Neuromuscular Transmission: New Insights

François Donati, PhD, MD, FRCPC

The receptor at the neuromuscular junction responds to the action of acetylcholine by undergoing a transformation that allows ions to pass freely. Thus, it is a ligand-gated ionic channel, the ligand being acetylcholine. It is a cholinergic receptor of the nicotinic type. It is made up of five subunits and belongs to a superfamily of receptors that includes the neural nicotinic receptor and also glycine receptors, γ-aminobutyric acid ($GABA_A$) receptors, and $5HT_3$ serotonin receptors (1). Thus, the neuromuscular junction represents a model for neurotransmission. Two types of nicotinic receptors, usually called fetal and adult, can be found in muscle, and those two types are slightly different from those described in the central nervous system. Each of the five subunits that make up the receptor is a protein made up of 437–499 amino acids (1). Two of these, called α, are identical. The rest are called β, δ, and either γ or ϵ. The γ subunit is characteristic of the fetal receptor. The ϵ is found in the adult receptor (Fig. 1). The nicotinic receptors found in the central nervous system are made up of only α and β subunits. The purpose of this review is to summarize the events involving acetylcholine synthesis, storage and release, with emphasis on the new findings. Particularly, development of the neuromuscular junction will be presented along with a discussion of the pharmacology of the junction and the effect of disease states. Finally, implications for monitoring will be presented.

Development

Origin of Nerve and Muscle

Muscle has a mesodermal origin. The nerve cells that innervate them originate from the ventral portion of the neural tube. The Schwann cells, which are the glia of the peripheral nervous system, also have a neural tube origin. Initially, a low density of acetylcholine receptors is found throughout the whole of the muscle membrane and only fetal receptors are present. Contact between a nerve cell and a muscle fiber triggers clustering of the receptors at the point of contact. In addition, there is regression of the other nerve terminals that might attempt contact with the same muscle fiber. As a result, a muscle cell is normally innervated by one nerve terminal and has one endplate, but exceptions occur. For example, one quarter of human facial muscles contains two or more endplates (2), but they function as if they had a single endplate. Conversely, each motor nerve may reach many muscle cells. Branching of axons usually occurs within the substance of the muscle. For example, the human flexor adductor pollicis is innervated by an average of 128 motor nerves, each of which branch into 106 terminals, for a total of 13,568 fibers (3).

Clustering

When a nerve terminal first makes contact with a muscle cell, the density of receptors is still small and the quantity of acetylcholine released is low. In the subsequent weeks, the density of receptors at the endplate increases markedly, folds develop, the size of the neuromuscular junction increases, and the number of extrajunctional receptors decreases. The switch from the fetal to the adult type receptor at the endplate occurs after birth in rodents but in the last trimester of pregnancy in humans. However, full maturity of the neuromuscular junction, with full formation of folds and an adequate density of receptors, probably does not occur until the end of the first year of life. In rodents, the size of the neuromuscular junction may increase from 100 μm^2 late in intrauterine life with a receptor density of 1,000 per μm^2 to 720 μm^2 2 wk after birth, with a density of 10,000 per μm^2 (4). Thus, the number of receptors at the junction increases from 100,000 to more than 7 million during that period.

Extrajunctional Receptors

Outside the junctional area, the number of receptors is <10 per μm^2, but even in the adult, the receptor type is still fetal, with a γ subunit. For this reason, the fetal receptor is also called extrajunctional. However, the total number of extrajunctional receptors is still large because the neuromuscular junction occupies only a small fraction of the muscle cell membrane. If anything, the size of the adult neuromuscular junction in humans is smaller than that of rodents, approximately 500 μm^2 (5). With a density of 10,000 to 20,000 per μm^2,

