WEEK 5: Muscle Contraction

Recommended Reading:
Pages 874-879 in Raven et al. (2005) – Muscle Physiology.

I. Introduction

For this week’s lab you will examine the main energy-consuming tissue in the bodies of animals – muscle. In particular, we will conduct an experiment to investigate conditions that demonstrate what chemicals in muscle fibers are necessary for contraction.

II. Muscle Structure and Physiology

The working elements of muscles are composed of actin filaments and filaments of myosin-II, an associated protein (myosin-I is found in other contractile structures that cause changes in cell shape and amoeboid movement). Myosin-II is a dimer of two identical polypeptides, each with a globular "head" that is attached to a longer "tail" region (see figure 5.1). The tails are wound together as a coiled coil, with the two heads sticking out at one end. Myosin-II dimers associate with one another to form a myosin filament, with the globular heads projecting outward at both ends to form "fuzzy" zones, with a "bare" middle zone consisting of tails only. Each myosin head is an ATP-hydrolyzing "motor", taking an ATP and splitting it into ADP and P\textsubscript{i}. The energy released from the phosphate bond drives a conformation change in the myosin molecule that results in the "walking" of the head along an actin filament. The simultaneous walking movements of many myosin heads can cause the myosin fiber to slide along the actin filaments, causing muscle contraction.

Figure 5.1. Structure of myosin molecules and their organization within a thick filament. (A) Myosin is a dimer – each of the two molecules has a head and a helix tail. (B) Myosin dimers associate with one another to form a ‘thick’ filament.
Skeletal (striated) muscle, cardiac (heart) muscle and smooth muscle all use myosin thick filaments to slide actin thin filament bundles to produce muscle contraction. Although the precise structures of these three muscle types differ, the basic contractile mechanism is the same. Skeletal muscle consists of long fibers, which are really enormous single cells that form when separate smaller cells fuse during development. Each muscle fiber (cell) is multinucleated, possessing the nuclei derived from the individual cells that fused during development; these nuclei are located just beneath the plasma membrane. The cytoplasm of a muscle fiber is packed with long, cylindrically-shaped structures about 1-2 µm in diameter – these so-called myofibrils are the contractile components of muscle. Each myofibril in striated muscle is organized as a linear chain of repeating, microscopic contractile units termed sarcomeres. Each sarcomere is about 2.5 µm long, in its relaxed state, and consists of myosin-II filaments (thick filaments), actin filaments (thin filaments) and associated proteins, organized into Z-discs, an A-band and I-bands (see figure 5.2). The plus ends of the thin filaments are embedded in and anchored to the Z-discs, which form the boundaries of the sarcomere. Centrally-located thick filaments overlap with the thin filaments but are not anchored at all. The A-band, located in the center of the sarcomere, consists of overlapping thick and thin filaments. The I-bands, located on either side of the A-band, contain regions of thin filaments only.

Under light microscopy, the A-bands appear dark because the presence of thick filaments scatters and deflects light from the objective lens; the I-bands appear light because the lack of thick filaments results in light going through to the lens. In the transmission electron microscope, A-bands are dark because the affinity of the thick filaments for heavy metal atoms like lead makes them electron-dense; I-bands are lighter because they are less electron-dense and more electron-transparent than A-bands.

During muscle contraction, the following events take place: At the beginning of a contraction cycle, the heads of myosin thick filaments are attached to and locked onto actin filaments in a state of rigor. In this locked state, the angle between the head of a myosin-II molecule and its tail is about
ATP molecules then bind to myosin globular heads, inducing shape changes which result in detachment of the heads from thin filaments. Next, the ATPase activities of myosin heads hydrolyze ATP to form ADP and P$_i$; the energy released is coupled to dramatic changes in the orientation of the heads. As a result, each myosin head “swings” along a thin filament a distance of about 5 nm, moving toward its plus end, and binds weakly to a new actin site. The heads of the thick filaments are now in a “cocked” state, with ADP and P$_i$ molecules still bound to them. In this cocked state, the angle between a myosin head and its tail is about 90°. The force-generating step that powers contraction is next. When the myosin heads bind weakly to the thin filaments, this causes the release of P$_i$ molecules, which in turn results in two events – stronger binding of the heads to actin sites on the thin filaments, followed by a power stroke in which the myosin heads, still bound to the thin filaments, snap back to their original 45° angle with the tails. This action drags the attached thin filaments toward the center of the sarcomere. During contraction, the thin filaments slide toward the center of the sarcomere, shortening both the distance between Z-discs (sarcomere length) and the size of the I-bands. The A-band remains constant in size during contraction.

