The role of Ca^{2+} binding in the activation of Synaptotagmin I

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Abstract

For 50 years it has been known that the rapid influx of Ca^{2+} into the presynaptic neuron triggers the fusion of synaptic vesicles with the neuronal plasma membrane[1]. This fusion releases neurotransmitters located within SVs from the presynaptic neuron that cause a cascade of signals that control nearly every physiological process. Soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins are required for this fusion, and Synaptotagmin I, a synaptic vesicle protein, is proposed to be the Ca^{2+} sensor that regulates neurotransmitter release. However, the mechanism by which SytI triggers fusion is still unknown. Ca^{2+} binding within SytI is coordinated by ten aspartic acid residues found in the cytoplasmic domain. By mutating the negatively charged aspartic acid residues into asparagine, glutamic acid, and alanine, thereby varying the size and charge of the binding pocket, we measured their molecular interactions so that we can begin to characterize the mechanism of SytI triggered neurotransmitter release. SytI activation will be measured using two assays: a phospholipid binding assay, and a reconstituted *in vitro* fusion assay. Using these assays, I will systematically complete the first comprehensive *in vitro* study of the Ca^{2+} requirements of neurotransmitter release.

Literature Review

For over 50 years it has been known that when action potentials arrive at an axon terminal and open the voltage gated Ca^{2+} channels, a rapid influx of Ca^{2+} into the presynaptic cell triggers the fusion of synaptic vesicles (SVs) with the plasma membrane of the presynaptic neuron, releasing neurotransmitters contained within the SVs[2]. After the neurotransmitters are released from the presynaptic neuron they diffuse across the synaptic cleft and bind to receptors on the postsynaptic neuron.

This process is called synaptic transmission [figure 1]. Neurotransmitters act as the brain's chemical messengers that stimulate physiological processes and influence hormones, thought processes, and muscle contraction, among many others[2]. This happens on a us-ms timescale, suggesting that a limited number of molecular rearrangements are occurring[2]. The minimum protein machinery required for SV exocytosis is a group of proteins called Soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins, however when only SNARE proteins are present SV exocytosis proceeds at such a slow rate that it is not physiologically relevant[3]. Since the rapid exocytosis of SVs is triggered by a rapid influx of Ca^{2+}, but SNARE proteins do not bind Ca^{2+}, there must be a presynaptic Ca^{2+} sensing protein that interacts with SNAREs drive exocytosis[2]. A protein named Synaptotagmin I (SytI) emerged as the probable Ca^{2+} sensor because it is the most abundant Ca^{2+} sensing protein found in synaptic terminals, it is localized to SVs and large dense core vesicles, it binds SNAREs, and knocking out SytI is lethal in mice because they are unable to suckle[2,4,5,8].

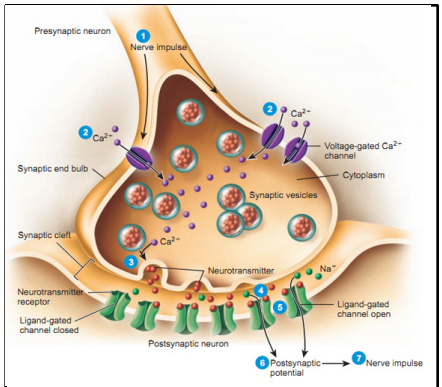

Figure 1. Schematic of a synapse. Upon rapid Ca^{2+} influx, SVs containing neurotransmitters fuse with the axonal membrane and release neurotransmitters across the synaptic cleft (Reece, 2001).

SV exocytosis is mediated by target membrane-SNAREs (t-SNAREs, Synxtaxin-1A [Syx1A] and Synaptosomal-associated protein B [SNAP-25B]) and a vesicular-SNARE (v-SNARE, Synaptobrevin-2 [VAMP2]) which together form a four-helix bundle in which SNAP-25B contributes two α-helices while VAMP2 and Syx1A are anchored to their respective membranes and contribute one α-helix each [figure 2A][2].

SytI was discovered in 1981[4] and since then a total of 17 Syt isoforms have been identified which are found throughout neuronal and non-neuronal tissues[6]. The structure of SytI is comprised of an intravesicular domain, a transmembrane domain, and a cytoplasmic domain where the tandem Ca^{2+} sensing domains, C2A and C2B are found [figure 2A][6]. In the cytoplasmic domain there are two eight stranded anti-parallel β-sandwiches, and protruding from each of the β-sandwiches are two flexible loops that make up the two Ca^{2+} binding domains[5]. There are five negatively charged aspartic acid (D) residues on each pair of loops, which Nuclear Magnetic Resonance studies have found to coordinate three Ca^{2+} ions on C2A and two Ca^{2+} ions on C2B [figure 2B][6]. Additionally, these residues are conserved throughout many species including Squid, *C. elegans*, Chickens, Mice, and humans, suggesting that they are physiologically relevant[2,7]. When Ca^{2+} binds to C2AB, the negative charge of the binding pocket is neutralized, which alters the electrostatic potential of the flexible loops,

