3.6 The Neuromuscular Junction and Excitation–Contraction Coupling

Learning Objectives

- Identify the major structural features of a motor neuron
- Distinguish between EPSP and IPSP
- Describe how the strength of EPSPs and IPSPs varies with time and distance
- Describe spatial and temporal summations
- Identify the major structural features of the neuromuscular junction
- List in chronological order the events of neuromuscular transmission
- Explain why the end-plate potential is not an action potential
- Define excitation–contraction coupling
- Identify the source of activator Ca^{2+} in E–C coupling
- List the sequential events in E–C coupling including the names of the major proteins
- Describe how the Ca^{2+} transient ends by reuptake of activator Ca^{2+} by SERCA
- Describe how Ca^{2+} binding to TnC activates cross-bridge cycling, include tropomyosin, TnI, and TnT
- Draw the relative time course of the action potential, Ca^{2+} transient, Ca^{2+} binding to TnC, and force
- Explain why force lags behind $[Ca^{2+}]$ and why it persists when $[Ca^{2+}]$ returns to normal
- Define tetany and tetanic frequency
- Explain how repetitive stimulation causes force summation

MOTOR NEURONS ARE THE SOLE PHYSIOLOGICAL ACTIVATORS OF SKELETAL MUSCLES

As described in Chapter 3.2, alpha motor neurons reside in the ventral horn of the spinal cord. They process synaptic inputs leading up to the production of an action potential in the motor neuron. The action potential propagates down the single axon toward the muscle cell where it makes a junction, variously called the neuromuscular junction, motor end plate, or myoneural junction. The process of conversion of the action potential on the motor neuron to an action potential on the muscle fiber is called neuromuscular transmission. The action potential on the muscle fiber then activates the muscle by a process called excitation–contraction coupling. This chapter covers all of the events from the formation of the action potential on the motor neuron to initiation of contraction in the muscle fiber.

THE MOTOR NEURON RECEIVES THOUSANDS OF INPUTS FROM OTHER CELLS

The motor neuron receives literally thousands of inputs from sensory cells, other cells in the spinal cord, or from higher centers in the central nervous system, and all of these inputs are integrated at the level of the motor neuron to determine its activity. These connections all occur through synapses or gaps between the cells. Upon excitation, the presynaptic cell releases a neurotransmitter which then binds to a receptor on the postsynaptic cell to eventually modulate its conductivity to ions. Neurotransmitters that directly alter ion conductances are called ionotropic. Other metabotropic neurotransmitters alter other targets within the postsynaptic cell that indirectly alter membrane conductances. Ionotropic neurotransmitters typically have effects with rapid onset and rapid decay, whereas metabotropic neurotransmitters generally take longer to have their full effect but the effect lasts longer.

POSTSYNAPTIC POTENTIALS CAN BE EXCITATORY OR INHIBITORY

The effect of ionotropic or metabotropic neurotransmitters depends on the receptors in the postsynaptic cell. If binding of neurotransmitter causes an increase in conductance to Na^+ ions, then the postsynaptic cell becomes depolarized. Because depolarization leads to excitation, this depolarization is called an excitatory postsynaptic potential or EPSP. Glutamate that is released from presynaptic terminals can bind to at least four different kinds of receptors: the kainate, AMPA, and NMDA receptors are all ionotropic, and mGluR is metabotropic. Each of these types exists in multiple isoforms. The kainate, AMPA, and NMDA receptors are named for the artificial agonists kainate, κ-amino-3-hydroxy-5-methyl/4-isoxazole propionic acid, and *N*-methyl D-aspartic acid, respectively. Binding of glutamate to either the kainate or AMPA receptors causes an increase in g_{Na} and g_K, resulting in EPSPs. Activation of the NMDA receptor by glutamate increases g_{Ca}, resulting in an influx of Ca^{2+} ions and depolarizing the cell.

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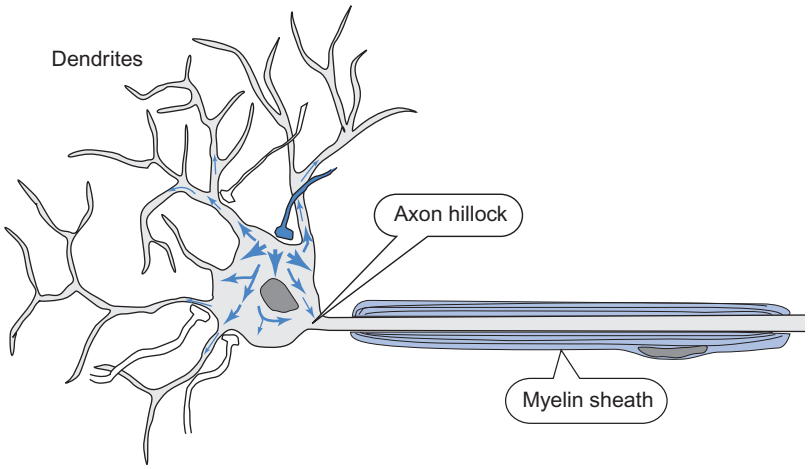

FIGURE 3.6.1 Spread of postsynaptic potential through a motor neuron. The postsynaptic potential intensity is indicated by the size of the arrows. It initiates at a terminal on the soma and spreads through the cytoplasm. The intensity falls off with distance away from the site of stimulation (the synapse) and also decays with time.

Certain other neurotransmitters bind to receptors that selectively increase the conductance of the postsynaptic membrane to Cl^- or K^+. Increased g_K decreases the membrane potential (makes it more negative) toward E_K. This hyperpolarization makes it more difficult to excite the membrane to form an action potential: it is called an **inhibitory postsynaptic potential** or **IPSP**. An example of a typical inhibitory neurotransmitter is **GABA** or gamma-aminobutyric acid. GABA binds to ionotropic $GABA_A$ receptors, which increase g_{Cl} and lead to an IPSP. GABA can also bind to metabotropic $GABA_B$ receptors that indirectly increase g_K to lead to an IPSP. Other examples of neurotransmitters are discussed in Chapter 4.2.

POSTSYNAPTIC POTENTIALS ARE GRADED, SPREAD ELECTROTONICALLY, AND DECAY WITH TIME

Both IPSPs and EPSPs can vary in magnitude, as opposed to the all-or-none character of the action potential. Their sizes depend on the number of neurotransmitter molecules that are released from the presynaptic terminal and on the number and kinds of receptors on the postsynaptic membrane. The postsynaptic potentials spread throughout the cell electrotonically or passively. As they spread, their magnitude decays with distance from the synapse because some of the current travels across the membrane as capacitative current. Because cytoplasmic resistance is little, EPSPs and IPSPs change little in the soma, but decay much more rapidly in the narrow dendrites because of their higher resistance. These EPSPs and IPSPs also decay with time, lasting 15–20 ms. Both are transient changes in the membrane potential (see Figure 3.6.1).

ACTION POTENTIALS ORIGINATE AT THE INITIAL SEGMENT OR AXON HILLOCK

The initial segment of the axon shows a clear area in histological sections and forms the most excitable part of the motor neuron because this area of the cell membrane contains high concentrations of the regenerative Na^+ channels. This part of the cell has the lowest threshold and for this reason it usually originates action potentials. Whenever this part of the neuron reaches threshold, an action potential is fired that propagates backward over the soma and forward down the axon toward the muscle fiber.

MOTOR NEURONS INTEGRATE MULTIPLE SYNAPTIC INPUTS TO INITIATE ACTION POTENTIALS

Usually a single EPSP on the motor neuron is insufficient to excite the trigger zone of the initial segment enough to reach threshold. Simultaneous stimulation of several excitatory connections can summate at the initial segment to produce a suprathreshold potential. The addition of simultaneous excitatory or inhibitory postsynaptic potentials is called spatial summation, because the different inputs originate at different locations on the motor neuron surface. Because EPSPs and IPSPs last some 15–20 ms, repetitive stimulation at a single synapse can cause repetitive EPSPs or IPSPs that can also add. This is called temporal summation, because the postsynaptic potentials are summed over time (see Figure 3.6.2). Spatial and temporal summations can occur together, so that repetitive stimulation of multiple inputs can be summated both temporally and spatially. A spinal motor neuron typically has 10,000 synaptic inputs, about 8000 on the dendrites and 2000 or so on the soma. The motor neuron integrates all of these inputs.

THE ACTION POTENTIAL TRAVELS DOWN THE AXON TOWARD THE NEUROMUSCULAR JUNCTION

Depolarization of the axon membrane in the middle of the axon initiates an action potential that propagates in both directions away from the stimulus. But under normal conditions, the action potential does not begin in the middle of the axon. Under normal physiological conditions, the action potential is unidirectional, beginning at the motor neuron soma and traveling down the axon toward its target, the muscle fiber.

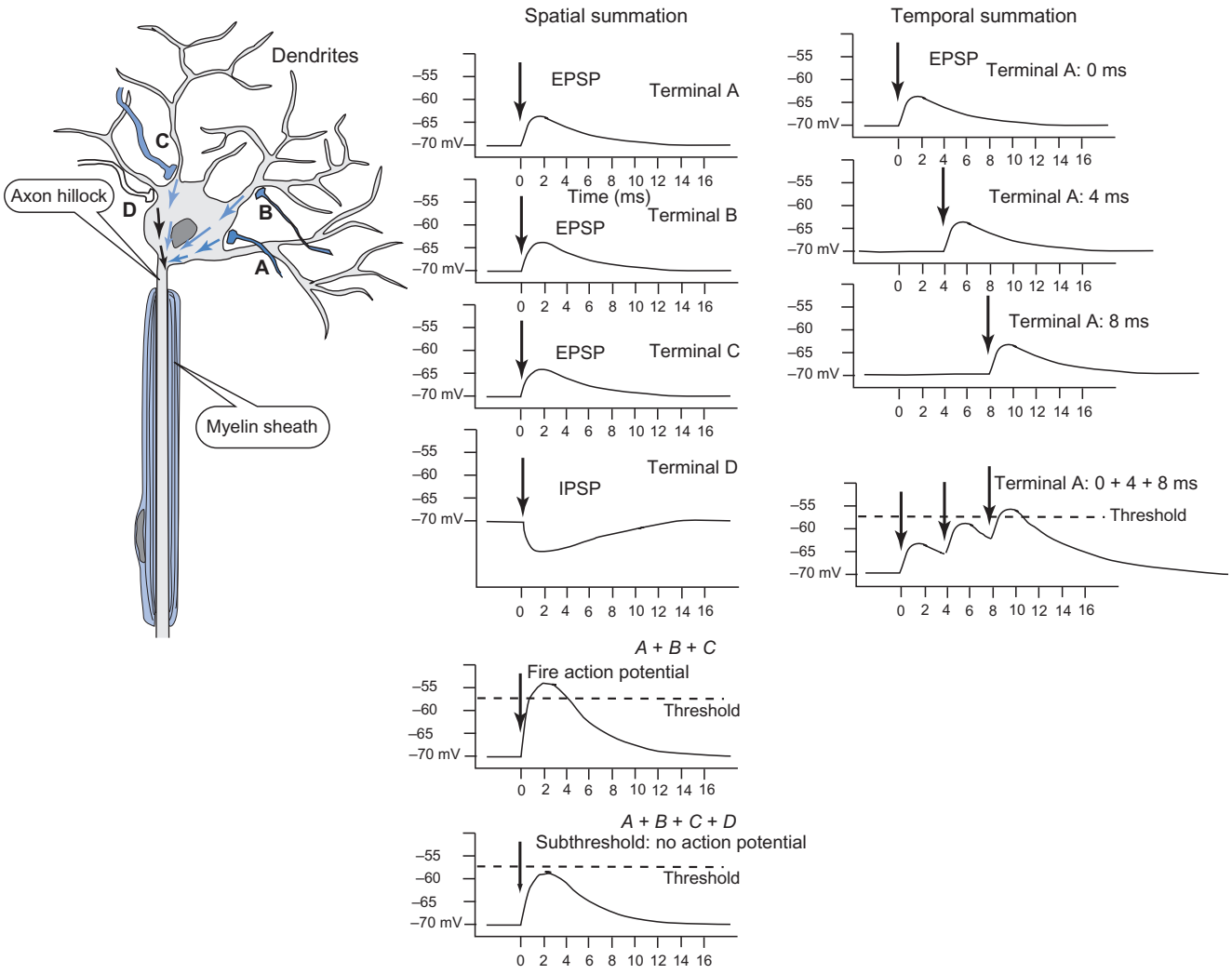

FIGURE 3.6.2 Hypothetical scheme illustrating spatial and temporal summations. Consider a motor neuron with four inputs. Inputs A, B, and C are excitatory inputs that produce EPSPs when excited, and D is an inhibitory input that produces an IPSP. Firing of A, B, or C alone results in EPSPs that are subthreshold. Simultaneous firing of A, B, and C summates to produce a suprathreshold potential that produces an action potential in the motor neuron. Simultaneous firing of A, B, C, and D is below threshold because the IPSP of connection D lowers the postsynaptic potential. This is spatial summation. Even though firing of A is insufficient to reach threshold, repetitive firing of A can summate to produce suprathreshold postsynaptic potentials. Summation of the postsynaptic potentials in time is temporal summation.