The hydrolysis of ATP to ADP and P$_i$ requires magnesium ions. In addition, muscle contraction has a specific requirement for calcium ions. The thin filaments are not bare actin filaments, but rather have associated with them two major actin-binding proteins: tropomyosin and complexes of troponin (see Figure 5.3). When a nerve impulse (action potential) is transmitted across a neuromuscular junction, it travels along the plasma membrane of the muscle fiber (cell) and along the contiguous transverse tubules (T tubules) that penetrate into the cell. This action potential causes the release of Ca$^{2+}$ ions from the sarcoplasmic reticulum (SR) into the myofibrils (the SR is a network of flattened membrane sacs that forms a sheath around each myofibril). Calcium ions then bind to the troponin complex, altering its shape and moving it slightly. Movement of the troponin complex results in a slight movement of tropomyosin, which in turn exposes myosin binding sites along the actin thin filament. Myosin heads cannot bind to thin filaments in the contraction cycle unless these sites are exposed. The contraction of smooth muscle also requires calcium ions, although the control mechanism is different from that described here for skeletal muscle.

**Figure 5.3.** Association of troponin and tropomyosin with actin fibers.  
**a** When intracellular calcium is low (<10$^{-8}$ M), tropomyosin blocks all actin sites along one turn of the actin helix. **b** When intracellular calcium is elevated (>10$^{-7}$ M), calcium binds to troponin which then alters the position of tropomyosin on the actin filament. Once tropomyosin has moved, the actin sites are exposed allowing myosin heads to attach.
III. Inhibition of Contraction

There are conditions within muscles that can affect contraction. For example, you may have experienced muscle fatigue after a long bout of exertion. Muscle strength will commonly decrease as a result of this fatigue, as well as the speed of contraction. It has been found that muscle fatigue is often associated with increased levels of free phosphate (P$_i$) and protons (i.e., lower pH) within muscles. (What might be the cause of these increases during fatigue?)

In addition, several chemicals and poisons act to reduce muscle contraction. One such chemical called BDM (2,3-butanedione monoxime) is a specific non-competitive inhibitor of myosin ATPase (Herrmann et al., 1992; Ostap 2002).

IV. Investigation of Muscle Contraction

In this experiment, you will examine the structure of muscle fibers, as well as examine investigate the chemical requirements (in terms of an organic energy source and inorganic ions) for muscle contraction. We will use glycerinated myofibrils from rabbit psoas muscle, a type of striated muscle in the loin area which rotates the hip joint and flexes the spine. Rabbit psoas muscle is a good model because the fibers are long and straight, and there is relatively little connective tissue associated with the muscle fibers. This is an in vitro model, in that the experiment is conducted outside the living organism.

The psoas muscle being used in lab today was removed from a rabbit and soaked in a 50 percent (v/v) glycerol at 0 °C for twenty-four hours. Glycerination disrupts the membranes of the muscle cells, ruptures mitochondria, and leaches out soluble constituents such as ATP and inorganic ions. However, glycerinated muscle retains the organized structural array of myosin thick filaments and actin thin filaments, actin-associated proteins like troponin and tropomyosin which regulate contraction, and the functional capacity for contraction.

