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Synaptotagmin Functions as a Calcium Sensor: How Calcium Ions Regulate the Fusion of Vesicles with Cell Membranes during Neurotransmission

Larry H Bernstein

New York Methodist Hospital, Brooklyn, New York, USA

Corresponding Author: Larry H Bernstein

New York Methodist Hospital, Brooklyn, New York, USA

larry.bernstein@gmail.com

Tel: 2032618671

Short Communication

This article is part of a series of articles discussed the mechanism of the signaling of smooth muscle cells by the interacting parasympathetic neural innervation that occurs by calcium triggering neuro-transmitter release by initiating synaptic vesicle fusion. It involves the interaction of soluble N-acetylmaleimide-sensitive factor (SNARE) and SM proteins, and in addition, the discovery of a calcium-dependsent Syt1 (C) domain of protein-kinase C isoenzyme, which binds to phospholipids. It is reasonable to consider that it differs from motor neuron activation of skeletal muscles, mainly because the innervation is in the involuntary domain. The cranial nerve rooted innervation has evolved comes from the spinal ganglia at the corresponding level of the spinal cord. It is in this specific neural function that we find a mechanistic interaction with adrenergic hormonal function, a concept intimated by the late Richard Bing. Only recently has there been a plausible concept that brings this into serious consideration. Moreover, the therapeutic drugs that are used in blocking adrenergic receptors are closely related to the calcium-channels. Interesting too is the participation of a phospholipid bound protein-kinase isoenzyme C calcium-dependent domain Syt1. The neurohormonal connection lies in the observation by Katz in the 1950's that the vesicles of the neurons hold and eject fixed amounts of neurotransmitters [1,2].

In Sudhof's Lasker Award presentation he refers to the biochemical properties of synaptotagmin were found to precisely correspond to the extraordinary calcium-triggering properties of release, and to account for a regulatory pathway that also applies to other types of calcium-triggered fusion, for example fusion observed in hormone secretion and fertilization. At the synapse, finally, these interdependent machines — the fusion apparatus and its synaptotagmin-dependent control mechanism — are embedded in a proteinaceous active zone that links them to calcium channels and regulates the docking and priming of synaptic vesicles for subsequent calcium-triggered fusion. Thus, work on neurotransmitter release revealed a hierarchy of molecular machines that mediate the fusion of synaptic vesicles, the calcium-control of this fusion, and the embedding of calcium-controlled fusion in the context of the presynaptic terminal at the synapse. The neural transmission is described as a biological relay system. Neurotransmission kicks off with an electrical pulse that runs down a nerve cell, or neuron. When that signal reaches the tip, calcium enters the cell. In response, the neuron liberates chemical messengers—neurotransmitters—which travel to the next neuron and thus pass the baton.

He further stipulates that synaptic vesicle exocytosis operates by a general mechanism of membrane fusion that revealed itself to be a model for all membrane fusion, but that is uniquely regulated by a calcium-sensor protein called synaptotagmin. Neurotransmission is thus a combination of electrical signal and chemical transport.

Several SMC types illustrate how signaling mechanisms have been adapted to control different contractile functions with particular emphasis on how Ca^{2+} signals are activated.

[1] Neural activation of vas deferens smooth muscle cells

Noradrenaline (NA) acts by stimulating α1-adrenoreceptors to produce InsP3, which then releases Ca^{2+} that may induce an intracellular Ca^{2+} wave similar to that triggered by the ATP-dependent entry of external Ca^{2+}. In addition, the α1-adrenoreceptors also activate the smooth muscle Rh_o/Rh_o kinase signalling pathway that serves to increase the Ca^{2+} sensitivity of the contractile machinery.

[2] Detrusor smooth muscle cells

The bladder, which functions to store and expel urine, is surrounded by layers of detrusor SMCs. The latter have two operational modes: during bladder filling they remain relaxed but contract vigorously to expel urine during micturition. The switch from relaxation to contraction, which is triggered by neurotransmitters released from parasympathetic nerves, depends on the acceleration of an endogenous membrane oscillator that produces the repetitive trains of action potentials that drive contraction.