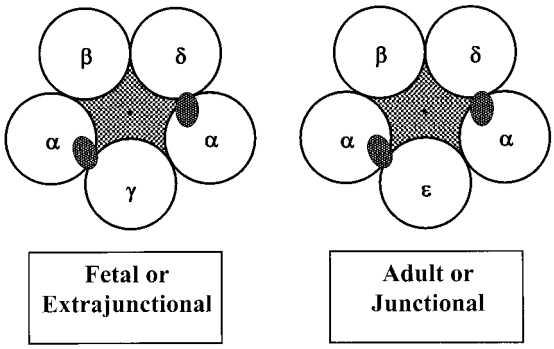

Figure 1. Schematic view of the muscle nicotinic receptor at the neuromuscular junction, as seen from the top. The location of the acetylcholine binding site is represented on both α subunits, close to either γ or ϵ, and δ.

there are 5 to 10 million adult type receptors at the junction. Muscle cells may be up to 100 mm in length and have a diameter of 50 μm or so, for a surface of $\pi \times 50 \times 100{,}000$ μm, or approximately 15,000,000 μm^2. With a receptor density of 10 per μm^2, the 150 million extrajunctional receptors may outnumber the junctional receptors by a factor of more than 10.

Release of Acetylcholine

Synthesis and Storage

Acetylcholine is synthesized in the nerve terminal from choline and acetyl coenzyme A (acetyl CoA). It is packaged, against a concentration gradient, into vesicles measuring 50 nm in diameter. The number of acetylcholine molecules per vesicle has been evaluated to be 10,000. The vesicles are coated with proteins, which play a role in storage, mobilization, and release. One of these proteins, synapsin, anchors the vesicle to the nerve terminal cytoskeleton. A vesicle-associated membrane protein, also known as synaptobrevin, plays a role in mobilization to a docking site on the nerve terminal membrane, made up of syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25) (Fig. 2) (6). Different forms of botulinum toxin prevent acetylcholine release by acting on a vesicle-associated membrane protein, syntaxin, or SNAP-25.

Active Zones

The docking sites are physically close to calcium channels, in an area called the "active zone." The density of active zones is approximately 2.5 per μm^2 of nerve terminal membrane (5). The architecture of the synaptic cleft is such that the active zones are opposite the crests of the postsynaptic synaptic folds, to minimize the distance between the release site and the postsynaptic receptor. These vesicles lying next to the active zones form the "immediately releasable pool," as they are preferentially released when an action potential invades the nerve terminal. Most of the vesicles, however, do not occupy this privileged position; these constitute the "reserve pool."

Depolarization

When an action potential invades the nerve terminal, as may occur during voluntary movement or as a result of peripheral nerve stimulation during an anesthetic, depolarization is manifest and calcium channels are activated. In the active zone, high intracellular calcium concentrations are achieved only near the inside of the channel itself, thus providing the signal for fusion of the nearby vesicle with the nerve terminal membrane and extrusion of its acetylcholine contents into the synaptic cleft. In rodents, an action potential will result in the release of 200–300 vesicles, with 2 to 3 million acetylcholine molecules released into the synaptic cleft. However, it has been suggested that humans release fewer vesicles (20 to 30), corresponding to 200,000 to 300,000 acetylcholine molecules (5). The membrane making up the vesicle is recycled.

Repetitive Stimulation

If stimulation is sustained, rapid exhaustion of the immediately releasable pool occurs unless the pool is replenished. The total number of vesicles in the nerve terminal, which may be in the hundreds of thousands, is large enough to sustain hundreds or thousands of stimulations. However, only a small fraction is immediately releasable, so the number of vesicles released per impulse decreases after the first impulse of repetitive stimulation. This phenomenon has been termed "fade" or "rundown." This explains why the margin of safety of the neuromuscular junction is less during repetitive (train-of-four or tetanic) stimulation.

