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Presynaptic mechanisms controlling calcium-triggered transmitter release at the neuromuscular junction

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Abstract

Calcium-triggered neurotransmission underlies most communication in the nervous system. Yet, despite the conserved and essential nature of this process, the molecular underpinnings of calcium-triggered neurotransmission have been difficult to study directly and our understanding to this date remains incomplete. Here we frame more recent efforts to understand this process with a historical perspective of the study of neurotransmitter release at the neuromuscular junction. We focus on the role of calcium channel distribution and organization relative to synaptic vesicles, as well as the nature of the calcium sensors that trigger release. Importantly, we provide a framework for understanding how the function of neurotransmitter release sites, or active zones, contributes to the function of the synapse as a whole.

Keywords

neurotransmitter release; neuromuscular junction; presynaptic; calcium channels; active zone

Introduction

More than a century has passed since the first studies of calcium-triggered acetylcholine release from the neuromuscular junction (NMJ) were published. Early reports by Locke [1] in 1894 and Overton [2] in 1904 provided some of the first pieces of evidence that calcium ions were required for communication between nerve and muscle cells. These observations were followed by more extensive investigations including a series of studies in the 1930s by T.P. Feng [3]. By measuring muscle contractions following nerve stimulation, Feng studied the effects of stimulus frequency, calcium ion concentration, and various pharmacological agents on neurotransmission at the NMJ. While these investigations provided important evidence for the role of calcium in neurotransmission at the NMJ, it was not until the invention of the microelectrode, and the demonstration that it could be used for recording membrane potential from the frog sartorius muscle [4], that detailed study of calcium-

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triggered chemical transmitter release at the NMJ became possible with sufficient temporal and spatial resolution to begin to elucidate underlying mechanisms.

In a series of publications in the 1950s and 1960s, Paul Fatt, Jose del Castillo, Ricardo Miledi, and Bernard Katz made a number of seminal discoveries which solidified the importance of the influx and timing of presynaptic calcium ions for chemical transmitter release [5,6]. Among other observations, they showed that varying the extracellular calcium concentration affected the size of the action potential-evoked endplate potential (EPP), but not the size of the spontaneous miniature endplate potentials (mEPPs) [7,8]. Katz and colleagues also discovered that calcium ions had to be present extracellularly during action potential invasion at the synapse for transmitter release to occur [9,10]. Katz and Miledi conducted complementary studies using the squid giant synapse to demonstrate that calcium ions enter the nerve terminal during the action potential to trigger transmitter release [11,12,13]. In addition, they also used recordings of action potential-evoked and spontaneous transmitter release from the frog NMJ in low extracellular calcium (a critical experimental variable) to formulate the quantal theory of chemical transmitter release [8]. Katz and coworkers' groundbreaking work also helped popularize the NMJ as a model system for studying calcium-dependent mechanisms of transmitter release. In recognition of the impact of this work, Bernard Katz shared the Nobel Prize in Physiology or Medicine in 1970.

These foundational studies have spurred a renewed effort over the last two decades to better understand the microscopic underpinnings of synaptic function at the NMJ. Aided by novel experimental techniques and computational approaches, recent studies have led to new insights at the NMJ and also provided a framework for better understanding of early discoveries. Below we highlight and discuss several key aspects of synaptic function, present recent discoveries, and discuss how these advances have helped to shed new light on the underlying microscopic principles of synaptic function. Finally, we identify and propose new avenues for future study.

Where are calcium channels located within the nerve terminal?

The NMJ has been an attractive preparation for the study of presynaptic function, due in part to the fact that its structural features make it amenable to experimental perturbation. It is a large synapse and therefore more accessible than small bouton-like central synapses. The NMJ also has a 1:1 innervation ratio between the presynaptic motor neuron and the postsynaptic muscle cell (in most cases), which simplifies data interpretation, and allows for experimental access to the peripheral nervous system. Anatomical studies in the 1970s also showed that the NMJ had a stereotypical presynaptic structure. Freeze fracture electron microscopy by Heuser, Reese, and others [14,15,16] demonstrated that the frog presynaptic terminal was subdivided into a large number of transmitter-releasing substructures called active zones. Each active zone contained a double row of synaptic vesicles flanking linear arrays of transmembrane particles, some of which were assumed to be voltage gated calcium channels [17]. The subdivision of the presynaptic motor terminal into well-organized active zones was subsequently confirmed at other NMJ preparations such as the rat [18], mouse [19], and human [20,21]. In each of these preparations, active zones appeared to contain

both synaptic vesicles and transmembrane particles (putative ion channels) arranged into short linear arrays (in contrast to the long linear arrays at the frog NMJ). The discovery of active zone organization spurred research into the density and spatial arrangement of voltage gated calcium channels, as well as the active zone material that participated in maintaining this organized arrangement [22].

