Bioc 415 – Experimental Physiology

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The concepts in this course will center on the physiology of membranes and of excitable cells. Please approach this field of study with the point of view that the cell membrane is a highly dynamic, structured, complex organelle, subject to the same physical laws as is any other structure. Cell membranes are probably responsible for more tasks than any other organelle. Such tasks include maintenance of steady state conditions within the cell and communication with the outside. Your understanding of every phenomenon addressed in this course begins with an appreciation for the role of the cell membrane.

In class we will discuss the concepts behind each study and will explore ways of learning about the structure and function of excitable cells through experimentation and observation. You will receive instruction and training in the use of specialized apparatus to collect data. You will design your own experiments based upon descriptions of the individual laboratory studies.

If you are like most biosciences majors your appreciation of the physical sciences and mathematics amounts to relief that you have made it past those "hurdles," and you are now doing your best to forget the experience. Try to appreciate the fact that cells, organs, and organisms live in a physical world, and that it is impossible to describe physiological processes or make predictions without applying mathematics and principles of physics and chemistry. Physics is probably the least appreciated of disciplines among students of biology. Table 1 presents some major topics that are covered in freshman physics, and how they apply to studies in physiology.

Prepare to take your skills to the next level. This course is designed to make you think (or, shall we say, to give you a much needed opportunity to exercise your ability to think).

Table 1. *Applications of physics to the study of physiology*

Concepts: Units, dimensions, vectors
Measurement of membrane potentials
Extracellular potentials
Force of muscle contraction
Units of conversion
Rates of changes
Precision and significant figures
Concepts: Work and energy
Nernst and Goldman equations
Isotonic, isometric muscle contraction
Concepts: Light and optics
Light microscopy (bright field)
Phase contrast microscopy
Muscle striations (diffraction)
Concept: Waves
Calibrating and troubleshooting instrumentation
Concepts: Electricity and magnetism
Membrane potential and measurement
Filters for signal conditioning
Compound action potentials
Electrocardiogram
Transducers
Amplifiers
Faraday cage (Gauss' law)

1 Origin of the Membrane Potential

Every cell membrane is a tiny power generator, maintaining an electrical potential difference, called a membrane potential (E_m), between the inside and outside of a cell. In the following study you will explore the origin of the membrane potential and test two historically significant attempts to explain it. The membrane potential is important to our studies because it is through changes to E_m that excitable cells produce and propagate signals. More fundamentally, the plasma membrane's selective permeability, its ability to maintain a chemical concentration difference, and its steady state membrane potential are fundamental and universal properties of living cells. Cells whose membranes do not have these properties are no longer living.

Measuring a membrane potential

All of the studies in this section require measuring membrane potentials from suitable tissue. We chose a crayfish preparation because of the general hardiness of the tissue, its long muscle fibers, and the thinness of the muscle sheet. In addition, crayfish tonic extensor muscle fibers do not contract when manipulated. Muscle contraction would break off the delicate microelectrode tip.

Apparatus

The components of the system include a stand to hold a dish for the crayfish preparation, a fiber-optic light to illuminate the preparation, a stereomicroscope (dissecting microscope), and of course a system for taking measurements and for recording data. You will measure membrane potentials by impaling the muscle with a glass microelectrode that is filled with 1M potassium chloride (KCl) and mounted on an active probe leading to an amplifier. The probe will be mounted on a micromanipulator so that the microelectrode can be precisely positioned and lowered into the tissue to impale a single cell.

Why the salt-filled microelectrode and active probe? Why not just stick a metal electrode into the cell and hook it to an oscilloscope? For one thing, poking something big through a cell membrane will usually kill the cell. Second, when a cell is impaled we have to complete a circuit in order to measure the voltage. Metal carries current through electron flow, but electric current in a cell is carried by ions. A third problem that to measure a voltage the cell membrane must act as a small battery, driving current through the circuit. A single cell hasn't the power with which to deliver a detectable signal to a digital recording device or to an oscilloscope.

To overcome these obstacles we pull glass microelectrodes with very small tips that can impale a cell without causing significant damage. The membrane readily re-seals around a symmetrical microelectrode tip of a few micrometers diameter. Second, we fill the microelectrode with concentrated KCl so that the KCl solution is contiguous with the cytoplasm and can carry current. Ion flow is converted to electron flow by placing the KCl in contact with a silver (Ag) electrode that is coated with silver chloride (AgCl). As current flows, chloride ions become deposited on the silver electrode or go into solution from the coating, depending on direction of the current. Thus, an Ag/AgCl electrode acts as a *transducer* to convert between ionic conduction and conduction by electrons.

A cell membrane can produce a very small electrical current without compromising its function. That tiny current, on the order of nanoamperes, must be amplified if it is to be recorded. Probe/preamplifier combinations are designed to condition the signal by greatly amplifying the current generated by the cell, either using an operational amplifier or a Wheatstone bridge circuit. Knowing the amplification factor, we can use Ohm's Law to measure E_m.

Traditionally, a physiologist would use an oscilloscope to measure bioelectrical phenom-

ena to overcome the problem of inertia with mechanical recording devices. Analog to digital (A/D) recording devices overcome that problem and add the advantage of storing all of the data on a computer drive.

Prepare a crayfish

Obtain a specially prepared dish containing a thick silicone layer for pinning the preparation and fill it with control saline solution. Remove the claws from a crayfish by twisting them off, then separate the abdomen from the cephalothorax by first cutting the exoskeleton and then the soft tissue as shown in figure 1.1. The rough treatment does not "torture" the animal. The crayfish nervous system is much less complex than ours, they do not feel pain in the way that vertebrate animals feel pain, in fact these animals are probably not even self-aware.

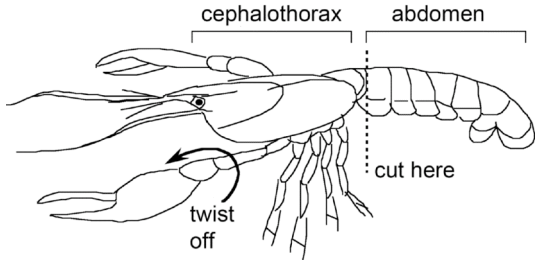

Fig. 1.1. Dissecting a crayfish. Twist off each claw, with care to prevent injury (to yourself, not the crayfish). Cut off the abdomen with care not to stretch the muscle.

Throughout the dissection, keep the muscle tissue moistened with crayfish saline to prevent it from drying out. Cut the upper exoskeleton parallel to the midline on both sides, just medial to the juncture between upper and lower exoskeleton (figure 1.2). Make sure you cut the connective tissue and not the muscle. Roll the underbelly back and gently undercut to remove the large mass of flexor muscles, exposing the deep extensors. Try not to contact the extensor muscles themselves. The intestine may remain attached to the upper shell or peel up with the flexors. If it remains attached gently remove it after completely removing the flexor muscles and cutting off the flipper.

You have now exposed the deep extensor muscles, attached to the upper shell. The deep extensors are the longitudinal muscle masses that are symmetrically disposed around the midline. The more medial, coiled muscle is the medial deep extensor while the more lateral mass composed of straight fibers consists of the lateral deep extensors.

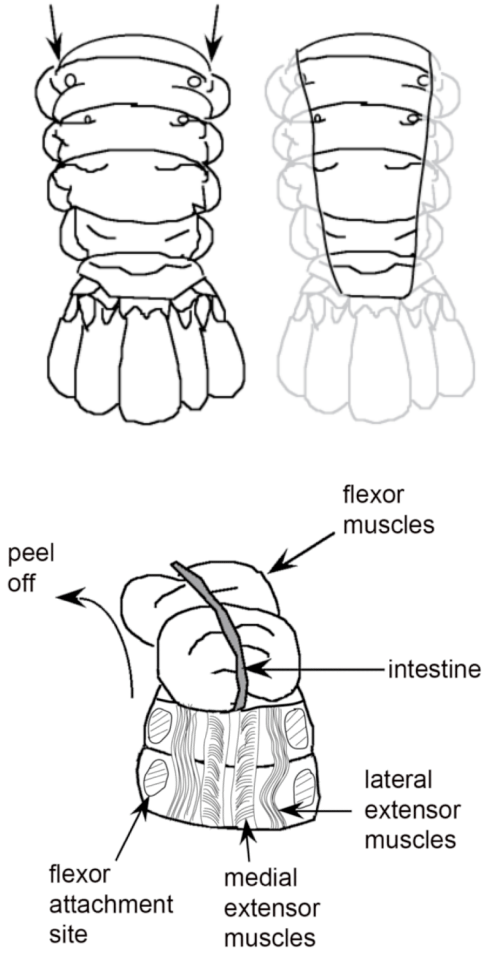

Fig. 1.2. Preparing the extensor muscles for microelectrode work. *[top figure]* Insert one blade of a sharp pair of heavy dissecting scissors into the cut end of the abdomen just medial to the place in which dorsal and ventral exoskeleton meet *(left)*. Cut the dorsal exoskeleton on both sides all the way to the flipper. Use fine dissecting scissors to remove the flexor muscles from the dorsal exoskeleton, leaving the piece outlined *(right)*. *[bottom figure]* Cut the flexors at their attachment sites, two per segment. Removing the flexor muscles reveals the medial and lateral deep extensor muscles.

Place the shell with muscle layers up in the dish and pin it to the bottom without stretching the muscles. Keep the tissue immersed from now on. Line up the dish and microscope so that you can clearly see two ribbons of medial extensor muscles on either side of the midline, flanked by the lateral extensors and by the cut ends of the flexor muscles. A good preparation will look very clean. If the solution is cloudy then it may be necessary to remove damaged tissue and/or change the solution.

Freshly cut tissues, particularly the cut ends of the flexor muscles, will release their contents into the medium, flooding it with excess potassium ion, which is the principle cation inside a cell. Until the tissue is fully equilibrated the excess potassium may influence your measurements. Soon you will explore the influence of extracellular potassium ion concentration on E_m in some detail.

Set up the recording system

The recording device consists of an active probe with gold plated tip on which we mount an electrode holder with KCl-filled glass microelectrode. The device includes a ground (reference) wire with alligator clip, a shielded cable with connector, and an electrode test circuit with button. Clip the reference wire to the probe tip to ground the input. Set up your screen with the trace in the middle of the channel and with a scale permitting measurements of ± 200 mV.

Now insert a 10 megohm (10M) resistor between probe tip and reference wire. The trace will remain in the same place if no current is flowing through the system. If it has moved, then disconnect and reconnect the probe (hold the connector, not the cable!) to discharge the circuit, re-center the trace, and check it again with and without the resistor in place.

Press the *electrode test* button and hold it for a second or two to record the voltage drop across the resistor. Knowing resistance and having measured voltage, you can use Ohm's Law to determine the current delivered through the probe tip when you press the *electrode test* button. The *electrode test* button will always deliver the same current, so now you can use the system to determine an unknown resistance.

Insert the end of the Ag/AgCl ground wire into the crayfish saline just under the surface. Clip the reference wire to the other end of the electrode, near the post. Obtain a microelectrode with holder and place it onto the probe. Use the micromanipulator to lower the tip into the saline, barely immersing the tip without contacting the tissue. You cannot see the actual tip, which is very thin, but you should see ripples as it contacts the saline. Repeat the test that you performed with the 10M resistor (press the *electrode test* button). You should obtain a square wave with voltage change proportional to the microelectrode tip resistance. A resistance between 10 and 40M is fine. Outside that range, the tip may be so large that it damages the cell membrane irreversibly, or so small that it clogs or breaks off when it contacts the tissue.

These are ambitious studies, and at first the "learning curve" will be steep. You likely will go through a lot of microelectrodes before you become proficient. Be patient. You have more than enough time to finish the project.

Obtain control membrane potentials

Careful technique requires that you record such information as electrode tip resistance. However, the recording system has an extremely high input resistance, so the precise value of the electrode resistance has very little bearing on determining the E_m.

One measures a membrane potential by lowering a microelectrode over a clear area of muscle until you see a deviation in the trace, indicating contact with the tissue. A gentle tap on the base of the micromanipulator is usually enough to make the cell surface bounce up into the electrode, the electrode will penetrate the cell, and the trace will suddenly drop. Wait a few seconds for it to become steady as the membrane seals around the microelectrode. Cell impalement is an acquired skill, so don't be disappointed if it takes you an hour or so to master the technique. You will obtain the most reliable measurements by gently entering tissue using the fine micromanipulator control, using the coarse control only for get-

ting close to the tissue or lifting the microelectrode out of the saline.

Record the E_m or simply mark the record and go back later to take the measurements. Do make sure that your measurements are reasonable, though, before proceeding too far. In control saline a well equilibrated preparation should give a potential on the order of 60-65 mV. After obtaining a measurement, raise the electrode above the tissue (but not out of the solution) so that the voltage returns to baseline. If baseline has drifted use the position control to reset it. Use the micromanipulator to move to a region a millimeter or two away, re-test the electrode, and penetrate a new cell. Move only very short distances with the electrode tip close to the tissue surface. Any lateral pressure on a tip will break it off. If you measure a significant drop in tip resistance it has probably broken and its performance will be compromised. If you measure a significant increase in resistance and/or the electrode test does not produce a "clean" square wave, then the tip may be clogged. Either way, the solution is to replace the microelectrode.

Data collection

Use your best judgment to determine how many replicate measurements you need. You'll need less if they are all identical, more if they are inconsistent. Have a plan for dealing with outliers (unrealistically high or low potentials).

Once you have sufficient control measurements you can change solutions. Simply pour off the saline solution, replace with the next solution, wait about five minutes and replace the solution again. While you wait for the tissue to equilibrate you can go back to your chart record and record your E_m measurements. Save the record. It is probably best to open a new file for each new series of measurements.

