechanics and low-angle interference X-ray diffraction in intact fibres of frog muscle, to record the structural changes in the filaments and in the myosin motors accompanying activation of contraction and stepwise change in force on the thick filament. During LS-2721 we have completed the experiments started with LS-2514, in which rapid changes in force were imposed on the resting fibre to determine the stress sensitivity of the myosin-based reflections in the absence of Ca^{2+}. Then, we have established a new protocol to verify if during Ca^{2+} activation, (*i*) the putative MyBP-C links responsible for the fast communicating path between thin and thick filament and (*ii*) the putative titin ability to switch of motors OFF-ON depending on the stress on the thick filament. For this it is necessary to record the structural changes in the filaments following a stepwise change in force imposed on the stimulated fibre without the confounding effects of myosin motors. During LS-2721 and part of LS-2791, we have tested

the effectiveness of the myosin inhibitor ParaNitroBlebbistatin (PNB)[8]. In the experimental visits LS-2992, LS-3085 we established that (i) 20 μM PNB arrest myosin motors in the OFF state during tetanic stimulation; (ii) force steps to 0.13-0.25 the isometric tension T_0 attained with tetanic contraction in control reveal a 100 times increase in the I-band titin stiffness upon stimulation; (iii) A-band titin under these conditions moves azimuthally myosin motors on the surface of the thick filament toward the lattice planes of the nearby actin filaments in a load dependent manner. This detailed description of the role of the titin as the mechanical link controlling in a load-dependent manner the switching OFF-ON of the myosin motors, published in PNAS on Feb 2023[9], sets the stage for future studies in demembranated cells from both mammalian models and human biopsies, aimed at defining the genotype-phenotype relation of titin variants in health and disease and developing specific therapeutical strategies. The next step, started with the experimental visit LS-3150, pursued the aim of defining the time course of the sequence of mechanical and structural events triggered by rise in internal Ca^{2+} to determine the relation between Ca^{2+}-dependent structural changes in thin and thick filaments and switch in titin stiffness.

Muscle fibre preparation and protocols. Frogs (*Rana esculenta*), cooled to 2-4 °C, were killed by a percussive blow to the head followed by destruction of the spinal cord in accordance with EU official regulations on Use of Laboratory Animals, and of the University of Florence Ethical Committee (in compliance with the rules of the Decreto Legislativo of Italian Government 4 marzo 2014, n. 26). Small bundles of 2-3 fibres were dissected from tibialis anterior muscles, taking care at minimising the length of the tendon attachment at the two ends. The bundles were then transferred to an experimental chamber containing Ringer's solution and mounted vertically at beamline ID02 between a capacitance force transducer and a loudspeaker-coil motor, carried by micromanipulators for adjustment of the bundle length and position in the X-ray beam path. Two mylar windows were moved as close as possible to the preparation to reduce the X-ray path in water. 2D diffraction patterns are collected in series of 29 time-windows of 2 ms exposure time and up to 200 Hz frequency (at temperature 4 and 14 °C, SL 2.15 μm) with the EIGER2-4M detector (2068x2162 pixels, active area 155x162 mm^2) first in control, during the rise of the isometric tetanus and during relaxation, and then in 20 μM PNB with the same temporal sequence. To mitigate the radiation damage from the high photon flux (10^{13} photons/s) at the ESRF-EBS, the bundle is shifted axially by 200 μm between contractions. Fast shutters are used to limit the exposure to the acquisition time. 4.8 m camera length allows the spatial resolution adequate to resolve the fine structure of the reflections marking the state of the thick filament, up to the M6. 31 m camera length is used to record the SL. Corresponding 2 ms frames in the time series from each protocol were added to improve S:N. One or two diffraction patterns per bundle were recorded with 31 m camera length to collect the sarcomeric reflections and check for SL changes and homogeneity during the protocol.

Results. Fig. 1 shows the time course of the intensities of the equatorial reflection 1,0 (A) originating from the lattice planes containing thick filaments, and the first myosin layer line ML1 (B), from the three stranded helical packing of the myosin motors on the surface of the thick filament following tetanic stimulation both in control and in 20 μM PNB.

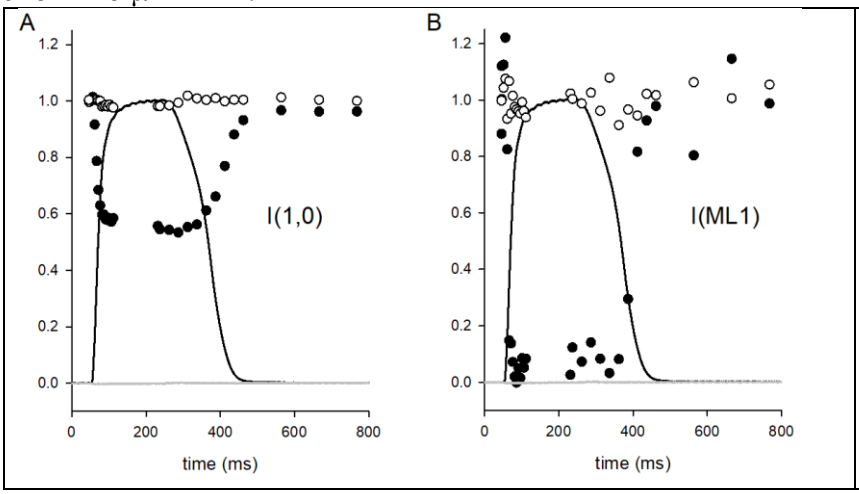

Fig.1. Superimposed X-ray signals and force trace in control (filled circles and black line respectively) and in 20 μM PNB (open circles and gray line respectively). A. Intensity of the 1,0 equatorial reflection. B. Intensity of the first myosin layer line (ML1). Bundle of 3 fibres from tibialis anterior of Rana esculenta. Temperature 14 °C, SL 2.15 μm

Conclusions. The results show the reliability of the PNB treatment in arresting the activation of myosin motors and the efficiency of the sequence and duration of X-ray exposures to describe the relevant structural signals.

References. 1. Linari *et al*. 2015, *Nature* **528**:276-9. **2.** Reconditi *et al.* 2017, *PNAS*.**114**:3240-45. **3.** Piazzesi *et al*. 2018, *Front Physiol* **9**:736-43. **4.** Powers *et al*. 2020, *J Physiol* **598**:331-45. **5.** Luther *et al*. 2011, *PNAS* **108**:11423-8. **6.** Reconditi *et al.* 2014, *J Physiol* **592**:1119-37. **7.** S. Labeit, B. Kolmerer, 1995, *Science* **270**:293–6. **8.** Kepiro *et al*. 2014, *Angew Chem* **53:**8211-15. **9.** Squarci *et al*. 2023, *PNAS* **120**:e2219346120