This mechanism of activation is also shared by [3,4], and uterine contraction. SMCs are activated by membrane depolarization (ΔV) that opens L-type voltage-operated channels (VOCs)

allowing external Ca^{2+} to flood into the cell to trigger contraction. This depolarization is induced either by ionotropic receptors (vas deferens) or a membrane oscillator (bladder and uterus). The membrane oscillator, which resides in the plasma membrane, generates the periodic pacemaker depolarization responsible for the action potentials that drive contraction.

The main components of the membrane oscillator are the Ca^{2+} and K^+ channels that sequentially depolarize and hyperpolarize the membrane, respectively. This oscillator generates the periodic pacemaker depolarization that trigger each action potential. The resulting Ca^{2+} signal lags behind the action potential because it spreads into the cell as a slower Ca^{2+} wave mediated by the type 2 RYRs.

Neurotransmitters such as ATP and acetylcholine (ACh), which are released from parasympathetic axonal varicosities that innervate the bladder, activate or accelerate the oscillator by inducing membrane depolarization (ΔV).

[3] The depolarizing signal that activates gastrointestinal, urethral and ureter SMCs is as follows:

A number of SMCs are activated by pacemaker cells such as the interstitial cells of Cajal (ICCs) (gastrointestinal and urethral SMCs) or atypical SMCs (ureter). These pacemaker cells have a cytosolic oscillator that generates the repetitive Ca^{2+} transients that activate inward currents that spread through the gap junctions to provide the depolarizing signal (ΔV) that triggers contraction.

[4] Our greatest interest has been in this mechanism. The rhythmical contractions of vascular, lymphatic, airway and corpus cavernosum SMCs depend on an endogenous pacemaker driven by a cytosolic Ca^{2+} oscillator that is responsible for the periodic release of Ca^{2+} from the endoplasmic reticulum. The periodic pulses of Ca^{2+} often cause membrane depolarization, but this is not part of the primary activation mechanism but has a secondary role to synchronize and amplify the oscillatory mechanism. Neurotransmitters and hormones act by modulating the frequency of the cytosolic oscillator.

Vascular or airway SMCs are driven by a cytosolic oscillator that generates a periodic release of Ca^{2+} from the endoplasmic reticulum that usually appears as a propagating Ca^{2+} wave.

Step 1. The initiation and/or modulation of this oscillator depends upon the action of transmitters and hormones such as ACh, 5-HT, NA and endothelin-1 (ET-1)that increase the formation of InsP3 and diacylglycerol (DAG), both of which promote oscillatory activity.

Step 2. The oscillator is very dependent on Ca^{2+} entry to provide the Ca^{2+} necessary to charge up the stores for each oscillatory cycle. The nature of these entry mechanisms vary between cell types.

Step 3. The entry of external Ca^{2+} charges up the ER to sensitize the RYRs and InsP3 receptors prior to the next phase of release. An important determinant of this sensitivity is the luminal concentration of Ca^{2+} and as this builds up the release channels become sensitive to Ca^{2+} and can participate in the process of Ca^{2+}-induced Ca^{2+} release (CICR), which is responsible for orchestrating the regenerative release of Ca^{2+} from the ER. The proposed role of cyclic ADP-ribose (cADPR) in airway SMCs is consistent with this aspect of the model on the basis of its proposed action of stimulating the SERCA pump to enhance store loading and such a mechanism has been described in colonic SMCs.

Step 4. The mechanism responsible for initiating Ca^{2+} release may depend either on the RYRs or the InsP3 receptors (I). RYR channels are sensitive to store loading and the InsP3 receptors will be sensitized by the agonist-dependent formation of InsP3.

Step 5. This initial release of Ca^{2+} is then amplified by regenerative Ca^{2+} release by either the RYRs or InsP3 receptors, depending on the cell type.

Step 6. The global Ca^{2+} signal then activates contraction.

Step 7. The recovery phase depends on the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), that pumps some of the Ca^{2+} back into the ER, and the plasma membrane Ca^{2+}-ATPase (PMCA), that pumps Ca^{2+} out of the cell.

