The C2A Domain of Synaptotagmin Alters the Kinetics of Voltage-gated Ca\(^{2+}\) Channels Ca\(_v\)1.2 (Lc-type) and Ca\(_v\)2.3 (R-type)\(^*\)

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Roy Cohen\(^\ddagger\), Lisa A. Elferink\(^\dagger\), and Daphne Atlas\(^\ddagger\)

From the \(^\ddagger\)Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel and the \(^\ddagger\)Department of Physiology & Biophysics and the Marine and Biomedical Institute, University of Texas Medical Branch, Galveston, Texas 77555-1069

Biochemical and genetic studies implicate synaptotagmin (Syt 1) as a Ca\(^{2+}\) sensor for neuronal and neuroendocrine neurosecretion. Calcium binding to Syt 1 occurs through two cytoplasmic repeats termed the C2A and C2B domains. In addition, the C2A domain of Syt 1 has calcium-independent properties required for neurotransmitter release. For example, mutation of a polylysine motif (residues 189–192) reverses the inhibitory effect of injected recombinant Syt 1 C2A fragment on neurotransmitter release from PC12 cells. Here we examined the requirement of the C2A polylysine motif for Syt 1 interaction with the cardiac Ca\(_v\)1.2 (L-type) and the neuronal Ca\(_v\)2.3 (R-type) voltage-gated Ca\(^{2+}\) channels, two channels required for neurotransmission. We find that the C2A polylysine motif presents a critical interaction surface with Ca\(_v\)1.2 and Ca\(_v\)2.3 since truncated Syt 1 containing a mutated motif (Syt 1* 1–264) was ineffective at modifying the channel kinetics. Mutating the polylysine motif also abolished C2A binding to Lc\(_{753–893}\) the cytosolic interacting domain of Syt 1 at Ca\(_v\)1.2 α1 subunit. Syt 1 and Syt 1* harboring the mutation at the KKKK motif modified channel activation, while Syt 1* only partially reversed the syntaxin 1A effects on channel activity. This mutation would interfere with the assembly of Syt 1/channel/syntaxin into an exocytotic unit. The functional interaction of the C2A polylysine domain with Ca\(_v\)1.2 and Ca\(_v\)2.3 is consistent with tethering of the secretory vesicle to the Ca\(^{2+}\) channel. It indicates that calcium-independent properties of Syt 1 regulate voltage-gated Ca\(^{2+}\) channels and contribute to the molecular events underlying transmitter release.

The synaptic vesicle protein Synaptotagmin I (Syt 1),\(^1\) is proposed to function as a Ca\(^{2+}\) sensor for neurotransmitter release (1, 2). Consistent with its proposed role as a calcium sensor protein, Syt 1 binds calcium via two repeating structures termed C2A and C2B domains (3).

A role for the C2A and C2B domains of Syt 1 in calcium-triggered neurosecretion is well established (4–7). For example, Ca\(^{2+}\) binding to the C2A domain enhances the association of Syt 1 with several proteins required for neurotransmission including syntaxin 1A (8, 9), SNAP-25 (10, 11), and AP2 (9, 12).

Furthermore, Ca\(^{2+}\) binding to the C2A domain promotes its insertion into membranes via an interaction with the acidic phospholipids (8, 9, 13, 14) consistent with the Ca\(^{2+}\) requirements of neurosecretion. Microinjection of recombinant C2A domains and antibodies specific for this region impair neurotransmitter release from neuroendocrine PC12 cells (15) and giant squid axons (16). Interestingly, the inhibitory effect of recombinant C2A fragments in PC12 cells occurs independently of its calcium binding properties and is mediated through a novel polybasic motif (17). Thus, the Syt 1 C2A domain contains calcium-dependent and -independent activities, which mediate Syt 1 function during neurotransmitter release. Furthermore, Syt 1 and Syt 4 were recently shown to promote transmitter release independently of Ca\(^{2+}\) binding to the C2A domain (18).

Interactions through the Syt 1 C2B domain are also functionally important for neurosecretion (19–26). Several studies have demonstrated that the activity of Ca\(^{2+}\) channels is modified by syntaxin 1A, Syt 1, and SNAP-25 (27–30). The syntaxin 1A or SNAP-25 inhibitory effects of Ca\(_v\)1.2, Ca\(_v\)2.2, and Ca\(_v\)2.3 activity are reversed by co-expression of Syt 1 (31–34). Recovery of channel activity by Syt 1 was directly proportional to the ratio of Syt 1 and syntaxin 1A, indicating that Syt 1 and syntaxin 1A regulate the Ca\(^{2+}\) channel directly (32, 33). Consistent with this, recombinant proteins comprising the C2A and C2B domains of Syt 1 bind to the II–III cytosolic domain of the a\(_{1L}, 2\), a\(_{2L, 1, 2}\), and a\(_{2L, 2}\) channel subunits (31–33, 35, 36).

Here we studied the functional interaction of Syt 1 with Ca\(_v\)1.2 and Ca\(_v\)2.3 by examining the relative contribution of the C2A polylysine motif on channel activity and binding to the cytosolic domain Lc\(_{753–893}\) of the a\(_{1L, 2}\) of Ca\(_v\)1.2. Our data indicate that the C2A domain of Syt 1 modulates the activation kinetics of Ca\(_v\)1.2 and Ca\(_v\)2.3. Mutation of the C2A polylysine motif abolished the binding to the cytosolic interaction domains of the channel. Moreover, this mutation altered the modulatory effect of Syt 1 on Ca\(_v\)1.2 and Ca\(_v\)2.3 activity, impairing the ability of Syt 1 to reverse the syntaxin 1A inhibition of channel activity.

