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Sr^{2+} Binding to the Ca^{2+} Binding Site of the Synaptotagmin 1 C_2B Domain Triggers Fast Exocytosis without Stimulating SNARE Interactions

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Summary

Sr^{2+} triggers neurotransmitter release similar to Ca^{2+}, but less efficiently. We now show that in synaptotagmin 1 knockout mice, the fast component of both Ca^{2+}- and Sr^{2+}-induced release is selectively impaired, suggesting that both cations partly act by binding to synaptotagmin 1. Both the C_2A and the C_2B domain of synaptotagmin 1 bind Ca^{2+} in phospholipid complexes, but only the C_2B domain forms Sr^{2+}/phospholipid complexes; therefore, Sr^{2+} binding to the C_2B domain is sufficient to trigger fast release, although with decreased efficacy. Ca^{2+} induces binding of the synaptotagmin C_2 domains to SNARE proteins, whereas Sr^{2+} even at high concentrations does not. Thus, triggering of the fast component of release by Sr^{2+} as a Ca^{2+} agonist involves the formation of synaptotagmin/phospholipid complexes, but does not require stimulated SNARE binding.

Introduction

When an action potential invades a nerve terminal, Ca^{2+} influx induces neurotransmitter release with an exquisite temporal specificity (Katz, 1969; Sabatini and Regehr, 1999). Release exhibits at least two components, a fast synchronous component that dominates at low-frequency stimulation, and a slower asynchronous component that dominates at high-frequency stimulation (Barrett and Stevens, 1972; Goda and Stevens, 1994; Atluri and Regehr, 1998; Hagler and Goda, 2001). Compared to other Ca^{2+}-regulated biological processes, both components of release are rapid since Ca^{2+} triggers synchronous release in as little as 100 μs, and asynchronous release in 10–50 ms (Goda and Stevens, 1994; Sabatini and Regehr, 1996). Both release components are Ca^{2+} dependent with similar apparent Ca^{2+} cooperativities but different apparent Ca^{2+} affinities (Goda and Stevens, 1994), suggesting that multiple Ca^{2+} sensors control release.

Sr^{2+} is a divalent cation that can substitute for Ca^{2+} in triggering neurotransmitter release (Miledi, 1966; Goda and Stevens, 1994; Capogna et al., 1997; Rumpel and Behrends, 1999; Xu-Friedman and Regehr, 1999, 2000). Although Sr^{2+}, similar to Ca^{2+}, induces both synchronous and asynchronous release, the properties of Sr^{2+}- and Ca^{2+}-evoked release are different. Synchronous and asynchronous release triggered by Sr^{2+} exhibit reduced peak amplitudes and longer time constants, suggesting that Sr^{2+} is a Ca^{2+} agonist for vesicle exocytosis that exhibits a lower efficiency than Ca^{2+} (Dodge et al., 1969; Goda and Stevens, 1994; Xu-Friedman and Regehr, 2000). In addition, Sr^{2+} causes a relative increase in asynchronous release (Goda and Stevens, 1994), probably because Sr^{2+} buffering and clearance is less effective than Ca^{2+} buffering and clearance (Rumpel and Behrends, 1999; Xu-Friedman and Regehr, 1999, 2000; Hagler and Goda, 2001). Furthermore, a relatively higher Sr^{2+} affinity of the Ca^{2+} sensor for asynchronous release may contribute to the Sr^{2+}-induced increase in asynchronous release (Goda and Stevens, 1994) .