Presynaptic Receptors

The mechanism by which the immediately releasable pool is replenished also involves calcium, which severs the bond between the vesicle and the actin skeleton, and allows migration to a docking site near the active zone. Regulation of this process is achieved through the action of presynaptic receptors, of which some provide a positive feedback system at the neuromuscular junction (Fig. 2), These presynaptic receptors depolarize in response to acetylcholine and other agonists such as succinylcholine and neostigmine. Depolarization makes more calcium available in the nerve terminal. Low doses of neuromuscular blocking

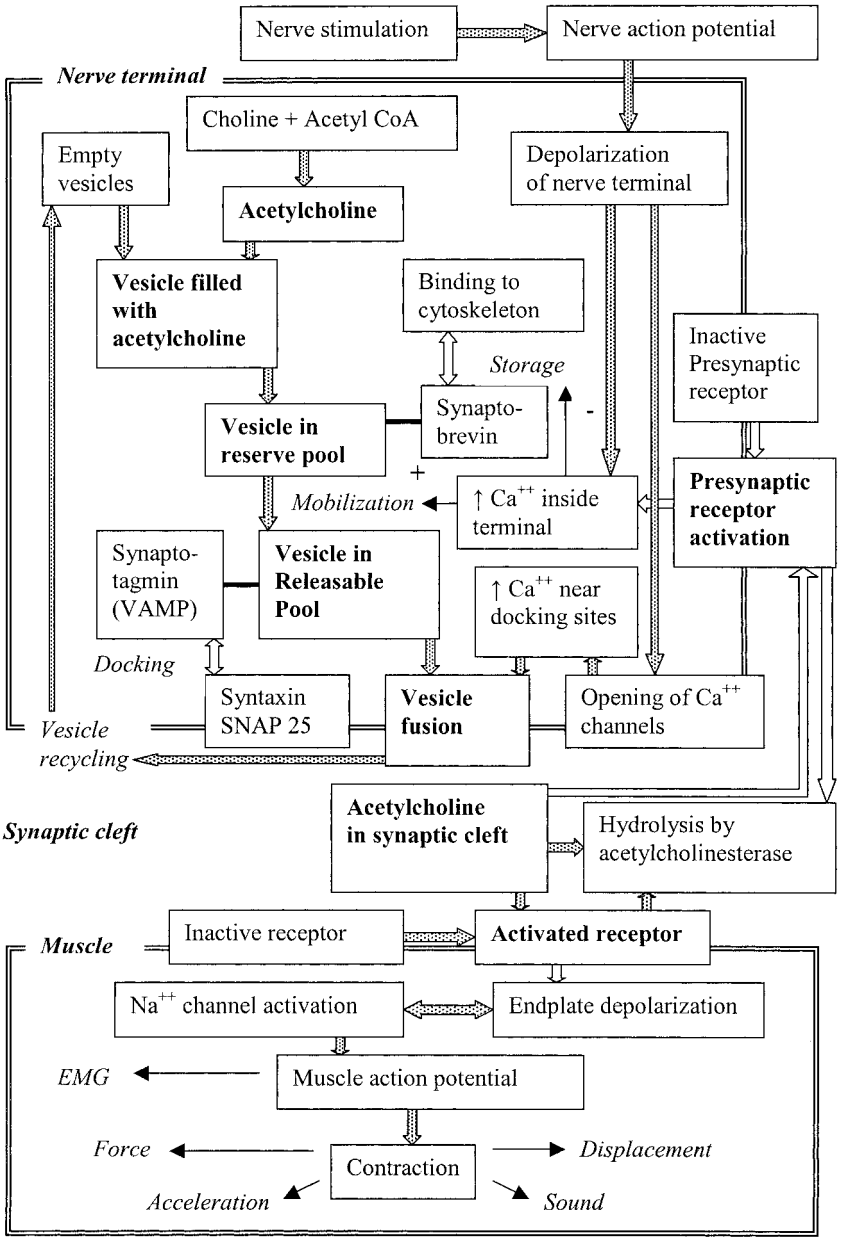

Figure 2. Diagram of acetylcholine synthesis, storage, release, binding, and breakdown at the neuromuscular junction. The path of acetylcholine is represented in **bold**, empty arrows represent presynaptic regulation, and words in *italics* represent processes or phenomena.

agents are sufficient to block these presynaptic receptors, accounting for fade. There is evidence that those "fade" receptors are neuronal nicotinic receptors made up of only α and β subunits and thus are different from the postsynaptic endplate receptors.