Two days before lab started, the muscle was transferred to a solution containing magnesium and calcium chelating agents (5 mM disodium EDTA; 5 mM EGTA; 1 mM DTT), soaked overnight, and washed several times by replacing the solution. The chelating agents increase the solubility of magnesium and calcium, allowing them to diffuse out of the muscle fibers, thereby removing them from the vicinity of the actin and myosin. The subsequent washings removed the Mg$^{2+}$ and Ca$^{2+}$ ions from the muscle fiber preparation. The muscle tissue was then homogenized with a high-speed blender to break up the muscle fibers into small fragments.

**Experimental Procedure**

Work in groups of two or three for this experiment. You will need eight transfer pipets, and at least eight clean microscope slides and cover slips. Clean the slides with water and a non-abrasive cleaner (for example, Windex or RBS), rinse them well with distilled water. Dry them thoroughly with Kimwipes, and blow away excess lint. You will also need a supply of at least eight Whatman No. 1 paper strips (about 3 x 8 cm each).

1. Obtain a 1.0 ml aliquot of muscle homogenate in a microfuge tube. Keep on ice at all times.

2. Use one Zeiss phase contrast microscope for the exercise (one microscope per group). If you haven't done so already, critically-focus the microscope (see page 5-10).

3. **Observation of Uncontracted Sarcomere**: Obtain the myofibril suspension from your TA – place this tube on ice. Gently agitate vial/tube by hand and then put **one drop** of the preparation to a microscope slide, using a transfer pipette. Apply a cover glass to the preparation. It is
important to use just one drop of the myofibril suspension to prevent the cover slip from floating and moving when you switch to the oil immersion objective. Focus in on the myofibrils, first using the low-power (10x) objective with the understage condenser set to "H" (= Brightfield). Then select the 40x objective, set the condenser to "Ph2" (for phase contrast), and focus with the fine-focus knob. Apply a drop of immersion oil to the illuminated area of the cover glass over the condenser; then swing and click the 100x objective into position (note: do not use this objective unless you have immersion oil on the cover slip). Use the fine-focus knob to obtain a sharp image; adjust light intensity with the voltage control for optimal clarity and contrast. The striated patterns of the myofibrils should now be clearly visible. If the cover glass is floating on too much liquid, the resolution of the myofibrils will be reduced significantly; in that case, touch a strip of filter paper briefly to the edge of the cover glass and withdraw excess fluid by capillary action; then, again adjust the fine-focus control. If oil is applied at or near one edge of the cover glass, oil may seep under it and ruin the preparation; apply oil only at or near the center of the cover slip.

4. Using figure 5.2 as a guide, prepare a thorough written description and a labeled sketch of the myofibrils. Base your descriptions and drawing on what you see under the microscope. Label as many parts as you can discern (for example, Z-lines, I-bands, and A-bands). Label and distinguish a single sarcomere. The A (anisotropic) bands are regions where the thick myosin filaments are localized. While A-bands include regions where thick and thin filaments overlap, H-bands contain only thick filaments. M-bands are located at the center of sarcomeres, at the center of H- and A-bands. M-bands and H-bands are resolved clearly only by transmission electron microscopy. Only thin actin filaments are found in the I (isotropic) bands. Dark Z-lines define the borders of the sarcomeres. Make sure that each person in your group has a chance to observe and sketch the sarcomeres.

5. Using the calibrated ocular micrometer with the oil immersion objective, measure the length (in µm) of a sarcomere in the relaxed state. The length of one sarcomere is the distance between two adjacent Z-lines. Take several measurements on different myofibrils and then record the average length.