**EXPERIMENTAL PROCEDURES**

\(\alpha_{1L, 2}\) (dN60-del1773; X15539) rat β2A (m80545); α\(_{2L}\) subunit cloned into pHBE239 (L27745) were obtained from Dr. L. Birnbaumer; a\(_{2L}\) rabbit skeletal (M86621) from A. Schwartz. Rat Syt 1 was obtained from M. L Bennett. The in vitro transcription kit was from Stratagene. Anti-syntaxin antibody was a kind gift of M. Takahashi and was prepared by us; anti-Syt 1 antibody was from Sigma; Anti-Lc\(_{753–893}\) antibody (32). CM5 sensor chip, N-hydroxysuccinimide, N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and ethanolamine-HCl were purchased from Biacore, AB (Uppsala, Sweden). Glutathione-agarose 4B beads were from Amersham Biosciences.

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† To whom correspondence should be addressed. Tel.: 972-2-658-5406; Fax: 972-2-658-5413; E-mail: datlas@vms.huji.ac.il.

‡ The abbreviations used are: Syt, synaptotagmin; EDC, N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride; GST, glutathione S-transferase.

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(K189A/K190A/K191A/K192A) was prepared by excising the C2B domain (amino acids 265–421) and religating the Pr/M1-XcRI fragment in directional cloning.

cRNA Injection and Protein Expression in Xenopus Oocytes—Stage V–VI oocytes were removed and defolliculated by collagenase (type I) treatment as described (37). Oocytes were injected with cRNA of α1.2 or α1.2,3 (5 ng/oocyte), α261 (5 ng/oocyte), β2α (10 ng/oocyte), and a day later with the C2B matrix using activated carboxyl matrix and EDC coupling in HBS-EP buffer (150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) 10 mM Hepes, pH 7.4, and surfactant P20) at a flow rate of 10 μl/min. The surface was activated for 7 min with a mixture of N-hydroxy succinimide (0.05 mM) and EDC (0.02 mM). His-tagged Lc753 was injected at a concentration of 20 μg/ml in 10 mM sodium acetate, pH 3.5, until the desired level of binding was achieved. Ethanolamine (1×, pH 8.5) was injected for 7 min to block the remaining activated groups. Control flow-cell surface was prepared by activating and then deactivating (blocking) the carboxyl groups as mentioned above. His6-Lc753 binding studies to wild type and mutant C2A domains were initiated by passing the recombinant fusion proteins GST-C2A wild type, GST C2A* mutant, and GST alone at increasing concentrations through the flow cells at a rate of 20 μl/min in HBS-EP running buffer. Surface regeneration was carried out after each binding assay by a 10-μl pulse of 1 M NaCl in 10 mM NaOH. The data were analyzed using the Kinetics Wizard of the Biacore control software with automatic corrections for nonspecific binding by subtraction of the responses obtained for the control surface from the data obtained. The kinetics of binding and affinity constants were calculated using the Biaevaluation software.

Binding of the cytoplasmic domain of Lc753 (100 nM) to GST fusion proteins, Syt 1, C2A, C2B, C2A*, and GST alone (100 pmol) using glutathione-agarose 4B beads (25 μl) was performed as described (31, 32). Immunoblots were probed using affinity-purified anti-Lc753 antibody and visualized by enhanced chemiluminescence (ECL system).
The C2A of Synaptotagmin Interacts with Ca\(^{2+}\) Channels

| TABLE I | Differential effect of syntaxin 1A Syt 1 wild type and Syt 1 mutants on the kinetic parameters of Ca\(_{1.2}\) and Ca\(_{2.3}\) |
| --- | --- |
| Ca\(_{1.2}\) | Ca\(_{2.3}\) |
| \(\tau_{\text{act}}\) | + Syntaxin 1A | + Syntaxin 1A |
| Channel | \(\tau_{\text{act}}\) | Peak | \(\tau_{\text{act}}\) | Peak | \(\tau_{\text{act}}\) | Peak |
| ms | nA | ms | nA | ms | nA | ms | nA |
| + Syt 1 | 2.9 ± 0.3 | 1924 ± 88 | 3.3 ± 0.3 | 2537 ± 347 | 5.8 ± 0.4 | 939 ± 46 | 7.8 ± 0.7 | 3328 ± 384 | 8.0 ± 0.5 | 3328 ± 384 |
| + Syt 1* | 3.7 ± 0.3 | 2268 ± 352 | 3.8 ± 0.3 | 2119 ± 484 | 5.5 ± 0.5 | 3771 ± 232 | 6.0 ± 0.6 | 2999 ± 208 |
| + Syt 1(–264) | 3.9 ± 0.3 | 2354 ± 396 | 4.4 ± 0.4 | 1409 ± 203 | 5.9 ± 0.3 | 3630 ± 272 | 9.0 ± 0.5 | 2951 ± 124 |
| + Syt 1(–264) | 3.2 ± 0.1 | 2325 ± 317 | 3.8 ± 0.4 | 1688 ± 221 | 4.7 ± 0.2 | 3297 ± 189 | 6.3 ± 0.5 | 2771 ± 127 |
| + Syt 1(–12) | 3.1 ± 0.3 | 1760 ± 467 | 5.4 ± 0.6 | 1419 ± 325 | 6.9 ± 0.2 | 3182 ± 145 | 7.6 ± 0.2 | 2638 ± 178 |

\(a\) \(-5 \text{ mV}\), \(b\) \(+5 \text{ mV}\), \(c\) \(+10 \text{ mV}\), \(d\) \(0 \text{ mV}\).