Keep track of the data as you go along and watch for systematic error, such as hysteresis. In this context hysteresis refers to the influence of the direction of change on a final result. For example, if you change solutions from control saline to solution containing ouabain, the deeper tissues may not be equilibrated by the time you take data. Similarly, when you attempt to wash out the ouabain the deeper tissues may remain inhibited for some time. Hysteresis will definitely complicate attempts to look at effects of changing extracellular ion concentrations in later experiments. In replicate experiments, experienced researchers vary the direction in which they change an independent variable or they may select values for independent variables at random. The object is to avoid bias that may be introduced by the direction in which a variable is changed.

Please do not conduct an extra rinse with saline containing oubain, which is very expensive. A second rinse is only necessary when we change the ionic composition of the saline and/or must wash out a substance. To remove most of the oubain you will want to conduct an extra rinse or two when you go back from a oubain-containing solution to control saline.

Troubleshooting

Recall that the direction of current is defined as the direction of movement of positive ions. You are working with a simple circuit in which current moves into the negatively charged interior of a cell. Chloride ions move from cell interior to the silver chloride interface and from there electrons move into the IC probe. The reference electrode is simply an Ag/AgCl electrode attached to the ground wire of the probe, with its tip immersed in the saline dish.

If you cannot find or center a trace, check that you have a complete circuit. Check that the ground wire is connected and immersed in saline. If you can't find a signal after tracing the circuit or is very noisy or unstable after checking for sources of interference, then conduct an electrode test. If the trace indicates a very high resistance and/or is unstable the tip is probably clogged or blocked by an air bubble. Get another microelectrode.

If during an experiment you find that the baseline moves when you simply move the microelectrode around in the solution without contacting tissue, or if you have trouble zeroing the baseline, then repeat the diagnostic procedures that you did when you initially set up to record, and troubleshoot the system accordingly. Chances are the problem is a faulty

microelectrode or microelectrode holder. We go through a lot of microelectrodes.

If you see a sine wave or complex wave with a frequency of 60 per second on a magnified section of record, then you have 60 cycle interference due to electric fields most likely generated by the fluorescent lights in the room, the fiber-optic power supply, or by a nearby computer. A common remedy is to work inside a Faraday cage, but we have found that with a well-grounded preparation a cage is unnecessary. You have low-pass filters built into your recording system. Condition the signal by filtering out frequencies of 50 Hz or more.

If you continually get low membrane potentials in your control preparation (< 55 mV or so) check that electrode resistance is at least 10M. Check for "leakage current" by grounding the probe tip and checking the signal with and without a 10M resistor inserted in the circuit. If the baseline signal shifts position with the resistor in the circuit, then discharge the device as described before. Use the ELECTRODE TEST button to check that the system measures the correct voltage for a 10M resistor.

If the system checks out, then examine the saline itself, which should be clear and not contaminated by bits of cut tissue and/or connective tissue. Change it if necessary. Try a completely new section of the preparation, away from the cut ends of the muscle. If you have been trying the lateral extensors, then switch to the medial, or *vice versa*. Try to enter each cell very gently. Poking through them quickly can clog or break the microelectrode, or enlarge the hole in the cell membrane so that it does not seal properly. Once you get a "good" reading, you need not move far away to take the second and later readings. With practice, you can get a dozen good readings in a couple of minutes.

If you can't find fault with your technique and you have gotten consistently low potentials in several areas of the preparation, don't waste any more of your time on it. Some preparations simply fail, perhaps from rough handling during the dissection or lack of stamina of the preparation due to parasitism of the animal. Obtain a new crayfish.

Above all, don't get frustrated. As soon as you get a good preparation, data collection will go very quickly.

Investigation: role of the sodium-potassium pump

Embedded in the membranes of living cells is a protein complex called sodium-potassium (Na^+-K^+) ATPase, often called the sodium-potassium pump, or simply the sodium pump (figure 1.3). The sodium pump maintains concentration gradients across the cell membrane by exchanging sodium ions for potassium ions, using free energy from the hydrolysis of ATP. For each ATP that is hydrolyzed, the pump expels three sodium ions from the cell, bringing two potassium ions into the cell. The potassium ion concentration in a cell interior is typically 20x greater than the sodium ion concentration, while the extracellular concentration of sodium ion is typically 40x that of extracellular potassium ion.

By exchanging three positive ions for two with each cycle, the pump also displaces a net positive charge to the outside of the cell. We refer to this net displacement as the "electrogenic" property of the sodium pump. The electrogenic effect continues as long as the pump is operating, with the magnitude of the contribution proportional to pump activity.

Membrane potentials vary with species and cell type, but typical values are -60 to -70 mV. The potential differences are described as negative values because we measure intracelluar potential with respect to an extracellular electrode. What is the reason for the potential difference? That is, what is the origin of the membrane potential?

Electrogenic pump activity alone can account for a small potential difference across a cell membrane. However, even if the pump exchanged positive ions one for one, it would produce a significant membrane potential. The next section will present a mechanism by which a semipermeable membrane maintains an electrical potential difference due to ion concentration gradients set up by the pump. For now, let's see how much of a typical membrane potential can be attributed to the electrogenic property.

Inhibiting the sodium pump

The poison oubain, a cardiac glycoside, shuts down the sodium pump almost completely. It was originally extracted from the seeds of *Strophanthus* species. Oubain inhibits the sodium pump by binding the complex on the extracellular side. Because binding is reversible and extracellular, we can quickly wash out ouabain from a preparation, allowing for well controlled experiments.

Sodium pump activity in oubain-sensitive tissues such as human heart is blocked with a concentration as low as 0.1 mM. Oubain-insensitive tissues such as frog or rat heart take up to 5 mM ouabain. Past experiments suggest that 1 mM oubain in normal crayfish saline will shut down nearly all sodium pump activity in muscles of *Procambarus clarkii*, the animal we will use for our studies. Normal (control) crayfish saline has the composition 205 mM NaCl, 1.0 mM $CaCl_2$, 12.3 mM $MgCl_2$, 5.4 mM KCl, and 2.4 mM $NaHCO_3$.

Experimental design: contribution of electrogenic activity to the membrane potential

Your first objective will be to determine the relative role of the electrogenic property of the sodium pump in maintaining a membrane potential. Consider the dual role of the pump. The electrogenic effect is present only while the pump is active, while the chemical gradients set up by the pump should last for a long time, even after the pump is shut down. We should be able to assess the relative contribution of the electrogenic property by comparing membrane potentials in ouabain-treated tissues with potentials in untreated tissues.

Your first assignment is to prepare an experimental design using the following outline as a model.

- Title
- Objectives: write objectives as statements that can be tested through experimentation.
- Data to be collected: what will you measure (include meaningful units), from what experimental preparations will you take data (species, tissues, treatments), how many samples will you take, will the experiment be repeated?
- Data analysis: how will you present the data, test any hypotheses, and/or apply statistics?
- Complications/controls: try to anticipate problems that might come up (this part will be easier after you have had some practice taking data); what control experiments must you conduct?

We treat all data collected in this laboratory as common property. We can be much more confident in the results from several experiments than the results from just one. Therefore we will attempt to collect data from all investigators for distribution to the team.

Equilibrium potential model

Around the beginning of the 20th century neither the existence of a sodium pump nor the fluid nature of a cell membrane were known. However, the ion-selective nature of membranes and the presence of large sodium and potassium gradients were recognized. Physiologists knew that $[K^+]$ inside a cell is much higher than it is outside a cell. They had evidence that the cell membrane is impermeable to Na^+ and somewhat permeable to K^+. On those bases, and inspired by the recent development of the Nernst-Planck equation by Walther Nernst, Julius Bernstein suggested that the cell membrane potential is the equilibrium potential (E_K) for potassium ion. In this model potassium ions move down the concentration gradient, carrying positive charges across the membrane. Movement of charge sets up an electrical potential difference that increasingly opposes further movement of K^+ from inside to outside. At equilibrium the electrical potential difference set up by the gradient is just sufficient to prevent further net K^+ movement in or out of the cell (figure 1.3). Ionic concentrations inside and outside the cell and the membrane potential all remain unchanged unless perturbed.

Julius Bernstein was the first to use the Nernst equation for potassium to predict the normal steady state potential. The terms "equilibrium potential model," "potassium electrode

hypothesis," and "Bernstein model" all refer to the use of E_K to predict E_m. The model was consistent with the direction of change in membrane potential in response to changes in concentration of extracellular potassium. That is, raising the extracellular potassium ion concentration reduced the potential difference and lowering the concentration increased the potential difference. At that time, though, the technology for penetrating a single cell to measure a membrane potential did not exist. Physiologists could not determine how close E_K comes to the actual magnitude of E_m.

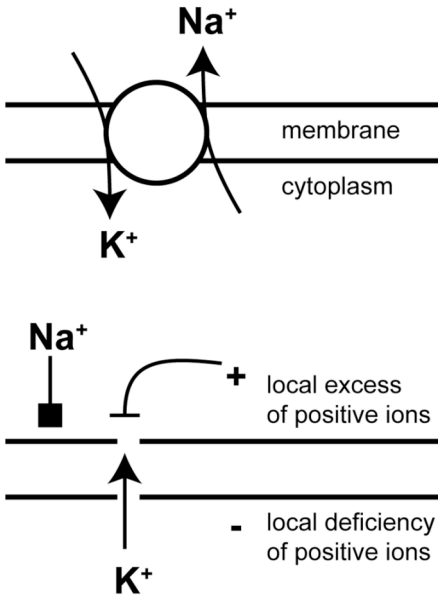

Fig. 1.3. A sodium-potassium pump maintains a high concentration of potassium ion and low concentration of sodium ion in the cytoplasm *(top)*. The equilibrium potential model of the resting membrane potential holds that the cell membrane is permeable to potassium ions but not to sodium ions. Potassium ions moving down the concentration gradient carry positive changes, creating a local excess of positive ions outside the membrane and a local deficiency of positive ions inside the membrane. At equilibrium *(bottom)* the electrical potential difference is just sufficient to prevent the net movement of potassium ions out of the cell.

Nernst-Planck equation for determining an equilibrium potential

Walther Nernst (1864-1941) was born in Prussia, received his doctorate in physics in 1887, and became a physical chemist while working with Wilhelm Ostwald. Nernst received a Nobel prize in chemistry in 1920. His life spanned a very productive era in the physical sciences. Nernst associated with the likes of Albert Einstein, Max Planck, and Niels Bohr, among other renowned scientists. He is best known for the development of the third law of thermodynamics and for his contributions to electrochemistry.

The Nernst-Planck equation (more commonly called simply the Nernst equation) can be derived from the equation for the free energy change (ΔG) for transporting 1 mole of cation (c^+) across a membrane. The two terms of the equation refer to osmotic work and electrical work, respectively.

$$\Delta G = RT \cdot \ln\left(\frac{[c^+]_i}{[c^+]_o}\right) + zFE$$

R = universal gas constant, 8.3 joules/mole ˚K
T = temperature in ˚K (273 plus ambient temp. in ˚C)
z = charge on the ion
F = Faraday's constant, 96,500 coulombs/mole
$[c^+]_i$, $[c^+]_o$ = Concentrations inside and outside of a closed membrane, respectively
E = Equilibrium potential

At equilibrium, ΔG for moving one particle across a membrane approaches 0, therefore

$$0 = RT\ln\left(\frac{[c^+]_i}{[c^+]_o}\right) + zFE,$$

$$zFE = -RT\ln\left(\frac{[c^+]_i}{[c^+]_o}\right), \text{ and}$$

$$E = -\frac{RT}{zF}\ln\left(\frac{[c^+]_i}{[c^+]_o}\right).$$

When applied to the equilibrium potential model in which $E_m = E_K$, the Nernst equation for potassium can be used in to predict the membrane potential in volts.

$$E_m = E_K = -\frac{RT}{zF}\ln\left(\frac{[K^+]_i}{[K^+]_o}\right)$$

E_K = equilibrium potential for K^+ across the membrane, in volts
$[K^+]_i$, $[K^+]_o$ = concentrations of K^+ inside and outside of a cell, respectively

Shortcut using log base 10

It is "good enough" for our experiments to use a quick method for estimating E_m. At 18˚C, which is close enough to the temperatures of our preparations,

$$E_m(mV) = -58 \bullet \log\left(\frac{[X]_{in}}{[X]_{out}}\right)$$

For example, log(10) = 1, so if you have a ten-fold gradient inside to outside for a cation, E_m is -58 mV. You must reverse the sign for an anion.

Experiment: influence of $[K^+]_{out}$ on E_m

In the mid-19th century came the development of the technology to impale single cells and measure a true membrane potential. You have already used similar technology to estimate the relative contribution to E_m by the electrogenic property of the sodium pump. What did you conclude from that experiment?

Now it is time to explore the contribution of ionic gradients to E_m. The ion with the greatest influence should be K^+. In fact, if the equilibrium potential model is correct then K^+ will be the only ion to affect E_m.

You will have time to conduct a complete experiment on one crayfish preparation. However, results from all experiments will be compiled and made available to you for the write-up. Plan to equilibrate tissues and conduct sampling as you did for the ouabain study.

Assignment

Prepare an experimental design to test the influence of K^+ on E_m by varying the K^+ concentration in the extracelluar solution. Table 1.1 describes test solutions that will be available in the laboratory. Start by writing down questions and/or hypotheses that are relevant to this study. Think about the simplest, most effective ways of analyzing and presenting the findings. Consider all factors that may complicate the experiment.

Suggestions for the experimental design

You will want to compare your measured E_m with calculated E_K values. To calculate E_K you need to know that the intracellular concentrations of potassium ion and sodium ion in crayfish muscle are 180 mM and 12 mM respectively. Check that your calculated values are reasonable. All should be well within an order of magnitude of 0.1 V.

Table 1.1. *Saline solutions with variable potassium concentrations for crayfish muscle. Sodium chloride concentration was adjusted as [KCl] was increased, so that each solution exerts the same osmotic pressure on the tissues.*

Solution[1]	[NaCl] (mM)	[KCl] (mM)
Normal	205	5.4
0.3 x K_{out}	210	1.6
2 x K_{out}	198	10.7
5 x K_{out}	178	26.8

[1]All crayfish saline solutions include 1.0 mM $CaCl_2$, 12.3 mM $MgCl_2$, and 2.4 mM $NaHCO_3$. Total sodium ion concentration includes the contributions of both NaCl and $NaHCO_3$.