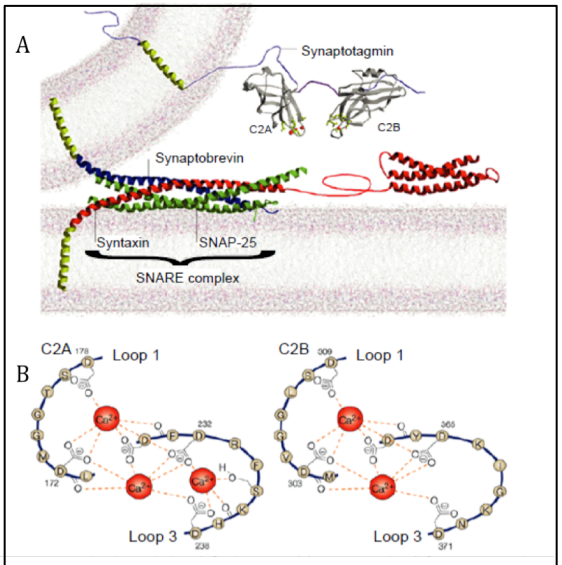

Figure 2. A) Schematic of SNARE proteins (Syntaxin-1A, SNAP-25B, Synaptobrevin-2) and SytI. SNARE proteins facilitate fusion of the synaptic vesicle with the plasma membrane, but the Ca^{2+} sensor SytI is required for fusion to proceed at physiological rates.
B) C2A and C2B domain of SytI and the ten coordinating aspartic acid residues. (Adapted w/ permission from Chapman, 2008).

allowing them to bind anionic phospholipids and SNAREs and penetrate the plasma membrane. In conjunction with the SNARE proteins, the penetrating loops of SytI induce a slight curvature in the membrane which changes the local phospholipid environment sensed by SytI and allows SytI to overcome the energy barrier, triggering the fusion of SVs with the neuronal plasma membrane, releasing the neurotransmitters[2].

Many cell-based studies revolving around the mutagenesis of SytI's Ca^{2+} ligands have proposed models that attempt to explain which residues are required for the activation of SytI's stimulatory fusion activity[9]. However, due to the unavoidable complexities of cell-based studies in neurons, there has been no underlying biochemical justification developed to tie together the overwhelming amount of data in the field.

If synaptic transmission is triggered by Ca^{2+} influx to the presynaptic neuron and SytI is the sensor, it would be expected that abolishing Ca^{2+} binding would alter synaptic transmission. However, early cell-based studies in mice and *Drosophila* neurons demonstrated that neutralizing all five Ca^{2+} ligands on C2A abrogated Ca^{2+} and phospholipid binding, but did not affect synaptic transmission[11,12]. A similar study by Mackler *et al* mutated analogous residues C2B and demonstrated that synaptic transmission was abolished[13]. Furthermore, a study by Striegel *et al* that disrupted Ca^{2+} binding in C2A using a mutation that sterically inhibited Ca^{2+} binding but maintained the negative charge of the native D residues resulted in a decrease in synaptic transmission by more than 80%[7]. Striegel posits that an "electrostatic switch" is flipped when Ca^{2+} ions bind and neutralize the negatively charged binding pocket, allowing the loops of SytI to interact with the anionic phospholipid phosphatidylserine (PS) and penetrate the plasma membrane. Further studies have shown that C2B is crucial for synaptic transmission in cell-based assays[10] but *in vitro* studies have repeatedly demonstrated that mutations in C2A negatively affect SytI C2AB's

ability to fuse membranes and fold SNARE proteins, both crucial events in the process of SV exocytosis[9,12,13]. The confusion surrounding the role of each C2 domain and the degree of importance of individual D residues is the result of attempts to establish a biochemical model based on physiological data without first understanding the highly specific coordination between SytI, SNARE proteins, phospholipids, and Ca^{2+}. In a process as tightly regulated as synaptic transmission, the molecular machinery is evidently too complex to untangle the underlying biochemical principles using electrophysiological recordings of Ca^{2+} ligand mutants (CLMs) without first stepping back and using a systematic approach in a simplified *in vitro* system.

Until there is a broad characterization of SytI's interactions with Ca^{2+} and phospholipids, progress will be disorganized and of limited validity. Studies have attempted to explain the activation of SytI based largely on isolated mutations and there has never been a comprehensive analysis of each SytI Ca^{2+} ligand. For this reason, it is necessary to first definitively characterize phospholipid-binding activity and vesicle fusion stimulating ability of SytI to reveal the basic biochemical interactions before this complex puzzle can be thoroughly understood under physiological conditions.

There are five aspartic acid (91 $Å^3$)[15] residues in SytI C2A and five in C2B. These ten residues will each be individually mutated into asparagine (N), glutamate (E), and alanine (A) and assayed for their phospholipid binding and fusion stimulation activity. The N (96 $Å^3$)[15] mutants maintain the relative size of the Ca^{2+} binding pocket but neutralize the charge. The E (109 $Å^3$)[15] mutants preserve the binding pocket's native negative charge, but overcrowd it with a bulky side chain. Lastly, A mutants neutralize the charge and increase the volume of the binding pocket relative to native D residues. By analyzing the Ca^{2+} ligand mutants (CLMs) response to varying the charge and size of the amino acid side chains, we can develop a clearer picture of SytI's

interactions with phospholipids and the requirements for activation of SytI's ability to trigger fusion of vesicles.

This study will provide the fundamental biochemical framework through which we can understand and gradually approach the complex nature of synaptic transmission *in vivo*. Characterizing amino acid substitutions through phospholipid binding assays and *in vitro* reconstituted fusion assays will allow us to determine what each of the mutants are biochemically capable of facilitating, and based on that we can begin to hone in on the structural characteristics of SytI that allow it to confer Ca^{2+} sensitivity to synaptic transmission.

Materials and Methods

Materials

Synthetic 1,2,-Dioleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (Phosphatidylserine, PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3phosphoethanolamine (Phosphatidylethanolamine, PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (Phosphatidylcholine, PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (PE-Rho) were obtained from Avanti Polar Lipids.