The Postsynaptic Receptor

Synaptic Cleft

The thin, 50-nm gap between the nerve terminal and the muscle endplate is called the synaptic cleft. If the neuromuscular junction were as large as a letter-size sheet of paper, the synaptic cleft would be approximately the thickness of the sheet. With such an arrangement, acetylcholine crosses the synaptic cleft quickly and very little escapes to the outside.

Shape of the Receptor

Each of the five subunits making up the nicotinic receptor is a protein (i.e., a series of amino acids) that enters and exits the membrane four times. Both the amino and the carboxy ends, as well as the majority of the amino acids, are on the outside of the membrane. Thus, the receptor looks more like a funnel than a doughnut, with the large end of the funnel on the outside. At rest, the

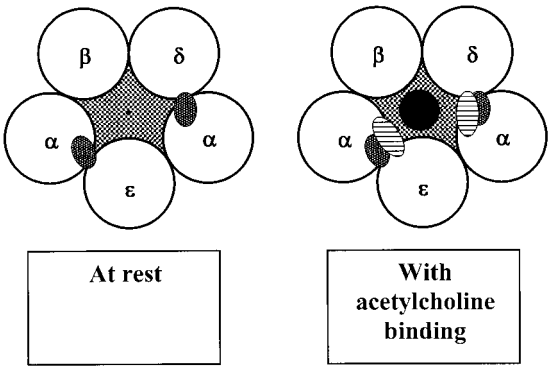

Figure 3. Junctional receptor at rest and bound to two molecules of acetylcholine, seen from the outside. The binding sites are located on the α subunits, near the δ and ϵ subunits respectively. When two molecules of acetylcholine bind to the receptor (hatched ellipses, right), the pore (black circle) opens.

receptor is closed; that is, the subunits touch each other in the thin part of the funnel.

Binding Site

Each α subunit is 437 amino acids long, with a long extra-cellular segment at the amino end. The acetylcholine binding site is located on this segment near amino acid 191 when counting from the amino end, adjacent to subunits δ and ϵ (or γ), respectively (Fig. 1). When two molecules of acetylcholine bind simultaneously to the receptor, a conformational change takes place in the protein structure of the subunits so that a pore is formed in the thin part of the channel, allowing ions to pass freely (Fig. 3).

Acetylcholinesterase

The acetylcholine molecules are hydrolyzed quickly by the enzyme acetylcholinesterase as soon as it leaves the receptor. The enzyme can be found attached to the basal membrane in the synaptic cleft and in the synaptic folds. The number of such enzyme molecules (3 million per endplate) is less than the number of receptors but is sufficient to make hydrolysis efficient.

Ionic Movements

During the short time (<1 ms) that the receptor stays open, sodium ions move inside the muscle cell along their concentration and ionic gradient; the inside is poor in sodium and electrically negative. This causes a depolarization. Potassium tends to come out of the cell, along its concentration gradient, but the potassium current is less than the sodium current because potassium does not move along its electrical gradient. Depolarization is further amplified by activation of sodium channels, which are concentrated in the depths of the folds. It appears that the presence of sodium channels, which open in response to a depolarization, is particularly important in humans, who have small endplates, but deep folds (5). When the membrane potential is raised to a certain threshold, typically 15 to 20 mV above resting potential, a muscle action potential can be generated.

Number of Vesicles Released

A single vesicle can depolarize the membrane by 0.5 mV. In rodents, as little as 30 to 40 vesicles are needed to bring the membrane to depolarizing threshold. This is much less than the 200–300 actually released. Also, the number of receptors activated is just a small fraction of the number available. The 2–3 million acetylcholine molecules released after an impulse do not open more than 1 million receptors, probably less. Two molecules are required per receptor, and some acetylcholine molecules either bind singly to a receptor or are destroyed en route. These estimates suggest that only a small percentage of the 10–20 million receptors are activated, and an even smaller proportion, perhaps as little as 2–3% of receptors, need to be activated to reach threshold. In humans, fewer vesicles are released, but there are more than needed thanks to the sodium channels. The proportion of receptors activated is also very small.