The potassium concentrations that we selected are among an infinite number of possible choices. That is, $[K^+]$ is a continuous independent variable. Likewise, both E_K and actual E_m are continuous dependent variables. E_m is directly related to the log of ion concentration, as can be seen from the Nernst equation. It is easier to compare observed with predicted values when one uses the logarithm of the appropriate variable to make the relationship linear. As you plan to analyze and present your results think carefully about how best to show the relationship between E_m and ion concentration and (preferably) at the same time show any deviations from predicted values.

Summary of the equilibrium potential model

To fully understand the nature of an equilibrium potential and to be able to discuss your findings, try to make sense of the following statements. Try to visualize the conditions and events that are described.

Observations

- Cell membranes maintain an electrical potential difference (E_m) between the exterior side and interior side, with the interior negative
- A cell interior has very high $[K^+]$ while $[K^+]$ is very low outside the cell
- An ion passively diffuses down its chemical concentration gradient, carrying charge with it, creating an electrical potential difference
- Passive diffusion of an ion down its concentration gradient has no significant influence on the concentration of that ion in or outside of the cell

Assumptions

- An asymmetrical distribution of ions across the membrane produces the E_m
- A typical cell membrane is permeable to K^+ but not to other ions
- Equilibrium is reached when the energy available to move K^+ against the electrical potential equals the available energy due to the chemical concentration difference

Hypothesis

- A cell membrane acts as a potassium electrode, so that $E_m = E_K$

Predictions based upon the model

- Changing $[K^+]$ outside the cell will change E_m
- Changing the extracellular concentration of other ions will not influence E_m
- E_K accurately predicts E_m under physiological conditions
- At equilibrium, the actual number of displaced ions is insignificant relative to the total number of ions in the system
- At equilibrium, potassium flux (movement across the membrane) continues, but there is no net flux

Diffusion potential model

Along with the technology to measure membrane potentials came the discovery that E_K is a very rough approximation of E_m. In fact the model completely broke down when attempting to explain action potentials. Part of the problem is that the Bernstein model only takes one ion into account and does not consider the relative permeability of cell membranes to other ionic species. Relevant ions include sodium, to which the cell membrane is permeable after all.

The current model for the E_m, (figure 1.4), holds that a constant diffusion of ions maintains an unequal separation of charge across a semipermeable cell membrane. The E_m is not a simple equilibrium potential after all. A passive 1:1 exchange of sodium and potassium ions down their respective concentration gradients maintains the E_m in a steady state, while the concentration gradients are maintained by sodium-potassium pump activity. Although sodium ions are present at higher concentration than potassium ions, membrane permeability to potassium ion is far higher than permeability to sodium ions (ratio 20:1 in crayfish muscle). Thus the membrane potential tends toward the equilibrium potential of potassium.

An ion's relative contribution to the membrane potential is influenced by it's conductance and its equilibrium potential (figure 1.4, bottom). Conductance, which is the inverse of resistance, refers to the tendency of an ion to traverse the membrane. Conductance is itself influenced by the ion's concentration in the system and permeability (P) of the membrane to the ion. For example, if permeability to sodium ion is reduced to zero then we have the equivalent of the equilibrium potential model, in which $E_K = E_m$. Opening sodium channels increases membrane permeability to sodium and thus its conductance, increasing the influence of sodium and driving E_m in the positive direction.

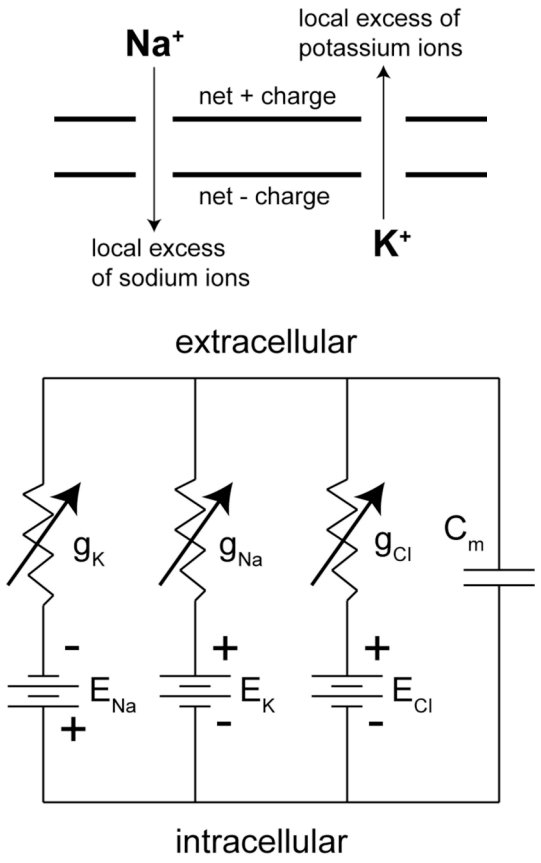

Fig. 1.4. *(top)* Ionic diffusion produces a "resting" membrane potential (E_m). *(bottom)* Equivalent electrical circuit of a resting membrane.

It should not be difficult to understand why the concentration difference is important, or why an ion will have little influence if either it's concentration or membrane permeability to the ions is very low relative to that of potassium ion. What is not intuitive for most of us is that in many cells, including crayfish muscle cells, chloride ion has no influence on E_m although chloride concentration is significant and the membrane is more permeable to chloride than to sodium. Chloride ion is not actively pumped and instead becomes passively distributed so that $E_{Cl} = E_m$.

To understand intuitively why chloride ion often fails to influence E_m you might consider the laws of thermodynamics. A membrane potential can perform work. It can drive ions against a concentration gradient, for example. Passive chloride ion movement can't set up a membrane potential because no work is done to distribute it across the membrane. The second law requires that to get work out of something we have to put more work into it than the system can return. We only consider a contribution by chloride ion it is actively pumped, expending energy.

The term "resting" as applied to a steady state membrane potential is misleading. Why? In the steady state ions are actively pumped, balanced by diffusion of sodium and potassium ions in both directions across the membrane. The cell membrane remains in a steady state until the concentration of one or more ions is changed or there is a change to the properties of the membrane.

Goldman constant field equation

The Goldman constant field equation was derived to predict the membrane potential (in volts) based upon the diffusion potential concept. It describes the result of contributions of three major electrolytes to the diffusion potential across a cell membrane. E_m is expressed by the Goldman-Hodgkin-Katz equation (its full name) as

$$E_m = -\frac{RT}{zF}\ln\left(\frac{P_{K^+}\left[K^+\right]_i + P_{Na^+}\left[Na^+\right]_i + P_{Cl^-}\left[Cl^-\right]_o}{P_{K^+}\left[K^+\right]_o + P_{Na^+}\left[Na^+\right]_o + P_{Cl^-}\left[Cl^-\right]_i}\right)$$

Terms are as described for the Nernst equation. Permeability of the membrane to potassium ion, P_{K+}, is arbitrarily set at 1, and P_{Na+} (relative to P_{K+}) is 0.05. Relative permeability of the membrane to chloride ion is estimated to be 0.5 relative to P_{K+}. Chloride ions are thought o follow the membrane potential passively, so that $E_{Cl-} = E_m$. Therefore to calculate E_m for crayfish muscle, one may use the abbreviated equation,

$$E_m = -\frac{RT}{zF}\ln\left(\frac{P_{K^+}\left[K^+\right]_i + P_{Na^+}\left[Na^+\right]_i}{P_{K^+}\left[K^+\right]_o + P_{Na^+}\left[Na^+\right]_o}\right)$$

Experiment: influence of $[Na]_{out}$ on E_m

You already have data with which to assess the predictive value of the Goldman equation. The diffusion potential model calls for potassium ion to have the greatest influence on E_m, so you incorporate your data from experiments with variable extracellular potassium

ion concentrations to this study. Estimates of E_m using the Goldman equation will differ from those using the Nernst equation because the diffusion model includes the influence of sodium ions.

Table 1.2. *Saline solutions with variable sodium concentrations for crayfish muscle. Choline chloride was substituted for NaCl so that each solution exerts the same osmotic pressure on the tissues.*

Solution[1]	[choline Cl] (mM)	[NaCl] (mM)
Normal	0	205
0.05 x Na_{out}	194	8
0.2 x Na_{out}	165	41
0.5 x Na_{out}	103	101

[1]All crayfish saline solutions include 1.0 mM $CaCl_2$, 12.3 mM $MgCl_2$, and 2.4 mM $NaHCO_3$. Total sodium ion concentration includes the contributions of both NaCl and $NaHCO_3$.

In the diffusion potential model, varying the extracellular sodium ion concentrations should affect E_m in a predictable way. Can you suggest what way? We cannot significantly raise extracellular $[Na^+]$ without throwing the cells into osmotic shock, but we can lower $[Na^+]_{out}$ if we substitute choline chloride for NaCl to maintain osmotic pressure (table 2). The cell membrane is impermeable to choline, thus its presence cannot influence the membrane potential.

Summary of the diffusion potential model

Have a look back at the summary of the equilibrium potential model. The original observations should remain true. Some of the other statements may be true as well, while others must be changed. What can you write up to summarize the principles of the diffusion potential model? You might incorporate such material into the discussion of your first research paper.

Research paper

The completed study is to be written up as a full research article according to the guidelines used by our courses in experimental biosciences (Bioc 211 and 311). Include full citations for any outside references that you use. You are strongly urged to review the guidelines and review instructors' comments on your past work, if available.

2 Properties of the Vertebrate Nerve Axon

Vertebrate nerves consist of cell bodies, axons, and specialized endings called synapses. They are responsible for a remarkable variety of communication functions, including delivery of sensory information to the brain, mediating local reflexes, and autonomic functions such as control of heart rate, breathing, and visceral function. Nerves also control voluntary muscle function, to be studied later in this course. The macroscopic structures that you identify as nerves during dissection of any vertebrate are really bundles of nerve axons without the cell bodies. The bundles are capable of conducting impulses *in vitro,* in fact the frog sciatic nerve bundle has been one of the most useful models for learning the properties of nerve conduction.

To be able to conduct, analyze, and interpret studies on vertebrate nerve you must be comfortable with the concept and mechanism of an action potential and be able to distinguish a compound (extracellular) membrane potential from a trans-membrane (intracellular) potential.

Action potentials

Membrane potentials of excitable cells are distinguishable from those of other cell types because they undergo dramatic transient changes in response to external or internal stimuli. Chemical, electrical, and mechanical stimuli all operate in nature to produce changes in membrane potential that we call an action potential.

Membranes of vertebrate nerve and muscle cells are populated by voltage-gated ion channels. A voltage-gated channel is triggered to open or close at some value of the membrane potential. In the resting phase (figure 2.1, A) ion movement is carried by chronically open rest channels. To begin an action potential an electrical stimulus partially depolarizes an excitable membrane to a threshold potential at which a population of voltage gated sodium channels open up. Open sodium channels dramatically increase membrane permeability to sodium ion, so that $g_{Na} >> g_K$. The membrane depolarizes very rapidly, producing the rising phase (B) of the action potential. Voltage-gated sodium channels are unstable in the open state and undergo a time dependent inactivation within a half millisecond or so. Before g_{Na} drops back to normal the E_m goes positive, producing what we call the overshoot phase (C). If the sodium channels remained open, then the relative contribution of the sodium ion gradient would continue to far exceed that of the potassium gradient, and E_m would approach E_{Na}, the equilibrium (Nernst) potential for sodium ion. Instead, though, the depolarization event is transient.

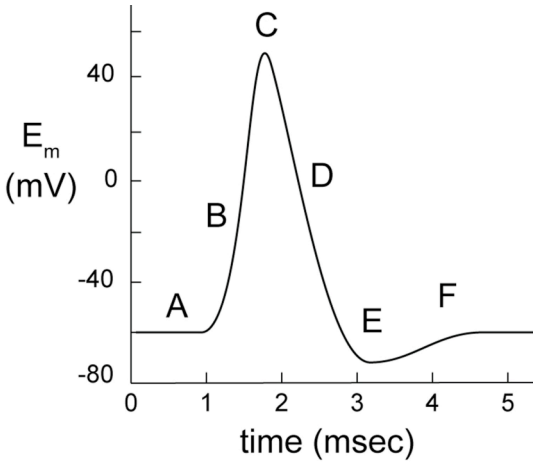

Fig. 2.1. Phases of an action potential as measured across a nerve axon membrane. *(A)* resting phase; *(B)* rising phase; *(C)* overshoot phase; *(D)* falling phase; *(E)* undershoot phase; *(F)* recovery phase.

Late in the rising phase of the action potential voltage-gated potassium channels open, increasing P_K by at least tenfold, causing g_K to increase to far above normal. Increased g_K produces the falling phase (D), characterized by a rapid drop of E_m toward the equilibrium potential for potassium ion. The falling phase follows the rising phase because, although voltage-gated sodium and potassium channels are triggered to open at the same threshold voltage, the sodium channels open immediately while potassium channels open after a

short delay. Voltage-gated potassium channels are slow to close, so that P_K and g_K remain high for a couple of msec, producing the undershoot phase (E).

The equilibrium model could not explain the undershoot phase, causing its eventual rejection when the technology to measure intracellular action potentials became available. Finally, during the recovery phase (F) the sodium channels reset and the potassium channels close, leaving the rest channels to again govern the membrane potential.

Passive current from an action potential in one region of nerve axon depolarizes adjacent regions of membrane, propagating action potentials down the length of the axon. An axon can conduct in either direction, although nervous systems are typically organized so that neurons are dynamically polarized in one direction only.

Compound action potential

Your experimental model will be a sciatic (also known as ischiatic) nerve from a large frog. You will stimulate one end of the nerve to produce action potentials in some or all of the axons, and measure action potentials from another part of the nerve in the direction of propagation. Measuring individual action potentials in vertebrate nerve axons is extremely difficult because of the tough extracellular matrix and small axon diameters. When recording activity from a nerve trunk such as a frog sciatic it is much easier to place extracellular recording electrodes on the nerve and measure what we call a compound action potential.