THE NEUROMUSCULAR JUNCTION CONSISTS OF MULTIPLE ENLARGEMENTS CONNECTED BY AXON SEGMENTS

As discussed in Chapter 3.4, typically a single motor neuron branches many times and each branch connects to a single muscle fiber. The total number of muscle fibers innervated by a single motor neuron is its **innervation ratio**, which can vary from 5 to 2000. Each motor neuron with its set of muscle fibers makes up a motor unit, whereas usually each muscle fiber typically is innervated by only one motor neuron. The junction of the motor neuron axon with the muscle forms a raised area on the surface of the muscle called a **motor end plate**. The axon breaks up into multiple branches, with enlarged areas called terminal boutons where neurotransmission takes place. Typically there are multiple terminal boutons in each motor end plate (see Figure 3.6.3).

NEUROTRANSMISSION AT THE NEUROMUSCULAR JUNCTION IS UNIDIRECTIONAL

Under normal conditions, an action potential on a motor neuron *always* results in an action potential on the muscle fiber, but an action potential begun on a muscle *never* results in an action potential on the nerve. The neuromuscular junction is designed for 100% fidelity and unidirectionality. This unidirectionality is assured by the highly asymmetric structure of the junction, as shown in Figure 3.6.4. The neuromuscular junction is a chemical synapse in which the action potential on the nerve is converted to a chemical signal, the release of the neurotransmitter acetylcholine. The

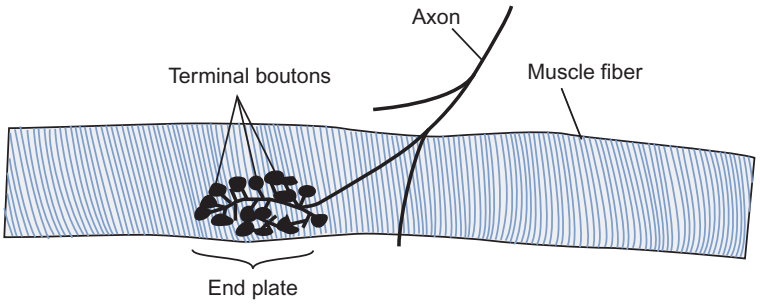

FIGURE 3.6.3 Motor end plate or neuromuscular junction. Motor neuron axons typically branch multiple times, and each branch ends in a neuromuscular junction or motor end plate. The end plate forms a raised area on the surface of the muscle fiber that involves multiple short branches of the axon that end in terminal enlargements of the axon called terminal boutons. These typically occur in a relatively small area of the surface of the fiber.

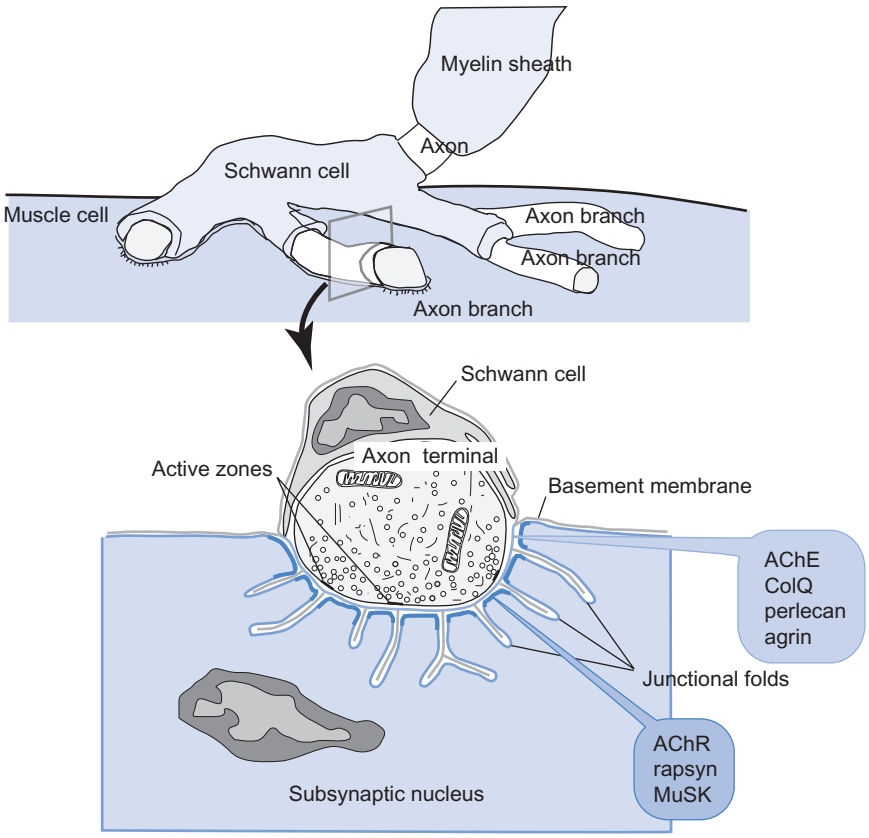

FIGURE 3.6.4 Schematic drawing of a neuromuscular junction. Branches of the motor neuron axon lie within a synaptic trough or gutter on the muscle membrane that has deep junctional folds. The axon terminal is filled with synaptic vesicles that contain neurotransmitter, acetylcholine, and that fuse with the presynaptic membrane only at specific active zones, seen in EM as darker areas on the presynaptic membrane. Particular proteins are located in specific places. AChR, the acetylcholine receptor, is located on the postsynaptic (muscle) membrane at the top of the junctional folds. AchE, acetylcholinesterase, is located in the basement membrane in the gap between nerve and muscle. Other proteins include agrin, which is secreted by motor neurons and aids in the signals that lead to the formation of the neuromuscular junction; rapsyn, which is receptor-associated protein of the synaptic membrane, is also associated with neuromuscular junction formation; MuSK, which is muscle-specific receptor kinase that binds to LRP4 (low-density lipoprotein receptor 4), which in turn is the receptor for agrin. ColQ and perlecan are basement membrane proteins.

chemical signal released into the narrow space between motor neuron and muscle fiber then is converted again into an electrical signal on the muscle. The unidirectionality is assured because the release of neurotransmitter, acetylcholine, occurs only by the presynaptic nerve axon, and receptors for the acetylcholine are localized only on the postsynaptic muscle fiber membrane.

MOTOR NEURONS RELEASE ACETYLCHOLINE TO EXCITE MUSCLES

The action potential on a motor neuron invades the terminal boutons of the neuromuscular junction. The depolarization of the action potential opens voltage-gated Ca^{2+} channels on the presynaptic membrane. Because the extracellular $[Ca^{2+}]$ is about 1.2×10^{-3} M and the intracellular $[Ca^{2+}]$ is less than 1×10^{-6} M, opening of the Ca^{2+} channels causes Ca^{2+} ions to rush into the synaptic terminal. This results in a short-lived, local increase in the cytoplasmic Ca^{2+} concentration.

The motor neuron axon terminal contains about 300,000 small vesicles, about 50 nm in diameter, that each contain between 1000 and 10,000 molecules of acetylcholine. A fraction of these vesicles forms a readily releasable pool (RRP) of neurotransmitter by docking at specific active zones on the presynaptic membrane. When the local $[Ca^{2+}]$ increases during neurotransmission, Ca^{2+} binds to Ca-sensitive proteins (synaptotagmin) that cause the synaptic vesicles to fuse with the presynaptic membrane, releasing their acetylcholine into the cleft between nerve and muscle.

The released acetylcholine diffuses to the postsynaptic membrane where it binds to two α-subunits of the **acetylcholine receptor** (AChR), which is also an ion