Effect of Neuromuscular Blocking Agents

Nondepolarizing Drugs

With so little acetylcholine required and so many receptors available, life is difficult for neuromuscular blocking agents. In addition, these drugs do not have access to the endplate directly but must sneak their way in through the edges of the neuromuscular junction. Theoretically, blockade can result from binding with one acetylcholine site, the other, or both because two molecules of acetylcholine are needed to activate the receptor. However, nondepolarizing neuromuscular blocking agents are bulky, rigid molecules. The funnel-shaped mouth of the receptor can probably accommodate only one of them at a time. Binding to a nondepolarizing drug occupies the site but does not induce the conformational change that opens the channel.

Succinylcholine

Depolarizing drugs like succinylcholine have an acetylcholine-like effect. However, acetylcholinesterase does not degrade succinylcholine in <1 ms as it does acetylcholine. As a result, depolarization persists. This produces desensitization of the nicotinic receptor, that is it fails to respond to the drug. In addition, the sodium channels that are initially activated become

inactivated within a few milliseconds, and are not reset, because the membrane potential does not return to its resting value. Potassium moves out of the cell because of the opening of the nicotinic receptors. It should be remembered that neuromuscular blocking agents bind to extrajunctional receptors as well as junctional receptors. With nondepolarizing agents, this extrajunctional binding has no effect, apart from sequestering some of the dose given. This diversion of the drug away from its intended site of action may explain why the dose has to be increased in cases of extrajunctional receptor proliferation, such as in burns. With depolarizing agents, the situation is different. They produce opening of the receptor, and this will occur at all receptors, whether junctional or extrajunctional. As a result, potassium exit from the muscle cell is increased markedly in conditions characterized by proliferation of receptors.

Margin of Safety

Receptor Occlusion Studies

More than 30 yr ago, it was recognized that the number of receptors activated by the acetylcholine released during normal neurotransmission was far in excess of what was needed to depolarize the endplate to threshold. Thus, it was predicted that a large proportion of receptors had to be occupied before neuromuscular blockade was manifest. An elegant set of experiments was therefore performed to quantify the proportion of receptors required based on receptor occlusion techniques. The principle is simple. Depolarization produced by an agonist (in this case succinylcholine) is measured at one endplate and increasing concentrations of d-tubocurarine are applied. The size of the depolarization is a measure of the number of free receptors, so a relationship between nondepolarizing neuromuscular blocking agent concentration and receptor occupancy can be derived. At each d-tubocurarine concentration, evoked twitch height is also measured. Thus, receptor occupancy can be derived for each degree of twitch depression.

Animal Studies

In the cat tibialis anterior, twitch height started to decline when 75% of the receptors were occupied and blockade was complete when 92% of the receptors were occupied (7). These figures have been quoted often, without considering that although the principle is entirely valid, the numbers are only approximations. It should be remembered that at, e.g., 60% blockade, each fiber in a muscle does not contract at 40% of its maximal strength. At 60% blockade, 40% of fibers function normally, whereas 60% of fibers have transmission failure at their endplates. In other words, neuromuscular blockade is graded in a whole muscle because there are slight differences in the concentration of drug required for blockade at each endplate. These differences are probably attributable to factors such as subtle differences in amount of acetylcholine released, number of receptors, or endplate size. This means that the margin of safety is different for each fiber within the same muscle. In the case of the cat tibialis anterior, the most sensitive fibers failed, on average, when 75% of receptors were occupied, whereas the most resistant required 92%.

Train-of-Four and Tetanus

The numbers change a bit when repetitive modes of stimulation are applied. The depletion of the immediately available pool of vesicles implies that if a second impulse arrives within a short time after the first, the number of available vesicles will be decreased. In the absence of neuromuscular blocking agents, this decrease has no consequence because the amount of acetylcholine released is far in excess of what is needed. However, in the presence of neuromuscular blocking agents, this is not the case and fade will be observed. For the cat tibialis anterior, it was found that fade of the train-of-four can be detected at 70% receptor occupancy (30-Hz fade at 70% occupancy and 100-Hz tetanic fade at 50% occupancy) (7). The original experiments showed considerable variation from one animal to the next.