**FIG. 3.** Syt 1 and Syt 1* interact with Ca\(_{1.2}\) in the presence of syntaxin 1A. A, superimposition of macroscopic \(\alpha\)-1,2, \(\alpha\)-261, and \(\beta\)-2a currents evoked from a holding potential \(-80 \text{ mV}\) by a single voltage step (160 ms) to \(+20 \text{ mV}\) in oocytes expressing the three-channel subunits in various combinations as indicated. B, peak-current amplitudes (data not shown) normalized to maximum current \((I/I_{\text{max}})\) plotted against test potentials were fitted according to Boltzmann; channel subunits (C) with syntaxin 1A (○), syntaxin 1A and Syt 1 (▲), or syntaxin 1A and Syt 1* (○). The mid-point of activation (\(V_{1/2}\)) and Boltzmann slope (k) of \(\alpha\)-1,2/\(\alpha\)/\(\beta\)-2a were \(V_{1/2} = -21.6 ± 1.2 \text{ mV}, k = 2.9 ± 1.9\), with syntaxin 1A, \(V_{1/2} = -7.5 ± 1.75 \text{ mV}, k = 7.2 ± 1.44\); with syntaxin 1A and Syt 1 \(V_{1/2} = -18.6 ± 2.1 \text{ mV}, k = 2.4 ± 0.8\); and with syntaxin 1A and Syt 1* \(V_{1/2} = -19.0 ± 2.2 \text{ mV}\) and \(k = 3.9 ± 2.1\). C, the activation time constants (\(\tau_{\text{act}}\) mean ± S.E., \(n = 8\)) are plotted against test pulses between \(-10\) and \(+30 \text{ mV}\); the channel alone (○), with syntaxin 1A (●), with syntaxin 1A and Syt 1 (▲) and D, with syntaxin 1A and Syt 1* (○). Two sample Student’s t tests were applied, and p values <0.05 were obtained from the two-tailed tests. See Fig. 2 for cRNA/oocyte of channel subunits, syntaxin 1A (2 ng/oocyte).

**RESULTS**

The C2A Domain of Syt 1 Is Required for Functional Interactions with the Voltage-gated Ca\(^{2+}\) Channels, Ca\(_{1.3}\)–Functional interactions of voltage-gated Ca\(^{2+}\) channels with the full-length Syt 1 have been previously described using the Xenopus oocytes expression system. To assess the role of the C2A polylysine motif (amino acids 189–192) on Syt 1 interactions with the Ca\(^{2+}\) channel, the C2A polylysine motif was substituted with alanine residues in full-length Syt 1 (Syt 1*) or a truncated form of Syt 1 (Syt 1\(_{1-264}\)) lacking the C2B domain (Fig. 1).

Ca\(_{1.2}\) currents were elicited in oocytes co-expressing \(\alpha\)-1,2, \(\alpha\)-261, \(\beta\)-2a, syntaxin 1A, and syntaxin 1A with Syt 1\(_{1-264}\), and syntaxin 1A with Syt 1\(_{1-264}\) from a holding potential of \(-80 \text{ mV}\) by voltage steps of 160 ms applied in 5-mV increments at potentials between \(-30\) and \(+45 \text{ mV}\). A, superposition of macroscopic \(\alpha\)-1,2, \(\alpha\)-261, and \(\beta\)-2a currents activated from a holding potential \(-80 \text{ by a single voltage step of 160 ms}\) to a test potential of \(0 \text{ mV}\) in various combinations as indicated. B, leak-subtracted peak current-voltage relationship; collected data from oocytes expressing the three-channel subunits (○) with syntaxin 1A (●), syntaxin 1A and Syt 1\(_{1-264}\) (▲), or C, syntaxin 1A and Syt 1\(_{1-264}\) (○). The data points correspond to the mean ± S.E. of current amplitude (\(n = 7\)). D, peak current amplitudes normalized to maximum current \((I/I_{\text{max}})\) are plotted against test potentials (data from B and C) and were fitted according to Boltzmann equation. The mid-point of activation (\(V_{1/2}\)) and the Boltzmann slope (k) of \(\alpha\)-1,2/\(\alpha\)/\(\beta\)-2a were \(V_{1/2} = -21.6 ± 1.2 \text{ mV}, k = 2.9 ± 1.9\), with Syt 1\(_{1-264}\), \(V_{1/2} = -20.6 ± 2.3 \text{ mV}, k = 5.1 ± 2.89\), and with Syt 1* \(_{1-264}\), \(V_{1/2} = -12.9 ± 3.1 \text{ mV}, k = 5.4 ± 2.1\). E, activation time constants of the channel (\(\tau_{\text{act}}\) mean ± S.E., \(n = 6\)) are plotted against test potentials (○) with syntaxin 1A (●), syntaxin 1A and Syt 1\(_{1-264}\) (▲), and F, syntaxin 1A and Syt 1* \(_{1-264}\) (○). Two sample Student’s t tests were applied, and p values <0.05 were obtained from the two-tailed tests. (See legend to Fig. 2 for cRNA injected per oocyte.)
The C2A of Synaptotagmin Interacts with Ca\textsuperscript{2+} Channels

Syt\textsuperscript{1} and Syt\textsuperscript{1*} interact with Ca\textsuperscript{2,3} (R-channel). Oocytes were injected with cRNA of n, 2.3 (5 ng/oocyte), z261 (5 ng/oocyte), β2a (10 ng/oocyte), and a day later, with Syt\textsuperscript{1} (5 ng/oocyte) or Syt\textsuperscript{1*} (5 ng/oocyte). Inward Ba\textsuperscript{2+} currents were elicited from a holding potential of −80 mV in response to an 80-ms pulse to various test potentials between −30 and +45 mV in 5-mV increments. A, leak-subtracted peak current-voltage relationship; collected data from oocytes expressing the three channel subunits (○) together with Syt\textsuperscript{1} (●) or Syt\textsuperscript{1*} (☆). The data points correspond to the mean ± S.E. of current (n = 8). B, the activation component of a typical current produced by a test pulse was fitted with a single exponential function between the lines marked by asterisks and was applied to determine the time constant of activation (τ\textsubscript{act}). C, activation time constants (τ\textsubscript{act}, mean ± S.E., n = 6) are plotted against potentials between −20 and +30 mV in the absence (○) and in the presence of Syt\textsuperscript{1} (●) or D, Syt\textsuperscript{1*} (☆). Two sample Student\textprime{}s t test were applied, and p values <0.05 were obtained from the two tailed tests.