Step 8. One of the effects of the released Ca^{2+} is to stimulate Ca^{2+}-sensitive K^+ channels such as the BK and SK channels that will lead to membrane hyperpolarization. The BK channels are activated by Ca^{2+} sparks resulting from the opening of RYRs.

Step 9. Another action of Ca^{2+} is to stimulate Ca^{2+}-sensitive chloride channels (CLCA), which result in membrane depolarization to activate the CaV1.2 channels that introduce Ca^{2+} into the cell resulting in further membrane depolarization (ΔV).

Step 10. This depolarization can spread to neighboring cells by current flow through the gap junctions to provide a synchronization mechanism in those cases where the oscillators are coupled together to provide vasomotor activity.

Synaptotagmin functions as a calcium sensor

Fifty years ago, Bernard Katz's seminal work revealed that calcium triggers neurotransmitter release by stimulating ultrafast synaptic vesicle fusion. But how a presynaptic terminal achieves the speed and precision of calcium-triggered fusion remained unknown. My colleagues and I set out to study this fundamental problem more than two decades ago.

How do the synaptic vesicle and the plasma membrane fuse during transmitter release? How does calcium trigger synaptic vesicle fusion? How is calcium influx localized to release sites in order to enable the fast coupling of an action potential to transmitter release? Together with contributions made by other scientists, most prominently James Rothman, Reinhard Jahn and Richard Scheller, and assisted by luck and good fortune, we have addressed these questions over the last decades.

As he described below, we now know of a general mechanism of membrane fusion that operates by the interaction of Snares (for soluble N-ethylmaleimide–sensitive factor (NSF)-attachment protein receptors) and SM proteins (for Sec1/Munc18-like proteins). We also have now a general mechanism of calcium-triggered fusion that operates by calcium binding to synaptotagmins, plus a general mechanism of vesicle positioning

adjacent to calcium channels, which involves the interaction of the so-called RIM proteins with these channels and synaptic vesicles. Thus, a molecular framework that accounts for the astounding speed and precision of neurotransmitter release has emerged.

Outlook

Our work, together with that of other researchers, uncovered a plausible mechanism explaining how membranes undergo rapid fusion during transmitter release, how such fusion is regulated by calcium and how the calcium-controlled fusion of synaptic vesicles is spatially organized in the presynaptic terminal. Nevertheless, many new questions now arise that are not just details but of great importance. For example, what are the precise physicochemical mechanisms underlying fusion, and what is the role of the fusion mechanism we outlined in brain diseases? Much remains to be done in this field.

How calcium controls membrane fusion

The above discussion describes the major progress that was made in determining the mechanism of membrane fusion. At the same time, my laboratory was focusing on a question crucial for neuronal function: how is this process triggered in microseconds when calcium enters the presynaptic terminal?

While examining the fusion machinery, we wondered how it could possibly be controlled so tightly by calcium. Starting with the description of synaptotagmin-1 (Syt1), we worked over two decades to show that calcium-dependent exocytosis is mediated by synaptotagmins as calcium sensors.

Synaptotagmins are evolutionarily conserved transmembrane proteins with two cytoplasmic C2 domains [5,6]. When we cloned Syt1, nothing was known about C2 domains except that they represented the 'second constant sequence' in protein-kinase C isozymes. Because protein kinase C had been shown to interact with phospholipids by an unknown mechanism, we speculated that Syt1 C2 domains may bind phospholipids, which we indeed found to be the case 5. We also found that this interaction is calcium dependent [6,7]and that a single C2 domain mediates calcium-dependent phospholipid binding [8,9]. In addition, the Syt1 C2 domains also bind syntaxin-1 and the SNARE complex. All of these observations were first made for Syt1 C2 domains, but they have since been generalized to other C2 domains.

As calcium-binding modules, C2 domains were unlike any other calcium-binding protein known at the time. Beginning in 1995, we obtained atomic structures of calcium-free and calcium-bound Syt1 C2 domains in collaboration with structural biologists, primarily Jose Rizo [10].

These structures provided the first insights into how C2 domains bind calcium and allowed us to test the role of Syt1 calcium binding in transmitter release [11].