6. Prepare a fresh wet mount of the myofibril suspension, as detailed in step 3, and focus on the myofibrils with the 40X objective. Agitate the tube containing the myofibril suspension just before you sample it. Then, using a transfer pipette, transfer about ten drops of Perfusion Solution I (MES; see table 5.1 below) along one edge of the cover glass. Place a 3 x 8 cm-strip of Whatman no. 1 filter paper against the opposite edge of the cover glass. The filter paper acts as a wick, drawing liquid under the cover slip by capillary action and perfusing the myofibrils with the test solution. It is important that the wick is 8 cm long so that it has enough capillary capacity to fully perfuse the slide. Observe the myofibrils as perfusion is taking place. Refocus with the fine-focus control as required. If the perfusion technique is working properly, within a few seconds you will see myofibrils rushing across the field of vision. MES solubilizes the myosin thick filaments, but not the actin thin filaments. After the myofibrils first start moving in the perfusion solution, wait one to two minutes and then remove the paper wick. Add additional drops of perfusion solution to the edge of the cover glass if all of the liquid is drawn under it before the wick is removed.

Bring the myofibrils back into sharp focus at 400X, apply a drop of immersion oil to the illuminated area of the cover glass over the condenser, and click the 100X oil immersion objective into position. Make your final observations of the effect of this treatment on the myofibrils at the maximum total magnification available in your microscope. Use the fine-
focus control to obtain a sharp image; adjust light intensity with the voltage control for optimal clarity and contrast. Make thorough written descriptions and a clear, labeled drawing of the appearance of a myofibril following treatment with MES. If possible, measure the length (in µm) of several sarcomeres to determine whether the perfusion solution has any effect on myofibril contraction or relaxation.

Calculate and record the mean sarcomere length. If resolution is poor, the cover glass is perhaps floating on too much material, and you may need to wick additional liquid from under it.

7. Repeat the procedure in step 6 for the other six perfusion solutions (II through VII) listed in table 5.1.

Table 5.1 Perfusion solutions used in the study of Glycerinated Muscle Myofibrils

Note: Use solutions at room temperature. Using a separate Pasteur pipette for each solution.

<table>
<thead>
<tr>
<th>Perfusion Solution</th>
<th>Designation of Test Solution</th>
<th>Ingredients of Test Solution</th>
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<tbody>
<tr>
<td>I</td>
<td>MES (Myosin Extraction Solution: Dissociates myosin from the myofibrils)</td>
<td>0.6 M KCl; 10 mM pyrophosphate; 10 mM Histidine-HCl; pH 7.0</td>
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<td>[Pyrophosphate solubilizes myosin. Histidine, an amino acid, functions as a buffer]</td>
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<tr>
<td>II</td>
<td>AMES (Actin and Myosin Extraction Solution: Dissociates both actin and myosin from myofibrils)</td>
<td>0.6 M KSCN; 10 mM Histidine-HCl; pH 7.0</td>
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<tr>
<td></td>
<td></td>
<td>[Potassium thiocyanate (KSCN) solubilizes both myosin and actin.]</td>
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<tr>
<td>III</td>
<td>No ATP / Mg(^{2+}) / Ca(^{2+})</td>
<td>100 mM KCl; 5 mM MgCl(_2); 5 mM CaCl(_2); 5 mM disodium EDTA; 5 mM EGTA; 1 mM DTT; 10 mM Histidine-HCl; pH 7.0</td>
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<tr>
<td></td>
<td></td>
<td>[Solutions III - V differ in the presence or absence of ATP and/or Ca(^{2+}) ion. DTT is a reducing agent that stabilizes myosin ATPase.]</td>
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<tr>
<td>IV</td>
<td>ATP / Mg(^{2+}) / No Ca(^{2+})</td>
<td>100 mM KCl; 1 mM ATP; 5 mM disodium EDTA; 5 mM EGTA; 1 mM DTT; 10 mM Histidine-HCl; pH 7.0</td>
</tr>
<tr>
<td>V</td>
<td>ATP / Mg(^{2+}) / Ca(^{2+})</td>
<td>100 mM KCl; 1 mM ATP; 5 mM MgCl(_2); 5 mM disodium EDTA; 5 mM EGTA; 1 mM DTT; 10 mM Histidine-HCl; pH 7.0</td>
</tr>
<tr>
<td>VI</td>
<td>ATP / Mg(^{2+}) / Ca(^{2+}) with BDM</td>
<td>100 mM KCl; 1 mM ATP; 5 mM CaCl(_2); 5 mM disodium EDTA; 5 mM EGTA; 1 mM DTT; 10 mM Histidine-HCl; pH 7.0; 10 mM BDM</td>
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<tr>
<td></td>
<td></td>
<td>[BDM (2,3-Butanedione monoxime) inhibits myosin ATPase.]</td>
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<tr>
<td>VII</td>
<td>ATP / Mg(^{2+}) / Ca(^{2+}) with P(_i), low pH</td>
<td>100 mM KCl; 1 mM ATP; 5 mM MgCl(_2); 5 mM CaCl(_2); 5 mM disodium EDTA; 5 mM EGTA; 1 mM DTT; 10 mM Histidine-HCl, 20 mM P(_i); pH 6.0</td>
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<tr>
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<td>[Excess phosphate (P(_i)) and low pH imitate conditions of muscle fatigue.]</td>
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Agitate the myofibril suspension briefly just prior to taking each sample. It is imperative that each test solution be thoroughly perfused under the cover glass. If the myofibrils are not completely perfused with the test solution, you will not be able to observe its effect on myofibril structure or contraction. For each perfusion solution, be certain to do the following:

☐ a. Observe the myofibrils at 400X as perfusion is taking place. Only when perfusion is complete and you’ve had a chance to observe whether or not the myofibrils contract, switch to 1000X under oil to make your final observations of myofibril structure. Complete contraction, when it occurs, takes place rapidly. If a rapid change does take place, you may want to repeat the perfusion so that each person in your group can observe it. Use the mechanical stage control to follow the wave of contraction across the cover glass as the active perfusion solution comes into contact with myofibrils. It may not be possible to measure the length of a partially or completely contracted sarcomere since in vitro contraction typically results in the muscle myofibrils contracting into tight, spindle-shaped structures. Record your observations of contraction in the form of sketches and written descriptions. Categorize the degree of contraction as none, light, moderate or strong.

☐ b. Make thorough written descriptions of the treated myofibrils and their responses and changes, if any, to the perfusion solution.

☐ c. Sketch and label the appearance of the myofibrils following treatment.

☐ d. Measure the length (in µm) of several sarcomeres to determine the effect, if any, of the perfusion solution on the contraction of myofibrils. Calculate and record mean sarcomere length.

Note: Much of the calcium in glycerinated muscle remains sequestered in the sarcoplasmic reticulum, making it unlikely that all of the endogenous calcium ions in the myofibrils can be removed even after an extended soaking in chelating solution containing EGTA. Please keep this point in mind as you interpret your results.

V. LABORATORY WRITE-UP

The details for the write-up will be discussed during lab.

Literature Cited:
**DATA SHEETS FOR LABORATORY 5**
**MUSCLE CONTRACTION**

*Chemical Requirements for the In Vitro Contraction of Glycerinated Myofibrils*

Labeled sketches, sarcomere lengths (µm), descriptions (no, slight, moderate, strong contraction)

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<th>Untreated</th>
<th>Treated with Perf. Soln. I: MES</th>
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<tr>
<th>Treated with Perf. Soln. II: AMES</th>
<th>Treated with Perf. Soln. III: No ATP / Mg$^{2+}$ / Ca$^{2+}$</th>
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| Treated with Perf. Soln. IV:  
  ATP / Mg$^{2+}$ / No Ca$^{2+}$ | Treated with Perf. Soln. V:  
  ATP / Mg$^{2+}$ / Ca$^{2+}$ |
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| Treated with Perf. Soln. VI:  
  ATP / Mg$^{2+}$ / Ca$^{2+}$ with BDM$^+$ | Treated with Perf. Soln. VII:  
  ATP/Mg$^{2+}$/Ca$^{2+}$ w/ P$i$ , low pH |
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I. INTRODUCTION
The Zeiss Axiostar microscope is set up for phase-contrast microscopy. This type of microscopy is ideal for viewing transparent specimens, such as living cells. The microscope capitalizes upon the fact that some structures, such as membranes and cell organelles, alter light waves passing them. These cell structures both refract (that is, bend) the light and alter the phase of the light waves (that is, move the wave peaks forward or backward). This phase change allows the phase-contrast microscope to highlight the refractive structures and make them easier to see.