Mutagenesis

Point mutations were introduced in *Rattus Norgevicus* SytI cDNA using a QuikChange Site-Directed Mutagenesis kit from Stratagene. In this study only the cytoplasmic domain containing tandem C2A and C2B was used, residues 96-421. This SytI fragment lacks the transmembrane domain found on full-length SytI, because the difficulty of purifying 30 full-length SytI CLMs with $n \geq 3$ would make this study impractical. SytI C2AB was subcloned into a pGEX-2T vector with a glutathione S-transferase tag.

Protein Purification

Recombinant proteins were purified as described[16]. Glutathione S-transferase tagged proteins were purified using glutathione-Sepharose (GST) beads (GE Healthcare) after disrupting the cell membrane with 25% Triton and sonification. Once the tagged proteins were tethered to the GST beads they were washed with a high salt buffer containing 50 mM Hepes, 1 M NaCl, 1mM $MgCl_2$, at pH 7.4 and 10 μg/ml DNase and 10 μg/Ml RNase. The proteins were the washed with a buffer solution of 50 mM Hepes, 100 mM NaCl, pH 7.4, and lastly a 50 mM Hepes, 100 mM NaCl, 10% glycerol solution at pH 7.4. SytI was cleaved from the GST bead via thrombin and quantified on a SDS-PAGE gel.

Phospholipid binding cosedimentation assay

The cosedimentation assay measures the extent of SytI's cytoplasmic domain binding to phospholipids. 15/55/30% PS/PC/PE liposomes (Avanti Polar Lipids) were extruded through 50 nm filters and final liposome size was measured using dynamic light scattering and was found to be 120 nm. The liposomes were titrated (0.1 uM-2 uM lipid) into the sample containing 4 uM protein, 1.2 mM Ca^{2+}, and 0.2 mM EGTA in 50 mM Hepes, 100 mM NaCl at pH 7.4 and incubated at room temperature for 15 minutes during which the Ca^{2+}/CLM/liposome complex forms. The sample was then spun in a centrifuge for 45 minutes at 100,000g at 4°C, and the supernatant containing the unbound protein was quantified on a SDS-PAGE gel while the protein bound to the liposomes sediments to the bottom of the centrifuge tube. The fraction percentage of unbound protein relative to the –liposome negative control was subtracted from 100 to calculate the percentage of protein bound to liposomes.

Reconstituted *in vitro* fusion assay[17]

The fusion assay tests the ability of soluble SytI to stimulate fusion between reconstituted v-SNARE vesicles and reconstituted t-SNARE vesicles, upon binding Ca^{2+}. Full-length syx-1A/SNAP-25 heterodimers were reconstituted as described[20] into 100 nM, 15/27/55/1.5/1.5 PS/PC/PE/NBD-PE/Rho-PE vesicles and labeled with a NBD(donor)/Rhodamine(acceptor) FRET pair covalently attached to the head group of PE. Full-length VAMP-2 was reconstituted into 100 nM 15/0/55% PS/PC/PE vesicles. The v-SNARE (0.5% total volume) and t-SNARE (4.5% total volume) vesicles were incubated in a white bottom 96-well plate at 37 °C

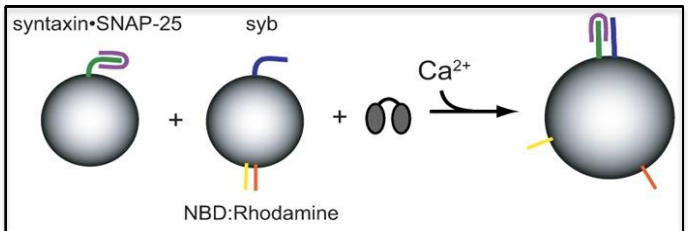

Figure 3. Schematic of reconstituted *in vitro* fusion assay. (Adapted w/permission from Yao, 2011)

for 20 minutes with 0.2 mM EGTA and 1μM SytI in a solution of 25mM Hepes, 100 mM KCl, and 10% glycerol at pH 7.8. At t=20 minutes 1.2 mM Ca^{2+} is spiked in, allowing SytI to trigger the fusion of vesicles [figure 3]. When the vesicles fuse the FRET pair is no longer in close spatial proximity, and the donors fluorescence is no longer quenched, leading to an increase in fluorescence which represents vesicle fusion. After 2 hours, 0.5% *n*-dodecylmaltoside was added to the reactions to solubilize the vesicles, resulting in maximum fluorescence. Fluorescence readings were taken in a BIO-TEK Synergy HT multi-detection micro plate reader.

DNA constructs

cDNA encoding rat SytI was provided by T.C. Sudhof (Stanford University, Menlo park, CA). Mouse VAMP2 was provided by J.E. Rothman (Yale University, New Haven, CT) and subcloned into a pTrcHIS-A vector[18]. Full-length t-SNARE heterodimers were generated by subcloning full-length rat SNAP-25B and rat syntaxin 1A cDNA into the pRSFDuet-1 vector, as described previously by Chicka *et al*[19].