As an action potential is propagated, the membrane potential is reversed, producing a "negative wave" that attracts positive ions away from any recording electrode in the vicinity. The electrode records a transient negative potential compared with the potential outside an inactive section of nerve. The negative wave that is generated by a single nerve axon is quite small, on the order of microvolts per axon. However, when hundreds of axons conduct action potentials simultaneously the extracellular response is readily measurable, on the order of millivolts. The magnitude of the recorded signal, called a compound action potential, is proportional to the number of axons that participate.

Prepare a sciatic nerve for stimulating/recording

Although we refer to a sciatic trunk as a "nerve," remember that it is really a bundle of many individual nerve axons and connective tissue. The longer the piece of nerve the easier it is to study nerve conduction and other properties. Nerves are remarkable in many ways, including the length of the cells themselves. The longest axons in a frog sciatic nerve extend the entire 5-6" length of the bundle. Others will cut off where they branch away from the sciatic trunk. You will notice in fact that the bundle becomes thinner toward the distal end (further away from the origin in the spinal column).

We place a nerve across a series of stimulating and recording electrodes in a specially constructed chamber in a moist atmosphere. We use a biological stimulator to depolarize one end of the nerve via a pair of stimulating electrodes while using a pair of recording electrodes placed further down the nerve to record the compound action potential.

The dissection

The longer the section of nerve the easier it will be to conduct your experiments, especially when you examine refractory properties and conduction velocities. With large frogs you may be able to salvage the experiment if the upper sciatic or the extreme lower part of the nerve are lost. There are two sciatics per frog, but to conserve on animals we typically use both of them, one per team. Refer to figure 2.2 during the dissection. An experienced, certified individual will ablate the brain and spinal cord of a frog with a needle, a procedure called pithing, and then will demonstrate the dissection.

Step 1. Lay the pithed frog on its belly and find the proximal end of the urostyle, an extension of the spinal column that is accessible from the dorsal side of the frog. The proximal end forms the "hump" in the middle of the frog's back. Use forceps to lift up the

skin at that site and make an incision through the skin only, Push your closed large scissors through the opening and spread the scissors so as to separate the skin from the underlying muscle.

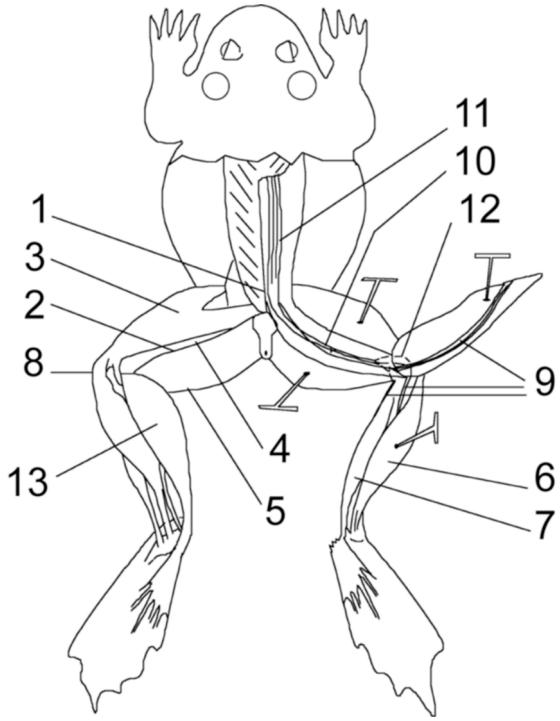

Fig 2.2. Dissecting a sciatic nerve from a bullfrog. *(1)* lift here with forceps and cut the muscles on both sides of the urostyle, reflecting the cut muscles to expose the upper sciatic. *(2)* separate the *iliofibrularis* and *semimembranosus* to reveal the sciatic nerve and companion artery; *(3) triceps femoris*; *(4) iliofibrularis*; *(5) semimembranosus*; *(6) tibialis anticus longus*; *(7)* Between the *tibialis posticus* and bone is one of the distal branches of the sciatic (with companion artery); *(8)* sciatic divides into 3 major branches in this region. The dissection is tricky - tough connective tissue must be removed to expose the nerves without cutting them. The best branch is probably the one that runs parallel to the *tibialis anticus longus*. The *gastrocnemius* branch is the most superficial, and is also relatively easy to recover; (9) sciatic branches. Dotted lines show path beneath connective tissue; (10) sciatic nerve and companion artery; (11) upper sciatic; (12) path of one distal branch runs beneath connective tissue on the kneecap, behind reflected *gastrocnemius*; (13) *plantaris longus* (*gastrocnemius*).

Continue freeing skin from muscle using blunt dissection, occasionally cutting the skin to give yourself more room, until you have cut all the way around the middle of the frog. Make an incision through the skin down the middle of the back to the groin.

Now grasp the frog's upper body and grab the skin with forceps or fingers. If you have not cut the muscles, a strong pull on the skin should remove it without damaging any of the structures underneath. Pull the skin all the way off the ends of the flippers as though you were removing a glove. Place the carcass on a moistened paper towel and wash hands and instruments.

Step 2. The sciatic nerves in the thighs lie along the femoral arteries, either of which can be reached from the lateral surface. Start with blunt dissection as above to begin to separate the *semimembranosus* muscle from the *iliofibrularis*. Cut through the fascia up and down the muscles and use blunt dissection (pushing and spreading the scissors) to reveal the artery and nerve. You may pin the muscles as in fig 6.1 to expose the nerve. ***Cautions*** (1) Avoid cutting any blood vessel, especially the femoral artery, as bleeding will obscure your view; (2) Always see where you cut (conversely, don't cut where you can't see) - it is a near certainty that if you violate this rule you will destroy the very structures that you are trying to preserve.

Step 3. Wherever you expose the nerve, keep it moist with frog Ringer's. ***Caution*** Do not stretch the nerve, crush it with forceps, or let it dry out at any time. Once you have located the position of the nerve in the upper thigh, trace it to the knee area. Here it branches and is obscured by connective tissue. Expose the branches by cutting the tendon at the insertion of the *gastrocnemius* and reflecting the muscle as in figure 2.2. Clear the connective tissue (carefully) to expose the branches through the knee area.

Step 4. Cut underneath the urostyle and cut the muscles on both sides while lifting the end of the urostyle. You can now trace a sciatic nerve to its exit point from the spinal column. Trace the nerve and artery through the hip region. Tie and cut the proximal end of the nerve at as high a point as possible. Now

carefully free the nerve past the hip and down past the knee as far as possible, cutting small branches as you go. You may want to tie the largest distal branch before cutting it off to facilitate placing it in the nerve chamber.

Step 5. The nerve should be free now and tied at least at one end. Remove it to a Petri dish with frog Ringer's (without stretching). Save the carcass on ice — the other sciatic will remain usable for some time.

Alternative dissection procedure

A "quick and dirty" way to remove a sciatic can produce a preparation almost as good as the result of the more tedious procedure described above. We free, tie off, and transect an upper sciatic as described above. We expose the femoral sciatic but do not try to dissect it out or expose it in the hip area. We cut off the flipper by transecting the distal tibia (end of the leg bone) to remove the entire flipper and ankle. With the distal connections severed we then apply a strong steady pull to the upper sciatic, breaking branches and eventually allowing extraction of the nerve.

Prepare a nerve chamber

Slide a pre-cut piece of filter paper into the bottom of your chamber. Be sure that it lies flat against the bottom, and pipet a liberal amount of frog Ringer's onto the paper (without letting the surface contact the electrodes).

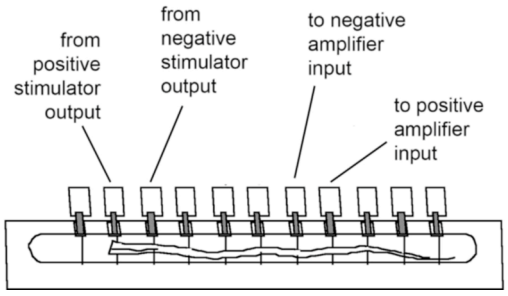

Fig. 2.3. Nerve chamber connections.

Pick up the nerve by the thread and place one end across an electrode near one end of the chamber. Allow the nerve to lay across the electrodes, covering as many as possible without stretching it. To ensure a clean signal see that no part of the nerve or thread contacts the bottom of the chamber. Now moisten a surface of the glass cover with water, and place it over the opening so the water forms a seal. It should not be necessary to disturb the nerve again for the remainder of the experiment.

Obtain and explore a compound action potential

Often the trickiest part of conducting nerve experiments is obtaining a signal in the first place. First you must be fairly comfortable with using the software to measure fast signals. You must select a suitable combination of voltage range and amplifier gain to record a compound action potential amplitude that can be anywhere from 5 to 50 mV peak-to-peak height, depending on the size and condition of the nerve. You must select a sampling rate that allows you to record components of a signal of perhaps 0.5 msec duration, start to finish. You'll need to set up your stimulator and triggering so that a sweep starts just before the stimulus is applied (include a delay), and so that you can see both the stimulus artifact and the compound action potential that follows it. Usually we set up so that the window covers 5 or 10 msec.

You will apply a stimulus in the form of a square wave output from your built in stimulator, and can vary both the pulse amplitude and the pulse duration. Effective pulse amplitudes typically range from 0.1V or so up to nearly 5V, the maximum output of many stimulators. You will work with pulse durations ranging from a few microseconds up to a msec or more. A good starting point is a pulse duration of 0.1 msec and a stimulus amplitude of 0.1V.

First, see if you can get a compound action potential. Figure 2.3 shows how to connect the chamber to a differential preamplifier. The stimulator cables come directly from the appropriate terminals of the A/D converter. The recording cables go to the appropriate positions on the amplifier lead block. A ground connection (green - not shown) may be necessary as well. Operate the amplifier in a high pass mode. This mode imposes a filter into the input so that only high frequency responses are recorded (e.g., stimulus artifacts,

nerve action potentials). The gain can remain at 1 since the software allows selection of a scale for display.

Apply a stimulus and note the position of the stimulus artifact. The artifact represents the passage of the passive wave past the negative and positive recording electrodes respectively (first spike in figure 2.4). If you do not see a waveform following the artifact then raise the stimulus voltage and try again. It is a good idea to raise voltage in large increments until you determine the range of effective stimuli. Every nerve will be different.

To determine the range of voltages that elicit a response, raise the stimulus voltage and record until you have an idea of the voltage needed to obtain the maximum action potential amplitude. Amplitude will fluctuate a bit at the peak, so don't try to be extremely precise. Reduce the stimulus voltage until you obtain an idea of the minimum voltage needed to obtain any action potential at all. You will likely need to change the voltage in small increments.

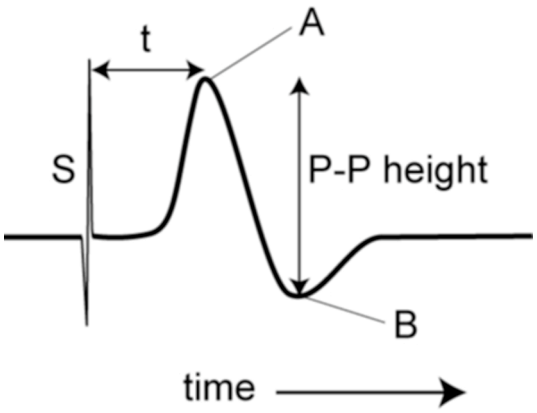

Fig 2.4. Typical compound action potential waveform. *S* stimulus artifact; *t* time for action potentials to reach the positive recording electrode; *A* peak of action potentials crossing the positive recording electrode; *B* peak of action potentials crossing the negative recording electrode; *P-P height* peak-to-peak height of the compound action potential.

Below are some terms that we will use when we discuss how to set up experiments. Assume that pulse duration is held constant.

- *maximal response* maximum obtainable peak-to-peak action potential magnitude
- *submaximal response* any recorded compound action potential that is less than maximal
- *threshold stimulus* minimum stimulus voltage needed to obtain an action potential
- *subthreshold stimulus* insufficient voltage for obtaining an action potential
- *suprathreshold stimulus* voltage that exceeds what is needed to obtain an action potential

Once you have determined your threshold stimulus and the voltage that gives you a maximal response you might want to try different combinations of recording electrode position to obtain the best (or most interesting) signal. For the next couple of experiments we would like to obtain a strong compound action potential with the negative recording electrode at least 1" away from the stimulating electrode. Remember: the stimulating electrode is the negative electrode, and should be toward the direction of propagation from the positive electrode. The positive electrode hyperpolarizes the membrane while the negative electrode depolarizes it.

Remember the following relationships. The compound action potential is propagated as a negative wave. This wave is recorded using a differential preamplifier in "normal" mode, so that when the wave crosses the negative electrode (cathode) a positive deflection is recorded. When the wave crosses the positive electrode (anode) a negative deflection is recorded. The relationship is multiplicative, that is, (-) times (-) = (+), (-) times (+) = (-), (+) times (+) = (+). The waveform that you see is due to a conducted pulse crossing both electrodes in sequence. To obtain a compound action potential wave form as shown in figure 2.4 the negative recording electrode must be the closest to the negative stimulating electrode, as shown in figure 2.3.

Stimulus thresholds and recruitment

This study will extend your initial exploration of the relationship between stimulus intensity and compound action potential amplitude in a systematic way. When we observe an increase in action potential amplitude as we raise stimulus voltage we refer to the increase as "recruitment" of nerve axons. Remember that compound action potential height is proportional to the number of axons conducting an action potential across the recording electrodes.

Now that you know the approximate range of stimulus voltages that will "work," you can make quantitative measurements of the relationship between stimulus voltage and proportion of axons that respond. Before you begin please keep the following considerations in mind.