Syt\textsuperscript{1} (Fig. 3B). Similarly, a complete reversal of the syntaxin 1A effect on Ca\textsuperscript{1,2} activation was observed with co-expression of Syt\textsuperscript{1} (Fig. 3C), and only partial reversal by Syt\textsuperscript{1*} (Fig. 3D).

The mutation at the polylysine motif impaired Syt\textsuperscript{1*} capacity to reverse the inhibitory effects of syntaxin 1A on Ca\textsuperscript{1,2} current amplitude and activation kinetics.

**Ca\textsuperscript{1,2} Interacts with Syntaxin 1A and the Truncated Syt\textsuperscript{1–264} Mutants**—The partial reversal of the syntaxin 1A effect on Ca\textsuperscript{1,2} activation by Syt\textsuperscript{1*} compared with Syt\textsuperscript{1} suggests that the polylysine C2A motif couples Syt\textsuperscript{1} to channel activation (Fig. 3, C and D). To isolate the contribution of C2A domain we co-expressed truncated Syt\textsuperscript{1} lacking the C2B domain (Syt\textsuperscript{1–264}) with Ca\textsuperscript{1,2} and syntaxin 1A. Syt\textsuperscript{1–264} whole cell currents were activated from a holding potential of −80 to 0 mV test pulse (Fig. 4A). Both the superimposed traces as well as the current-voltage relationships (Fig. 4, A–C) showed diminished current amplitudes by syntaxin 1A that were only partially reversed by Syt\textsuperscript{1–264} and Syt\textsuperscript{1*–1–264}. Syt\textsuperscript{1*–1–264} was significantly less effective than Syt\textsuperscript{1–264}.

Furthermore the large shift in the half-maximal voltage induced by syntaxin 1A (see above) was shifted back to \(V_{1/2} = −20.6 \pm 2.3\) mV by Syt\textsuperscript{1–264} and only to \(−12.9 \pm 3\) mV by Syt\textsuperscript{1*–1–264} (Fig. 4D).

A more striking difference between Syt\textsuperscript{1–264} and Syt\textsuperscript{1*–1–264} was observed on channel activation (Fig. 4, E and F). The marked slowing effect of activation kinetics by syntaxin 1A was fully reversed by Syt\textsuperscript{1–264}. (Fig. 4C; Table I). In contrast, Syt\textsuperscript{1*–1–264} was completely ineffective (Fig. 4E). Together, these results suggest the involvement of the C2A polylysine motif in the interaction with the channel.

K189–192A Mutations Abolish Lc\textsubscript{753–893} Binding to the C2A Domain of Syt\textsuperscript{1}—The polylysine motif (189–192) at the C2A
demonstrated by current-voltage relationship (Fig. 2A). The activation component of Ca\textsuperscript{1,2} current was measured at each test pulse and was fitted with a single exponential function between the lines marked by asterisks (Fig. 2B). Under these experimental conditions both Syt\textsuperscript{1} and Syt\textsuperscript{1*} slightly reduced activation rate at voltage range of −15 to −5 mV, while at more positive potentials \(t_{\text{max}}\) approached control values (Fig. 2C; Table I). Lysates of oocytes co-injected with Syt\textsuperscript{1}, Syt\textsuperscript{1*}, and Ca\textsuperscript{1,2} were prepared and analyzed for Syt\textsuperscript{1}/Syt\textsuperscript{1*} expression by Western analysis using anti-Syt\textsuperscript{1} antibody (see “Experimental Procedures”). As shown in Fig. 2D no significant difference in the expression of Syt\textsuperscript{1} and Syt\textsuperscript{1*} in injected oocytes was observed.

**Syt\textsuperscript{1} and Syntaxin 1A Are Functionally Coupled to Ca\textsuperscript{1,2} Activity**—We previously demonstrated that Ca\textsuperscript{1,2} as well as Ca\textsubscript{2,2} (neuronal N-type channel) activities are inhibited with co-expression of syntaxin 1A (28, 32, 37). Since the inhibitory effect of syntaxin 1A on these channels is reversed by Syt\textsuperscript{1} (31, 32, 33) we next examined the Syt\textsuperscript{1*} mutant for reversal of syntaxin 1A inhibitory effects on channel activity. Fig. 3 shows the results of co-expressing Ca\textsuperscript{1,2} and syntaxin 1A with Syt\textsuperscript{1} and Syt\textsuperscript{1*} in Xenopus oocytes. Superimposed traces of macroscopic whole cell Ba\textsuperscript{2+} currents showed an 80% inhibition of current amplitude by syntaxin 1A, which was fully reversed in the presence of Syt\textsuperscript{1} and partially by Syt\textsuperscript{1*} (Fig. 3A; Table I). Furthermore, peak current amplitudes normalized to maximum current (\(I/\text{I}_{\text{max}}\)) showed a large voltage shift in the half-maximal voltage (\(V_{1/2}\)) induced by syntaxin 1A from \(V_{1/2} = −21 \pm 1.2\) mV to \(V_{1/2} = −7.5 \pm 1.8\) mV. This voltage shift was reverted to \(V_{1/2} = −18.6 \pm 2\) mV by Syt\textsuperscript{1} and −19 ± 2.2 mV by Syt\textsuperscript{1*} (Fig. 3B). Similarly, a complete reversal of the syntaxin 1A effect on Ca\textsuperscript{1,2} activation was observed with co-expression of Syt\textsuperscript{1} (Fig. 3C), and only partial reversal by Syt\textsuperscript{1*} (Fig. 3D).