Since the location of presynaptic voltage gated calcium channels relative to docked synaptic vesicles was suggested to be important for synaptic function, investigators performed a series of studies using intracellular exogenous calcium buffers to shed light on the distance between the two [23]. The strategy was to compare the effects of two different buffers: EGTA, a relatively slow binding calcium buffer; and BAPTA, a faster acting calcium buffer. The expectation was that if calcium channels were positioned close to docked synaptic vesicles, only the fast, exogenous buffer would be able to interfere with the release process. Indeed, when the effect of these two buffers were compared at the squid giant synapse, BAPTA greatly reduced transmitter release while EGTA showed little or no effect. Similar effects of BAPTA on transmitter release were subsequently found at the frog [24] and neonatal rat NMJs [25]. These findings led to the conclusion that calcium channels must be close to calcium binding sites on synaptic vesicles and therefore that calcium ions entering the terminal through voltage gated calcium channels must bind to vesicular calcium sensors in less than 200 microseconds. Based on a spatially realistic computer model of the frog NMJ active zone that included endogenous calcium buffers, the buffered diffusion coefficient of presynaptic calcium was estimated to be $2 \times 10^{-7} \text{ cm}^2/\text{sec}$ [26]. Therefore, voltage gated calcium channels must be spatially close to synaptic vesicles in order to provide sufficient calcium ions to trigger transmitter release.

That calcium channels were located close to transmitter release sites within active zones was further supported by experiments that used calcium-sensitive dyes to visualize the spatial localization of calcium entry sites. This approach was initially applied to the squid giant synapse [28,29] and revealed that calcium entry during nerve stimulation did not occur throughout the whole presynaptic nerve terminal. Rather, calcium entry was restricted to active zones where synaptic vesicles were located and active zone transmembrane particles had previously been identified via freeze fracture. That calcium entry was restricted to active zones was subsequently confirmed at the frog NMJ using optical techniques [27,30] (see Figure 1). By labeling postsynaptic acetylcholine receptors, these studies also showed that active zones were directly opposed to postsynaptic acetylcholine receptors (see Figure 1).

Direct labeling of presynaptic calcium channels further supported the hypothesis that presynaptic calcium channels are restricted to transmitter release sites. Using a fluorescently-tagged toxin (ω -conotoxin GVIA) that bound selectively to the N-type voltage-gated calcium channels (the type of calcium channels predominately expressed at the frog motor nerve terminal), investigators showed that calcium channels were present only in presynaptic active zones (Figure 2). In particular, at the frog NMJ, N-type voltage gated calcium channels were restricted to “bands” that co-localized with postsynaptic acetylcholine receptors. [31,32]. Since presynaptic active zones directly oppose postsynaptic receptors at the NMJ, these data provided some of the first direct evidence of calcium channel localizations within the presynaptic nerve terminal.

While all of these studies have provided important insights into the location of presynaptic calcium channels within active zones, and with respect to synaptic vesicles, the precise nature, location, and stoichiometry of calcium and other voltage gated ion channels in active zones remains an open question.

Synaptotagmin is the Vesicular Calcium Sensor Triggering Vesicle Release

The quantitative relationship between the calcium ion concentration in the presynaptic terminal and the magnitude of transmitter release was first examined at the frog NMJ [33,34] and termed the calcium-release relationship (CRR). Strikingly, in the frog NMJ the CRR was found to be a highly non-linear, 4th order power law. In other words, a small change in the presynaptic calcium concentration leads to a large change in transmitter release. Remarkably, every synapse that has been studied to date (across all species and synapses) displays a similar non-linear relationship between calcium concentration and transmitter release with exponents ranging from 3–5 [35,36,37,38,39,40,41,42]. Therefore, the non-linear CRR first elucidated at the frog NMJ is thought to be a conserved feature of synaptic vesicle fusion. However, the molecular mechanisms that underlie this CRR remain an active topic of inquiry.