As soon as you remove a sciatic from the animal the axons begin to deteriorate. Although an isolated sciatic nerve in a sealed moist chamber remains functional for hours it becomes increasingly refractory to stimuli with the passage of time. Refractory means failing to respond to a stimulus. What this means for you is that if you take measurements then go back and repeat the experiment you will discover that the stimulus thresholds are higher. Because the system will be changing it is recommended that throughout these studies you take the following approach to your experiments.

- decide exactly how you will conduct the experiment
- plan to collect more rather than less data
- explore the properties of the nerve preparation so that you know roughly what to expect
- change the independent variable in a systematic way
- take more data points in ranges in which the change is most rapid
- take fewer data points in ranges in which there is little observed change
- conduct the experiment, recording data as quickly as you can
- after you have recorded data, then go back and obtain quantitative measurements from the record

Suggestions for your experimental design

Your objective is to determine the relationship between stimulus intensity (voltage) and the height of the resulting compound action potential Recall of course that the latter quantity represents the number of axons that conduct an action potential, and that changes in compound action potential height have nothing to do with the size of action potentials of individual axons. Collect enough data to reveal possibly subtle changes in slope of the relationship. Keep in mind that you need the most data over the range(s) for which the compound action potential changes most rapidly with changes in stimulus intensity. A dozen or so data points will not be sufficient. A very good set of data may reveal heterogeneity within the nerve bundle, something that we will explore more thoroughly when we look at conduction velocities.

Decide how best to present the analysis in your results section. Remember that the object is to choose the single, most effective way of representing the relationship between compound action potential height and stimulus intensity. Raw data seldom serve that purpose.

Physiological significance of the recruitment curve

A sciatic nerve is a bundle of individual axons of different types. Control of activity is accomplished by varying the number of axons that depolarize in response to a stimulus. Under the present experimental conditions, a single nerve axon will "fire" if the stimulus intensity is great enough, otherwise it shows no activity at all. This is what is meant by the "all or none" property of nerves. Axons of a particular type, best characterized by axon diameter, share common properties such as conduction velocity and sensitivity to a stimulus. If you see a huge jump in compound action potential amplitude over a short range of stimulus intensities then you are probably stimulating a large number of nerves of a par-

ticular type. You will very likely encounter a voltage range over which little additional nerve activity can be detected. This can mean that few or no axons in the bundle have stimulus thresholds in that range. Once you apply a stimulus that exceeds the threshold for every nerve in the bundle, further increases in voltage will have no effect. Your recruitment curve will reach a plateau.

Suggestions for discussion

What is the relationship between an action potential of a single nerve axon and the compound action potential that you recorded? Can you define the phenomenon of recruitment in your own words? What is the importance of recruitment to fine control of nerve and nerve/muscle activity? Explain the recruitment curve, including any discontinuities or plateau regions. Do the data suggest that the sciatic nerve is a homogeneous bundle of axons? What would the recruitment curve look like if that were the case? Is there any evidence for the presence of different groups containing axons with similar properties?

Strength-duration relationship

Strength-duration relationships provide another means of studying the generation of an action potential. This time the focus will be on the properties of a stimulus needed to initiate a response, and the properties of nerve that dictate the requirements.

The study is based on the observation that extracellular electrical stimulation of an excitable membrane is accomplished by drawing positively charged ions away from the outer surface. If the extent of depolarization reaches a threshold level, voltage-gated sodium channels open momentarily, resulting in a dramatic increase in permeability of the membrane to sodium. A shift in distribution of sodium ions occurs in response to the permeability change. Momentarily, the membrane potential approaches the equilibrium potential for sodium, and the sodium terms dominate in the Goldman constant field equation. The change in trans-membrane potential, called the action potential, is transient of course.

Once an action potential is triggered it is self-propagating, that is, the action potential is of sufficient magnitude to depolarize the membrane further along the axon, and thus a signal is conducted.

A high voltage stimulus draws more charge per unit time than a low voltage stimulus. Therefore if one starts with a threshold stimulus for an axon or a number of axons, then reduces the voltage or reduces the time interval over which the voltage is applied, the response should disappear (or become reduced in the case of a compound action potential). Increasing the time interval or voltage should increase the compound action potential height or cause an absent response to return. Voltage and duration for a minimum effective stimulus might be expected to be inversely proportional. For example, if you stimulate a nerve axon and obtain a 10 mV peak to peak response, intuitively you would expect to obtain the same response if you halve the voltage and double the duration. A simplistic model would hold that for a given stimulus electrode the minimum effective stimulus is the product of the voltage and duration.

There is nothing simple about the design of a nerve axon, of course. Please design an experiment to determine the relationship between stimulus voltage and stimulus duration for a frog nerve axon.

Suggestions for your experimental design

With a maximal response, all excitable axons in the bundle are stimulated. Starting with the minimum effective stimulus needed to obtain a maximal response, raising either the voltage or the duration should do nothing, since all axons already are participating. Lowering either the voltage or the duration should cause some axons to drop out, thus reducing the overall height of the compound action potential. The result is a submaximal response.

The object of collecting data will be to estimate the relationship between duration and voltage for all stimuli that produce an identical response. A half maximal response occurs when the measured compound action potential is half the maximum height. Half of the excitable axons in the bundle will be responding. A straightforward way of starting these ex-

periments is to first select an arbitrary duration and find the voltage that gives you a half maximal response. You can then systematically vary the stimulus duration (the independent variable) and determine the voltage needed to restore the compound action potential to half maximal (see figure 2.5).

There are a couple of good reasons for working with half of the nerve axons instead of the full bundle. For one thing, recall that your preparation becomes more refractory with time. By working with a half maximal response you are selecting the most responsive, and presumably most healthy, axons in the bundle. Second, by working with a half maximal compound action potential you ensure that no matter in which direction you change a stimulus parameter, you will see a change. If you work with the maximum response you won't see a change when you increase duration or voltage.

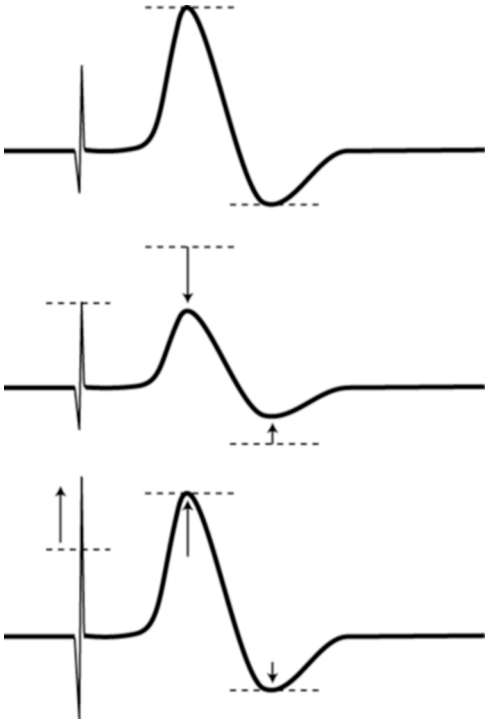

Fig 2.5. Response of a compound action potential to changes in duration or voltage of stimulus. *(top)* initial recording of a compound action potential (c.a.p.); *(center)* c.a.p after reducing stimulus duration; *(bottom)* original peak-to-peak height is restored by increasing stimulus voltage; note the resulting increase in height of the stimulus artifact.

As you plan your experiment remember that you are not measuring the actual compound action potential this time. You are to determine what stimulus voltage is required to give an identical response over a wide range of stimulus durations. Also apply the same principles that were recommended in designing your experiment on recruitment.

Keep records of how you set up the experiment, including how you went about testing the limits and how you decided to increment the independent variable. After you generate data, do a quick analysis to see if you have enough data points with which to address the questions. If there are any regions you would like to explore further, do it right away before the preparation deteriorates. As you fill in data points, keep in mind that they won't fit perfectly into the original excitability relationship because of changes in the preparation itself. Do not be overly meticulous about gathering "perfect" data. The object is to address the questions, not to make the curve picture-perfect.

Here are some questions that you will want to address with your study. Can you answer all of them? Why or why not?

- Is the excitability curve linear within all or part of its range? What is its actual shape?
- Is there a minimum voltage, or is any voltage, no matter how low, effective if the duration is prolonged enough? That is, is there an asymptote parallel to the voltage axis?
- Is there a minimum duration, or is any duration, no matter how short, sufficient if you raise the voltage sufficiently? That is, is there an asymptote parallel to the duration axis?

Interpreting the results requires that you think about the effect of a voltage change on ion movement at the surface of the nerve, how a stimulus draws charge, how that charge might be distributed, and how the parameters of a stimulus might affect the state of the sodium channels. Sufficient background information will be presented in the lecture component of the course.

Complication

A problem with recording compound action potentials when you have a very long stimulus pulse width is that the artifact interferes with the waveform. You can observe the problem by increasing the pulse width from about 0.1 msec to 1 msec or so. Eventually the stimulus artifact "walks up" the rising portion of the action potential, so that the peak can't be seen. Select a placement for your recording electrodes that will minimize the effect but still allow you to measure a strong compound action potential.

Analysis

As always, select the single most effective way to represent the relationship. If you plot the data, you may have to prepare separate graphs for different ranges of durations, since you will be spanning two orders of magnitude.

Suggestions for discussion

Prepare to explain in your own words how an electrical stimulus triggers an action potential. Consider what must happen to ions at the level of the cell membrane. Remember that current is proportional to voltage, and it is actually current (ion movement) that triggers an action potential. Remember that to obtain an action potential enough sodium channels must open at one time to allow the cell membrane to reach a threshold membrane potential. The open state of a sodium channel is transient, as discussed in lecture and text. After a brief time the open channel becomes inactivated. In addition to the biology, consider the physics of the stimulus event. For example, increasing the duration of a stimulus increases the effective distance of the electrode. That is, an electrode draws charge from farther away if the duration is increased, so the current density (number of charges moved per unit volume) is reduced for a given voltage. Then less charge is moved per unit area of membrane as well.

Keeping all of this in mind, explain the significance of the three major portions of the strength-duration relationship: asymptotic at long pulse widths; asymptotic at short pulse widths; middle, or somewhat linear, portion. Discuss in terms of ion movements and channels.

Conduction velocities

Different types of nerve axons conduct an action potential at different velocities, ranging from 0.5 m/sec in unmyelinated axons to 200-300 m/sec in the fastest myelinated axons. By cleverly arranging the stimulating and recording electrodes you can detect the presence of different types of nerve axons in the nerve trunk. You can also measure their actual conduction velocities and attempt to identify them.

A talk will present the concept of saltatory conduction. Fiber diameter, stimulus threshold, and conduction velocity have been found to be interrelated, in that conduction velocity increases and stimulus threshold level decreases with increasing fiber diameter. This gives some clue as to what factors affect the different properties.

Experimental design

You will have to work out your experimental design in the laboratory itself. How you set it up depends on length of nerve and how it responds to stimuli. There may be "dead spots" or even a point beyond which the nerve doesn't conduct at all.

So far you have worked with a single set of recording electrodes, positioned at a fixed point away from the stimulating electrodes. The present study requires that you estimate how fast the signal is conducted. That will require that you measure the time it takes the compound action potential to pass two points. The physical distance between those two points (electrodes are 1/2 inch apart) divided by the time between stimulus artifact and compound action potential peak gives the velocity of conduction for the average axon in the bundle. Here are several suggestions for optimizing the determination of conduction velocity and for discriminating among waveforms.

A rough approximation of conduction velocity can be obtained by placing the pair of recording electrodes at some measured distance from the stimulating (negative) elec-

trode, and measuring the time from the stimulus artifact to the peak of the first wave. The stimulus artifact is, for practical purposes, conducted instantaneously, so its appearance at a recording electrode marks the exact time of the stimulus itself, and the delay from artifact to first peak is the conduction time.

One catch to this method of measurement is that if there is any delay at the stimulating electrode the delay will be figured into your calculation and you will record too slow a velocity. Since the delay is a constant, you can factor it out by determining conduction times at several distances. Try it, and determine for yourselves how to analyze the data so as to factor out the delay.

Another way to get a more accurate determination is to separate the negative and positive recording electrodes and measure the time between the biggest positive peak and the biggest negative peak. That value is the time it takes the potential to travel the distance between electrodes. There is a drawback to this method and to the previous method of measurement, however.

As you should know by now, the action potential is propagated as a negative wave. The negative recording electrode inverts the signal, so you see a positive wave on the screen. The positive electrode does not invert, so you see a negative wave. The nerve bundle consists of different fiber types with different conduction velocities. Therefore, even with the two recording electrodes well separated the negative wave from a slow-conducting fiber group can interfere with the positive wave of a fast-conducting fiber group. In practice, the wave forms shift as electrodes are moved to different locations, adding experimental error to measurement of the conduction velocity. In addition, the presence of discrete groups of fibers is masked because of the interference. The conduction velocity you measure for the whole bundle is an average velocity for all of the fibers, weighted toward the group with the largest number of axons.

The problem of an interfering wave can be eliminated using a "killed end" preparation. Both recording electrodes must be in contact with the preparation, since each electrode serves as a reference voltage for the other. Remember, potentials are only measured relative to something. The differential recording also nullifies interference from electric fields in the room, so that if we didn't have a Faraday cage we could still do the study. The positive electrode still serves as a perfectly good reference if it is in contact with a portion of nerve that does not conduct. To set up a killed end preparation simply attach the positive electrode to the most distal wire that is in contact with nerve. Verify that you can obtain a positive wave. Block conduction just proximal to that point by crushing the nerve with forceps and/or hanging several drops of lidocaine over a half cm distance of the nerve trunk. Now you will record only a positive wave, and you can be confident that you have no interfering negative wave.

A drawback to the killed end preparation is that we often see artifacts due to capacitance of various components of the system. No single method of estimating conduction velocities will be perfect. It is recommended that you try all available methods and base your estimates on the most reliable data.