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Furthermore the large shift in the half-maximal voltage induced by syntaxin 1A (see above) was shifted back to \(V_{1/2} = −20.6 \pm 2.3\) mV by Syt\textsuperscript{1–264} and only to \(−12.9 \pm 3\) mV by Syt\textsuperscript{1*–1–264} (Fig. 4D).

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Fig. 7. Syt 1 and Syt 1* interact with Ca_{2,3}. Inward Ba^{2+} currents were elicited in oocytes co-expressing α_{2,3}, α2δ1, and β2a along with Syt 1_{1–264} and Syt 1*_{1–264}, in response to voltage steps of 80 ms to test potentials between −30 and +40 mV in 5-mV increments. Holding potential was −80 mV. A, superimposition of α_{2,3}, α2δ1, and β2a current traces, either alone or in combination with Syt 1_{1–264} and Syt 1*_{1–264} activated from a holding potential of −80 mV in response to a 0-mV test pulse. B, leak-subtracted peak current-voltage relationship: collected data from oocytes expressing the three channel subunits alone (○), with Syt 1_{1–264} (■) or C, with Syt 1*_{1–264} (□). The data points correspond to the mean ± S.E. of current (n = 8). D, peak current-amplitudes normalized to maximum current (I_{max}) are plotted against test potentials (data from B and C) were fitted according to Boltzmann. The mid-point of activation (V_{1/2}) and the Boltzmann slope (k) of α_{2,3}/α2δ1/β2a were V_{1/2} = −8.8 ± 0.1 mV, k = 2.3 ± 0.1; with Syt 1_{1–264}, V_{1/2} = 0.5 ± 0.1 mV, k = 2.9 ± 0.1; and with Syt 1*_{1–264}, V_{1/2} = −6.2 ± 0.1 mV and k = 2.5 ± 0.1. E, activation time constants (τ_{act}, mean ± S.E., n = 6) are plotted against test potentials in the absence (○) and in the presence of Syt 1_{1–264} (■) and F, Syt 1*_{1–264} (□). Two sample Student’s t tests were applied, and p values <0.05 were obtained from the two-tailed tests. cRNA of channel subunits injected per oocyte, see Fig. 5; Syt 1*_{1–264} (5 ng/oocyte); Syt 1_{1–264} (5 ng/oocyte).

Fig. 8. Syt 1 and Syt 1* modify Ca_{2,3} properties in the presence of syntaxin 1A. A, superposition of macroscopic α_{2,3}, α2δ1, and β2a current traces evoked in response to an 80-ms pulse from a holding potential of −80 mV by a single voltage step to a 0-mV test pulse in oocytes co-expressing the three-channel subunits alone and together with either Syt 1 or Syt 1*. B, leak-subtracted peak current-voltage relationship: collected data from oocytes expressing the three-channel subunits (○) with syntaxin 1A (●), syntaxin 1A and Syt 1 (▼), and C, syntaxin 1A and Syt 1* (▲). The data points correspond to the mean ± S.E. of current (n = 8). D, peak current amplitudes normalized to maximum current (I_{max}) (data from B and C) are plotted against test potentials displayed with a Boltzmann fit. The mid-point of activation (V_{1/2}) and the Boltzmann slope (k) of α_{2,3}/α2δ1/β2a were V_{1/2} = −8.8 ± 0.1 mV, k = 2.3 ± 0.1; with Syt 1, V_{1/2} = −2.8 ± 0.8 mV, k = 3.9 ± 0.25, and with Syt 1*_{1–264}, V_{1/2} = −2.7 ± 1.38 mV, k = 4.2 ± 0.4. E, the activation time constants (τ_{act}, mean ± S.E., n = 6) are plotted against test potentials between −20 and +25 mV: the channel alone (○), with syntaxin 1A (●), syntaxin 1A and Syt 1 (▼), or F, syntaxin 1A and Syt 1* (▲). Two sample Student’s t tests were applied, and p values <0.05 were obtained from the two-tailed tests. cRNA/oocyte, see Fig. 3; Syntaxin 1A (2 ng/oocyte).

domain is exposed on the surface of the β-sandwich of Syt 1 where they are accessible for interacting with potential effector molecules (7). To determine whether the loss of functional interaction with the channel is related to impaired binding to L_{C2A–2a}, the II–III linker of the Ca_{1,2} α1 subunit (32). Two types of binding studies of C2A and mutant C2A* domains
were preformed. (i) Recombinant GST-C2A, GST-C2A*, GST-Syt 1, and GST proteins were immobilized to GSH-agarose beads and incubated with equimolar concentrations of recombinant His\textsubscript{Lc753-893} (0.5 \(\mu\text{M}\); 2.5 \(\mu\text{M}\)). As shown by Western analysis using anti-Le\textsuperscript{C753-893} antibody (32), C2A, C2B, and Syt 1 bind Le\textsuperscript{C753-893}, but no binding of C2A* was observed (Fig. 5A).

(ii) The affinity of C2A and C2A* to Le\textsuperscript{C753-893} was tested using the Biacore technology (Biacore; see “Experimental Procedures”). His\textsubscript{Lc753-893} was immobilized on a sensor chip surface. Recombinant samples of GST-C2A, GST-C2A* at the indicated concentrations were injected into the flow cell of the system (Biacore), and changes in resonance units were recorded as a function of time to yield sensorgrams as shown in Fig. 5B. The C2A binding to Le\textsuperscript{C753-893} is manifested as large amplitude of the surface plasmon resonance signal while no resonance signal was obtained by C2A*. GST alone showed no binding (data not shown). The calculated affinity of C2A was 0.213 \(\mu\text{M}\) (5.9 ± 0.46; Fig. 5B). Hence the C2A mutant does not bind to the intracellular domain of the channel that comprises the Syt 1 interaction (31, 32, 35, 40, 47).