A number of hypotheses have been put forth to explain the non-linear CRR. The non-linearity suggests that the fusion of a synaptic vesicle is triggered by the combined action of more than one calcium ion in the nerve terminal. An early hypothesis that remained popular until fairly recently was that the 4th order CRR was due to the presence of only four cooperative calcium binding sites on synaptic vesicles. This theory, however, runs counter to more recent biochemical and proteomic studies of synaptic vesicle proteins [43,44]. These studies showed that the protein synaptotagmin (type 1 or 2) is a primary component of the vesicular calcium sensor apparatus for synaptic vesicle release [45]. Since synaptotagmin molecules are present in numbers of 7–15 per vesicle [43,44] and since each synaptotagmin molecule can bind up to 5 calcium ions, the total number of available calcium binding sites per vesicle could be as high as 35–75. Such a large number of calcium binding sites does not fit well with the 4 assumed cooperative binding sites from earlier physiological models.

The number of calcium ions binding to individual synaptotagmin molecules during release, and the number of synaptotagmin proteins per vesicle that need to be calcium-bound to cause synaptic vesicle fusion is currently under active investigation. One recent modeling study at the frog NMJ that is consistent with both biochemical and physiological data [46] argues that a 4th order CRR could be achieved with an excess of synaptotagmin proteins on synaptic vesicles (~6–8 synaptotagmins positioned near the base of a synaptic vesicle in association with SNARE proteins, containing 30–40 calcium binding sites) with only a small subset requiring calcium to be bound for release to take place (3–4 synaptotagmin proteins each binding only 2 ions to screen C2 domain charges, for a total of 6–8 bound calcium ions). Dittrich et al.'s excess-calcium-binding-site model predicted the experimental non-linear CRR in the absence of any inherent cooperativity in calcium binding and fits well with other known biochemical and physiological data. Further, Dittrich et al. demonstrated that the presynaptic calcium dynamics during release exhibited complex spatial and temporal dynamics that were driven by the presynaptic action potential and the probabilistic opening

of voltage gated calcium channels during the action potential. These findings highlighted that studies and models that treat presynaptic calcium concentration during synaptic vesicle release as spatially and temporally homogeneous may be missing key aspects of the vesicle release process. Additional validation of the excess-calcium-binding-site model, and the elucidation of the specific details of this calcium-synaptotagmin-membrane fusion reaction, will require further study.

Recent studies have also shed additional light on the nature of the vesicular calcium sensor. While synaptotagmin-1 and 2 are thought to be the primary calcium sensors responsible for triggering fast synchronous neurotransmitter release, burgeoning evidence suggests other synaptotagmin isoforms may be involved in mediating different forms of neurotransmission [47,48]. In particular, at a number of synapses synaptotagmin-7 has been shown to mediate the mechanism by which calcium ions trigger short-term facilitation of transmitter release during short trains of activity [47]. Synaptotagmin-7 has higher affinity for calcium and slower binding kinetics than synaptotagmin-1 or 2. Interestingly, at other synapses, synaptotagmin-7 may perform other calcium-dependent roles [48].

Action potential shape, calcium-activated potassium channels, and the control of calcium-triggered transmitter release

During an action potential, a rapid but brief depolarization activates a fraction of the calcium channels present in the nerve terminal before quickly repolarizing back to resting membrane potential. Evidence from the frog NMJ indicates that only about 20% of functional calcium channels open in response to any given action potential, as we discuss in more detail below [49]. As the action potential is repolarizing, calcium channels that have opened experience a rapidly increasing driving force for calcium. This increase in driving force, in turn, generates a brief, but large influx of calcium into the nerve terminal that provides the majority of calcium ions responsible for vesicle fusion. The correlation of the shape of the action potential waveform with calcium current is effectively a balance between opening more calcium channels, the driving force for calcium ion entry, and calcium channel closure as the stimulus to open calcium channels is removed near resting membrane potential. The delicate interplay between the shape of the action potential waveform and presynaptic calcium current suggest that action potential shape may have substantial impact on synaptic vesicle release.

A major factor which influences action potential shape are potassium channels (voltage-gated potassium channels and calcium-activated potassium channels; K_{Ca}), which shape the repolarization and after-hyperpolarization phases. K_{Ca} channels have been shown to be co-localized with voltage-gated calcium channels at active zones in the NMJ [50]. At the mouse NMJ, Katz et al. [51] showed that K_{Ca} current is dependent on calcium entry through the Cav2.1 (P/Q-type) channels that are localized within the active zone [52]. Further, experiments using calcium chelators, EGTA and BAPTA, demonstrated that K_{Ca} and calcium channels were spatially close to one another because exogenous BAPTA but not EGTA prevented K_{Ca} channel activation [53, 54].