Synaptotagmins constitute a large family of membrane proteins that are candidate Ca^{2+} sensors for exocytosis (reviewed in Adolfsen and Littleton, 2001; Südhof, 2002). Synaptotagmins contain a single N-terminal transmembrane region, a linker sequence, and two conserved C_2 domains. In most synaptotagmins, both C_2 domains bind multiple Ca^{2+} ions in a complex with phospholipids, and additionally form Ca^{2+}-dependent complexes with SNARE proteins and other molecules implicated in exocytosis. Synaptotagmins 1 and 2 are the most abundant synaptotagmins that are enriched on synaptic vesicles and differentially expressed in brain (Matthew et al., 1981; Perin et al., 1990; Geppert et al., 1991; Ullrich et al., 1994; Marqueze et al., 1995). Studies in mutant mice have demonstrated that synaptotagmin 1 functions as the Ca^{2+} sensor for the fast component of release in hippocampal neurons (Geppert et al., 1994; Fernández-Chacón et al., 2001, 2002). Direct evidence for this conclusion was derived from a point mutation (R233Q) that was introduced by homologous recombination into the murine synaptotagmin 1 gene (Fernández-Chacón et al., 2001). The R233Q mutation, although localized to the C_2A domain, decreases the apparent Ca^{2+} affinity of the double C_2 domain fragment of synaptotagmin 1 and the apparent Ca^{2+} affinity of neurotransmitter release approximately 2-fold. In contrast, point mutations in the Ca^{2+} binding sites of the C_2A domain that partly abolish Ca^{2+} binding have no effect in vivo (Fernández-Chacón et al., 2002; Robinson et al., 2002), probably because they impair only Ca^{2+}-dependent phospholipid binding to the isolated C_2A domain but not to the double C_2 domain fragment (Fernández-Chacón et al., 2002). Mutations in Ca^{2+} binding sites of the C_2B domain, conversely, abolish Ca^{2+}-triggered release (Mackler et al., 2002). Together these results suggest that Ca^{2+} binding to the C_2B domain of synaptotagmin 1 is essential for Ca^{2+} triggering of fast exocytosis. However, these results do not clarify the precise contribution of the C_2A domain to Ca^{2+} triggering of release and do

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not reveal whether Ca^{2+} binding to the C_2B domain is sufficient for inducing exocytosis.

Mouse mutants of synaptotagmin 1 exhibit no change in the slow, asynchronous component of release or in Ca^{2+}-independent forms of exocytosis, suggesting that synaptotagmin 1 function is selective for the fast component (Geppert et al., 1994). Other synaptotagmins, possibly synaptotagmins 3 and 7 (the most abundant after synaptotagmins 1 and 2), may act as Ca^{2+} sensors for asynchronous release (Südhof, 2002). This suggestion was raised because these synaptotagmins bind Sr^{2+} comparatively better than synaptotagmins 1 and 2 (Li et al., 1995a), and because they exhibit a higher apparent Ca^{2+} affinity than synaptotagmins 1 and 2 (Sugita et al., 2001, 2002). In addition, the C_2 domains of synaptotagmins 3 and 7 are effective inhibitors of Ca^{2+}-dependent exocytosis in neuroendocrine PC12 cells where synaptotagmin 1 is not required for release (Shoji-Kasai et al., 1992; Sugita et al., 2001; Shin et al., 2002), although additional synaptotagmins (such as synaptotagmin 9) (Fukuda et al., 2002) may also be involved.

A key question in understanding neurotransmitter release is how Ca^{2+} binding to synaptotagmin 1 triggers fast release. Two major interactions mediated by synaptotagmin 1, Ca^{2+}-dependent binding to phospholipids and to SNARE proteins, have been suggested as the triggering mechanism (Südhof, 1995). However, the nature and importance of these interactions continue to be disputed. Based on indirect data in cracked PC12 cells, it was proposed in one study that Ca^{2+}-induced SNARE binding is essential for exocytosis (Zhang et al., 2002). In contrast, a second study using a similar approach observed a better correlation of exocytosis with phospholipid binding, although SNARE binding was not excluded as a mechanism (Shin et al., 2002). At the synapse (where exocytosis is quite different from PC12 cells), the importance of phospholipid binding was supported by the fact that changes in the apparent Ca^{2+} affinity of synaptotagmin 1/phospholipid complexes correlate with release, but are not accompanied by equivalent changes in SNARE binding (Fernández-Chacón et al., 2001). However, no direct test of the importance of Ca^{2+}-induced SNARE binding to synaptotagmin 1 has been reported. We have now tried to address this question at the synapse under conditions that do not require overexpression or dominant-negative effects, but make use of the insights gained in electrophysiological studies on Sr^{2+} as a low-affinity Ca^{2+} agonist in neurotransmitter release (Miledi, 1966; Goda and Stevens, 1994; Rumpel and Behrends, 1999; Xu-Friedman and Regehr, 1999, 2000; Hagler and Goda, 2001). Our data show that Sr^{2+} binding to the synaptotagmin 1 C_2B domain is sufficient to trigger fast exocytosis. This action involves the formation of Sr^{2+}-induced complexes of synaptotagmin with phospholipids but not with SNAREs, demonstrating that synaptotagmin/SNARE complexes induced by divalent cations are not essential for triggering fast neurotransmitter release.