**The C2A Domain of Syt 1 Is Required for Functional Interactions with C\textsubscript{a}2.3 (R-channel)—**C2A, C2A* currents were elicited in oocytes co-expressing \(\alpha\text{C}2.3/\beta 2a/\alpha 2B1\) subunits (41) and Syt 1 or Syt 1* (Fig. 6). Syt 1 or Syt 1* modified neither C\textsubscript{a}2.3 current-voltage relationship nor peak-current amplitude (Fig. 6A). Conversely, Syt 1 strongly accelerated C\textsubscript{a}2.3 activation in the range of −20 to +5 mV, converging at more depolarized values (>−5 mV) (Fig. 6B; Table I; Ref. 30). Interestingly, Syt 1 was previously shown to accelerate the activation kinetic of Ca\textsubscript{a}2,2 (N-type channel; Ref. 28). The effect of Syt 1* on Ca\textsubscript{a}2,3 was more complex, showing mixed effects of this mutant on channel activation (Fig. 6C). At negative potentials between −20 and −10 mV, the rate was accelerated by Syt 1* similar to Syt 1, but between −5 and 0 mV an abrupt decrease in the rate was observed, which was slower than the channel (Fig. 6D). At more positive potentials, in the range of +5 to +30 mV, \(\tau_{\text{act}}\) approached control values (Fig. 6, C and D).

**Activation of C\textsubscript{a}2.3 Requires the C2A Polylysine Motif—**We next examined the requirement of the Syt 1 C2A polylysine motif on channel activation using the truncated Syt 1 mutants. C2A, C2A* currents were elicited in oocytes co-expressing the three channel subunits along with Syt 1\textsuperscript{1-264} and Syt 1*\textsuperscript{1-264}. In both mutants the C2B domain is missing, and in Syt 1*\textsuperscript{1-264} the polylysine motif was substituted with alanine residues. The effects of the truncated mutants on channel activity are shown in Fig. 7, A and B.

Superimposed traces of whole cell current were activated from a holding potential of −80 mV by a single voltage step to 0-mV test pulse (Fig. 7A). Syt 1\textsuperscript{1-264} appeared to inhibit current amplitude by 60% at 0 mV, while Syt 1*\textsuperscript{1-264} displayed no effect on current amplitude but significantly slowed channel inactivation (inactivation kinetics were not explored in the present study). Current-voltage relationships were significantly shifted in the presence of Syt 1\textsuperscript{1-264} but not Syt 1*\textsuperscript{1-264} (Fig. 7, B and C). Peak current amplitudes normalized to maximum current (\(I_{\text{max}}\)) show that the half-maximal voltage activation (\(V_{1/2}\)) was significantly displaced by Syt 1\textsuperscript{1-264} from −8.8 ± 0.1 mV to 0.5 ± 0.1 mV and only marginally to −6.2 ± 0.1 mV by Syt 1*\textsuperscript{1-264} (Fig. 7D). This voltage shift can account for the apparent reduction in current amplitude. The slope factors were directly comparable between control conditions and those expressing Syt 1\textsuperscript{1-264} or Syt 1*\textsuperscript{1-264} (Fig. 6D). Syt 1\textsuperscript{1-264} was also efficient at accelerating Ca\textsubscript{a}2,3 activation in the −20 to −5 mV range similar to full-length Syt 1 (Fig. 7E, Fig. 6C). In contrast, the acceleration of activation by Syt 1*\textsuperscript{1-264} was smaller and was detected only in −20 to −10 mV range (Fig. 6F). The effects of Syt 1\textsuperscript{1-264} and Syt 1*\textsuperscript{1-264} on Ca\textsubscript{a}2,3 currents were specific for this channel as co-expression of these proteins result in no effect on Ca\textsubscript{a}1,2 activation kinetics (see Table I). Together, these data suggest that Ca\textsubscript{a}2,3 activation involves the C2A domain of Syt 1. Moreover, mutation of the polylysine motif modifies the interaction of the C2A domain with the channel.

**A Cross-interaction of Syntaxin 1A with Syt 1 Mutants and C\textsubscript{a}2.3—**Superimposed traces of macroscopic whole cell C\textsubscript{a}2,3 current elicited from a holding potential of −80 mV by a single voltage step to 0-mV test pulse showed a partial reversal by Syt 1 (from 50% to 18%) of the syntaxin 1A-mediated current inhibition but not by Syt 1* (Fig. 8A). Current-voltage relationships obtained in the presence of syntaxin 1A or syntaxin 1A with either one of the Syt 1 mutants indicated a shift toward more positive potentials by Syt 1* (Fig. 8, B and C) that could account for the reduction in current amplitude at 0 mV (Fig. 7A). Peak current amplitudes normalized to maximum current (\(I_{\text{max}}\)) showed no shift in the half-maximal voltage of Ca\textsubscript{a}2,3 (\(V_{1/2}\) = −8.8 ± 0.1 mV) by syntaxin 1A (\(V_{1/2}\) = −9.0 ± 2.1 mV) (Fig. 8D), unlike the large shift induced by syntaxin 1A in Ca\textsubscript{a}2,2 (Fig. 3B). In the presence of syntaxin 1A, \(V_{1/2}\) was shifted toward more positive potentials to −4.3 ± 1.2 mV by Syt 1 and to −0.4 ± 0.7 mV by Syt 1* (Fig. 8D). Hence, Syt 1 and Syt 1* differentially modify the syntaxin-associated channel.