Different Species and Muscles

The margin of safety depends on the muscle or the species considered. Not unexpectedly, the cat diaphragm was found to require greater receptor occupancy (approximately 85%) for blockade. Dogs have a greater margin of safety (80%–90%) for both tibialis anterior and diaphragm (7). Using other experimental methods, widely different estimates (from 45% to 92%) have been obtained depending on muscle and species (5). In humans, the margin of safety might be smaller than in other species. Thus, the actual receptor occupancy where neuromuscular blockade becomes detectable in humans is not known, but it is highly probable that for the most sensitive human muscles, such as the adductor pollicis, the figure is $<75\%$. Large variations are likely to occur between individuals and between muscles.

Effect of Reversal Drugs

It is now accepted that not all the effects of reversal drugs are explained by the inhibition of acetylcholinesterase. However, acetylcholinesterase inhibition

certainly represents a major effect. Studies of mice lacking the gene for acetylcholinesterase have demonstrated effects that are similar to those of neostigmine (8). Twitch height is greater than in control mice. Duration of contraction is longer, and fade occurs during high frequency stimulation. These effects are reversed by administration of d-tubocurarine. The most likely explanation for the reversal effect of anticholinesterase drugs is a prolongation of the half-life of acetylcholine, which can bind repeatedly to many receptors. There cannot be more than 100% enzyme inhibition; this effectively sets a limit on the dose of neostigmine, pyridostigmine, or edrophonium. In the absence of neuromuscular blocking agents, anticholinesterase drugs cause fade, but this fade is an accentuation of the first few twitches rather than a depression of subsequent twitches. Activation of presynaptic receptors directly by the drug or via cholinesterase inhibition produces back-firing of the terminal branches of the axon, thus triggering repetitive stimulation.

Effect of Disease

Myasthenia

In myasthenia gravis, antibodies against the nicotinic receptor at the neuromuscular junction produce a curare-like state, with characteristic fade. In this case, the margin of safety is reduced and may be zero. Not unexpectedly, patients with myasthenia gravis have an increased sensitivity to nondepolarizing neuromuscular blocking agents. They show resistance to succinylcholine. This suggests that the intensity of succinylcholine blockade depends on the degree of depolarization, which in turn depends on the number of receptors available. Recently, there has been growing interest in myasthenic syndromes, a collection of heterogenous congenital or acquired conditions involving abnormal ionic channels or acetylcholinesterase activity at the neuromuscular junction. Neuromuscular function and treatment depend on the specific disorder.

Denervation

The integrity of the neuromuscular junction is maintained by the nerve. The presence of a healthy and active nerve terminal is required to keep the high density of receptors at the junction and suppress the formation of extrajunctional receptors. Lack of nerve activity results in axonal sprouting with the emergence of contacts with the muscle cell. This increases the number of receptors at the neuromuscular junction (up-regulation), but there is also proliferation of extrajunctional receptors. As a result, there is resistance to the effect of nondepolarizing neuromuscular blocking agents. More receptors need to be occupied at the neuromuscular junction to produce an effect. Muscle atrophy probably makes the junction more resistant to the effect of nondepolarizing neuromuscular blocking drugs. When muscle cells are reduced in size, the neuromuscular junction does not shrink as much as muscle itself. This means that there are more receptors per unit surface area of muscle; therefore depolarization is easier to accomplish and more difficult to block. Resistance to the effect of nondepolarizing neuromuscular blocking agents occurs in upper motor neuron lesion, such as cord transection, and in burns. In both these conditions, the dose of nondepolarizing agent must be increased markedly for the same effect. In cases of prolonged immobilization and in patients receiving chronic infusions of neuromuscular blocking agents, up-regulation and a need for a greater dose of non-depolarizing drug also occur but to a lesser extent.

Depolarizing Agents

Because it is an agonist, succinylcholine activates all receptors, both junctional and extrajunctional. If the latter predominate, they will be responsible for some of succinylcholine's effects. A relatively high density of extrajunctional receptors can produce depolarization along the whole length of the muscle cell, and this may be manifest as a contracture, which is an increase in tone without action potential propagation. More importantly, opening of the receptors will produce an outgoing movement of potassium. Severe hyperkalemia has been documented in burns and cord transection. It has also been observed when succinylcholine was given in patients with muscle dystrophy, even if the disease was not manifest at the time of administration (9). In that case, proliferation of extrajunctional receptors is probably not involved, but succinylcholine is thought to cause breaks in the fragile membrane of muscle, causing potassium to leak out.