Results

To investigate the role of Ca^{2+} binding in the activation of SytI, each of the ten aspartic acid (D) residues in SytI's Ca^{2+} binding domains that are thought to coordinate Ca^{2+} ions were individually mutated to N, E, and A for a total of thirty mutants which were tested for their lipid binding ability and vesicle fusion activity. The native D residues are negatively charged at physiological pH and occupy a volume of 91 $Å^3$[15]. The N mutants have a neutral charge at physiological pH and occupy a volume of 96 $Å^3$[15], nearly identical to native D residues. The amino acid E maintains the native negative charge but is much larger (109 $Å^3$)[15] and thus decreases the volume available for Ca^{2+} to enter and bind. The A mutant has a neutral charge and occupies a small volume of the pocket (67 $Å^3$)[15], increasing the volume of the Ca^{2+} binding pocket. Analysis of these D, E, and A mutants allowed us to investigate the effects of neutralizing the Ca^{2+} binding pocket, altering the size of the pocket, and simultaneously altering the charge and size of the native Ca^{2+} binding pocket. To test the CLMs' ability to bind to the negatively charged phospholipids found in the neuronal plasma membrane, cosedimentation assays were performed (n≥3). To measure the affinity of the CLMs toward the anionic phospholipid, phosphatidylserine (PS), the dissociation constant (K_d) was calculated. Wild type (WT) SytI had a K_d value of 253 ± 25 uM phospholipid [table 1]. On the C2A domain of

Kd (μM)

	Protein	D	N	E	A
	WT	253 ± 25			
C2A	172		460 ± 68	644 ± 85	404 ± 50
	178		556 ± 14	391 ± 80	630 ± 170
	230		463 ± 28	310 ± 18.	418 ± 35
	232		416 ± 32	462 ± 29	562 ± 100
	238		461 ± 48	458 ± 43	266 ± 41
C2B	303		475 ± 60	340 ± 36	492 ± 47
	309		434 ± 55	424 ± 73	674 ± 100
	363		828 ± 329	347 ± 35.	559 ± 130
	365		363 ± 32	407 ± 26	565 ± 140
	371		382 ± 22	388 ± 11	n/a

%Fmax

	Protein	D	N	E	A
	WT	37.6 ± 5.19			
C2A	172		4.51 ± 1.50	33.5 ± 6.13	2.16 ± 0.55
	178		2.85 ± 1.06	47.3 ± 5.35	1.99 ± 0.53
	230		3.30 ± 0.86	34.6 ± 5.37	2.23 ± 0.48
	232		11.4 ± 1.97	7.82 ± 1.58	2.69 ± 1.21
	238		5.00 ± 0.40	28.7 ± 5.12	16.3 ± 7.24
C2B	303		28.8 ± 7.27	22.2 ± 5.62	22.3 ± 5.52
	309		29.2 ± 3.20	32.5 ± 7.75	9.76 ± 4.63
	363		8.78 ± 2.06	23.7 ± 7.81	6.16 ± 1.97
	365		31.5 ± 2.72	31.3 ± 7.77	24.0 ± 2.60
	371		38.5 ± 10.9	29.6 ± 6.02	N/A

Rate of fusion (min^{-1})

	Protein	D	N	E	A
	WT	0.117 ± 0.028			
C2A	172		0.004 ± 0.004	0.040 ± 0.016	0*
	178		0.001 ± 0.001	0.075 ± 0.020	0*
	230		0.003 ± 0.002	0.100 ± 0.007	0.0004 ± 0.0003
	232		0.048 ± 0.019	0.033 ± 0.017	0.017 ± 0.016
	238		0.011 ± 0.002	0.064 ± 0.014	0.018 ± 0.010
C2B	303		0.037 ± 0.013	0.060 ± 0.022	0.042 ± 0.011
	309		0.060 ± 0.013	0.073 ± 0.019	0.015 ± 0.007
	363		0.015 ± 0.005	0.088 ± 0.026	0.017 ± 0.005
	365		0.058 ± 0.007	0.082 ± 0.021	0.035 ± 0.002
	371		0.047 ± 0.015	0.108 ± 0.026	N/A

Table 1. Summary of K_d, $\%F_{max}$, and rate of fusion data.

SytI C2AB residues 172N, 230N, 232N, and 238N had the lowest K_d values, i.e. highest affinity for phospholipids [table 1]. 172N, 230N, and 238N are each thought to interact with two Ca^{2+} ions while 232N interacts with three Ca^{2+} ions [figure 2B][6]. Residue 178N, which interacts with one Ca^{2+} ion[6], had the highest K_d.

The K_d values for the CLMs on the C2B domain of SytI C2AB varied noticeably from the analogous mutations in C2A as shown in Table 1. The CLMs 309N, 365N, and 371N had a greater affinity for PS than their counterparts on C2A, differing by -122, -53, and -79 uM respectively. While 365N has only a slightly stronger affinity for phospholipids than its analogous residue on C2A, 232N, they have very different $\%F_{max}$ values [table 1, figure 5].

Testing the C2A CLMs using the D-E mutation resulted in a similar overall spread of K_d values to the D-N mutants, however the relative ranking of residues with respect to their K_d values differed from the order of the same residues with the D-N mutants [table 1, figure 4]. Residue 230E on C2A of SytI C2AB performed most similarly to WT [table 1, figure 4]. The only residue at which a D-E CLM had a K_d within the error of its D-N counterpart was at residue 238E.