Ca\textsubscript{a}2,3 activation was accelerated in cells expressing syntaxin 1A and was not modified further by Syt 1 (Fig. 8E). In contrast, Syt 1* slowed the activation kinetics in the presence of syntaxin 1A, in particular in potentials between 0−15 mV (Fig. 8F). Thus, mutation of the polylysine motif increased the current voltage shift of Ca\textsubscript{a}2,3 and was less effective than Syt 1 at reversing the syntaxin 1A inhibition. In addition, the mutation appeared to affect the interaction of the channel with syntaxin 1A, slowing the activation kinetics. Together, these data suggest that the Syt 1 C2A polylysine motif participates in the syntaxin 1A modulation of Ca\textsubscript{a}2,3 activation.

Since Syt 1 and Syt 1 mutants affect syntaxin 1A modulation of the channel, their effect on syntaxin 1A expression in oocytes was tested (Fig. 9). Oocytes were injected with syntaxin 1A cRNA (5 ng/oocyte) and cRNA encoding the various Syt 1 mutants (5 ng/oocyte) as indicated. At day five after injection, oocytes were lysed and proteins were separated on SDS-PAGE and analyzed using monoclonal anti-syntaxin 1A antibody (Fig. 9). As shown by the Western blot analysis there were no significant changes in syntaxin 1A expression in the presence of either one of the four Syt 1 mutants (Fig. 9).

The contribution of the KKKK motif to the channel interaction with syntaxin 1A was examined by using the two tran-

**Fig. 9. The effect of Syt 1 wild type and mutants on syntaxin 1A expression in oocytes.** Xenopus oocytes were injected with cRNA of \(\alpha\text{C}2.3\) (5 ng/oocyte), \(\alpha\text{C}2.3\) (5 ng/oocyte), \(\beta 2a\) (10 ng/oocyte), and \(\alpha\text{C}1.2\) (5 ng/oocyte) with the corresponding subunits, syntaxin 1A (5 ng/oocyte), and with Syt 1, Syt 1*, Syt 1\textsuperscript{1-264}, or Syt 1*\textsuperscript{1-264} (5 ng/oocyte) as indicated. Five days later, oocytes were lysed and proteins were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The level of syntaxin 1A expressed in the oocytes was determined in a Western analysis by using monoclonal anti-syntaxin 1A antibody and detection by ECL.
FIG. 10. Syt 1*–264 and Syt 1*1–264 modify Ca,2,3 kinetics in the presence of syntaxin 1A. A, superposition of macroscopic α,2,3, αβ2, and β2a current traces evoked in response to an 80-ms pulse from a holding potential −80 mV by a single voltage step to a 0-mV test pulse in oocytes co-expressing the three-channel subunits alone and together with Syt 1*–264 or Syt 1*–264 as indicated. B, leak-subtracted peak current-voltage relationship; collected data from oocytes expressing the three-channel subunits (○), syntaxin 1A (●), or with syntaxin 1A and Syt 1**–264 (■) or syntaxin 1A and Syt 1**–264 (▲). The data points correspond to the mean ± S.E. of current amplitude (n = 9). C, peak current amplitudes normalized to maximum current (I/I(max)) (data from B) are plotted against test potentials and displayed with a Boltzmann fit. The mid-point of activation (V1/2) and the Boltzmann slope (β) of α-2,3/αβ2/β2a were V1/2 = −8.8 ± 0.01 mV, k = 2.3 ± 0.1; with syntaxin 1A, V1/2 = −8 ± 2.1 mV, k = 3.9 ± 0.7; with syntaxin 1A and Syt 1*–264, V1/2 = −2.8 ± 0.8 mV, k = 3.9 ± 0.25; and with syntaxin 1A and Syt 1*–264, V1/2 = −2.1 ± 1.4 mV, k = 4.2 ± 0.4. D, the activation time constants (τact, mean ± S.E., n = 6–8) of the channel (○) or with syntaxin 1A (●) were plotted against test potentials between −20 and +30 mV as indicated. E, the activation time constants were measured in oocytes expressing both syntaxin 1A and Syt 1*–264 ( ■) and F, syntaxin 1A and Syt 1**–264 (▲). Two sample Student’s t tests were applied, and p values <0.05 were obtained from the two-tailed tests. cRNA (ng/oocyte) injected, see Figs. 6 and 7.

FIG. 11. A schematic model illustrating putative cross-talk interfaces of the voltage-gated calcium channel, syntaxin 1A (SX), and the C2A domains of synaptotagmin (Syt 1) and synaptotagmin mutated at the C2A* polylysine motif (Syt 1*). Transmembrane IIa and IIIa are the boundary of Lc(753–893), the cytosolic domain of Ca,1.2.

cated Syt 1 mutants lacking the C2B domain. Currents were evoked from a holding potential of −80 mV to 0 mV test pulse (Fig. 10A). As shown current amplitude was reduced by syntaxin 1A and was partially reversed by the two mutants. Current-voltage relationships showed that Syt 1*–264 and Syt 1**–264 were equally effective at reverting syntaxin 1A inhibition of Ca,2,3 current amplitude (Fig. 10B). The half-maximal voltage (V1/2) of the channel was not affected by syntaxin 1A (see Fig. 8D), but a small shift toward more positive potentials was observed by Syt 1*–264 to 2.7 ± 1.4 mV and to −2.8 ± 0.8 mV by Syt 1**–264 (Fig. 10C). Syntaxin 1A accelerated Ca,2,3 activation (Fig. 10D), which was further increased in the presence of Syt 1*–264 (Fig. 10E). In contrast, Syt 1**–264 lost the ability to accelerate Ca,2,3/syntaxin 1A activation (Fig. 10F). Together, these data show that the mutant failed to modify Ca,2,3 interaction with syntaxin and suggest that the C2A polylysine motif participates in the syntaxin 1A cross-talk with Ca,2,3.