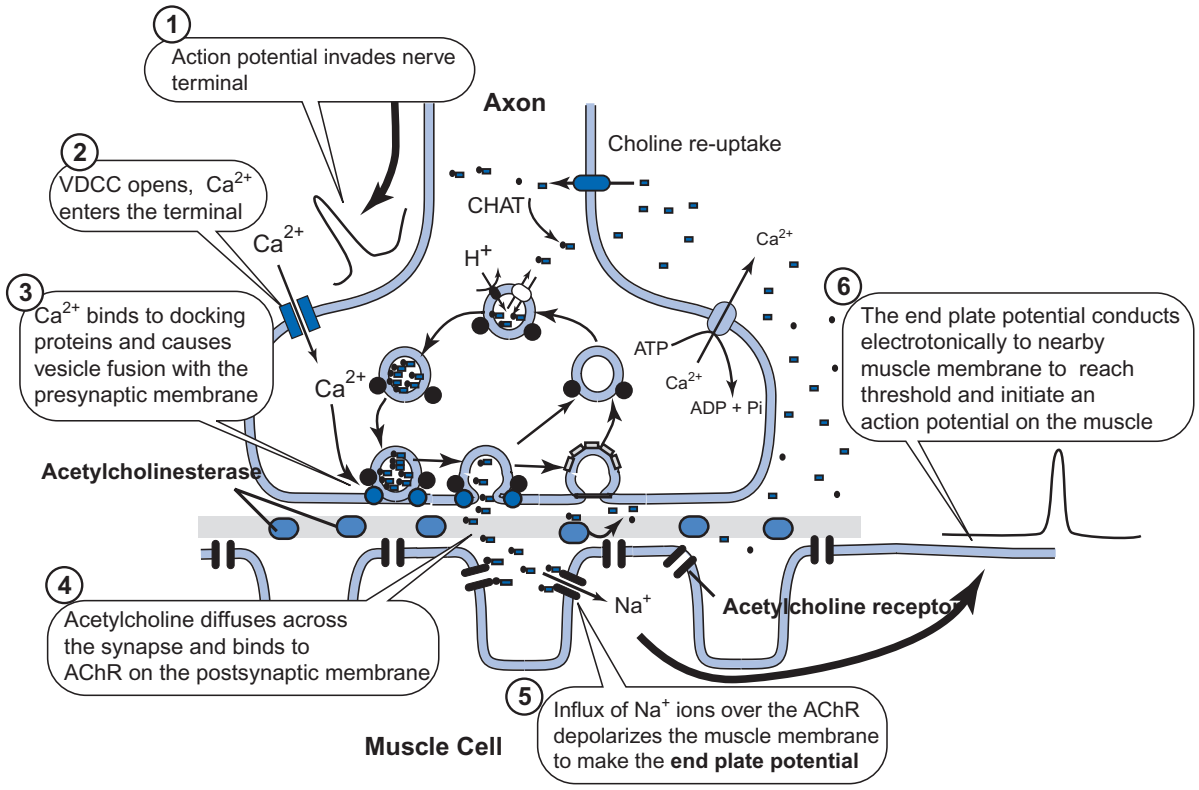

FIGURE 3.6.5 Steps in neuromuscular transmission. The overall process converts an action potential on the motor neuron to an action potential on the muscle fiber membrane. The action potential on the motor neuron propagates along the axon and opens voltage-gated Ca^{2+} channels, leading to an increase in cytoplasmic [Ca^{2+}] in the terminal. The [Ca^{2+}] binds to proteins that dock synaptic vesicles at the active zone, leading to fusion of the vesicles with the presynaptic membrane and release of neurotransmitter, acetylcholine, into the synapse. ACh diffuses across the gap to bind to ACh receptors located on the postsynaptic membrane. ACh binding to this receptor increases the K^+ and Na^+ conductance, which in turn depolarizes the muscle cell membrane. This depolarization is called the end-plate potential. It is conveyed passively to a nearby patch of muscle membrane where, if the end-plate potential is sufficient, it initiates an action potential. Vesicle release is shut off by removal of the synaptic Ca^{2+} by Ca^{2+} pumps or Na^+/Ca^{2+} exchange. The acetylcholine signal is terminated by hydrolysis of acetylcholine in the synapse by acetylcholinesterase. *VDCC* = voltage-dependent calcium channel.

channel consisting of five subunits. This ligand-gated channel increases its conductance to both Na^+ and K^+, but because Na^+ is so much further away from its equilibrium potential, the main current is an inward current carried by Na^+. This inward current depolarizes the cell, forming the **end-plate potential**. The depolarization spreads passively, or **electrotonically**, to the nearby patch of sarcolemma. This end-plate potential usually drives the muscle cell membrane to threshold, causing an action potential on the muscle membrane.

Ca^{2+} EFFLUX MECHANISMS IN THE PRESYNAPTIC CELL SHUT OFF THE Ca^{2+} SIGNAL

As long as there is a high [Ca^{2+}] in the region of the vesicles and the active zone of the presynaptic membrane, there will be continued fusion of synaptic vesicles and release of their contents into the gap. The Ca^{2+} signal is shut off in several ways: (1) influx into the terminal is stopped, because the voltage-gated channels close when the membrane repolarizes soon after the action potential passes; (2) Ca^{2+} diffuses away or is bound to intracellular Ca^{2+}-binding proteins; (3) the plasma membrane Ca-ATPase pump and the Na^+-Ca^{2+} exchanger remove Ca^{2+} that enters the cytoplasm during the brief opening of the Ca channels. The plasma membrane Ca-ATPase is shown in Figure 3.6.5.

ACETYLCHOLINE IS DEGRADED AND THEN RECYCLED

Muscles at rest show spontaneous slight depolarizations that are due to the spontaneous release of a single vesicle full of acetylcholine. These slight depolarizations are all about equally large, each due to the fusion of a single vesicle, and they are called **miniature end-plate potentials**. The size of end-plate potentials is the result of many of these miniature end-plate potentials. In human neuromuscular junctions, some 20–25 vesicles fuse with each motor neuron action potential. At each active zone, the probability of a vesicle fusion event is about 0.7, so that human neuromuscular junctions have about 30–35 active zones. Continuous excitation and subsequent fusion of this number of vesicles with the presynaptic membrane would expand the area of the membrane and deplete it of vesicles. There are two ways to avoid this: (1) prevent full vesicle fusion in the first place and (2) recycle vesicle material that fuses by endocytosis. The first method uses a transient "fusion

pore" to connect the vesicle lumen with the extracellular space, and full vesicle fusion with the membrane does not occur. In the second case, an elaborate mechanism involving specialized proteins such as clathrin and dynamin pinches off the fused vesicle membrane area and endocytoses it. Empty vesicles are refilled with acetylcholine through a transport channel that exchanges acetylcholine for H^+ ions. The H^+ ions are accumulated within the vesicles through a vacuolar H-ATPase in the vesicle membrane.

Acetylcholine is also partially recycled. Acetylcholine is degraded to acetate and choline in the synapse through the action of acetylcholinesterase. The acetate is not recycled, but the choline is taken back up into the presynaptic cell through a transporter on the presynaptic cell membrane. In the cell, acetylcholine is resynthesized from acetyl CoA and choline through the enzyme **choline acetyltransferase**. The events occurring in the axon terminal that initiate and shut off neuromuscular transmission are shown in Figure 3.6.5.

THE ACTION POTENTIAL ON THE MUSCLE MEMBRANE PROPAGATES BOTH WAYS ON THE MUSCLE

The end-plate potential is a graded potential (it is not all-or-none) that propagates electrotonically to the neighboring patch of muscle fiber membrane where it initiates an action potential on the muscle much like it does on unmyelinated nerves. Because it begins at the neuromuscular junction, at the belly of the muscle fiber, the action potential propagates in both directions so that all of the muscle is activated within a short interval. The action potential is conducted down the T-tubules to provide nearly simultaneous activation of all parts of the muscle fiber.

Clinical Applications: Diseases of the Neuromuscular Junction

Myasthenia gravis (MG) translates from Greek and Latin to mean, literally, "grave muscle weakness." It is the most common disease associated with the neuromuscular junction but it remains an uncommon disease. The reported prevalence of MG has been increasing with time: in the 1950s the mean prevalence was 22 per million persons and in the 1990s it was 94 per million persons. Reported worldwide prevalence ranges from 40 to 180 per million persons, with an annual incidence of 4–12 per million persons. The increase may be due to improved identification of the disease, prolonged survival of afflicted persons due to improved treatment, and aging of the population at risk. The number of persons with MG in the United States in 1995 was estimated to be 38,000. In 2000, it was estimated to be 59,000. Thus it is a relatively rare disease. It generally affects women younger than 40 and men after turning 60. The ratio of women to men with the disease is about 3:2.

MG is characterized by muscle weakness that worsens with activity and improves after rest. People usually present to their physicians with complaints of specific muscle weakness and not general fatigue. Ocular motor disturbances and ptosis (drooping eyelids) are the initial symptoms in some two-thirds of patients; another one-sixth complain of oropharyngeal muscle weakness, difficulty in chewing, swallowing, or talking; only 10% complain of limb weakness.

The most common cause of MG is an acquired autoimmunity to the AChR in the neuromuscular junction. The antibodies block, alter, or destroy AChRs so that neuromuscular transmission in some muscle fibers fails. Because muscular contractions rely on thousands of muscle fibers, and only some of the junctions fail, the result is weakness rather than paralysis. Treatment aims at removing the antibody attack or boosting the acetylcholine signal to increase the opening time of the remaining active AChRs. Autoimmune suppression using corticosteroids (prednisone) improves or eliminates symptoms in more than 75% of patients. Patients who do not respond to corticosteroids may respond to the immunosuppressant azathioprine. Some patients, particularly those who develop the disease later in life, have a thymoma (tumor of the thymus gland, which is involved in processing of autoimmune antibodies). Removal of the thymus in these cases improves their condition markedly. The acetylcholine signal in the neuromuscular junction can be lengthened by anticholinesterase drugs such as neostigmine bromide or pyridostigmine bromide.

Some people with antiacetylcholine receptor (AChR) antibodies also have antibodies directed against intracellular muscle proteins such as titin and the ryanodine receptor (RyR) (see Chapters 3.6 and 3.7). These patients typically have thymomas and their disease is more severe. The presence of titin and RyR antibodies is a proposed subclassification of MG that correlates with myopathy.

Some 10% of patients with generalized MG do not have anti-AChR antibodies at all. Instead, they have antibodies to a muscle-specific protein kinase (MuSK), low-density lipoprotein receptor 4 (LRP4), or some unidentified protein. The condition is commonly referred to as seronegative MG or SNMG. MuSK is a tyrosine kinase that is involved in the signaling for formation of the neuromuscular junction. Nerve cells release agrin that binds to LRP4 that initiates a signal mechanism that leads to AChR clustering in the neuromuscular junction.

In the Lambert–Eaton Myasthenic Syndrome (LEMS), antibodies are directed against the voltage-gated calcium channels in the presynaptic nerve terminal. There are a variety of such voltage-gated Ca^{2+} channels in the body. Those on the presynaptic nerve terminal are the P/Q-type. Immunoglobulins from LEMS patients bind to P/Q-type Ca^{2+} channels and decrease the Ca^{2+} influx caused by the action potential. Since this Ca^{2+} entry triggers fusion of the vesicles containing acetylcholine, a smaller spike of cytoplasmic $[Ca^{2+}]$ causes fewer vesicles to fuse and a smaller and shorter acetylcholine release. In some cases, neurotransmission fails and muscle weakness results. It is distinguishable from MG because muscle strength increases with repetitive stimulation in LEMS and decreases in MG.

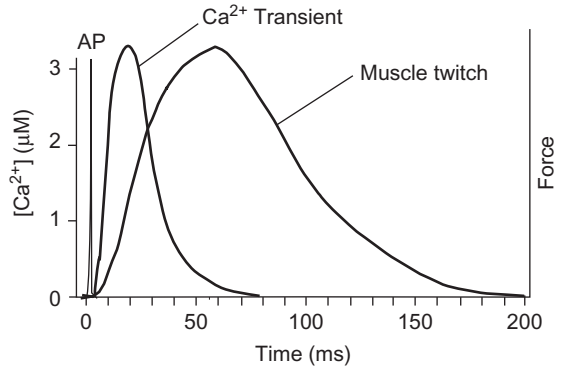

FIGURE 3.6.6 Duration of the action potential (AP), Ca^{2+} transient, and force in a muscle twitch. The action potential occurs first and is the shortest event, lasting some 2–5 ms. The Ca^{2+} transient follows the action potential and force is developed later. Ashley and Ridgway used aequorin, a protein isolated from a luminescent jellyfish, *Aequorea aequorea*, to demonstrate the Ca^{2+} transient. A variety of artificial fluorescent probes can follow the Ca^{2+} transient, and all of these show a Ca^{2+} transient that follows the action potential but precedes force development.

THE MUSCLE FIBER CONVERTS THE ACTION POTENTIAL INTO AN INTRACELLULAR CA^{2+} SIGNAL

The process by which the action potential on the surface of the muscle fiber signals muscle contraction is called excitation–contraction coupling or E–C coupling. The first clue to its mechanism was provided by Christopher Ashley and Ellis Ridgway, who demonstrated that Ca^{2+} transients follow the action potential but precede force in barnacle muscle. This time course is consistent with the action of Ca^{2+} as a second messenger between action potential and contractile activity (force or shortening). Figure 3.6.6 shows the time course.