Systematically move the negative recording electrode to different positions on the nerve trunk. Examine the waveform produced as you vary stimulus intensity. Can you identify groups of axons that conduct at different velocities? Can you relate velocity to stimulus threshold? Try to measure conduction velocities for alpha, beta, and gamma fibers of the A (myelinated) group. If you have a particularly good preparation, you may be able to identify these three or more distinct groups. The farther away from the stimulus you place the recording electrode, the more time the waveforms have to separate. However the signal is attenuated because there are fewer nerve fibers at the distal end. You'll have to find the best trade-off situation.

In order to detect slower conducting γ fibers, you may have to slow your recording speed. You may even be able to identify type B or C fibers (α, β, and γ are subtypes of type A fibers). In frogs, the type B fibers conduct at 4-5 m/sec, and type C at 0.4-0.5 m/sec. You may need to use the averaging method to detect gamma or type B or C fibers. Averaging will clean up any signal, and give you a

more accurate picture of nerve conduction under any conditions. Instead of stimulating with a single pulse, simply deliver and average 20 or so pulses.

Analysis

Decide for yourself how best to present the results. You can compare methods if you wish, analyze in order to factor out any possible delays at the stimulus, etc. You make the choices - just make sure that your results are scientifically acceptable.

Suggestions for discussion

What are typical diameters, conduction velocities, functions of the different fiber types? Were you able to identify all of the fiber types? Suggest experimental reasons why you might have been unable to detect some of them. Explain any inconsistencies among velocity determinations. Did you see a relationship between fiber type and stimulus threshold? Why should conduction velocity and threshold be related? Although you did not measure fiber diameter, diameters have been determined for fiber types in frog sciatic nerve. How does fiber diameter relate to velocity and stimulus threshold? Why should there be such a relationship?

Refractory period

The refractory period places a limit on the rate at which a nerve axon can propagate action potentials. During the refractory period potassium conductance is too high to permit generation of an action potential and the voltage-gated sodium channels may still be inactivated. You can explore the phenomenon of a refractory period by applying pairs of stimuli to the nerve trunk.

A talk will present the concept of refractory period, including the distinction between absolute versus relative refractory period. Since you will be working with a nerve trunk and not individual nerves, there are limits to how much information on refractory properties of nerve that you can obtain from the preparation. Nevertheless, by carefully choosing stimulus parameters you can estimate a refractory period for the most refractory fibers (those with the highest thresholds) and for the least refractory fibers. You may also be able to demonstrate the presence of a relative refractory period.

To measure refractory period, you need to set up the stimulator to deliver pairs of identical stimuli, one pair at a time. If the time interval to the second stimulus is long enough you will get two compound action potentials of equal height, one following each stimulus. As you reduce the interval you should find a point in which the height of the second potential is reduced. At that point you are within the refractory period for some of the axons in the trunk. Some of the fibers will not have recovered in time to respond to the second stimulus, and so they drop out.

As you bring the stimuli close together the second potential will continue to drop until there is no response at all. That marks the beginning of the refractory period for all of the fibers. It can difficult to tell if you have reached that point, since the second stimulus artifact may interfere with the waveform.

Suppose that you obtain a maximal response and then reduce the interval between the two stimuli so that the second wave almost disappears. If you then apply a suprathreshold stimulus you may be able to restore part of the action potential, that is, the recorded waveform is bigger.

Decide on an appropriate placement for the stimulating and recording electrodes. Should they be close to the stimulating electrode or far away? Should you work with a bipolar wave, a killed end preparation, or does it matter? Should the stimulus be subthreshold, maximum effective stimulus, or suprathreshold? Select an appropriate stimulus duration. Set up the stimuli and do some pilot experiments to determine the approximate range of intervals within which you can demonstrate the refractory period. Then, starting with paired stimuli with an interval that is on the edge of the refractory period, begin reducing the interval as you record interval and compound action potential height. Repeat with your choice of one or more different stimulus voltages. As with the earlier experiments, choose increments of change so that

you can zip through each experiment very quickly.

Analysis

Figure out an effective way to illustrate the relationship between height of the second compound action potential and stimulus interval. Remember, it is a continuous relationship between two variables. It would be good to illustrate the effect of using a suprathreshold stimulus. You might be able to accomplish both objectives with the same analysis.

Suggestions for discussion

What is the physiological basis of a refractory period? For a single nerve axon, what is the difference between the absolute and relative refractory periods and the basis for the difference? How is the ability to explore refractory period limited by studying a nerve trunk rather than an individual fiber, and why? How much information were you able to obtain, and what were you unable to determine about refractory properties of nerve?

Channel-blocking agents

This last study will further explore nerve conduction and sensitivity to a stimulus. The results will be essentially qualitative, so you get to relax a bit.

An action potential is generated when a region of membrane is partially depolarized so that voltage gated sodium channels open, increasing permeability of the membrane to sodium and allowing a shift in distribution of the ion. The extent to which the membrane must be depolarized in order to trigger an action potential is called its excitability threshold. An action potential is propagated when it partially depolarizes an adjacent region of bare membrane, allowing that region to reach the excitability threshold, and so on. Since the nerves you are studying are myelinated, sensitive regions are located at discrete points on the nerve fibers, called nodes of Ranvier.

Obviously, propagation of the action potential can only occur if the height of the passively conducted wave is greater than the excitability threshold of the nerve. That height diminishes as it is propagated, since the current from the wave is distributed throughout a larger volume the further it is from the source. Therefore any defect in the nerve or agent that reduces sensitivity of the nerve to a stimulus should decrease the effective range of a conducted stimulus and decrease the conduction velocity or block conduction altogether. Local anesthetics can block conduction by reducing excitability for a sufficient distance along a nerve. Since no nerve is "perfect", the ratio of action potential height to excitability threshold must be considerably greater than 1 to allow the wave to be passively conducted past defects in the nerve trunk. This ratio, called the "safety factor", is generally 5 or greater.

Lidocaine is a local anesthetic that blocks fast sodium channels. It works most efficiently on unmyelinated fibers. Since pain fibers are unmyelinated and motor neurons are myelinated, pain can be deadened without loss of motor function.

Perform this study last, since you will be deadening parts of the nerve. Without blood circulation the nerve doesn't recover readily from the anesthetic. Design an experiment to determine if the slower conducting fibers drop out first following treatment with lidocaine. This would lend credence to the concept that slow conducting fiber types are less sensitive to a stimulus, that is, the effective range of a passive wave is shorter than with faster conducting fibers.

Lidocaine is most effective if used on a section of nerve that is relatively thin. It definitely won't penetrate the thick proximal region of nerve. A good approach to treatment with lidocaine is to hang several drops over a cm or so of nerve, so that a passive wave can't "jump" the deadened region. You may have to inject the stuff using a syringe with small needle to get under the sheath.

Analysis

Hey, this is a no-brainer. Look at the pictures and decide which fiber groups drop out first. If you get multiple fiber types and can demonstrate a progressive dropping out of fibers, then you may wish to include a figure or two in your paper. You can create some very informative figures if your recording application allows you to superimpose records.

Suggestions for discussion

How does lidocaine (xylocaine, procaine) reduce membrane excitability? Should the safety factor be the same for all axons in the nerve trunk? Why or why not? Explain why you might expect smaller diameter, slower conducting fibers to drop out first when treated with lidocaine.

Troubleshooting the nerve recordings

As emphasized in the membrane potential study, check over your entire set-up before yelling to an instructor for help. There are several circuits to check during recording, and you can save time by checking circuits that can be responsible for the symptom.

Suppose you cannot get a sweep to show up at all...

If you get no record on the computer screen, then you aren't triggering a sweep. Check that your pulse width is at least 50 μsec, your pulse spacing is around 200 msec. Make sure that the stimulator control is set up to trigger a sweep and that it is activated.

You get a sweep but you see no qualitative changes when you turn up the stimulus intensity...

If you see a deflection at the start of the sweep (stimulus artifact), then your recording system is working. If you do not see a stimulus artifact, then check the time parameters for your sweep. Is the artifiact over before the sweep starts? Check the sensitivity of the channel. The height of the compound action potential and artifact varies with the quality of contact of electrodes with nerve. You should set the sensitivity so that you can detect a 25 mV signal or less.

Nothing yet? Then check that your stimulus electrodes are plugged in to electrodes that contact the nerve. Sometimes the electrodes are bent down, and there is air between the electrode and the nerve. Still nothing?

Check your recording system. The recording electrodes should be right next to the stimulating electrodes when you first set up — not at the end of the nerve. Again make sure the nerve makes good contact with the electrodes. Make sure the amplifier is on, and in the proper mode. Make sure that you are recording from the correct channel - check all of the connections. Check the settings. Make sure that the input is not grounded.

You've tried all of this and still no artifact...

It is almost a certainty that you've missed something, since the stimulus artifact can be obtained from a wet string - you don't even need the nerve! Get help, but be prepared to be embarassed at your lack of expertise.

You get an artifact but no action potential...

You know that you are delivering a stimulus and recording properly, otherwise there would be no stimulus artifact. Place the recording electrodes right next to the stimulating electrodes. Make the sensitivity 5 volts full screen, and the sweep duration 5 msec, and turn up the stimulus intensity. You should see a deflection from baseline as you raise intensity. If you not, then increase the gain. If nothing happens, get some help.

Once you obtain the bipolar deflection that corresponds to a nerve action potential adjust the sensitivity and sweep speed so that the trace resembles that illustrated in figure 2.4.

If you cannot get an action potential despite all of your efforts then ask the instructor to replace your nerve chamber with one that has been tested.

Research paper

You are responsible for preparing a proper research paper according to the guidelines presented in the introductory and advanced experimental biosciences courses. For each section, start with an outline. Present converted data in the results section, as described for the first report. This time, it will be up to you to decide what should be included in the write-up and how to present it, although you should heed the guidelines for analysis and discussion that were presented in each section. When you have completed the report, then write your abstract and assemble all parts in proper order.

3 Contractile Mechanisms of Vertebrate Skeletal Muscle

From your course work you should be familiar with the mechanism of striated muscle contraction at the molecular level. But do you have a functional understanding of muscle contraction? Can you list the mechanisms by which the strength of contraction is varied? Can you explain how a weightlifter can use the same muscles to curl 150 lbs., lift a 5 lb. infant, or raise a styrofoam cup to her lips, all using the same muscle groups? How do you vary the intensity of contraction of muscle to control your movements?

This study will employ the frog *plantaris* muscle, which is innervated by a branch of a sciatic nerve. While control of the *plantaris* is not nearly as precise as that of such versatile structures as the muscles of the human larynx, the frog nerve-muscle preparation is nevertheless a durable and inexpensive model for muscle function. With this model you will be able to study most of the important mechanisms for control of skeletal muscle contraction.

The lecture material is indispensable for organizing your thoughts as well as for clear explanations (I hope) of some of the phenomena to be observed. Talks will review excitation-contraction coupling, anatomy of vertebrate skeletal muscle, and mechanical properties of muscle.

Skeletal muscle structure and function

The tension generated by a muscle can be graded by recruiting motor neurons that connect to (innervate) the bundle of cells that we call a single muscle. The more motor neurons recruited, the greater number of contracting muscles and the greater the tension. The biochemical and anatomical properties of muscle also allow for control based on frequency of stimulation and even by the starting position of the muscle.

The motor unit

Adult vertebrate skeletal muscle is composed of a bundle of single cells (fibers), that are innervated by a motor neurons. Each individual motor nerve terminates at anywhere from three to 1000 individual muscle cells with each cell receiving only one nerve terminal as illustrated by figure 3.1. The set of muscle fibers innervated by a single motor neuron is called a motor unit. In general a single muscle such as the *plantaris* contains a large number of motor units, thus it consists of thousands of individual fibers.

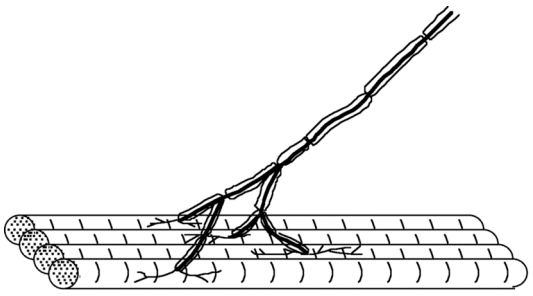

Fig. 3.1. Representation of a motor unit.

Myelinated fibers terminate in unmyelinated nerve endings. No fiber receives information from more than one motor nerve, however each nerve terminates at a number of fibers. Figure 3.1 is stylized, of course. Fibers may be more dispersed, with overlap among fibers of several motor units.

Generating a muscle action potential

The connection between nerve and muscle is made at a single specialized site on the muscle fiber known as the neuromuscular junction. The membrane of muscle fiber is modified to form a specialized structure, the motor end-plate, and the motor nerve axon forms a specialized unmyelinated terminal. The action potential conducted by a motor nerve is carried to the end-plate where the neurotransmitter acetylcholine is released by the nerve terminal into a space between muscle and nerve

called the synaptic cleft. Acetylcholine binds receptors on the endplate, causing a small localized depolarization called the end-plate potential.

The end-plate potential gives rise to an action potential "spike" that is actively propagated along the length of the muscle fiber, and into the interior via the transverse tubules, a network of extensions of cell membrane that surrounds each sarcomere, the basic unit of muscle contraction. The action potential triggers a series of events that causes muscle contraction. An important observation, key to understanding some of the phenomena that you will investigate, is that electrical events precede mechanical events. A muscle cell depolarizes first, then it contracts.

In the individual muscle cell the action potential is an all or none event just as with nerve, meaning that it either takes place or doesn't. There are no "partial" action potentials. If the extracellular potential from the entire muscle is recorded, however, the compound action potential is observed to vary in amplitude in accordance with the number of active motor units.

Excitation-contraction coupling and sliding filament theory

The muscle sarcomere (the unit of force generation) contains two types of filaments, thick and thin. The thick filaments are composed of the protein myosin and occupy the center of each sarcomere, forming the A-band (anisotropic). The thin filaments are composed of the protein actin. The actin filaments form the structures identified as I-bands (isotropic) in the light microscope. A filamentous protein called tropomyosin is closely associated with the actin filaments.