Monitoring

Fade

Current modes of monitoring rely heavily of the fade properties of nondepolarizing neuromuscular blocking agents. Train-of-four and tetanic fade are the result of decreased acetylcholine release with repetitive stimulation, a phenomenon which is unmasked and accentuated when receptors are blocked. For reasons that are unclear at this time, fade is less during onset than during recovery, so it is unnecessary to use train-of-four stimulation to monitor onset. Depolarizing drugs such as succinylcholine produce little fade, at least during short-term administration.

Posttetanic Facilitation

With nondepolarizing blockade, tetanic stimulation (50–100 Hz) produces a period of post-tetanic facilitation, when the response to any stimulation mode is accentuated compared with the pretetanic situation. This phenomenon is the basis for the posttetanic count, where, in the absence of any response to train-of-four stimulation, a 50-Hz tetanus causes twitches to be seen. Similarly, if tetanic stimulation is used, the response to train-of-four stimulation is spuriously elevated when applied soon after the tetanus. In the past, it was recommended to allow 5 min to elapse before the neuromuscular junction was considered to return to its normal state. Recent evidence suggests that 1–2 min is sufficient. The term "facilitation" is preferred to "potentiation," which refers to an accentuated response observed in the absence of neuromuscular blocking agents. Traditionally, posttetanic facilitation was explained by the massive influx of acetylcholine on the competitive balance between the neurotransmitter and the blocking agent. However, it is surprising that a neurotransmitter that remains in the synaptic cleft <1 ms has an effect which extends for 5 orders of magnitude as long (100 seconds or so).

Electrical Events

The methods used to assess neuromuscular blockade clinically rely on some aspect of muscle response. When depolarization to threshold occurs in a muscle cell, an action potential travels along the whole length of the muscle. Because the neuromuscular junction is most often near the midpoint of the muscle fiber, the action potential propagates to both ends simultaneously, with a typical velocity of 3–5 m/s. The event is complete in approximately 10 ms (50 mm at 5 m/s), and it is possible to record it by measuring the electromyogram (Fig. 2). This is best accomplished by placing one electrode in the midpoint of the muscle (near the neuromuscular junction) and another electrode at one of the muscle extremities. The recorded signal is the sum of many action potentials coming from several thousand muscle fibers.

Contraction

The action potential triggers calcium entry into the muscle fiber from stores in the sarcoplasmic reticulum. A contraction is then allowed to take place. Most commonly, this contraction is evaluated manually or visually. Mechanomyography, or measurement of force of the isometric contraction, is more accurate. Accelerometry is a surrogate measurement, which is based on the assumption that force = mass $\times$ acceleration, with mass being constant. Contrary to mechanomyography, which involves measurement against a resistance, accelerometry requires a freely moving muscle, and is more susceptible to changes in position. Displacement is also measured in other monitors. Contracting muscle also emits sounds of uncertain origin. Two main hypotheses have been proposed: the muscle fibers might vibrate like the string of a violin or the spatial displacement of the muscle might compress surrounding tissue. Applying a stethoscope to the adductor pollicis during train-of-four stimulation will convince skeptics. The principle has been applied for phonomyography measurements. Recent evidence suggests that the sounds emitted are of low frequency and most of the signal is below hearing threshold. Specialized equipment with special microphones is thus needed to record and process the signal.

Summary

- Acetylcholine is packaged in vesicles, which are released in response to depolarization. Calcium is required for release.
- The postsynaptic receptor requires two acetylcholine molecules to be activated.
- Sodium receptors amplify the depolarizing effect of nicotinic receptors at junction.
- There is more acetylcholine released compared with what is needed for depolarization. This "margin of safety" is species-dependent and muscle-dependent and decreases with repetitive stimulation.
- Humans probably have a lower margin of safety than other species.

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