THE CA^{2+} DURING E–C COUPLING ORIGINATES FROM THE SARCOPLASMIC RETICULUM

Because the rise in myoplasmic [Ca^{2+}] upon depolarization of the sarcolemma in skeletal muscle can occur in the absence of extracellular Ca^{2+}, the intracellular Ca^{2+} must originate from some cellular store. This is the sarcoplasmic reticulum or SR. The SR is a network of a sac-like, membrane-bound structures that surrounds the myofibrils and contains loads of Ca^{2+}. The SR makes close contact with the T-tubules in structures called triads, consisting of one T-tubule and two terminal cisternae. The terminal cisternae are the specialization of the SR that forms a continuous sac near its junction with the T-tubule. The triads are typically found at the junction of the A-band and I-band in skeletal muscles. In electron micrographs, foot structures can be seen connecting the T-tubule with the terminal cisternae. The SR does not form a continuous sleeve but instead it is discontinuous, with "windows" in its surface to allow for transport of metabolites such as ATP, ADP, and Pi into and out of the myofibril. The structure of the SR has been presented in Figure 3.5.4.

CA^{2+} RELEASE FROM THE SR AND REUPTAKE BY THE SR REQUIRES SEVERAL PROTEINS

A protein called the dihydropyridine receptor (DHPR) senses the membrane potential on the T-tubule membrane and relays this to another protein, the Ryanodine receptor (RyR), on the SR membrane inside the fiber. The DHPR consists of five subunits, one of which forms a Ca^{2+} channel. The DHPR is associated with L-type Ca^{2+} channel activity, but Ca^{2+} influx through the DHPR is unnecessary for E–C coupling in skeletal muscle. There is close physical contact between the DHPR and the RyR in the SR terminal cisternae. The RyR provides a channel for rapid Ca^{2+} release from the SR. It thus appears that the DHPR acts as a voltage sensor that in some way triggers Ca^{2+} release from the SR. Although there continues to be some uncertainty as to the mechanism, current results favor a direct interaction of the DHPR and the RyR in E–C coupling.

The RyR is so named because it binds a plant alkaloid, ryanodine, with high affinity. There are several different isoforms. **RyR1** is the type in skeletal muscle; **RyR2** is in the heart and brain; and **RyR3** is present in epithelial cells, in smooth muscle, and also in the brain. RyR1 is a large protein of 565 kDa. It associates as tetramers in the SR membrane, forming a structure of about 2 million Da. The purified RyR1 forms channels in lipid bilayers with large conductances and gating characteristics consistent with its role in E–C coupling. It is believed that part of the DHPR interacts either directly with RyR1 or through another protein to trigger the opening of RyR1 and subsequent release of Ca^{2+} stored within the lumen of the SR. The sequential events in EC coupling and relaxation are shown in Figure 3.6.7.

REUPTAKE OF CA^{2+} BY THE SR ENDS CONTRACTION AND INITIATES RELAXATION

To relax the muscle, activator Ca^{2+} is taken back up into the SR by a calcium pump. This is a membrane protein of 110 kDa that links the hydrolysis of ATP to ADP and Pi with the pumping of 2 Ca^{2+} atoms back into the SR lumen. The SR Ca pump is also referred to as the SR Ca-ATPase. There are several different isoforms in different tissues. The one in fast-twitch muscle is SERCA1a for smooth endoplasmic reticulum calcium ATPase. Slow-twitch muscle has the SERCA2a isoform.

Calsequestrin is a 63-kDa protein in the SR lumen that binds Ca^{2+} with low affinity (dissociation constant, $k_D = 10^{-3}$ M) but high capacity. Calsequestrin allows the SR to store a lot of Ca^{2+} at relatively low concentrations. Thus the SR can release a lot of Ca^{2+} yet it can reaccumulate the Ca^{2+} rapidly to allow relaxation. Without calsequestrin, the same amount of Ca^{2+} in the tiny volume of the SR would reach concentrations so high that the Ca^{2+} pump would reach its thermodynamic limit before all the Ca^{2+} could be reaccumulated. Calsequestrin may also directly participate in E–C coupling by interacting with

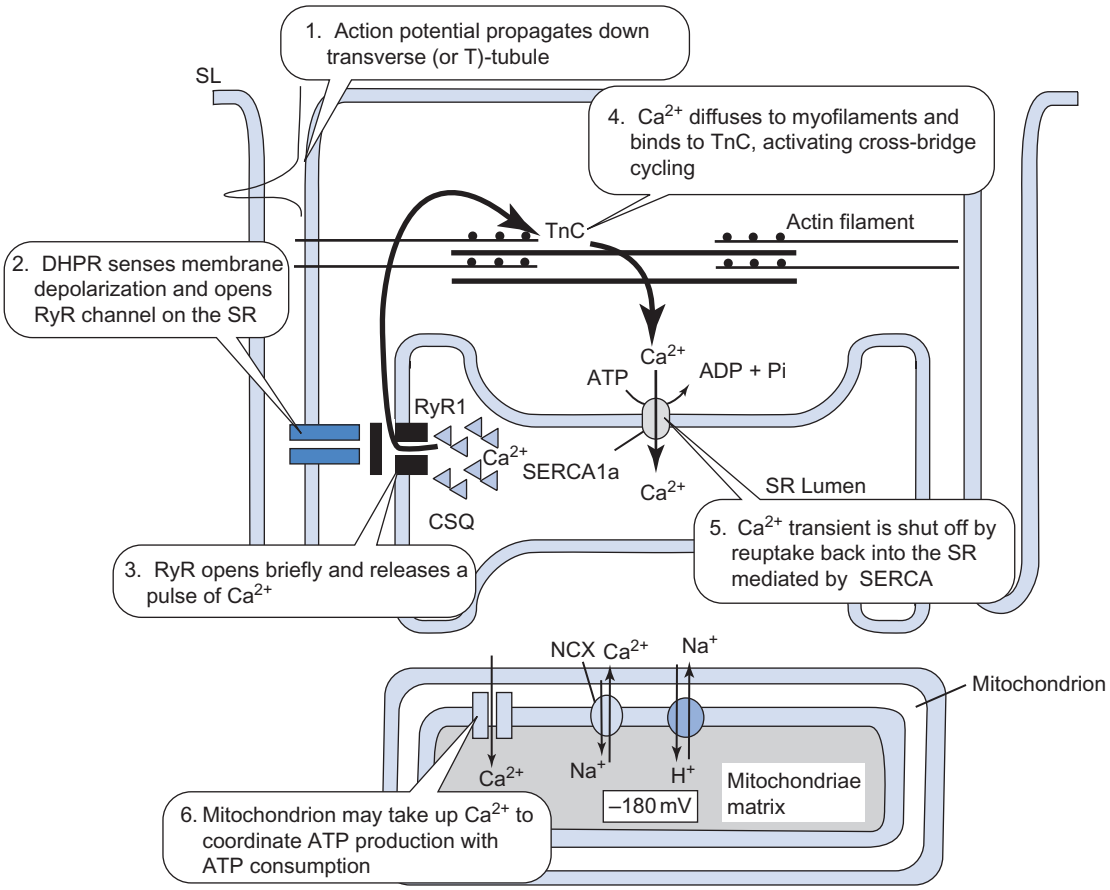

FIGURE 3.6.7 Cartoon schematic of the events that cause the rise and fall of the Ca^{2+} transient in skeletal muscle. The action potential propagates down the T-tubule where its depolarization is sensed by the DHPRs in the T-tubule membrane. These interact with the ryanodine receptors (RyR1) in the membranes of the terminal cisternae of the SR that are in close physical contact with the DHPR. The RyR is the Ca^{2+} release channel that opens briefly, allowing a pulse of Ca^{2+} to leave the SR. This released Ca^{2+} can bind to the myofilaments and activate cross-bridge cycling because the SERCA pumps on the SR membrane do not immediately remove activator Ca^{2+} back into the SR. During relaxation, the SERCA Ca^{2+} pumps remove cytosolic Ca^{2+} from the myofilaments and restore the SR to its resting state of high luminal [Ca^{2+}]. Mitochondria can also take up Ca^{2+} but probably do so to regulate their own rate of ATP production commensurate with ATP consumption rather than to regulate the Ca^{2+} transient.

the RyR1. In this way, calsequestrin may be a "Ca^{2+} wire" that feeds Ca^{2+} directly to the RyR1 Ca^{2+} release channel.

CROSS-BRIDGE CYCLING IS CONTROLLED BY MYOPLASMIC [CA^{2+}]

During relaxation, there is some overlap of the thin and thick filaments, but they do not interact because tropomyosin is in the way. Recall the structure of the thin filament (see Figure 3.5.7): it is a double strand of actin molecules with a half-repeat of about seven actins spanning some 38.5 nm. Tropomyosin is a long rod-shaped molecule 38.5 nm long that lies along the filament. At one end is the troponin complex of TnT, TnI, and TnC. At low myoplasmic [Ca^{2+}], the tropomyosin blocks the myosin head from interacting with the actin on the thin filament, and there is no cross-bridge formation. Figure 3.6.8 shows the steric interference of tropomyosin on myosin binding to the thin filament.

The fast-twitch isoform of troponin C has four binding sites for Ca^{2+}. Two of these are called high-affinity or "Ca–Mg" sites. The other two sites have a lower affinity for Ca^{2+} and a much lower affinity for Mg^{2+} and are therefore called "Ca-specific sites." The Ca–Mg sites appear to be always occupied and therefore they do not regulate TnC; Ca^{2+} binding to the Ca-specific sites regulates TnC configuration. TnC binds Ca^{2+} cooperatively, which means that the binding of one Ca^{2+} to an empty Ca-specific site greatly increases the binding to the second empty site. Thus Ca^{2+} tends to bind to both Ca-specific sites or to none at all, and the result is a very steep dependence on [Ca^{2+}] (see Figure 3.6.9). When Ca^{2+} binds to TnC, it changes its conformation. Because TnC connects TnT with TnI, all three shift their position. Because TnT binds tropomyosin, tropomyosin also shifts its position. This shift has the effect of uncovering the binding site for myosin on the actin, allowing cross-bridges to form. Binding to actin activates myosin ATPase activity and cross-bridge cycling, producing force or shortening.

Thus Ca^{2+} binding to TnT has its effect amplified: instead of regulating the interaction of only one actin with the thick filament, it regulates the interaction of one entire half-turn of F-actin. This is more economical of energy. If every actin–myosin interaction required Ca^{2+} binding, it would necessitate a larger Ca^{2+} release

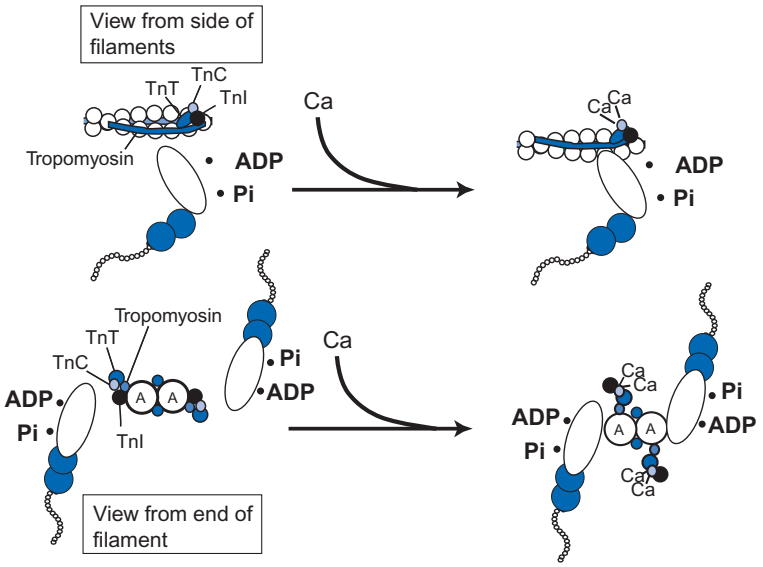

FIGURE 3.6.8 Calcium control of actin–myosin interaction. In relaxed muscle, tropomyosin blocks actin–myosin interaction and is held in place by TnT, which binds tropomyosin, TnC, which binds calcium, and TnI, which inhibits the interaction of actin and myosin. In the presence of micromolar Ca^{2+} brought about by release of Ca^{2+} from the SR, TnC changes its conformation and rearranges TnI and TnT so that tropomyosin moves out of the way of the myosin binding site on actin. Thus actin and myosin can interact, engage in cross-bridge cycling to consume ATP, and shorten or develop force.