A muscle action potential triggers a release of calcium from the sarcoplasmic reticulum (SR) of the muscle cell, which in turn binds troponin, a regulatory protein associated with tropomyosin. The troponin molecules undergo a conformational change, causing the tropomyosin to twist, exposing binding sites for myosin on the actin filaments. Actin-myosin cross-bridges are then made and broken, causing the muscle to contract until the calcium is re-sequestered by the SR. Current theory holds that contractile force is generated in the area of overlap (interdigitation) between the thick and thin filaments. The region of overlap can be detected within the A-band, using a light microscope.

As long as calcium and ATP are present, cross bridges are continuously made and broken. There are enough cross bridges that the effect is that of uninterrupted tension. As long as this condition is maintained, the muscle is said to be in the *active state.* Cross bridge binding and release take place in a cyclic manner as illustrated in figure 3.2.

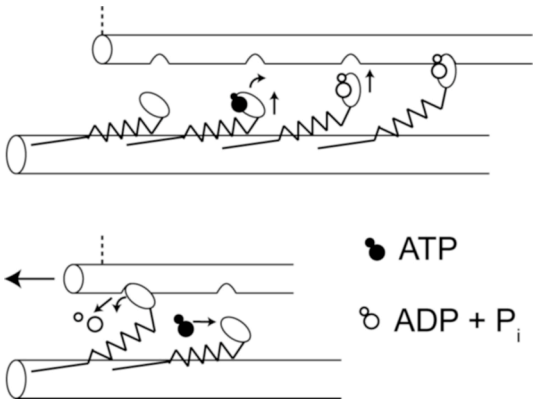

Fig. 3.2. Functional model of cross bridge activity in the sliding filament theory of muscle contraction.

Figure 3.2 represents an actin filament alongside a myosin filament with cross bridges. ATP spontaneously binds open sites on the myosin heads and is immediately hydrolyzed to ADP and inorganic phosphate (P_i). Some of the free energy of hydrolysis of ATP is used to reconfigure the myosin head in to a high energy "cocked" position. The cross bridge remains in this state until the binding sites on the actin filament are unmasked.

Upon attachment of the cross bridge to its binding site on actin the myosin head relaxes into its low-energy state, propelling the actin filament in the direction shown. ADP and P_i are then released, causing release of the myosin head from its binding site on actin. ATP binds immediately, restarting the cycle. Cross bridge activity continues, even if the muscle has fully contracted, as long as calcium levels remain high enough to keep the myosin bind-

ing sites open and as long as ATP is present. This equivalent of "running in place" permits the muscle to maintain and even increase tension after contracting completely. In this active state of contraction the generated force is proportional to the number of participating cross bridges, which in turn should be proportional to the amount of actin-myosin overlap.

Muscle shortening is thus due to a rapid making and breaking of actin-myosin bonds which propel the thin filaments further into the A-band. When the resting muscle is stretched and held at a fixed length, the thin filaments slide out of the A-band and the region of overlap is diminished. In a completely isometric contraction, further stretching and holding reduces the number of apposed actin and myosin reactive sites and thus reduces the tension the muscle is able to develop.

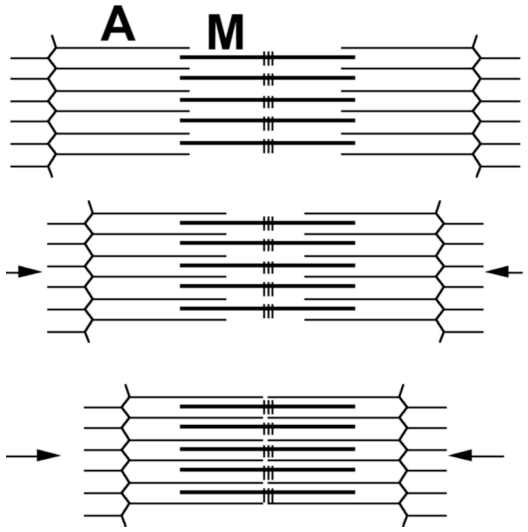

Fig. 3.3. Shortening of the sarcomere by interdigitation of actin *(A)* and myosin *(M)* filaments.

Measuring muscle tension and compound action potentials

Tension in grams must be "translated" into a signal that can be delivered to a recording system such as your analog/digital conversion system. The "translator" that converts grams to volts is called a transducer. There are many types of transducers that serve many purposes. We will make use of a device called a force transducer. The transducer, when deflected, delivers a DC output to the recording device.

Force transducer calibration

Prior to dissecting the nerve-muscle preparation calibrate your force transducer with weights and set up the software so that the scale of one channel is calibrated in grams tension rather than in volts. Referring to figure 3.4, hang weights of 10 and 100 grams on the transducer blade and record the pen deflection (in volts) at an appropriate amplification (gain). With a traditional chart recorder, an appropriate gain is usually the largest possible amplification that allows the record to remain on scale. If the record goes off scale, the gain is simply reduced. For the data acquisition system you can simply select a gain that enables you to clearly see a deflection from baseline. The program should let you amplify the signal later, after all data have been acquired. The program is designed to scale the record directly in grams, so you won't need a separate standard curve. Use a recording speed of 1 mm/sec. Before starting your work, determine how accurately the system records tensions between 0.5 and 100 grams.

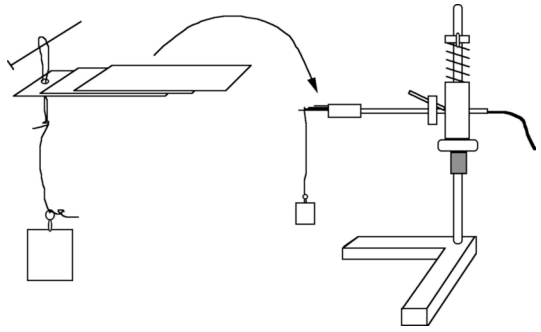

Fig. 3.4. Calibrating a force transducer. Hang weights on the force transducer by pushing the loop through the hole in the transducer leaf, then pushing a pin through the loop. Gently lower the weight and record tension.

Careful recordkeeping is absolutely necessary since all of the listed facts must be known in order to convert your raw data to figures and/or tables. Record in the chart file and/or your notebook each and every change made to the parameters, such as changing speed or gain

of the recording, as soon as you perform the change. Don't rely on memory, or you'll wind up repeating perfectly good experiments. The record itself should have all of the information you need to complete the results section of the paper.

The dissection

Do not allow instruments that have come in contact with skin to touch muscle or nerve (rinse before use). The instructor will pith a leopard or grass frog. Peel off the skin on one leg from the base of the thigh. Measure and record the *in situ* length of the *plantaris* with leg fully extended then with leg fully bent. Free the *plantaris* from the plantor surface of the foot by following the Achilles tendon to its insertion and severing it at that point. Then free the *plantaris* from the tibia along its entire length. Tie a tight ligature around the upper end of the tibia just below the kneecap. Cut the tibia just below the ligature.

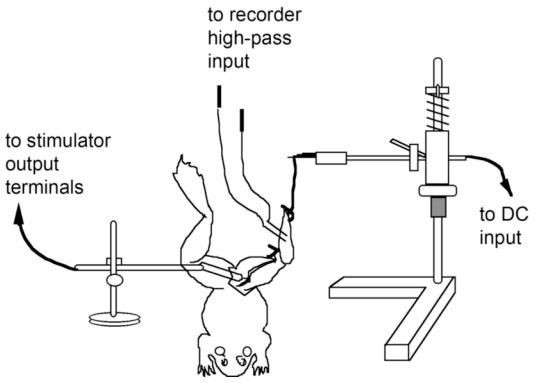

Fig. 3.5. Arrangement for stimulating a sciatic nerve to record muscle tension and muscle compound action potential. The frog board with posts for pinning the knee in place is not shown.

Expose the appropriate femoral sciatic nerve and free it from the fascia and adjacent femoral blood vessel. Be careful not to damage the vessels. Tie a suture around the nerve and transect at the most proximal site obtainable. Keep the nerve and muscle moist by frequently pipetting Ringer's solution over the preparation (soak up the run-off, please). Place the frog dorsal side up on the mounting board and run a pin through the holes in the wooden columns and through the femur (proximal to the kneecap). Cover the frog with Ringer's-moistened paper towels. Mount the proximal end of the nerve on the stimulating electrodes, tucking the rest of its length between the thigh muscles to keep it from drying out. All stimuli shall be applied to the nerve, thus the electrical events that you record are compound action potentials, not stimulus artifacts.

Using surgical thread and a straight pin, connect the muscle to the force transducer as illustrated (figure 3.5). The length of thread used should be such that with the muscle at rest length the manipulator's vertical drive can permit the muscle to be shortened to 3/4 its rest length or stretched to 1.5 times rest length. The thread must be perpendicular to the plane of the mounting board and the force transducer parallel to the plane of the table (why?). At rest length the line between the muscle and transducer should be taut but no passive tension should be observable on the chart recorder.

We record a compound action potential using two pin electrodes, each connected to two lengths of insulated wire. The two pins should be placed about 3 mm apart near the center and stuck well into the muscle. A ground wire may be necessary to reduce noise. It can be placed anywhere in contact with the animal. Wires from the pin electrodes are taken to an AC-coupled input of a preamplifier for high-pass recording.

Set up the stimulator to deliver square wave pulses of duration 1 msec, frequency 1 Hz. Start at 0.1V and increase stimulus intensity in 0.1V increments until you elicit a muscle twitch. Once you have verified that the muscle responds to stimuli you may wish to protect the nerve from drying out by placing a small dab of petroleum jelly over the nerve where it contacts the electrodes.

Recording data

You should be set up now for recording two channels. While getting started don't bother to record. You can easily see if the muscle does or does not twitch. Once you have a working preparation, then you can work on getting a good recording. A healthy preparation should respond with clearly sepa-

rated twitches with continuous pulses at one stimulus per second. Stimulate the nerve at one pulse per second using the minimum available pulse width. Find a stimulus voltage that elicits a twitch. Once you have a twitch start recording twitch tension using a relatively slow chart speed so that stimuli that are one second apart are clearly separated on the record. As you know by now, you will need to select a sampling rate that is sufficiently fast to record the fast events of a muscle twitch. To ensure that the rate is sufficient, you may wish to stimulate with single pulses and zoom in on the window to enlarge the twitch recording. You should get a smooth curve, not a spike, when you spread out the signal. You can then adjust the window resolution so that the screen scrolls by at a reasonable rate.

If you have a visible twitch but nothing shows up on the record you may have to adjust the gain (however, having calibrated the transducer with the same system, the present gain should be appropriate). As the intensity of the stimulus is increased one or both of your recording channels may go off scale. Records are not valid if the trace "pegs," that is, goes beyond the limits you set for the sensitivity. When this happens simply change gain and/or the recording range. Sometimes the smaller twitches simply take up slack in the thread and do not show up on the record. You can fix that situation by raising the force transducer to take up the slack.

Once the twitch record is established, set up to record the muscle compound action potential. It is likely to be a noisy signal, so you should filter it. However, the action potential is a fast event, so be careful that you don't attenuate the signal. You may have to live with some noise. Do use AC coupling on the action potential channel, since the signal tends to drift quite a bit. When you filter the signal, use either the filter feature in the software or a filter in the preamplifier, but not both. When you have the best recording you can produce, it is time to find the threshold for a measurable twitch and the minimum stimulus needed to produce a maximum twitch.

Regulation of contractile strength by recruitment

Physiologists refer to vertebrate muscle contraction as "all-or-none", just as they refer to an "all-or-none" nerve action potential. This means that if a motor nerve initiates contraction in the muscle cells it innervates, each cell responds maximally. The individual cells of a single motor unit act as one, all either contracting at once or not contracting at all. In vertebrates, there is nothing about the quality of an individual nerve impulse that will give variable degrees of contractile strength.

Recall that a sciatic nerve is composed of many individual nerve fibers, many of which are motor nerves. Recall from your previous studies that different nerves have different thresholds to a stimulus. Then by varying the stimulus intensity you should be able to control how many motor nerves are depolarized with each stimulus, and thus control the number of active motor units. More active motor units means a greater force of contraction. This mechanism for regulation of tension development in skeletal muscle is called recruitment.

The plantaris is composed of thousands of muscle cells that make up hundreds of motor units. If you were to stimulate just one motor unit, how much tension would you expect to see? Suppose you stimulated all of the motor units, that is, every muscle cell was made to contract? Remember that electrical events precede mechanical events. Knowing that the height of the compound action potential is directly proportional to the number of muscles that are depolarized, how would you expect the height of the compound action potential in the first case to compare to that for the latter case?

For the experiments on recruitment and effects of stimulus frequency you should simultaneously monitor the muscle compound action potential and the muscle tension. For experiments related to starting length of muscle (length-tension relationships) you will only need to monitor tension.

Staircase phenomenon

Before starting the recruitment experiment, set your stimulus frequency at 1 Hz and see if the twitch height stays the same over time. If you have a weak muscle it may decline. If it is fresh and healthy, you may see something called Treppe (the staircase phenomenon). Twitch tension will drop at first, then it will rise well above initial levels. This phenomenon is related to the release and reuptake of $Ca2^{+}$ by the sarcoplasmic reticulum. You will explore this phenomenon more thoroughly when you look at the effects of stimulus frequency on strength of contraction.

Stimulus frequency is a way of varying contractile strength without changing the number of participating motor units. Now, to look strictly at recruitment you want to control any variables related to stimulus frequency, right? When you design your experiment, think about how to do that.

Experimental design

Remember to explore the limits before proceeding. That is, determine threshold stimulus voltage and the voltage needed for a maximal response, so that you can plan your experiment fairly completely and collect the data over a short period of time. Consider the following questions as you plan your analysis and design an experiment to study recruitment.

Is there a linear relationship between twitch tension and compound action potential amplitude? Why? Is there a plateau? Why or why not? Knowing what it was that you recorded, should there be a plateau?