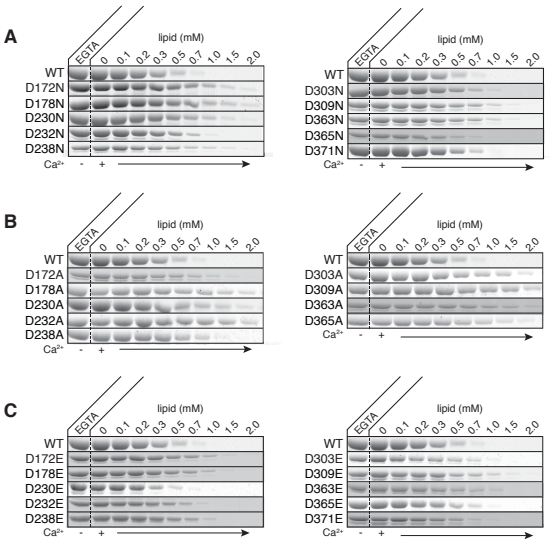

Figure 4. Cosedimentation assay. Representative SDS-PAGE gels show quantification of depletion of unbound protein in supernatant as a function of increasing lipid concentration (n≥3).

The C2B D-E CLMs had relatively uniform K_d values regardless of which residue was mutated and all mutants performed very similarly to WT SytI C2AB. C2B D-E K_d values ranged from 340 uM to 424 uM [Table 1]. Residue 303E showed the largest discrepancy in K_d values between itself (K_d=340 ± 36 uM) and the analogous

mutation on C2A (172E: K_d=644 ± 85 uM), while the same comparison of analogous residues with D-N mutations resulted in nearly identical lipid binding at residues 172N and 303N [Table 1]. Both D-N and D-E mutations of residue 371 on C2B had identical effects because 371D is not believed to be a Ca^{2+} ligand in WT SytI[2].

The D-A mutation drastically alters the size of the Ca^{2+} binding pocket along with neutralizing the negative charge of the native D residues. Therefore, D-A mutations generally disrupt SytI's ability to bind PS regardless of which Ca^{2+} ligand is altered. On C2A, residues 172A, 230A, and 238A bound PS similarly as the lipid concentration increased [figure 4]. The D-A CLMs with the lowest affinity for PS were the 178A and 232A mutants, although they each have a large error [table 1].

On the C2B domain of SytI, D-A mutants yielded poor K_d values in comparison to WT and the D-N and D-E mutants. Residues 303A, 309A, 363A, and 365A had a range of K_d values from 492 uM to 674 uM [Table 1]. Residue 371A was not tested because it is not a Ca^{2+} ligand[2] and is difficult to purify.

To test whether the CLMs could be activated to fuse vesicles bearing SNARE proteins upon Ca^{2+} binding, *in vitro* reconstituted fusion assays were performed[17]. From the fusion assays we calculated the extent of fusion by measuring the percent maximum fluorescence ($\%F_{max}$) and the rate of fusion (min^{-1}) [Table 1]. The K_d values from the cosedimentation assays were plotted against extent of fusion and the rate of fusion from the fusion assays [figure 6].

Figure 6A,B shows the relationship between K_d and the extent of vesicle fusion and rate of fusion for the D-N CLMs, separated by domain. The proteins that trigger fusion to the greatest extent and that have a strong affinity for binding phospholipids are closer to the lower right quadrant of the plot, while those that are unable to trigger vesicle fusion and have a low affinity for PS are in the upper left quadrant. If a protein's phospholipid affinity were indicative of its ability to fuse vesicles, it would be expected that there would be a strong negative correlation between the protein's K_d and $\%F_{max}$. However, many sets of mutants in this study had poor correlations between K_d and $\%F_{max}$, suggesting that there are additional necessary steps between the SytI/PS complex forming and vesicle fusion [figure 6].

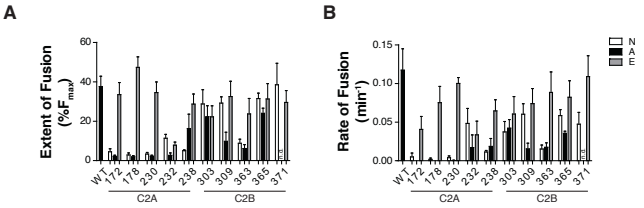

Figure 5. Extent of fusion and rate of fusion results from *in vitro* reconstituted fusion assays (n≥3) (Evans, unpublished).

The D-N mutants on C2B, excluding 363N, of SytI C2AB had significantly greater extents of fusion than the D-N mutations on C2A, while the K_d values for mutants on each domain are within the error of each other [figure 6A]. Interestingly, 363N deviates from this generalization, with a high K_d and low $\%F_{max}$ [figure 6A]. 363N's $\%F_{max}$ value was only slightly greater than the analogous mutation on C2A (230N), yet 363N's K_d value was significantly greater than 230N. It is notable that a 363N has a lower affinity for binding phospholipids but is capable of stimulating a similar extent of fusion as 230N, which has a greater affinity for PS.

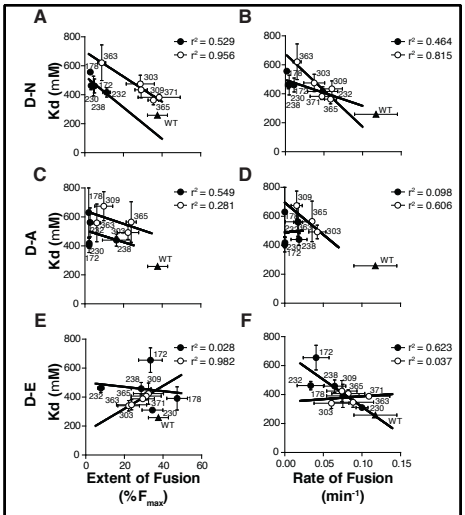

Figure 6. K_d values from cosedimentation assays plotted against extent of fusion ($\%F_{max}$) and rate of fusion data from fusion assays. Closed circles indicate C2A residues and open circles indicate C2B residues.