The biochemical properties of Syt1 suggested that it constituted Katz's long-sought calcium sensor for neurotransmitter release. Initial experiments in *C. elegans* and *Drosophila*, however, disappointingly indicated otherwise. The 'synaptotagmin calcium-sensor hypothesis' seemed unlikely until our electrophysiological analyses of Syt1 knockout mice revealed that Syt1 is required for all fast synchronous synaptic fusion in forebrain neurons but is dispensable for other types of fusion [12]. These experiments established that Syt1 is essential for fast calcium-triggered release, but not for fusion as such.

Although the Syt1 knockout analysis supported the synaptotagmin calcium-sensor hypothesis, it did not exclude the possibility that Syt1 positions vesicles next to voltage-gated calcium channels (a function now known to be mediated by RIMs and RIM-BPs; see below), with calcium binding to Syt1 performing a role unrelated to calcium sensing and transmitter release. To directly test whether calcium binding to Syt1 triggers release, we introduced a point mutation into the endogenous mouse *Syt1* gene locus. This mutation decreased the Syt1 calcium-binding affinity by about twofold. Electrophysiological recordings revealed that this mutation also decreased the calcium affinity of neurotransmitter release approximately two fold, formally proving that Syt1 is the calcium sensor for release [11].

The three Ca2+-binding sites are formed by two loops 1 and 3 at the top of the C2A domain. Five aspartate residues, one serine residue, and two backbone carbonyl groups coordinate the three bound Ca^{2+} ions, which are labelled Ca1, Ca2 and Ca3. Arginine 233 (R233) and lysine 236 (K236), positively charged residues that surround the Ca^{2+}-binding sites and that were mutated in this study, are highlighted by a grey box. Residues are shown in single-letter amino acid code, and are identified by number [12].

In addition to mediating calcium triggering of release, Syt1 controls ('clamps') the rate of spontaneous release occurring in the absence of action potentials, thus serving as an essential mediator of the speed and precision of release by association with SNARE complexes and phospholipids [11].

It was initially surprising that the Syt1 knockout produced a marked phenotype because the brain expresses multiple synaptotagmins [6]. However, we found that only three synaptotagmins—Syt1, Syt2 and Syt9—mediate fast synaptic vesicle exocytosis [13]. Syt2 triggers release faster, and Syt9 slower, than Syt1. Most forebrain neurons express only Syt1, but not Syt2 or Syt9, accounting for the profound Syt1 knockout phenotype. Syt2 is the predominant calcium sensor of very fast synapses in the brainstem [14], whereas Syt9 is primarily present in the limbic system [13]. Thus, the kinetic properties of Syt1, Syt2 and Syt9 correspond to the functional needs of the synapses that contain them.

Parallel experiments in neuroendocrine cells revealed that, in addition to Syt1, Syt7 functions as a calcium sensor for hormone exocytosis. Moreover, experiments in olfactory neurons uncovered a role for Syt10 as a calcium sensor for insulin-like growth factor-1 exocytosis [15], showing that, even in a single neuron, different synaptotagmins act as calcium sensors for distinct fusion reactions. Viewed together with results by other groups, these observations indicated that calcium-triggered exocytosis generally depends on synaptotagmin calcium sensors and that different synaptotagmins confer specificity onto exocytosis pathways.

We had originally identified complexin as a small protein bound to SNARE complexes [16]. Similar to complexins, synaptotagmin

I competes with α-SNAP for syntaxin binding. Do complexins also compete with synaptotagmin I for syntaxin binding, or do these molecules bind to distinct sites that are both contacted by α-SNAP? To test this question, we performed immunoprecipitations of synaptotagmin I from brain. These demonstrated that complexins coprecipitate with synaptotagmin, suggesting that synaptotagmin I and complexins interact simultaneously with syntaxin and do not compete with each other. In contrast with complexins, a-SNAP was not present in the synaptotagmin I immunoprecipitates (data not shown). The coprecipitation of syntaxin I and complexins with synaptotagmin I was disrupted by exogenous α-SNAP in a concentration dependent manner, confirming that the coprecipitation of complexins with synaptotagmin is mediated via an interaction with syntaxin. Addition of exogenous complexin, on the other hand, had no effect on the binding of synaptotagmin to syntaxin, and parallel immunoprecipitations of syntaxin confirmed these interactions. These data suggest that complexins compete for syntaxin binding with α-SNAP but not with synaptotagmin.