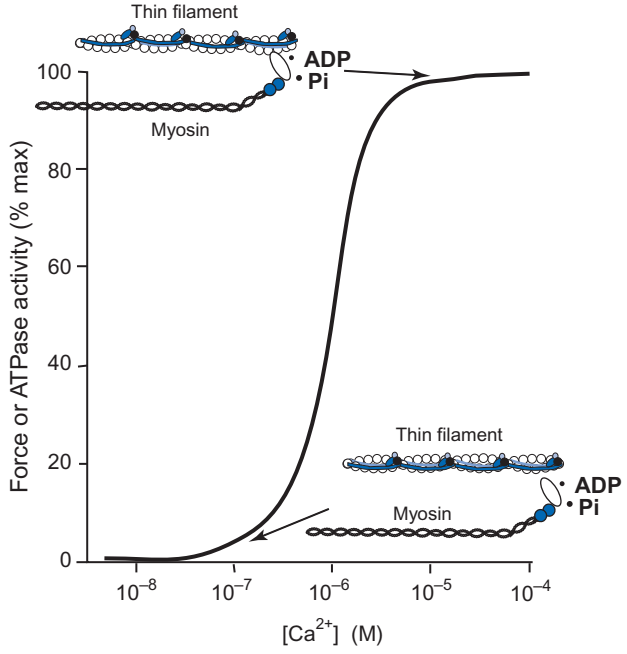

FIGURE 3.6.9 Ca-control of actomyosin ATPase activity or force. No force is generated at resting myoplasmic $[Ca^{2+}]$ of 10^{-7} M, whereas maximum force is generated at peak $[Ca^{2+}]$ of about 10^{-5} M. The steep dependence on $[Ca^{2+}]$ is a consequence of the highly cooperative nature of Ca binding and interaction of the filaments. It makes Ca appear to be a switch between the two states of no force and maximum force.

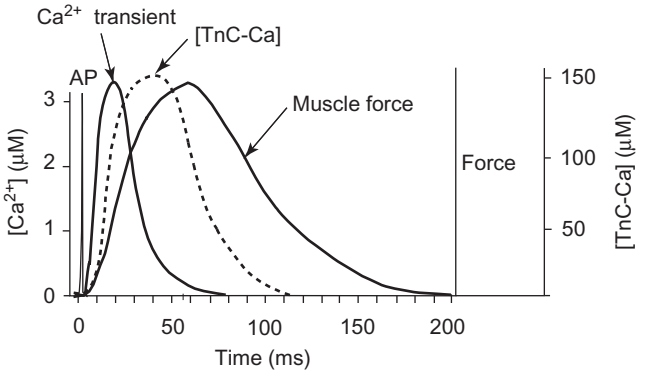

FIGURE 3.6.10 Time course of Ca^{2+} transient, [TnC–Ca], the concentration of TnC with bound Ca^{2+}, and force during the muscle twitch. The presence of elastic elements in series with the force generators is used to explain the discrepancy in the time courses of [TnC–Ca] and force generation.

from the SR, and a greater expenditure of energy to pump the Ca^{2+} back in to relax the muscle.

SEQUENTIAL SR RELEASE AND SUMMATION OF MYOPLASMIC $[CA^{2+}]$ EXPLAINS SUMMATION AND TETANY

Because it takes some time for TnC to bind Ca^{2+}, there is a delay between the rise in the Ca^{2+} transient and TnC binding with Ca^{2+} during a single twitch. TnC also retains some Ca^{2+} even when the Ca^{2+} transient has fallen. The drop in the myoplasmic $[Ca^{2+}]$ is brought about by active transport of free Ca^{2+} back into the SR through the operation of the SERCA Ca pump. This pump can transport only that Ca^{2+} that diffuses to it—the free Ca^{2+}. As free $[Ca^{2+}]$ drops, Ca^{2+} dissociates from the TnC–Ca^{2+} complex. This dissociation reaction is much slower than the binding reaction. Therefore, TnC remains bound with Ca^{2+} after the Ca^{2+} transient has peaked and fallen. Thus we would expect force to remain elevated even after the Ca^{2+} transient has fallen. We might expect that force at any time is proportional to the occupancy of TnC with Ca^{2+}. Figure 3.6.10 shows the hypothetical time course of $[Ca^{2+}]$, [TnC–Ca], and force. The force is delayed compared to the occupancy of TnC with Ca and remains elevated when TnC–Ca has returned to baseline.

THE ELASTIC PROPERTIES OF THE MUSCLE ARE RESPONSIBLE FOR THE WAVEFORM OF THE TWITCH

Experiments with rapid stretching of fibers upon initiation of contraction or rapid shortening of fibers during

contraction reveal an elastic behavior that appears to be in series with the contractile elements. Rapid stretch produces a much stronger force, and rapid release during contraction reduces the force to zero. The elements of the muscle that produce force appear to be in series with a spring, as shown in Figure 3.6.11, called the series elastic element. Force developed by the contractile elements (the myofilaments engaged in cross-bridge cycling) first stretches the spring. The force transmitted to the outside of the muscle depends on the length of the spring and is determined by its length–tension characteristics. The necessity of stretching the spring to transmit force to the outside of the muscle causes the delay in the force from its origin in the cross-bridges. Similarly, the series elastic element remains stretched when Ca^{2+} dissociates from TnC and the cross-bridge cycling stops. Thus the force transmitted to the outside of the muscle remains elevated for the time required for the series elastic element to return to its normal length.

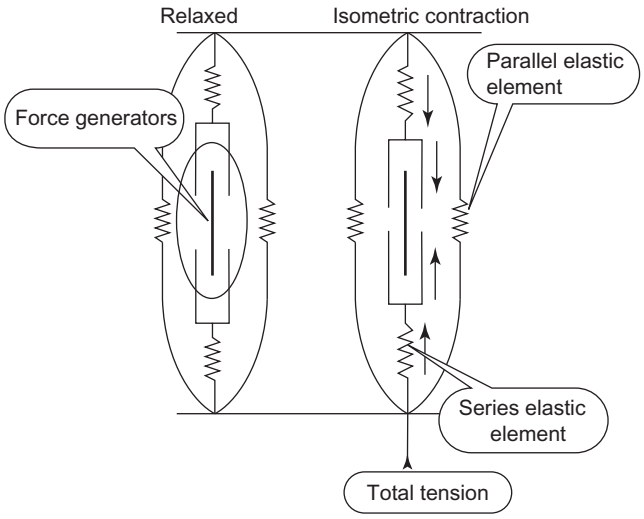

FIGURE 3.6.11 Series and parallel elastic components of muscle contraction. The muscle behaves as if there are elastic components in series with the contractile filaments and other elastic components in parallel with it. Force transmitted to the outside of the muscle lags behind the force generators because they must first stretch out the series elastic elements. After the force generators stop generating force, the total tension remains elevated because the elastic elements remain stretched. What proteins are responsible for these elastic behaviors is not known, although titin is hypothesized to contribute part and some of the series elastic behavior appears to reside in the cross-bridges.

REPETITIVE STIMULATION CAUSES REPETITIVE CA^{2+} RELEASE FROM THE SR AND WAVE SUMMATION

The twitch force lags behind the [Ca^{2+}] and [TnC–Ca] transients because of the delay in stretching the series elastic elements. On repetitive stimulation by its motor neuron, the SR repetitively releases Ca^{2+} and the Ca^{2+} transients meld to form a more continuous high myoplasmic [Ca^{2+}]. By simple mass action, the prolonged [Ca^{2+}] prolongs the time that TnC remains bound with Ca^{2+}, and this prolongs the activation of the cross-bridges. This in turn allows more time to fully stretch the series elastic elements. When the series elastic elements are stretched fully, maximum tension is transmitted to the outside of the muscle. Maximum tension is reached at the tetanus. Thus higher force seen in tetanus originates in the summation of the Ca^{2+} transients. Figure 3.6.12 illustrates this schematically.

SUMMARY

Motor neurons receive thousands of synaptic inputs, either on the soma or on the dendrites, from sensory cells, interneurons, and cells higher up in the CNS. Stimulation of excitatory connections depolarizes the

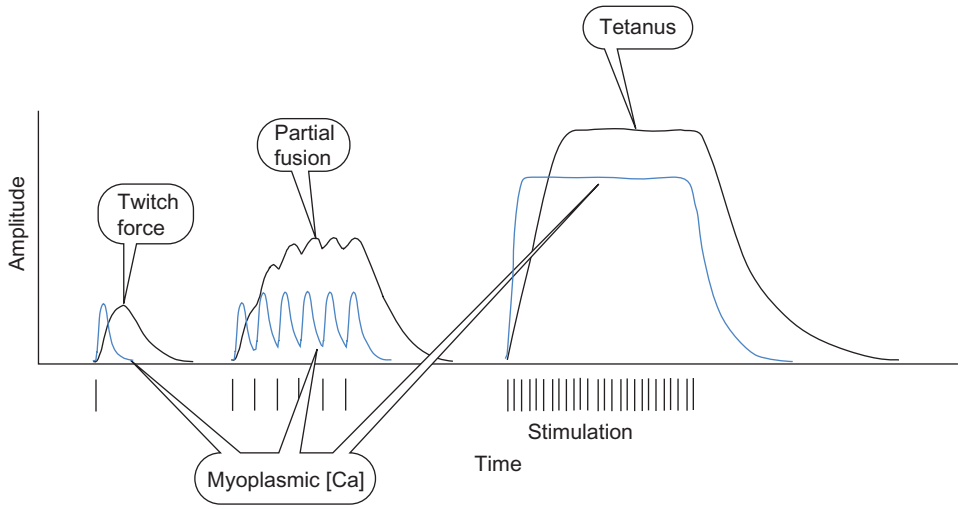

FIGURE 3.6.12 Summation of Ca^{2+} transients and tetanus. The Ca^{2+} transient is shorter than force development in the twitch. Rapid repetitive stimulation can elicit a second release of Ca^{2+} from remaining SR Ca^{2+} stores before the Ca^{2+} transient is finished. Although the amount of released Ca^{2+} in subsequent releases may diminish, because of diminished SR Ca^{2+} remaining in the SR, the total myoplasmic [Ca^{2+}] increases. Rapid repetitive stimulations result in a transfer of Ca^{2+} from the SR to the myoplasm. During the twitch the cross-bridges are fully activated, but there is insufficient time for the force generators to stretch the series elastic elements. During prolonged and rapid repetitive stimulation (tetanus), these force generators have time to stretch these elements and to fully transmit the force to the outside of the muscle. At intermediate stimulation frequencies, muscle force is partially fused.

motor neuron; this is an EPSP. Other connections hyperpolarize the cell (IPSP). Both IPSPs and EPSPs decay with distance and time. If the axon hillock reaches threshold, it fires an action potential back over the soma and down the axon. Multiple simultaneous EPSPs and IPSPs can add by spatial summation. Repetitive EPSPs and IPSPs add by temporal summation.