Overall, despite having comparable K_d values, C2B D-N mutants were able to facilitate fusion to a greater extent than analogous mutations on C2A.

Figure 6B shows the relationship between K_d and the rate of fusion for D-N mutants. Despite having similar extents of fusion to WT [figure 6A], D-N mutations on C2B significantly decrease the rate at which they facilitate vesicle fusion, with rates approximately half as large as WT SytI C2AB. The same trend exists for D-N mutations on C2A, although unlike C2B mutants they were unable to stimulate a high percentage of fusion. A D-N mutation at residue 232 of C2A does not negatively affect the rate of fusion to the extent of the rest of the C2A mutations. Although 232N has low extent of fusion, it achieves that maximum relatively quickly with a rate of 0.048 ± 0.018 min^{-1} [figure 6A,B].

Although D-E mutants on C2A showed a range of K_d values, there was no correlation between those values and $\%F_{max}$ [figure 6E], that is, CLMs with similar affinities for PS facilitated fusion to very different extents. For example, 178E and 232E have K_d values within error of each other, but 178E had an event greater extent of fusion than WT while 232E was the most detrimental D-E mutation with respect to extent of fusion [figure 6E]. Conversely, 172E and 23E have similar extents of fusion but 172E has a high K_d while 230E has a relatively low K_d [figure 6E]. Not only can CLMs with similar K_d values have significantly different $\%F_{max}$ values (C2A D-N versus C2B D-N, figure 6A) but CLMs with significantly different K_d values can have similar $\%F_{max}$ values.

Although D-E mutations on C2B had a positive correlation between K_d and $\%F_{max}$, that correlation was not reflected in the relationship between K_d and rate of fusion (r^2=0.037, figure 6E,F). In contrast to 232N, which facilitates a small extent of fusion very quickly, 172E facilitates an extent of fusion comparable to WT but it does so very slowly [figure 6E].

Additionally, the activity of 172E is even more interesting because of its low affinity for phospholipids.

As expected, the D-A mutants generally had low $\%F_{max}$ values relative to WT as well as slow rates of fusion [figure 6C,D]. Similar to the trend in the comparison of D-N mutations on C2A versus C2B, the D-A mutations on C2B are slightly more tolerated with respect to extent of fusion than mutations on C2A. CLMs 303A and 365A stand out as the mutants able to reach the greatest extent of fusion. Based on a study by Ubach *et al*[6], residues 303 and 365 are each involved in coordinating both of the two Ca^{2+} ions bound by C2B, which prompts the question of why SytI tolerates impairing a Ca^{2+} ligand involved in coordinating multiple ions, more than a mutation that impairs a ligand involved in coordinating only a single Ca^{2+} ion?

Although the 303A and 365A CLMs had intermediate $\%F_{max}$ values relative to WT, their rates of fusion indicate that they do so very slowly [figure 6C,D]. The D-A mutations on C2A had very slow rates of fusion compared to the rates of fusion of D-E mutants on C2A, but the rates were similar to the D-N mutations on C2A, which also neutralize the binding pocket but maintain a similar pocket volume to the native D residues.

To investigate the greater tolerance of SytI for neutral residues on C2B than C2A, we combined the data points for the D-N and D-A mutations into one data set and plotted the K_d values of both types of mutation against extent of fusion, separated by domains. When the data from the D-N and D-A mutations were merged,

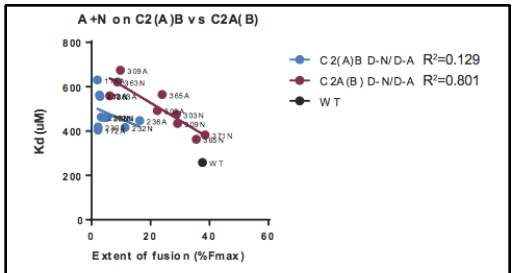

Figure 7. K_d values from cosedimentation assays of all D-N and D-A CLMs plotted against $\%F_{max}$ values from fusion assays.

the CLMs with mutations on C2B exhibited a greater extent of fusion for comparable K_d values than CLMs on C2A [figure 7]. The strong correlation (r^2=.801) suggests that there is a difference in the ability of CLMs on C2B versus C2A to facilitate vesicle fusion *in vitro*.

Several overarching patterns became evident from these *in vitro* studies. Based on cosedimentation assays, mutations that neutralize the native D residues on C2A or C2B greatly decrease the protein's affinity for the negatively charged phospholipid, PS [table 1]. However, the same D-N and D-A mutations are in fact capable of fusing vesicles if the mutation is on the C2B domain [figure 6A-D], with some mutants performing almost identically to the much more tolerated D-E mutations. The D-E mutation, which decreases the volume of the Ca^{2+} binding pocket but maintains the negative charge had very little effect on the phospholipid affinity of C2B mutants, and less dramatic effects on C2A mutants than the neutralizing mutations [table 1]. Most D-E mutants on C2A and C2B were still able to facilitate efficient fusion, based on the extent and rate of fusion.

In summary, mutations that decrease the size of the binding pocket are much more tolerated on both domains than mutations that alter the charge of the Ca^{2+} ligands. However, the C2A domain is much more sensitive to these mutations that neutralize the negative charge of the binding pocket than the C2B domain is. Additionally, some residues have similar K_d values but very different $\%F_{max}$ values and vice versa, suggesting that there are other intermediate steps between phospholipid binding and vesicle fusion that these point mutations are affecting.