II. THE ZEISS AXIOSTAR MICROSCOPE
The Zeiss Axiostar microscope is very similar to the Nikon microscopes you used two weeks ago – see Figure 1 below.

The main differences between the Nikon Compound microscope and the Zeiss Axiostar are:
1. The lamp brightness knob (#5 on figure below) is above the lamp on/off switch.
2. The lamp has a diaphragm (#11 on figure).
3. The condenser has both a "brightfield" setting ('H' on the condenser dial) and a phase-contrast setting ('Ph' on the condenser dial). The 'H' setting illuminates your specimen just like on the Nikon microscopes. There is also a "darkfield" ('DF' on the condenser dial) setting which creates a black background.
4. There are two special phase-contrast objectives (40x and 100x) – these objectives will only work when the dial on the condenser dial is set to "Ph2". Use the "H" setting for the 10x objective.

Figure 1. The Zeiss Axiostar Microscope.

Key to figure components:
1. Eyepieces
2. Binocular Tube
3. Arm
4. Knob for locking binocular tube
5. Brightness Control
6. On/Off Switch for lamp
7. Fine Focus Knob
8. Coarse Focus Knob
9 & 10. Stage Motion Knobs
11. Lamp Diaphragm
12. Condenser Diaphragm holder
13. Diaphragm lever
14. Condenser
15. Centering screw
16. Stage
17. Objective
18. Nosepiece
III. USING THE ZEISS AXIOSTAR

A. Adjusting the Zeiss Microscope
(Critical Focus adjustment)

1. Plug in the microscope and turn on the light source. Adjust the brightness control so that the lamp is set to a medium intensity.

2. If it isn't already in position, rotate the nosepiece until the "low-power" (i.e., 10x) objective is selected.

3. Set the diaphragm iris lever about 2/3 open – i.e., so that the lever is about 1/3 from the brightest position.

4. Adjust the condenser so that it is at its highest position under the stage (turn the small knob on the left side of sub-stage condenser).

5. Put a specimen slide in the slide holder and focus on the specimen. Alternatively, you may use a slide on which you put a mark with a permanent marker.

6. Look through both oculars and widen or narrow the distance between the oculars to match your interpupillary distance. If necessary, adjust the ocular diopter as in the directions in the lab manual for the Nikon compound microscope.

7. While looking through microscope, turn the knurled ring on the lamp (#11 in the figure) until you can see a hexagon. You may need to open or close the iris lever under the stage to see this hexagon.

8. Locate the small knob on the left side of sub-stage condenser (this knob adjusts the height of the condenser). Using this knob ONLY, focus the hexagon until the sides of the hexagon are as sharp as possible.

9. The hexagon should be in the center of the field of view. If it is not, turn one or both of the centering screws (#15 in the figure) to put the hexagon in the center.

10. Now turn the knurled ring on the lamp until the hexagon just disappears from view.

Your microscope is now ready for use.

B. Using Phase-Contrast

1. If it isn't already in position, rotate the nosepiece on your microscope until the 10x objective is selected.

2. Set the dial on the condenser to 'H'.

3. Insert your slide into the slide holder.

4. Focus your slide. If you are having trouble seeing your specimen, you can try setting the dial to 'PH2' (Phase contrast). This setting works with the 10x objective and may help increase contrast. If you're still having trouble finding your specimen, try focusing on the edge of your cover slip first.

5. Now turn the nosepiece and select the 40x objective.

6. Set the condenser dial to 'Ph2' - this is the phase setting that matches your objective (the phase setting is written on the objective).

7. Use the fine adjustment knob to focus your specimen.

If you see black, try opening the diaphragm lever on the condenser.