An action potential in the motor neuron propagates down the axon to the neuromuscular junction. There, axon terminals are filled with thousands of tiny synaptic vesicles that contain acetylcholine (ACh). The action potential activates voltage-gated Ca^{2+} channels in the membrane that open briefly to let in Ca^{2+}, which then binds to special proteins that activate fusion of the synaptic vesicles with the active zone of the nerve terminal. The acetylcholine is released into a gap between muscle and nerve, and diffuses to the muscle membrane where it binds to the AChR, causing it to increase g_{Na} on the muscle membrane. Influx of Na^+ produces an endplate potential which is conveyed electrotonically to nearby patches of muscle membrane, where an action potential begins and propagates in both directions away from the neuromuscular junction. The ACh signal is shut off by removal of Ca^{2+} from the nerve terminal cytoplasm and by destruction of ACh by acetylcholinesterase.

The muscle membrane action potential propagates along the muscle membrane and penetrates deep into the muscle along transverse tubules (T-tubules). Depolarization of the T-tubule is sensed by DHPRs on the T-tubule membrane. The DHPRs are mechanically linked to RyR located on the SR membrane immediately adjacent to the SR. RyR1 is a large tetramer that forms a gated Ca^{2+} channel across the SR membrane. These RyRs briefly open in response to depolarization of the T-tubules that is sensed by the DHPR. The terminal cisternae contain Ca^{2+} free in solution and bound to calsequestrin. Opening of the RyR1 channel releases the stored Ca^{2+}, which increases cytosolic $[Ca^{2+}]$.

The released Ca^{2+} diffuses throughout the myofibril and binds to troponin C (TnC). Ca^{2+} binding alters the conformation of TnC, which in turn alters the disposition of TnI, TnT, and tropomyosin. At rest, tropomyosin inhibits the interaction of actin and myosin so that cross-bridge cycling cannot occur. Ca^{2+} binding to TnC removes the inhibition of tropomyosin so that cross-bridge cycling occurs. The result is actomyosin ATPase activity and force development or shortening of the muscle. Reuptake of the released Ca^{2+} by the SR Ca pump causes relaxation of the muscle because Ca^{2+} dissociates from TnC by mass action. Removal of Ca^{2+} from TnC causes tropomyosin to move back to its inhibitory position, cross-bridge cycling stops, and the muscle relaxes.

Muscle force on the outside of the muscle fiber lags behind the Ca^{2+} transient. The lag is due to a delay in TnC binding of Ca^{2+} and a delay in the transmission of the force from the cross-bridges to the exterior of the muscle. The muscle behaves as if the force generators have a spring arranged in series with them. Force is transmitted through the spring only to the extent that the spring is stretched. This takes time, so the force transient (the twitch) is both delayed and prolonged compared to the Ca^{2+} transient. At least some of the spring characteristics are in the myofilaments themselves.

Repetitive action potentials can cause additional Ca^{2+} release before the muscle relaxes from the first twitch. The prolonged elevation of cytosolic $[Ca^{2+}]$ gives the myofilaments more time to completely stretch the series elastic elements to fully transmit their force to the exterior of the muscle. Force increases with increasing frequency of stimulation until tetany is reached, in which there is maximum force and no waviness in the force.

REVIEW QUESTIONS

1. Could neuromuscular transmission occur in the absence of extracellular Ca? Why or why not?
2. What is spatial summation? Temporal summation? Can both occur simultaneously?
3. Would you expect some synaptic vesicles to fuse with the presynaptic motor neuron membrane without an action potential? Why or why not?
4. What effect would an inhibitor of acetylcholinesterase have on neuromuscular transmission?
5. Curare blocks the AChR from binding ACh. What effect would this have on neuromuscular transmission?
6. What protein senses the voltage on the T-tubule? What protein releases Ca^{2+} from SR stores? What protein takes Ca^{2+} back up into the SR?
7. What protein confers Ca^{2+} sensitivity on the myofilaments?
8. Why is tetanic force greater than twitch force?
9. Why does rapid stretch of a muscle during its activation increase the force?

Clinical Applications: Malignant Hyperthermia and Central Core Disease

On April 14, 1960, a 21-year-old student sustained compound fractures of his tibia and fibula when he was run over by a car in Melbourne, Australia. In the casualty department, he was more worried about anesthesia than his injuries. He said that 10 of his close relatives had died during anesthesia for minor procedures. Ether had been used in all these anesthesia-associated deaths, so the attending physicians used a new anesthetic, halothane. After 10 minutes of anesthesia, the student's blood pressure fell, his heart rate rose, and he felt very hot. The anesthetic was stopped and the student was rubbed down with ice packs. He recovered. Further clinical examination of the student showed no known abnormality, but study of his family history indicated a previously undescribed inborn error of metabolism inherited as an autosomal dominant trait. The syndrome was characterized

by hyperthermia, a rapid and large rise in body temperature, and it was malignant: fully 70% of the early cases were fatal. This first case of malignant hyperthermia was reported in *The Lancet* in 1960 by Michael Denborough.

Persons with malignant hyperthermia are usually clinically inconspicuous. Their condition is brought on by a triggering event, usually exposure to **volatile anesthetics** or to depolarizing muscle relaxants such as **succinylcholine**. The increased metabolism is due to increased cytoplasmic [Ca^{2+}] within muscle cells that hydrolyzes ATP by the activation of cross-bridge cycling and the activation of glycogenolysis, glycolysis, and oxidative metabolism. The muscles become rigid and may undergo **rhabdomyolysis**, in which the muscle membranes lose their integrity and leak myoglobin, creatine kinase (CK), and potassium into the blood. The high plasma [K^+] can cause lethal cardiac arrhythmias. The condition is not uniform: some cases of MH are brought on by exertion, overheating, or infections. Persons suspected of being MH susceptible can be diagnosed using the response of muscle biopsies to halothane or caffeine. Early treatment with **dantrolene sodium** reduces the crisis and reduces the mortality rate from 70% to 10%. Dantrolene inhibits Ca^{2+} release from the SR through the RyR1 Ca^{2+} release channel. This prevents the rapid rise in cytoplasmic [Ca^{2+}] that causes MH.

Central core disease (CCD) derives its name from the histological appearance of type I muscle fibers in affected individuals. In this case, RyR1 exhibits increased Ca^{2+} permeability even in the resting state. In these fibers, the central core of the muscle fibers is damaged, with unstructured and damaged contractile filaments and destruction of the mitochondria, probably due to too much [Ca^{2+}] in the interior of the cell. At the periphery of the cell, the surface membrane presumably pumps out excess Ca^{2+} across the SL membrane, thereby preventing injury to the mitochondria in the peripheral regions of the muscle fiber.

Mutations of the RyR1 make up the most frequent cause of MH and CCD. Over 20 distinct mutations of the RyR1 cause MH. In some other persons, MH is caused by mutations in the DHPR. The DHPR makes close physical contact with RyR1 and during normal excitation–contraction coupling the DHPR provides RyR1 with the signal it needs to release Ca^{2+}.

APPENDIX 3.6.A1 : MOLECULAR MACHINERY OF THE NEUROMUSCULAR JUNCTION

NEUROMUSCULAR JUNCTIONS CONSIST OF A COMPLEX ARRAY OF INTERACTING PROTEINS

As described in Chapter 3.6, neuromuscular transmission involves a series of processes that begin with an action potential on the nerve axon and end with an action potential on the muscle fiber. The main sequence is (1) conversion of the electrical signal, the action potential, to a cytosolic Ca^{2+} signal within the nerve terminal; (2) fusion of docked vesicles with the presynaptic membrane and release of neurotransmitter; (3) diffusion of the neurotransmitter, acetylcholine, to the postsynaptic, muscle fiber membrane; (4) conversion of the chemical signal, acetylcholine, into an electrical signal by opening ligand-gated channels on the muscle membrane; (5) conduction of the resulting end-plate potential to the area of muscle membrane outside of the end plate; (6) initiation of the action potential on the muscle fiber membrane. These events are all mediated by proteins and their spatial arrangement is crucial to the sequence of events. This appendix considers the proteins involved on both the presynaptic and postsynaptic membranes that produce these events. This is a second level of detail that is not generally required for introductory courses. A third level of structural detail involves the parts of the proteins that interact with other proteins' parts, and the detailed structure of these proteins. This third level is the concern of structural biologists and we will not consider it here.

Electron micrographs of the neuromuscular junction shows electron-dense material on the presynaptic membrane near where synaptic vesicles congregate and attach to the presynaptic membrane. These areas of the membrane are called **active zones**, and vesicle fusion occurs only at these areas. The presynaptic membrane at the active zone contains several protein constituents that specifically bind to proteins on the vesicular membrane. These proteins are collectively known as **SNARE** proteins for **SNAP receptor**. These names derive from other factors that were found to be required for synaptic transmission: **NSF** is the *N*-ethyl maleimide sensitive factor and **SNAP** is the **soluble NSF attachment protein**; SNARE is for SNAP receptor. **Synaptobrevin** (also called VAMP, for vesicle-associated membrane protein) is a v-SNARE that binds to synaptic vesicles and to another protein on the vesicles, **synaptotagmin**. Synaptotagmin binds Ca^{2+} and confers Ca^{2+} sensitivity to vesicle fusion. Synaptobrevin interacts with two t-SNARE proteins (t for "target"), **SNAP-25** and **syntaxin** on the presynaptic membrane. The v-SNARE protein synaptobrevin and the t-SNARE proteins SNAP-25 and syntaxin spontaneously coil up in an exergonic reaction that forces the vesicle towards the target membrane. There are a variety of t-SNARE and v-SNARE proteins that interact selectively, which allows for the same general machinery to be used in vesicle trafficking throughout the cell. Neuromuscular transmission is a specific example of it. The priming of the vesicle for fusion is regulated by several other proteins. **SM proteins (Munc-18** is one example) bind to syntaxin and are essential for membrane fusion in the cell. Another protein, complexin, activates the complex but clamps the complex to prevent premature fusion. Lastly, synaptotagmin binds Ca^{2+} when it rises during neurotransmission and reverses the complexin clamp. The location of the voltage-gated calcium channel is crucial here: typically only a few Ca^{2+} channels open at any active zone, and the Ca^{2+} concentration increases locally, limited by binding and diffusion of Ca^{2+} away from the Ca^{2+} channel. Other proteins, such as RIM (Rab-3 interacting molecule), are thought