Since K_{Ca} channels are in close physical proximity to voltage gated calcium channels and contribute to the shape of the action potential, and thus calcium influx, it follows that K_{Ca} channels may play a pivotal role in the subtle regulation of neurotransmitter release. Indeed, when K_{Ca} channels were blocked at the adult frog or mouse NMJ, an increase in transmitter release was observed [53,55,56]. This increase in transmitter release was interpreted by the authors to be a result of greater calcium channel activation and influx due to a broadened action potential. These data, support the conclusion that K_{Ca} channels are closely associated with voltage-gated calcium channels in the active zone. Depending on how tightly K_{Ca} channels are coupled to voltage-gated calcium channels, their impact on the action potential waveform may vary. Variations in the K_{Ca} channel contribution to action potential shape may change their impact on the temporal profile of the resultant calcium influx [57, 58, see Figure 3].

What remains to be determined is the number of K_{Ca} channels present among the active zone proteins, their stoichiometric relationship with presynaptic calcium channels, and the specific impact these K_{Ca} channels have on the presynaptic action potential within the NMJ. Looking back at the linear array of transmembrane particles observed in the frog NMJ via freeze fracture [14], one may speculate that these particles may correspond to both voltage gated calcium and K_{Ca} channels, among others (see Figure 4).

Mechanisms that control the probability of transmitter release from active zones at the NMJ

Above, we reviewed evidence that neuromuscular synapses are built from many individual active zones (Figure 1, 2), but what governs the probability of transmitter release at each single vesicle release site within these active zones? Here, a single vesicle release site is defined as an individual synaptic vesicle and closely associated voltage gated calcium channels [59]. Since vesicle fusion is triggered by calcium influx, it is useful to ask if this calcium flux is homogeneous across the synapse or if calcium influx is sparse and heterogeneous. This question was addressed at the frog NMJ active zone using calcium imaging techniques that could resolve the spatial calcium profile within 1 msec of an action potential depolarization. Wachman [27] showed that the calcium influx during a single action potential does not fill the entire active zone, but rather is sparse, distributed, and variable in location. These data suggested that there are relatively few calcium channels in each active zone, and that each channel opens with a relatively low probability. Using a high-resolution single-pixel optical fluctuation analysis, Luo et al. [30] tested this hypothesis and concluded that there are relatively few calcium channels in the frog NMJ active zone, perhaps 20–40 among the 200 or so active zone proteins identified in freeze fracture. In addition, Luo et al. determined that the probability that each of these channels opened during an action potential was low ($p = 0.2$). Luo et al. [30] further concluded that the frog NMJ active zone had an approximate 1:1 stoichiometry of synaptic vesicles to functional voltage gated calcium channels. These findings suggested that relatively few calcium channels open in each active zone during an action potential, and that the calcium flux through only few channels were sufficient to trigger vesicle fusion. This conclusion is supported by data from Shahrezaei et al. [42] who used experimental and modeling studies to propose that few

calcium channels opened within an active zone during an action potential, and that the calcium ion flux that triggers each vesicle fusion event originated from only one or two calcium channels. To shed additional light on these findings, Luo et al [49] used a combination of calcium imaging, measurements of transmitter release, and MCell [60] computer simulations to show that opening of only a single calcium channel can in fact provide enough calcium ions to trigger a vesicle fusion event, but that vesicle fusion under these conditions occurs with very low probability. They further estimated that the probability of vesicle fusion after a nearby calcium channel opens is approximately $p \sim 0.05$.

Taken together, these studies led to the conclusion that transmitter release at the frog NMJ is governed by two low probability events: a low probability that individual voltage-gated calcium channels open during the very brief presynaptic action potential; and a low probability the neighboring docked synaptic vesicle will fuse with the plasma membrane when a calcium channel does open.

The NMJ is a large and reliable synapse, but how are NMJs built to function this way?

The NMJ is known as a strong and reliable synapse. Following each presynaptic action potential enough acetylcholine containing vesicles are released to depolarize the postsynaptic muscle cell far beyond the threshold for firing a postsynaptic action potential (the so-called safety factor; [61]). With so much acetylcholine being released (100s of quanta at the frog NMJ and 10s to 100s of quanta at the mammalian NMJ), it is tempting to assume that the NMJ possess high probability transmitter release sites. Evidence that this might indeed be the case was provided by a binomial estimate of the number of transmitter release sites (N) and probability of release from those sites (P) using a variance-mean analysis at the mouse NMJ in 2 mM extracellular calcium [62]. Wang et al. [62] concluded that the mouse NMJ had about 70 release sites with a probability of transmitter release greater than 0.9 following each action potential.