Discussion

There is an abundance of cell-based mutagenesis studies that investigate the role of SytI as the Ca^{2+} sensor involved in the exocytosis of SVs, which contain neurotransmitters[2]. The inherent complexity of *in vivo* systems has led to several opposing ideas regarding the molecular mechanism by which SytI triggers the rapid synchronous release of neurotransmitters. To address the discrepancies between the physiological results of these cell-based studies, we used a simplified *in vitro* system to investigate the nature of SytI's interactions with Ca^{2+} and the phospholipids of synaptic vesicles.

Other studies have utilized similar *in vitro* biochemical approaches, however these studies often only address a few isolated mutations[7,8-13] but this is the first comprehensive biochemical characterization of all ten Ca^{2+} ligands in SytI C2AB, testing the effect of altering the charge and size of the binding pocket simultaneously and independently using three different point mutations. A presumed chronology of events leading up to the fusion of SVs with the neuronal plasma membrane would be that in order for exocytosis to occur, SytI must first be able to bind the anionic phospholipids of the membrane. Therefore, it was expected that CLMs that are able to facilitate efficient vesicle fusion would also have a high affinity for anionic phospholipids, that is, as extent and rate of fusion increase the K_d values should decrease.

When we compared the K_d values to the extent and rate of fusion, mutants that substituted a native D residue with a bulky amino acid, E, uncoupled this relationship and resulted in the absence of a linear relationship between phospholipid affinity and vesicle fusion efficiency [figure 6E,F] Notably, residues 172E and 230E both reached an extent of fusion within error of each other and WT SytI C2AB, despite 172E having the lowest phospholipid binding affinity of all the D-E mutants. Conversely, residues 178E and 232E had phospholipid binding affinities within error of each other yet displayed the greatest and least extent of fusion, respectively, of the D-E mutants.

If CLMs that achieve a similarly large extent of fusion have significantly different phospholipid affinities, and vice versa, there must be additional molecular mechanisms at work in between the events of SytI binding phospholipids and vesicle fusion. It should be noted that the r^2 value (0.982) of the D-E CLMs of the C2B domain in figure 6E is not considered significant because each point is within both the K_d and $\%F_{max}$ error, and thus the limited spread of the data resulted in a potentially misleading r^2 value. Additionally, figure 6F shows the absence of a correlation between phospholipid affinity and rate of fusion for D-E CLMs on the C2B domain, which is consistent with the extent of fusion data with regard to the spread of K_d values and $\%F_{max}$. The apparent correlation of the D-E CLMs on the C2A domain in the rate of fusion in figure 6F is difficult to decipher due to a lack of context, since they are the only type of mutation on C2A that resulted in any substantial fusion activity. Considering the noticeable correlation between the C2A D-E mutants' phospholipid affinity and rate of fusion, and the absence of a correlation with extent of fusion, this supports the premise that the C2A and C2B domains are not functionally symmetric in WT SytI[2], which may only be noticeable when the protein can facilitate fusion as in the case of the D-E mutations.

While the only mutations on C2A that yielded any substantial extent of fusion were the D-E CLMs, several C2B D-N mutants achieved an extent of fusion within error of WT SytI C2AB [figure 6A,E]. Given the strong linear relationship between C2B D-N CLMs' phospholipid binding affinity, extent of fusion (r^2=0.956), and rate of fusion (r^2=0.815) [figure 6A,B], lipid binding appears to be a decent indicator of the protein's overall ability to stimulate efficient fusion.

Residue 363N had a markedly lower phospholipid affinity, extent of fusion, and rate of fusion relative to the other C2B CLMs [figure 6A,B]. One plausible explanation for the poor

performance of the 363N CLM in our assays is that residue 363 is centrally located on loop 3 of C2B where it interacts with both Ca^{2+} ions that would bind to C2B[6] [figure 2B], and the native negative charge at that location may be necessary to coordinate the Ca^{2+} ions. Residues 303 and 365 of C2B also interact with two Ca^{2+} ions, but it is unclear why they are less impaired by D-N mutations than 363N.

A cell-based study that measured the excitatory postsynaptic current (EPSC) of cultured mouse hippocampal neurons expressing a mutant SytI with all five of its native D residues on C2A neutralized to N, reported that there was no decrease in synaptic transmission as measured by EPSC amplitude, despite this mutant's inability to bind Ca^{2+}[9]. This result is confounding because if half of the proposed Ca^{2+} sensor (C2A) does not bind Ca^{2+}, but synaptic transmission is unaffected, it raises the question of what role, if any, does the C2A domain play in synaptic transmission if it is not necessary for C2A to sense Ca^{2+}?

While the cell-based system that this study was done in is inherently more complex than our *in vitro* biochemical approach can address, it is notable that all five of our C2A D-N mutants failed to stimulate fusion. In a simplified system with all the necessary protein and lipid components, it would be reasonable to expect our assays to err on the side of overestimating fusion capabilities since it lacks much of the regulatory machinery found in neurons, but our C2A D-N mutants are unable to produce any significant extent of fusion at a physiologically relevant rate.

However, an emerging model can reconcile the differences between our *in vitro* single residue D-N mutants and Stevens'[9] cell-based approach using a quintuple D-N mutant. Despite earlier studies that reported that C2A is not required for Ca^{2+} triggered synaptic transmission[12], it is now generally accepted that C2A does play a role in the process of synaptic transmission.