Analysis of complexin-deficient neurons showed that complexin represents a cofactor for synaptotagmin that functions both as a clamp and as an activator of calcium-triggered fusion [17]. Complexin-deficient neurons exhibit a phenotype milder than that of Syt1-deficient neurons, with a selective suppression of fast synchronous exocytosis and an increase in spontaneous exocytosis, which suggests that complexin and synaptotagmins are functionally interdependent [18].

How does a small molecule like complexin, composed of only ~130 amino acid residues, act to activate and clamp synaptic vesicles for synaptotagmin action? Atomic structures revealed that, when bound to assembled SNARE complexes, complexin contains two short α-helices flanked by flexible sequences. One of the a-helices is bound to the SNARE complex and is essential for all complexin function [19-24].

The Albert Lasker Basic Medical Research Award has gone to two researchers who shed light on the molecular mechanisms behind the rapid release of neurotransmitters—findings that have implications for understanding the biology of mental illnesses such as schizophrenia, as well the cellular functions underlying learning and memory formation. By systematically analyzing proteins capable of quickly releasing chemicals in the brain, Genentech's Richard Scheller and Stanford University's Thomas Südhofadvanced our understanding of how calcium ions regulate the fusion of vesicles with cell membranes during neurotransmission. Among Scheller's achievements is the identification of three proteins—SNAP-25, syntaxin and VAMP/synaptobrevin—that have a vital role in neurotransmission and molecular machinery recycling. Moreover, Südhof's observations elucidated how a protein called synaptotagmin functions as a calcium sensor, allowing these ions to enter the cell. Thanks to these discoveries, scientists were later able to understand how abnormalities in the function of these proteins contribute to some of the world's most destructive neurological illnesses. The Lasker-DeBakey Clinical Medical Research Award went to three researchers whose work led to the development of the modern cochlear implant, which allows the profoundly deaf to perceive sound. During the 1960's and 1970's Greame Clark of the University of Melbourne and Ingeborg Hochmair, CEO of cochlear implant manufacturer MED-EL, independently designed implant components that, when combined, transformed acoustical information into electrical signals capable of exciting the auditory nerve. Duke University's Blake Wilson later contributed his "continuous interleaved sampling" system, which gave the majority of cochlear implant wearers the ability to understand speech clearly without visual cues. Bill and Melinda Gates were also honored this year with the Lasker-Bloomberg Public Service Award. Through their foundation, the couple has made large investments in helping people living in developing countries gain access to vaccines and drugs. The Seattle-based Bill & Melinda Gates Foundation also run programs to educate women about proper nutrition for their families and themselves. The organization has a broad mandate in public health; one of its most well-known projects is the development of a low-cost toilet that will have the ability to operate without water. The full collection of Lasker essays, as well as a Q&A between Lasker president Claire Pomeroy and the Gateses, can be found here.

VSM of the artery and related the action of calcium-channel blockers (CCMs) to the presynaptic interruption of synaptic-vesicle fusion necessary for CA+ release that leads to neurotransmitter secretion. Under the circumstance neurotransmitter activation, the is VSM contraction (associated with tone). The effect of CCB action on neurotransmitter action, there is a resultant vascular dilation facilitating flow. In this section, we extend the mechanism to other smooth muscle related action in various organs [25-27].

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SMCs are activated by membrane depolarization (ΔV) that opens L-type voltage-operated channels

This mechanism of activation is also shared by [3], and uterine contraction. SMCs are activated by membrane depolarization (ΔV) that opens L-type voltage-operated channels (VOCs) allowing external Ca2+ to flood into the cell to trigger contraction. This depolarization is induced either by ionotropic receptors (vas deferens) or a membrane oscillator (bladder and uterus). The

membrane oscillator, which resides in the plasma membrane, generates the periodic pacemaker depolarizations responsible for the action potentials that drive contraction.

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