This result is difficult to reconcile with the number of quanta released per action potential, and the findings presented in the previous section that suggested transmitter release at the NMJ is fundamentally governed by two low probability events: opening of calcium channels and calcium-triggered vesicle release. It also contradicts information obtained from anatomical studies of active zones, and the short-term plasticity of transmitter release during pairs of stimuli. For example, immunohistochemical staining for the active zone protein bassoon [63] coupled with freeze-fracture studies of mouse active zones [18], showed that each mouse NMJ has about 500–900 active zones [64] with an average of two docked synaptic vesicles in each active zone [65]. Therefore, if one considers each docked synaptic vesicle to be a functional unit (single vesicle release site, the “secretosome”; [66]) each mouse NMJ has 1000–1800 single vesicle release sites. Assuming that single vesicle release sites are mostly independent of one another, this suggests an N of around 1000–1800 in the context of a binomial vesicle release model. The assumption that single vesicle release sites function independently excludes consideration of a within active zone “lateral inhibition” hypothesis proposed by Stevens and Wang [67] which has yet to be thoroughly tested at the

NMJ. Independent of the interpretation of N as active zones or single vesicle release sites, the binomial analysis from the mouse NMJ by Wang et al [62] ($N \sim 70$; $P > 0.9$) is difficult to reconcile with the fact that there are 500–900 active zones, unless most of them are silent and only a small subset (< 10 –20%) participate in action potential evoked transmitter release nearly every time. Additional evidence that argues against the probability of transmitter release being close to 1 at small number of active transmitter release sites in the mouse NMJ comes from studying short-term plasticity following multiple stimuli at short inter-stimulus intervals. If transmitter release following an action potential were derived from a small subset of active zones, each with a very high probability of release, one would expect strong short-term depression during repeated stimuli as vesicle depletion would be significant. A range of studies at the mouse NMJ showed that there is very little change in the magnitude of transmitter release during short trains of stimuli [68,69]. Thus, either the probability of transmitter release from each release site is very low, or the subsequent action potentials in a short train recruit transmitter release from a different subset of active zones than the first action potential in the train. The latter would be a striking finding if it could be substantiated.

The large number of vesicle release sites across the synapse raises the question of homogeneity of release - or lack thereof - across the NMJ. In other words, are all single vesicle release sites equally likely to release a vesicle or is there heterogeneity in release probability across the NMJ? To date there have been no single vesicle-resolution studies to investigate this question at the vertebrate NMJ, but several studies with intermediate resolution have contributed to our understanding. Using the optical reporter synaptopHluorin, Tabares et al. [70] reported hot spots of vesicle fusion at the mouse NMJ during low frequency activity at room temperature. However, the transmitter release distribution was more uniform when studied at body temperature [71]. Heterogeneity in transmitter release across NMJ active zones was also observed in studies in the garter snake NMJ [72]. At the drosophila NMJ, high resolution imaging studies of single vesicle release events following action potential stimulation also support the conclusion that there is heterogeneity in the probability of release across active zones [73]. In this context, there is evidence that the number of single vesicle release sites that participate in action potential-evoked transmitter release varies with stimulus frequency, and is different during low frequency versus high frequency activity [74]. Furthermore, Ruiz et al. [75] showed that an estimation of the size of the readily releasable pool (thought to represent the total number of docked synaptic vesicles that could be released during action potential activity) changes at different stimulus frequencies. These data lead to the hypothesis that there may be a pool of silent active zones that are recruited during high frequency activity, which was recently confirmed in an elegant study at the drosophila NMJ [76].

Based on the preponderance of evidence to date, we conclude that the NMJ is a large and reliable synapse constructed from hundreds or thousands of primarily low probability release sites. Further, there is increasing evidence of significant heterogeneity amongst these single vesicle release sites, and some may release transmitter with much higher probability. Low probability release sites have the advantage of providing a mechanism for maintaining synaptic reliability during trains of action potentials, as low probability release sites are not

called upon to release transmitter very often, avoiding significant synaptic depression due to synaptic vesicle depletion.