DISCUSSION

A role for the C2A domain of Syt 1 in calcium-triggered neurotransmitter release has been well established in neurons and neuroendocrine cells (15–17, 42). Mutation of a polylysine motif distal to the calcium coordination site reverses the inhibitory effect of injected Syt C2A fragments on calcium-regulated secretion (17, 43). Since mutation of the polylysine motif does not affect the overall structure or Ca,2 binding properties of the C2A domain, calcium-independent properties involving the polylysine motif are important for the Syt-mediated steps leading to neurotransmitter release (17). However the nature of
these interactions remained unknown.

We addressed the possibility that the voltage-gated Ca\(^{2+}\) channel (Ca\(^{1.2}\), Ca\(^{2.1}\), Ca\(^{2.2}\), and Ca\(^{2.3}\)) is an established effector for Syt 1 (30–32, 34, 40), may be functionally coupled through the polylysine C2A domain. Using the Xenopus oocytes expression system we examined the functional consequences of mutating the C2A polylysine motif on Ca\(^{1.2}\) (Le-type) channel that supports evoked secretion in PC12 cells and the neuronal Ca\(^{2.3}\) (R-type) channel. The changes induced in the activation kinetics and current amplitude of voltage-sensitive Ca\(^{2+}\) channels demonstrate that the C2A polylysine motif participates in the interaction of Syt 1 with both Ca\(^{1.2}\) and Ca\(^{2.3}\).

Modulation of Ca\(^{1.2}\)—Since the Syt 1/syntaxin 1A interaction occurs independently of the C2A polylysine motif (17), the observed differences in syntaxin 1A modulation of channel activity in the presence of Syt 1 may result from either a direct interaction with the channel or with a new site formed by the association of syntaxin 1A with the channel. The full-length Syt 1 reversed syntaxin 1A inhibition of Ca\(^{1.2}\) activity, while Syt 1\(^{1–264}\) was significantly less effective. The marked slowing of activation kinetics of Ca\(^{1.2}\) by syntaxin 1A was reversed by Syt 1\(^{1–264}\) was significantly less effective. The marked slowing of activation kinetics of Ca\(^{1.2}\) by syntaxin 1A was reversed by Syt 1\(^{1–264}\) (Le-type) the channel that supports evoked secretion in PC12 cells and the neuronal Ca\(^{2.3}\) (R-type) channel. The changes induced in the activation kinetics and current amplitude of voltage-sensitive Ca\(^{2+}\) channels demonstrate that the C2A polylysine motif participates in the interaction of Syt 1 with both Ca\(^{1.2}\) and Ca\(^{2.3}\).

Modulation of Ca\(^{2.3}\)—Previously, induction of faster activation by Syt 1 was observed for the neuronal Ca\(^{2+}\) channels, Ca\(^{2.2}\) and Ca\(^{2.3}\), in contrast to no effect on Ca\(^{1.2}\) (28–30). Here we show that truncated Syt 1 (Syt 1\(^{1–264}\)) accelerated Ca\(^{2.3}\) activation suggesting that C2A and not C2B domain is responsible for the observed effects. Mutation of the polylysine motif in C2A abolished the stimulatory effect on Ca\(^{2.3}\) activation, indicating the role of this motif in the interaction of the vesicular protein with the channel as well as with Ca\(^{2.3}\) associated with syntaxin 1A.

The truncated mutant Syt 1\(^{1–264}\) partially restored (−75%) current amplitude but did not reverse the syntaxin 1A effect on activation. Thus modulation of the syntaxin/Ca\(^{2,3}\) kinetics was affected by Syt 1\(^{1–264}\) but was lost in Syt 1\(^{1–264}\). In contrast, Syt 1 and Syt 1\(^{1–264}\) effectively reversed the syntaxin 1A inhibition of Ca\(^{2.3}\) current amplitude. These results propose that the C2B domain partially compensates for the mutation in the C2A polylysine motif.

Together, the data indicate that the C2A polylysine motif affects the activation of the channel and modulates the kinetics of syntaxin 1A-associated channel. In Fig. 11a we showed that in a schematic model of putative interactions of the channel, syntaxin 1A, and Syt 1.

In summary, our studies provide compelling evidence that the Syt 1 Ca\(^{2.3}\) domain is involved in a functional coupling of the vesicle with the voltage-gated Ca\(^{2+}\)-channels, Ca\(^{1.2}\) and Ca\(^{2.3}\). The C2A polylysine motif appears to participate in this interaction and likely functions independently of the Syt 1 Ca\(^{2+}\)-mediated interactions with phospholipids or syntaxin 1A (17). The effects of C2A polylysine motif on transmitter release in PC 12 cells as previously reported, may result from a direct modification of the activation kinetics of the Ca\(^{2+}\) channel or function indirectly by competing with endogenous Syt 1 for interactions with the channel. The ability of Syt 1, syntaxin 1A, and the Ca\(^{2+}\) channel to interact is consistent with the formation of a functional exocytotic unit, the excitosome (32). The excitosome complex composed of the Ca\(^{2+}\) channel, syntaxin 1A, SNAP-25, and Syt 1 displays distinct kinetic properties required for calcium-triggered secretion (28, 29, 32, 33, 39, 46).

Therefore, inhibition of neurotransmitter release by C2A domain might occur by interfering with generating the excitosome complex and the ensuing propagation of the signal from the channel to the fusion/docking machinery rather than Ca\(^{2+}\) binding to Syt 1 (30). The physiological relevance and the consequences of the different modulation of neuroendocrine (Ca\(^{1.2}\)) and neuronal (Ca\(^{2.3}\)) Ca\(^{2+}\) channels by Syt 1 during the steps leading to transmitter release will require further studies.

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The C2A Domain of Synaptotagmin Alters the Kinetics of Voltage-gated Ca$^{2+}$ Channels Ca$\gamma$1.2 (Lc-type) and Ca$\gamma$2.3 (R-type)

Roy Cohen, Lisa A. Elferink and Daphne Atlas

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