What are the fundamental differences between your recruitment data for the nerve-muscle preparation with data for the nerve axon that you collected previously? [Hint: for the recruitment study you won't bother to measure the stimulus parameters at all; you will measure muscle compound action potential and muscle tension] How can you best present the relationship between muscle compound action potential and twitch tension?

You may find that within some ranges of stimulus intensity both the muscle compound action potential and contractile response don't change very much. Within other ranges of stimuli, there is tremendous change. Particular groups of motor nerve axons share similar stimulus thresholds, thus you can expect such clustering.

Based upon your observations related to recruitment, would you say that the frog has a great deal of fine control over the contractile strength of the *plantaris*?

Regulation of contractile strength by stimulus frequency

You have seen how the anatomy of the motor unit determines how the contractile strength of voluntary muscle is regulated. There are other ways of varying tension, however. Treppe is one example of how tension can vary without changing the number of active motor units. The application of repeated stimuli with increasing frequency produces contractions that we call incomplete and complete tetanus. The mechanisms behind Treppe and both types of tetanus are related.

After a rest a muscle becomes stiff. The first contraction after a rest typically produces more tension than the second contraction. The decline continues for a few more contractions until a second phenomenon becomes apparent. The decline in tension with repeated contractions can be compared with repeatedly stretching a rubber band. It is stiff at first, then as it warms up it is progressively easier to stretch.

Muscle contracts actively of course, unlike a rubber band. The trigger is an elevated cytoplasmic calcium ion concentration, which causes cross bridges on the actin filaments to become unmasked and active contraction to begin. Treppe refers to the rise in twitch tension with continued stimuli, following the initial decline. It takes place because the SR does not sequester all of the calcium it releases in the brief interval between stimuli. Thus with each stimulus, calcium concentration starts off at a higher level, and it doesn't take as long to reach the threshold level to initiate active contraction. The active state lasts longer, and so more tension develops. Eventually, a steady state should be reached and twitch height remains constant provided that stimulus frequency doesn't change.

What happens, though, if you increase the frequency? At frequencies much higher than 1 Hz, tension does not return to baseline prior to the next contraction. You see a twitch with each stimulus, but the twitch doesn't start from zero each time. What you are seeing is the result, again, of intracellular calcium cycling. Following each release of calcium by the SR, there is less time to take it back up. The duration of active tension is increasingly prolonged with each stimulus, until a steady state is reached. The condition in which individual twitches can be seen but the tension doesn't return to baseline is called incomplete tetanus.

In complete tetanus, stimuli come so fast that calcium levels do not drop below the threshold and the active state is maintained constantly. No individual contractions can be seen. There is just one enormous contraction, a constant pull that often produces many times the tension that is attainable with a single twitch.

Experimental design

What are the relationships between peak tension and compound action potential amplitude and frequency of stimulation? Does the compound action potential amplitude predict changes in tension due to changing stimulus frequency, as it did for the recruitment experiment? From what you have learned about the anatomy of muscle and recruitment, you should know what to expect, and be properly skeptical when you interpret the data.

Decide how best to present the relationships of compound action potential and tetanus tension to stimulus frequency. After deciding how to collect data you should be able to look at the data and address the following questions.

What happened to total tension as the frequency of stimulation was increased? Were individual twitch tensions observable with high frequency stimulation? Did the muscle have time to "relax?" Are the tension and action potential amplitudes correlated? If so, under what conditions does the correlation hold and where does it break down?

You'll need to decide what frequencies to apply to the muscle, how long to apply each stimulus, what strength of stimulus to apply, etc.

Regulation of isotonic contractions by varying rest length

A muscle contraction in the form of a twitch closely resembles an *isotonic* contraction, that is, the muscle is allowed to shorten while pulling against a constant opposing force. Complete tetanus resembles an *isometric* contraction, in which the muscle builds tension while remaining at some fixed length. To remember the meaning of each term, remember that the prefix "iso-" means "constant," and that the suffixes "-tonic" and "-metric" refer to tension and length, respectively. All muscle movements in reality are some combination of isotonic and isometric contraction, and even in the laboratory it is difficult to separate the two.

You have seen that developed tension depends on both the number of fibers activated and in some circumstances on the frequency of stimuli. Now you will explore how extending a muscle before applying a stimulus can influence the strength of contraction.

The isotonic relationship as applied to heart muscle is known as the Frank-Starling law of the heart. In exercise, blood returns to the heart at a greater rate than at rest. Heart rate goes up with exercise as well, but even with the faster heart rate the filling volume is greater with each heartbeat, or *stroke*. The myocytes (muscle cells) and their sarcomeres are increasingly stretched between contractions in proportion to the increase in filling volume. The Frank-Starling law describes an increased force of ventricular contraction that results from increased filling of the ventricle. More blood is ejected per stroke so that in exercise the ventricles retain less blood at the peak of contraction than they do during rest. *Stoke volume* increases in exercise. Without such a response blood would pool in the heart since the ejection fraction (fraction of blood in the ventricle that is actually pumped out with each beat) would be too low to keep up with demand.

Applied to skeletal muscle, the law simply states that within physiological limits the

greater the resting length of the muscle the greater the resulting force of contraction, as the actin and myosin filaments reach a more optimal degree of interdigitation for achieving contraction. Serious weightlifters are very aware of the phenomenon (but probably not at the molecular level).

Experimental design

You will need a little help designing these experiments. You aren't studying recruitment at all, so use a stimulus strength that produces the maximum possible twitch tension. Increase the strength by half again because the corresponding nerve will become increasingly refractory.

You will need to calibrate your muscle stand so that you can raise the transducer and stretch the muscle by known increments. A good increment to use is 0.5 mm per record.

Start with the shortest rest length for which you can measure a twitch and set up to stimulate at one stimulus per second. Record about one dozen twitch tensions then stop the stimulus, allowing the muscle to fully relax. The staircase phenomenon may influence data collection, but you don't want to prolong the recording until a steady state is reached each time. The muscle may not last that long. Increase rest length and take another record.

Repeat the recording with increasing rest length. Under these conditions the muscle is permitted to shorten completely each time, and the active state is terminated with each twitch. When active tension reaches an obvious plateau or begins to decline, terminate the experiment. Overstretching the muscle will damage it and you won't be able to conduct the next study.

At first, the tension the resting muscle applies to the transducer (the passive tension) will remain the same as you increase rest length. Passive tension will begin to increase as you apply a significant stretch to the muscle. When you apply a stimulus, the tension that the muscle develops over and above passive tension is called the active tension. Total tension is the sum of the two.

For your experimental design, fill in the blanks. For example, how will you analyze and present the results? How will you incorporate rest length, passive tension, active tension, and possibly total tension into the analysis? As always, pick the single most effective way to present the findings.

Suggestions for discussion

What determines the degree of isotonic tension? Since the muscle is permitted to shorten, maximum cross bridge overlap is achieved, therefore number of active cross bridges is not a factor. Consider that the muscle bundle is held together by connective tissue and that the ends of the muscle bundle are anchored by tendons. Those structures, and the bending transducer leaf in the experiment, provide a passive elastic force that opposes the tension developed by contraction. A simple model for the mechanism is presented in Figure 3.6.

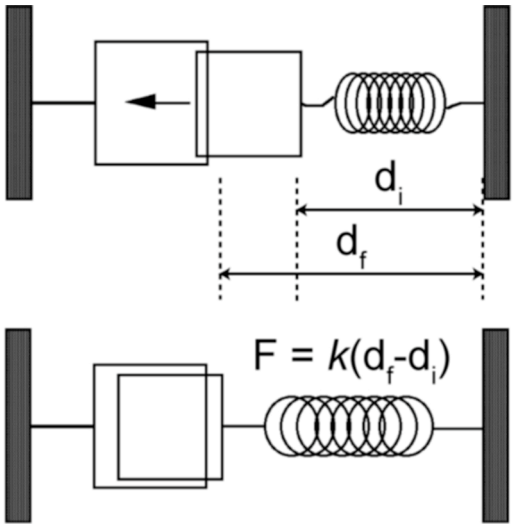

Fig. 3.6. The force of contraction of a skeletal muscle pulling on the series elastic elements can be likened to a piston pulling on a spring. The opposing force is directly proportional to the distance (d_f - d_i) over which the spring shortens.

Recall your *in situ* rest length measurements for the muscle. They represent the maximum and minimum lengths attainable by the muscle as the leg is extended or bent. Does the difference between maximum and minimum lengths appear to fall within the isotonic portion of the length-tension curve for this muscle? From your observations, does varying rest length of skeletal muscle appear

to be an effective means of controlling movement?

Regulation of isometric contractions by varying rest length

Complete tetanus represents an *isometric* contraction. The muscle is held at a fixed length at some point while active contraction continues to build tension. You can test the sliding filament theory by studying an isometric length-tension relationship. Refer back to figure 3.3. Suppose we hold the muscle at some fixed length so that when tension is fully developed the filaments partly overlap as in (B). In sliding filament theory the ability to develop tension is directly proportional to the number of active cross bridges. How do you think isometric tension in (B) should compare with isometric tension produced by a fully contracted saromere (C)? What do you suppose should happen to tension if we hold the muscle at a fixed length so that filament overlap resembles the illustration in (A)?

Experimental design

To examine an isometric length-tension relationship you will need to produce the greatest tension obtainable without causing the muscle to fatigue. Look back at the experiment that studied regulation of contraction through frequency of applied stimuli. How can you obtain the isometric tension that you need?

Start your experiment in the same way as you started the isotonic length-tension experiment, and conduct it in a similar way. The difference is that the muscle will produce an isometric rather than isotonic contraction. At first, the muscle will shorten completely, so that actin-myosin overlap will be the same with increasing rest length. Often, we obtain a better isotonic length-tension response with this experiment than we do with the isotonic length-tension study! As you increase rest length (and passive tension) the muscle will eventually be unable to shorten, and you should see the isometric part of the relationship.

You will have to put a lot of passive tension on the muscle to complete these experiments, and the muscle will at some point exert considerably more tension than in the isotonic tension study. Make sure that you have it tied well, or the knot might very well pop off!

Complete your experimental design as you did for the previous experiment.

Suggestions for discussion

Can you distinguish specific regions of the length-tension curve that represent different physiological conditions? Considering that the sarcomeres of a whole muscle do not necessarily contract in unison, and that individual muscle cells may be stretched to different lengths, how do you suppose the length-tension relationship for a muscle bundle differs from one for a single muscle fiber? Why would it not be suitable to perform this experiment using single stimuli, in order to generate twitch tensions rather than tetanus tensions?

Does the isometric length-tension relationship support the sliding filament theory? What does theory predict for this relationship?

Fatigue

So, you've had a hard day. You're feeling a bit tired, not quite ready to run the 100 yard dash. If you aren't too bushed, try the following experiment to determine if it is likely that fatigue of the neuromuscular junction contributes to this "weak" feeling. Set up a second stimulus to stimulate the muscle directly. You may require a separate stimulator, as the output from the built-in stimulator may not be enough.

With just enough tension on the muscle to measure a satisfactory twitch, stimulate the nerve at the minimum frequency previously required in order to obtain complete tetanus. Do the same for the muscle to verify that both stimuli are adequate.

Stimulate the nerve while measuring both tension and compound action potential, until active tension drops to zero. Record at a fairly slow rate of speed. If you have trouble getting a decent action potential, then just pull out the pins and record tension only.

Stimulate the muscle without turning off the nerve stimulus. Is the action potential re-

stored? How about the tension? What is the likely source of fatigue, then? Report this result appropriately.

Suggestions for discussion

What would you have expected to see if fatigue is due to depletion of neurotransmitter? Would developed tension and the height of the compound action potential drop proportionally? What if fatigue is due to depletion of energy within the muscle? What would you expect to see in one case versus the other when you stimulate the muscle directly after achieving fatigue?

Troubleshooting the nerve/muscle preparation

Below is a list of things to try just in case you run into an unresponsive preparation.

The muscle doesn't twitch in response to a stimulus...

Never mind the chart record for now. You should be able to see the muscle twitch when you stimulate the nerve. If it does not twitch, check that the stimulator is set up properly, as with the nerve study (check amplitude, pulse width, frequency). You can connect the stimulator output directly to a recorder input channel and make sure you are getting an output. Another possibility is that the nerve is damaged and/or does not make good contact with the hook electrodes. Check that the electrodes do not touch anything but the nerve (the stimulus may be grounded out). Check that the nerve directly contacts the electrodes and that they are separate. Try reversing the leads — the negative electrode should be distal (toward the muscle). Move to another part of the nerve if you can. If you still can't get a twitch, ask the instructor for a preparation with which to test the system.

The muscle twitches but you see nothing on the recorder...

Watch the transducer leaf as the muscle twitches. Does it move? Gently bend the leaf with a finger. If you get no action on the record check that everything is on including both recording channels. Check the gain. The position control for your force transducer should move the trace on the record. If not, check that the input is not grounded. Check that the trace isn't "pegged" or bottomed out. It will not respond if its rest position is far off the scale.

The muscle twitches, but it will not "settle down"...

Between twitches the muscle should quickly return to baseline tension and remain still. If the muscle fails to return to baseline or appears to "squirm" or twitch between stimuli, a stray stimulus or bias voltage is probably reaching the sciatic. Check the ground connections throughout the system. An older preparation may show the symptoms due to fatigue. Change preparations.

No compound action potential...

Check that the channel receiving the signal is indeed in record mode, and check sensitivity. Check that the amplifier is on. Check all connections. You are bound to get some kind of signal, even if the pin electrodes aren't in the muscle. By the way...

If the signal on your action potential channel is going crazy...

One or both of your pin electrodes is out of the muscle. Stop the recording and secure the electrode(s) in the muscle.

You get a compound action potential but it is noisy...

This is the most persistent problem in the muscle lab. The electrodes are small and the bare wires pick up all kinds of electrical interference. Check the filter settings on your amplifier. Check that the preparation is grounded. Try putting a ground clamp on the force transducer stand. If you are using a Faraday cage make sure that it is properly grounded. The preparation should be well to the back of the cage. If the signal is still too noisy, scream very loudly. You will get your instructor's attention.