Results

Role of Synaptotagmin 1 in Synaptic Responses to Ca^{2+} or Sr^{2+}

We cultured hippocampal neurons from wild-type and synaptotagmin 1 knockout mice, and recorded glutamatergic synaptic responses in autaptic neurons. Neurons were stimulated by action potentials in the presence of 4 mM extracellular Ca^{2+} or Sr^{2+} (Figure 1A). As described previously (Goda and Stevens, 1994), in wild-type neurons, Sr^{2+} induced synaptic responses that exhibited longer time constants and lower peak amplitudes than Ca^{2+}-induced responses, although the total amount of release measured after 1 s was similar. Curve fitting revealed that Sr^{2+} triggered both components of release with slower time constants, and caused a relative decrease in the fast, and a relative increase in the slow component (Figure 1B; Table 1) (see also Goda and Stevens, 1994; Xu-Friedman and Regehr, 2000).

We then examined synaptotagmin 1-deficient synapses. Again as reported previously (Geppert et al., 1994, 1997), the fast component of Ca^{2+}-dependent synaptic responses was severely impaired in the absence of synaptotagmin 1 (3.8-fold slower), whereas the time constant of the slow asynchronous component was unaffected, although its relative amplitude was increased (from 16% to 50%; Figure 1 and Table 1). The total amount of vesicles released during the first 100 ms after an action potential was 233 $\pm$ 31 vesicles ($n = 30$) in wild-type and 150 $\pm$ 18 vesicles ($n = 31$; $p < 0.05$) in synaptotagmin 1-deficient neurons. The total number of release-ready vesicles between both groups as measured by stimulation with hypertonic sucrose, however, was similar (wild-type 4036 $\pm$ 367, $n = 30$; mutants 4513 $\pm$ 531, $n = 31$). In agreement with previous measurements (Geppert et al., 1994, 1997), these data suggest that synaptotagmin 1 acts predominately as a catalyst to accelerate release upon Ca^{2+} influx.

We next compared the responses of synaptotagmin 1-deficient and wild-type synapses to Sr^{2+}. Similar to Ca^{2+}-induced release, deletion of synaptotagmin 1 decreased the relative contribution of the fast component to the total observed release (from 44% to 23%), and additionally slowed the apparent time constant of the fast component in Sr^{2+}-induced release (Table 1). In contrast, the time constant of the slow component of Sr^{2+}-induced release was indistinguishable between wild-type and synaptotagmin 1-deficient synapses, as observed above for Ca^{2+}-induced release. Thus synaptotagmin 1-deficient synapses exhibit similarly selective impairments in the fast component of release in the presence of Ca^{2+} and Sr^{2+}, demonstrating that synaptotagmin 1 is essential for fast release mediated by both divalent cations.

Sr^{2+}-Dependent Phospholipid Binding to Synaptotagmin 1 C_2 Domains

Sr^{2+} does not support phospholipid or syntaxin binding by the C_2A domain of synaptotagmin 1, suggesting that Sr^{2+} cannot trigger release by mediating the interaction of the synaptotagmin 1 C_2A domain with phospholipid membranes or syntaxin 1 (Li et al., 1995a, 1995b; Sugita et al., 2002). Since recent data revealed that the C_2B domain of synaptotagmin 1 serves as a Ca^{2+}/phospholipid binding module equivalent to the C_2A domain (Fernandez et al., 2001) and is essential for release (Mackler et al., 2002), we tested the possibility that the C_2B domain may mediate a Sr^{2+}-dependent function of synaptotagmin 1.