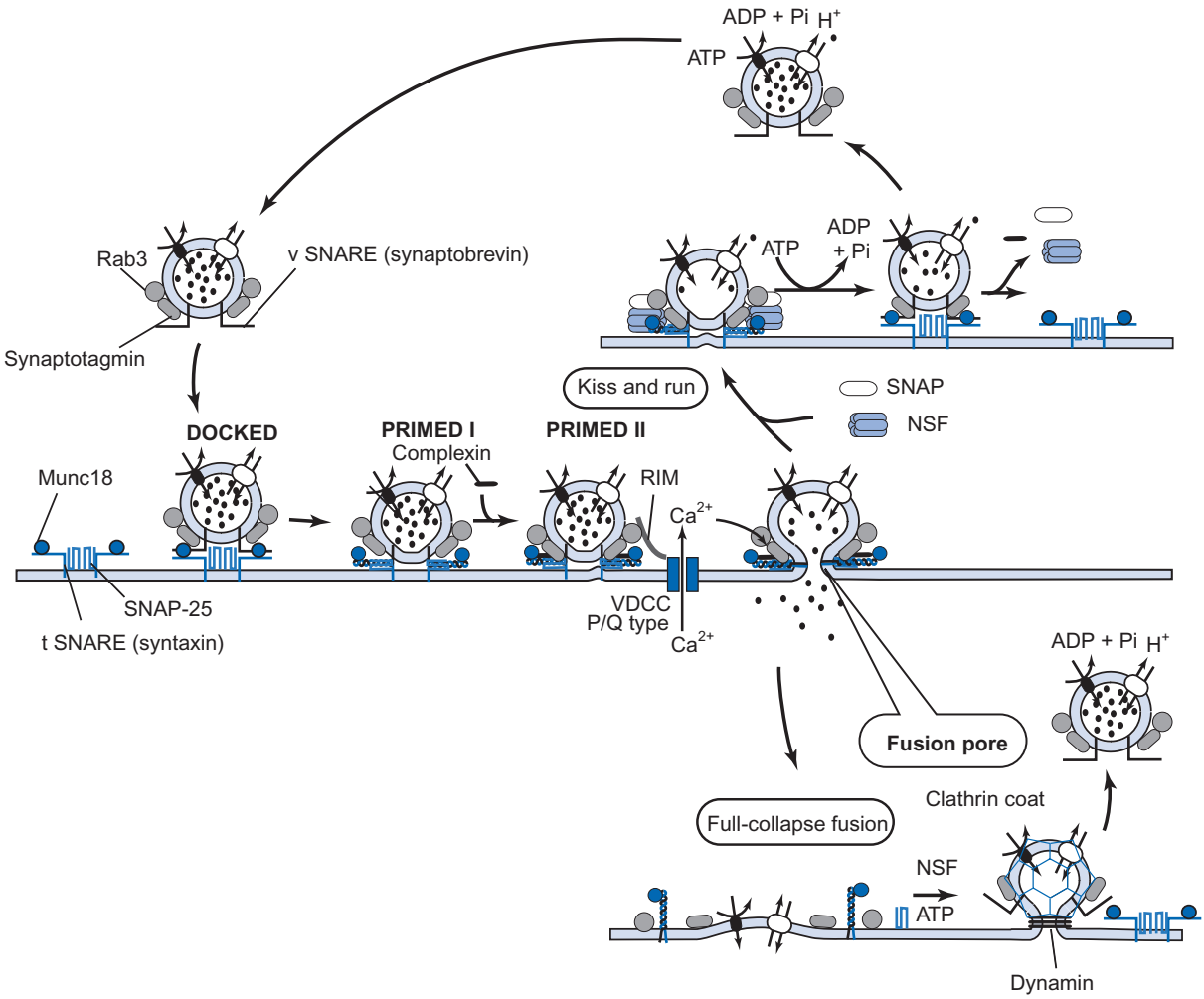

FIGURE 3.6.A1.1 Proteins involved with vesicle fusion and recycling in the neuromuscular junction. Vesicles have a v-SNARE protein (synaptobrevin or VAMP) whereas the presynaptic membrane has t-SNARES, syntaxin, and SNAP-25. These proteins interact and coil up spontaneously in a series of reactions that first dock the vesicle and then prime it in two stages. The reaction requires S/M proteins, in this case Munc-18. The coiling of the SNARES produces a force normal to the membrane that draws the vesicle to the membrane. Premature fusion is prevented by complexin that interacts with the SNARES and prevents the completion of the reaction. RIM, Rab-3 interacting molecule, interacts with Rab-3 on the vesicle and P/Q-type voltage-gated Ca^{2+} channels on the presynaptic membrane to draw these together so that the Ca^{2+} channel is close to the active zone. When the action potential propagates over the presynaptic membrane, the Ca^{2+} channel opens briefly and Ca^{2+} enters the terminal in narrow space, increasing the local [Ca^{2+}]. Synaptotagmin binds the Ca^{2+}, and in this form displaces complexin from its inhibitor position, allowing the vesicle to fuse with the presynaptic membrane and empty its content of acetylcholine into the synapse. Fused vesicles may undergo full-collapse fusion, in which case the vesicle membrane proteins are incorporated into the presynaptic membrane, or form a fusion pore that does not expand. This fusion pore is thought to be reversible, so that the vesicle fusion is "kiss and run." Recycling of the membrane component requires the *N*-ethylmaleimide sensitive factor, NSF, and ATP, and the soluble NSF-associated protein (SNAP, distinguished from SNAP-25).

to position the P/Q-type voltage-gated Ca^{2+} channels near the docked and primed vesicles. This process is illustrated in Figure 3.6.A1.1.

Although neurotransmission at each active zone results in the fusion of at most a few vesicles, transmission at physiological frequencies (50 Hz or more) can result in the fusion of a large number of vesicles. If synaptic vesicles fully fuse with the presynaptic membrane, the readily releasable pool of vesicles will become depleted, the area of the presynaptic membrane will increase, and also the character of the presynaptic membrane will change because of incorporation of vesicle proteins having a different composition than presynaptic membrane proteins.

There are two ways of avoiding these problems. First, the vesicles may not fuse completely with the presynaptic membrane. Instead, the vesicles "kiss and run": they form a transient fusion pore that allows neurotransmitter to escape to the synapse, but the fusion pore does not expand and instead closes off. The vesicle is released by an ATP-requiring reaction that uncoils the t-SNAREs and v-SNARES from each other, mediated by NSF (N-ethyl maleimide sensitive factor), a hexamer that presumably splits 3–6 ATP molecules with each catalytic cycle to uncoil the SNARE complex. Second, complete fusion of vesicles can be reversed by slow endocytosis involving segregation of the vesicle and presynaptic proteins, clathrin

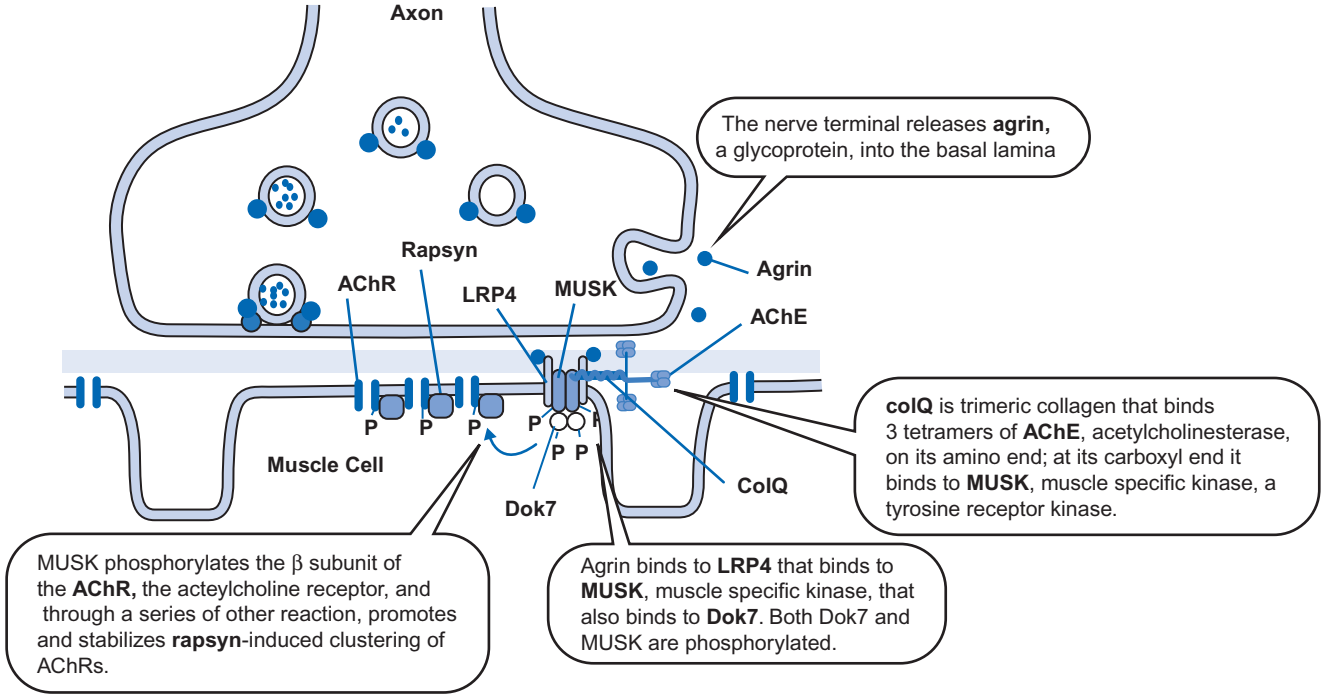

FIGURE 3.6.A1.2 Proteins involved in the formation and maintenance of the neuromuscular junction. Signals pass between the motor neuron and muscle fiber to form and maintain the neuromuscular junction, and only one of these signals is shown here. Neurons release agrin, a glycoprotein, into the extracellular space adjacent to the muscle fiber. Agrin binds to a receptor, LRP4, on the muscle membrane. LRP4 binds to MUSK, muscle-specific kinase, which is a receptor tyrosine kinase. MUSK phosphorylates Dok7, a dimer that binds to MUSK, and also autophosphorylates itself in multiple tyrosine residues. MUSK phosphorylates the β subunit of the acetylcholine receptor, AChR, that promotes its association with Rapsyn, receptor-associated protein of the synapse. Rapsyn and acetylcholine receptor cluster at the neuromuscular junction. This clustering acts on a prepattern of AChR that exists without agrin. Further organization of the neuromuscular junction is provided by colQ, collagen type Q. This consists of a trimer of collagen that binds three tetramers of acetylcholinesterase, AchE, on the amino ends of the collagen. The carboxyl ends bind to MUSK. Thus rapsyn, AChR, MUSK, Dok7, LRP4, and AChE are all colocalized within the neuromuscular junction.

coats of the vesicles and pinching off of the endocytosing vesicles by use of dynamin. These processes are also shown in Figure 3.6.A1.1.

SPECIALIZATIONS OF THE MUSCLE FIBER MEMBRANE PREPARE IT TO RECEIVE THE ACETYLCHOLINE SIGNAL

The key protein on the muscle fiber membrane is the acetylcholine receptor, **AchR**, which consist of 5 subunits: 2 α, and one each of β, γ and ε or γ. Both α subunits must bind acetylcholine in order to open the receptor's high conductance path. Clustering of AchR in the end-plate region is the result of cross-talk between the neuron and the muscle fiber. Nerve cells secrete **agrin** into the extracellular space, and agrin binds to **LRP4**, low-density lipoprotein receptor 4, which binds to **MUSK** (Muscle-specific kinase) a receptor tyrosine kinase on the surface of the muscle fiber. Activation of MUSK requires **DOK7**, a cytoplasmic protein. DOK7 not only activates MUSK, but is one of its substrates. MUSK phosphorylates the β subunit of the AChR, causing it to bind to another protein, **rapsyn** (receptor associated protein of the synapse). Rapsyn appears to mediate the clustering of the AchR at the neuromuscular junction. The extracellular domain of MUSK interacts with the carboxyl end of collagen Q (colQ) while the amino terminus of colQ interacts with **acetylcholinesterase** (AChE) in the extracellular space. Other signals pass between neuron and muscle fiber to maintain the neuromuscular junction. The main proteins associated with neuromuscular transmission are shown in Figure 3.6.A1.2.