Striegel *et al*[7] reported that a D229E mutation in *Drosophila melanogaster* SytI (corresponding to a D178E mutation in rat SytI) abolished Ca^{2+} binding on C2A and resulted in an 80% decrease in the excitatory junction potential (EJP), suggesting that C2A is involved in synaptic transmission. Striegel justified these results by proposing that C2A may serve as an electrostatic switch whose negatively charged binding pocket must be neutralized in order for SytI to bind to the anionic phospholipids and negatively charged SNARE proteins. Ca^{2+} binding would be sufficient to neutralize the negative charge of the binding pocket, but it is apparently not necessary if the negatively charged D residues are neutralized to N, as reported by Stevens *et al*[9] who used mutagenesis to artificially neutralize the residues to mimic Ca^{2+} binding. The finding that the C2A quintuple D-N mutant results in no decrease in synaptic transmission[9] while the D229E *Drosophila* mutant results in >80% decrease[7], despite neither mutant binding Ca^{2+}, is strong evidence that *in vivo,* the Ca^{2+} binding pockets of C2A need to be neutralized in order to facilitate synaptic transmission.

The impaired phospholipid binding activity of individual C2A D-N CLMs that we observed is supported by several studies[9,11]. If quintuple C2A D-N mutants are able to achieve efficient synaptic transmission in cell-based studies, but the same mutations tested independently *in vitro* resulted in a decrease in phospholipid affinity, extent of fusion, and rate of fusion, it is possible that the single D-N mutants not only inhibit sufficient Ca^{2+} binding, but also fail to neutralize the overall charge of the Ca^{2+} binding pocket, effectively preventing SytI from undergoing the change in electrostatic potential that is necessary for synaptic transmission. This interpretation of our results is supported by another *in vitro* study, which reported that mutating the four Ca^{2+} ligands on the C2B domain of SytI C2AB from D-N resulted in no apparent decrease in phospholipid affinity[14], possibly because the quadruple D-N mutant neutralizes the binding pocket. While our study does not test the C2B quadruple D-N mutant, the broad range of K_d

values for the individually mutated D-N residues is consistent with the electrostatic switch model because mutants with single D-N mutations cannot bind Ca^{2+} or flip the electrostatic switch.

Based on the electrostatic switch idea, D-E mutants should largely prevent efficient fusion or synaptic transmission since they inhibit Ca^{2+} binding and maintain the negative charge of the binding pocket. In relation to Striegel's model that, in general, D-E mutants have impaired synaptic transmission because they are unable to "flip" the electrostatic switch, some of the D-E CLMs in our study are consistent with that logic, while some D-E CLMs in our *in vitro* assays were fully functional. In particular, the C2A CLM 178E (analogous to *Drosophila* 229E) achieves an extent of fusion similar to WT SytI C2AB. The discrepancy between Striegel's cell-based study that mutating residue D229E in *Drosophila* SytI results in an 80% decrease in synaptic transmission[7], and our study which shows that the analogous mutant in rat SytI achieved a large extent of fusion within error of WT [figure 6E] has several possible explanations. Considering the absence of a correlation between K_d with either $\%F_{max}$ or rate of fusion, along with the different behavior of Streigel's 229E mutant and our 178E mutant, the most intriguing explanation for the large range of fusion capabilities of D-E mutants is that perhaps these mutants are interfering with a molecular mechanism that takes place in between the lipid binding and synaptic transmission events, which might only occur when the native negative charge is conserved. Alternatively, the discrepancy could be due to the dramatically different environments that SytI is studied in when using an *in vitro* or cell-based approach.

In order to address the gaps in our understanding of the phospholipid binding and vesicle fusion ability of activated SytI, further studies are needed to address the chemical thermodynamics and biophysical properties of the CLMs. Specifically, the next step for us is to understand exactly how many Ca^{2+} ions the CLMs are capable of binding using Isothermal Titration Calorimetry and

to investigate the ability of CLMs to penetrate phospholipid membranes using a membrane penetration assay using fluorescent reporter probes.

In summary, a mutation that decreases the volume of the Ca^{2+} binding pocket (D-E) in SytI's C2 domains generally uncoupled the apparent linear relationship between the protein's phospholipid affinity and ability to efficiently fuse vesicles. Despite the absence of correlation between phospholipid affinity and vesicle fusion capabilities, D-E substitutions, which alter the size of the binding pocket, were generally the most tolerated mutation in this study. In contrast, a single D-E substitution in the C2A domain of *Drosophila* SytI reduced synaptic transmission by more than 80%. Based on the overall inability of D-N and D-A CLMs to facilitate vesicle fusion, these neutralizing substitutions seem to be the most deleterious *in vitro*, especially on the C2A domain. In contrast, cell-based studies found the opposite effect, that is, D-N mutations on C2A do not impair synaptic transmission in most neurons. We believe that a single neutralized ligand only partially neutralizes the C2A Ca^{2+} binding pockets (such as our individual D-N and D-A mutants), and therefore the pockets don't sufficiently cross the electrostatic potential threshold to mimic Ca^{2+} binding. It remains unclear why D-N mutations are much more tolerated on C2B than C2A, although this observation reinforces the principle that the domains are not functionally equivalent.

This biochemical mutagenesis study has narrowed the focus of the molecular mechanism of SytI activation. The discrepancies between *in vitro* and cell-based data have directed our interest toward elucidating the step in between phospholipid binding and vesicle fusion *in vitro* that our D-E mutants influenced, indicated by the absence of a correlation between K_d and $\%F_{max}$ as well as extent of fusion. The intermediate steps between phospholipid binding and vesicle fusion that are influenced by the character of Ca^{2+} ligands in SytI C2AB may be membrane

penetration, ability to fold SNARE proteins, and SytI oligomerization, which will be the focus of future studies.

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