Conclusions

The organization of neurotransmitter release sites, or active zones, has a large impact on their function and on the function of the synapse as a whole. Historical and more recent studies have shown that a key determinant of synaptic vesicle release is the organization and stoichiometry of voltage gated calcium channels with respect to synaptic vesicles. In addition, the shape of the action potential itself has a significant impact on the spatiotemporal calcium dynamics. Calcium activated potassium channels in particular have been shown to play a key role in shaping the action potential waveform and thereby indirectly regulating the vesicle release process. Recently, experimental and computer modeling efforts have also succeeded in providing a unified view of the vesicle release process (excess-calcium-binding-site model [43]) which integrates known physiological and biochemical evidence. Overall, it has become increasingly obvious that the spatio-temporal calcium dynamics during synaptic vesicle release is highly heterogeneous and complex both within and across individual active zones. This heterogeneity across active zones likely underlies variations in neurotransmitter release between active zones that has begun to be characterized across different preparations. Whether this functional heterogeneity is underpinned by structural heterogeneity (as in the number and density of voltage gated calcium channels or expression of different isoforms of vesicle release sensors and proteins) will be integral to our understanding of calcium-triggered neurotransmitter release and will prove to be an exciting area of investigation for synaptic physiologists in the years to come.

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• of special interest

•• of outstanding interest

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Highlights

The positioning of Ca²⁺ channels relative to synaptic vesicles determines function

The Ca²⁺–release relationship can be explained by an excess of calcium binding sites

Action potential shape indirectly influences calcium entry at the synapse

Low probability of Ca²⁺ channel opening and Ca²⁺ ion-triggered fusion governs release

Active zone heterogeneity likely characterizes neuromuscular junction function

Figure 1.

Calcium imaging at the frog NMJ. A. Calcium dye loaded into the nerve terminal. B. During a single action potential stimulation, the calcium-induced increase in fluorescence is restricted to defined locations along the length of the synapse. C. When the intensity of the fluorescence change is converted to a pseudo-colored scale to more clearly represent site of high calcium concentration. D. The left panel is an enlargement of the area in C shown by the white box demonstrating restricted sites of calcium entry. The right panel shows the same region stained with a label for the postsynaptic receptor bands (predictive of the location of presynaptic active zones). Comparing the left and right panels it is clear that calcium entry is restricted to active zone regions of the frog nerve terminal. Scale bars = 2 μm . (Adapted from [27])

Figure 2.

Frog NMJ labeled with two fluorescent toxins that demonstrate the alignment of presynaptic voltage-gated calcium channels with postsynaptic acetylcholine receptors (predictive of the location of presynaptic active zones). In this image, the same nerve terminal is stained with ω -conotoxin GVIA (orange) to localize presynaptic N-type calcium channels, and α -bungarotoxin (green) to localize postsynaptic receptors. The top and bottom panels of this figure represent the same nerve terminal imaged first in orange (top) and then in green (bottom). A comparison of these two panels reveals that the calcium channel label (orange) is perfectly aligned with the acetylcholine receptor label (green). (Adapted from [31])

Figure 3.

Differential effects of broadening an action potential repolarization on calcium current. A. When a control action potential shape (thin black line) is broadened starting just after the peak of the action potential (thick black line), the normal calcium current (thin red line) is increased in amplitude and broader (thick red line). B. When a control action potential shape (thin black line) is broadened starting just at 50% of action potential repolarization (thick black line), the normal calcium current (thin red line) is decreased in amplitude and broader (thick red line). Adapted from [57].

Figure 4.

Graphic representation of a portion (about 1/3) of a frog NMJ active zone during an action potential stimulation. The active zone structure is highly organized such that there are two double rows of proteins that run down the middle, and two rows of docked synaptic vesicles that are positioned lateral to the active zone proteins. The active zone proteins are known to include both voltage-gated calcium channels (solid black circles) and calcium-activated potassium channels (blue circles). However, their exact density and position relative to one another and to docked synaptic vesicles within the double rows of active zone proteins is unknown and remains a focus for future study. In this diagram we have assumed that both represent only a small subset of active zone proteins, and that voltage-gated calcium channels are positioned between docked synaptic vesicles and calcium-activated potassium channels. With this structural arrangement the local nanodomain (green) of calcium entry through an open calcium channel can access binding sites on both vesicles and voltage gated potassium channels efficiently. The low probability that voltage-gated calcium channels open during an action potential is represented by the small number of open calcium channels and associated calcium nanodomains. Similarly shown is the low probability that calcium entry through a calcium channel will trigger a neighboring synaptic vesicle to fuse by the presence of only a single fusion event (red).