APPENDIX 3.6.A2 : MOLECULAR MACHINERY OF THE CALCIUM RELEASE UNIT

THE CALCIUM RELEASE UNIT CONSISTS OF MULTIPLE PROTEINS ON T-TUBULES AND SARCOPLASMIC RETICULUM MEMBRANES

The Calcium Release Unit of muscle consists of the terminal cisternae of the sarcoplasmic reticulum and its adjacent transverse or T-tubule that is the invaginated part of the surface membrane. The two major proteins are the voltage sensor on the T-tubule, the **DHPR (dihydropyridine receptor)**, and the calcium release channel, **RyR (ryanodine receptor)**, on the sarcoplasmic reticulum. The DHPR is composed of five subunits. The α1 subunit acts as a voltage sensor and changes its conformation when the T-tubule depolarizes during the excitation–contraction coupling. This conformational change is transmitted to the RyR, causing it to open and release Ca^{2+} stored in the lumen of the SR.

Clinical Application: Congenital Myasthenic Syndromes

Myasthenia gravis is an autoimmune disease in which antibodies are directed against proteins of the neuromuscular junction: AchR, MUSK, and LRP4, and some unidentified ones. These antibodies reduce neuromuscular transmission and produce muscle weakness. Antibodies against the voltage-gated Ca^{2+} causes the Lambert–Eaton Myasthenic Syndrome. Mutations in genes encoding the proteins involved in neuromuscular transmission produce **congenital myasthenic syndromes** (CMS), which are a group of rare and heterogeneous disorders.

Genetic disorders of the AChR cause Slow Channel Syndrome, in which the AChR opening is prolonged. This causes desensitization blockade of the receptor and damage to the muscle fiber due to cation overload. Fast Channel CMS is rare, causes severe weakness, and is treated with AChE blockers to help keep channels open longer. Mutations in MUSK produce proximal limb and facial weakness without ocular involvement. Mutations in Dok7 is usually present with limb-girdle myasthenia, accounting for some 20% of CMS cases. Mutations in CHAT (choline acetyltransferase, the enzyme that catalyzes the synthesis of acetylcholine from acetyl CoA and choline) cause a neonatal CMS with life-threatening failure of the respiratory muscles. CMS can also be caused by mutations in LRP4 and ColQ.

Macromolecular complexes such as those involved in neuromuscular function can fail if any of their components fail. Thus it is not surprising that a variety of mutations can produce similar symptoms.

Clinical Application: Botox Treatment

Botox treatment is the most popular cosmetic treatment, with over 6 million treatments in the United States each year in 2013. It removes facial wrinkles by paralysis of the facial muscles and also produces a flat effect—a severe reduction in the external expression of emotions. Typically injection of picogram quantities of the toxin can relax muscles for 2–4 months. It is also used for other muscular disorders, including strabismus (misalignment of the eyes), achalasia (spasm of the lower esophageal sphincter), and blepharospasm (uncontrolled and sustained contractions of the muscles around the eye).

Botulinum toxin (botox) originates from *Clostridium botulinum*, a gram-negative bacterium. Botulism can be fatal, and most often results from ingestion of food contaminated with the toxin. The toxin is inactivated by heating to more than 85°C for more than 5 minutes. Fatal botulism kills by arrest of the respiratory muscles. *Clostridium botulinum* produces seven related toxins, called botulinum toxin types A, B, C1, D, E, F, and G. The main commercial types are A and B. The toxin is synthesized as a protoxin of 150 kDa, which is subsequently cleaved to light (L) and heavy (H) chains that remain linked by a disulfide bond. Nerve terminals have receptors for both the H-chain and L-chain. The L-chain is transported across the nerve terminal membrane by endocytosis, and it becomes incorporated into endosomes. The various L-chains are **metalloproteinases**—proteases that require metal ions for activity. The L-chains bind Zn^{2+}. Their substrates are one of several proteins that make up the **SNARE** complex that is required for neurotransmitter vesicle fusion with the presynaptic nerve membrane. At least three proteins are involved in this process: v-SNARE (synaptobrevin) is associated with neurotransmitter vesicles; t-SNARE (syntaxin) is on the presynaptic membrane; and SNAP-25, another t-SNARE. All of these proteins are required for the docking of vesicles in the active zone of the presynaptic terminal and for subsequent neurotransmission. Each of the botulinum toxins cleaves one of these three proteins and thereby interferes with neurotransmitter release. Synaptobrevin is cleaved, at different loci, by toxins B, D, G, and F; SNAP-25 is cleaved by A, C, and E; syntaxin is cleaved by toxin C. Thus botulinum toxin causes paralysis of the muscles by cutting up the proteins that are essential for the release of acetylcholine at the neuromuscular junction.

The RyR is present in three isoforms: **RyR1**, **RyR2**, and **RyR3**. RyR1 is present in all skeletal muscles. RyR2 is expressed in cardiac muscle and RyR3 is located in a variety of tissues. The RyR consists of a homotetramer, with single copy molecular weight of 565 kDa. The RyR is regulated by phosphorylation/dephosphorylation at several phosphorylation sites. These are phosphorylated by **protein kinase A (PKA), protein kinase G (PKG), and calmodulin-dependent protein kinase (CAM kinase II)** and are dephosphorylated by **protein phosphatases (PP1 and PP2a)**. Each monomer of the tetrameric RyR receptor binds **calmodulin**, either with or without bound Ca^{2+}, and **FKBP-12 (FK506 binding protein)**. FKBP is named for its binding of FK506, an immunosuppressor drug. Binding of FK506 to FKBP causes FKBP to dissociate from the RyR.

The RyR1 also binds **triadin** and **junctin**, two proteins of similar structure that bind each other, RyR and **calsequestrin (CASQ)** within the lumen of the SR. Calsequestrin has two main isoforms: CASQ1 and CASQ2. Fast-twitch muscle expresses only CASQ1. Slow twitch expresses both CASQ1 and CASQ2, and cardiac muscle contains only CASQ2, CASQ binds luminal Ca^{2+} at low affinity ($K_D = 1$ mM) but high capacity. At high $[Ca^{2+}]$, the CASQ polymerizes and forms extended strings within the SR lumen.

Rapid release of Ca^{2+} from the SR entails a movement of positive charges from the lumen to the cytosolic compartment. If there were no compensatory charge movements, this would cause a negative potential inside the SR that would oppose further Ca^{2+} release. This is

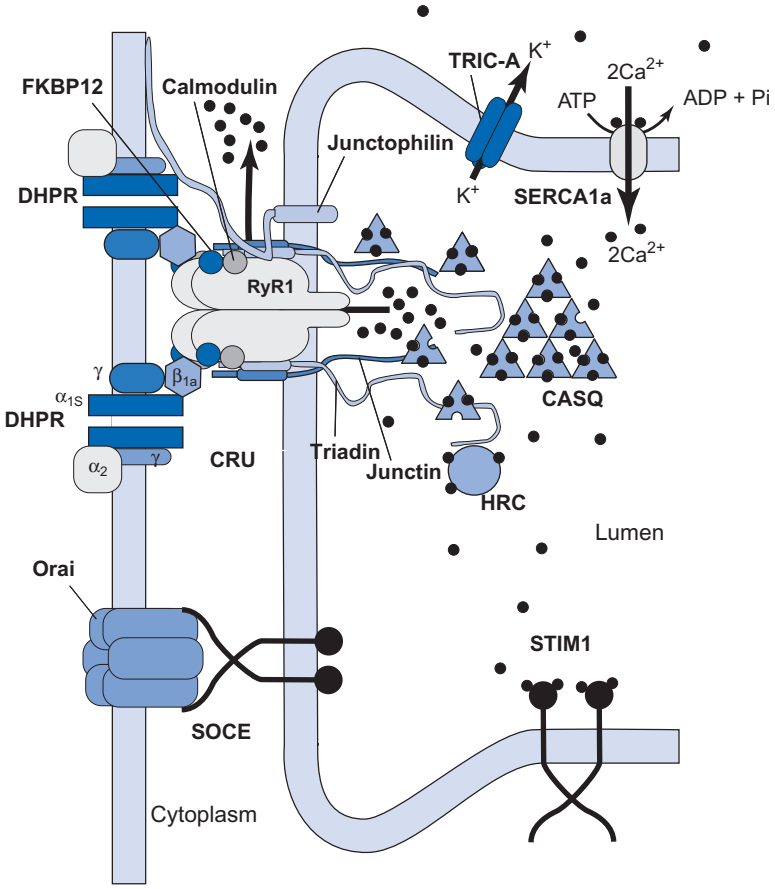

FIGURE 3.6.A2.1 Proteins involved in the release of calcium at the triad in skeletal muscle. Calcium is accumulated into the lumen of the SR by the operation of the SERCA1a calcium pump, a primary active transporter, that pumps in 2 Ca^{2+} atoms for each ATP hydrolyzed. The Ca^{2+} inside the SR is largely bound to calsequestrin (CASQ) and histidine-rich Ca^{2+}-binding protein (HRC). CASQ polymerizes when luminal [Ca^{2+}] is high. Opening of a conductance pathway through the RyR1 releases Ca^{2+} during excitation–contraction coupling. The RyR1 is present as a homotetramer that binds triadin, junction, junctophilin, FKBP, and calmodulin. Triadin and junctin also bind to each other and to CASQ and HRC. These may inform the RyR1 about the level of Ca^{2+} stores in the SR, and they modify the gating behavior of RyR1. Opening of the RyR1 is typically accomplished by conformational changes in the dihydropyridine receptor, DHPR, on the T-tubule membrane. There is a direct mechanical connection between the β_{1a} subunit of the DHPR and RyR1. This requires close apposition of DHPR and RyR1 which is stabilized by junctophilin that binds both SR and T-tubule membranes, DHPR and RyR1. When Ca^{2+} stores are depleted, STIM1 alters its conformation and opens Orai, a store-operated Ca^{2+}-entry channel. Rapid movements of Ca^{2+} across the membrane entail currents that produce a membrane potential that inhibits further Ca^{2+} current. This potential is short-circuited by TRIC-A that allows for counter currents of K^+—outward during Ca^{2+} accumulation by the SERCA1a and inward during Ca^{2+} release by the RyR1.

avoided by **TRIC-A (trimeric intracellular cation-selective channel)** in the SR membrane. These allow K^+ entry into the SR in response to any negative potential that develops.

The Ca^{2+} store in the SR is maintained by the operation of SERCA in the membranes of the SR. This 110-kDa protein directly couples ATP hydrolysis to the transport of 2 Ca^{2+} atoms into the SR. The **SERCA (smooth endoplasmic reticulum Ca-ATPase)** comes in different isoforms. Fast-twitch fibers express SERCA1a, whereas slow-twitch and cardiac muscle express SERCA2a. Slow-twitch and cardiac muscle express a small protein, **phospholamban (PLN)** that associates as pentamers in the membrane and inhibits the SERCA2a pump. Phosphorylation of phospholamban by CAM kinase II or PKA relieves the inhibition of the SERCA2a pump by phospholamban.

The triad junction includes a Store-operated Ca^{2+} entry (SOCE) mechanism. This complex of proteins senses the Ca^{2+} content of the SR and, when it is low, opens a pathway to refill the SR from the extracellular space. The sensor of the SR content is **STIM1 (stromal interacting molecule)** that is present as a homodimer and contains luminal sites for binding Ca^{2+}. Upon depletion, STIM1 undergoes a conformational change and clusters near the triad where it directly interacts with a plasma membrane hexameric channel called **Orai1**. Orai1 opens allowing Ca^{2+} to enter the restricted space between T-tubule and SR.

A cartoon illustrating the main features of the interactions of these proteins in the triad is shown in Figure 3.6.A2.1. The main point of this is to remind the student that these functions are carried out by macromolecular assemblies of proteins and that mutations or deficiencies of any of these participatory proteins could have serious consequences for the operation of the entire assembly.