We incubated liposomes composed of 25% phospha-

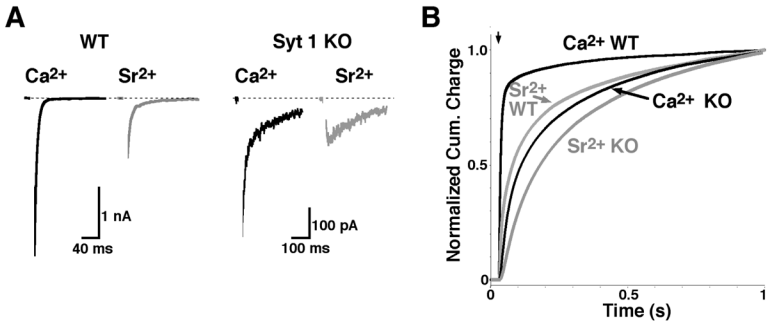

Figure 1. Ca^{2+}- and Sr^{2+}-Induced Synaptic Responses in Hippocampal Neurons from Wild-Type and Synaptotagmin 1-Deficient Mice

(A) Hippocampal neurons from wild-type and synaptotagmin 1 knockout mice were grown in microisland cultures in which isolated neurons form autapses. Excitatory postsynaptic currents (EPSCs) were recorded in response to action potential stimulation in the presence of 4 mM Ca^{2+} or Sr^{2+} and 4 mM Mg^{2+}. Note the difference in scale between wild-type and knockout responses.

(B) Time course of the normalized total release in response to stimulation by action potentials. EPSC charges from wild-type (Ca^{2+}, dark gray trace; Sr^{2+}, light gray trace; n = 7) and synaptotagmin 1 knockout neurons (Ca^{2+}, black trace; Sr^{2+}, gray trace; n = 36) were integrated and averaged over 1 s. The amplitudes, time constants, and fractions of synchronous and asynchronous release components analyzed by fitting the integral of individual traces to two exponentials are summarized in Table 1.

tidylserine and 75% phosphatidylcholine with purified GST fusion proteins containing the individual C_2A or C_2B domain or the double C_2A/B domain fragment of synaptotagmin 1 at different concentrations of Ca^{2+}, Sr^{2+}, or Ba^{2+}, isolated the liposomes by centrifugation, and analyzed bound proteins by SDS-PAGE and Coomassie blue staining (Figures 2A–2C). As described previously (Fernández-Chacón et al., 2002; Shin et al., 2002), Ca^{2+} stimulated binding of both separate C_2 domains at the same Ca^{2+} concentration (~10 μM), and of the double C_2 domain fragment at a lower Ca^{2+} concentration (~3 μM), indicative of cooperativity. In contrast, Sr^{2+} or Ba^{2+} induced only marginal binding of the isolated C_2A domain to liposomes even at high concentrations (>300 μM), but effectively stimulated binding of the isolated C_2B domain and the C_2A/B domain fragment at much lower concentrations (~10 μM). Although the relatively loose Sr^{2+}/ and Ba^{2+}/phospholipid complexes made a precise analysis of the binding constants difficult, the increase in apparent Ca^{2+} affinity observed in the double C_2A/B domain fragment compared to the isolated C_2A and C_2B domains (Figure 2A) was not detectable for the apparent Sr^{2+} and Ba^{2+} affinities (Figures 2B and 2C), suggesting that Sr^{2+} and Ba^{2+} binding to the double C_2A/B domain/phospholipid complex exhibits less cooperativity than Ca^{2+} binding. Furthermore, the binding of the double C_2A/B domain fragment of synaptotagmin 1 to phospholipids induced by Sr^{2+} and Ba^{2+} exhibited an increased sensitivity to NaCl, suggesting that it was not as tight as Ca^{2+}-induced binding, although the amount of lipids bound at saturation were equivalent (Figures 2D and 2E).

In spite of the apparent inability of the isolated C_2A domain to bind Sr^{2+} in phospholipid complexes, it is possible that in the context of the double C_2A/B domain fragment, the C_2 domain/phospholipid interaction nucleated by Sr^{2+} binding to the C_2B domain could allow Sr^{2+} to bind to the C_2A domain secondarily. To test this possibility, we exploited the observation that mutations in the Ca^{2+} binding site of the C_2A domain abolish Ca^{2+}-dependent phospholipid binding of the isolated C_2A domain, but have little effect on the apparent Ca^{2+} affinity of the double C_2A/B domain fragment (Fernández-Chacón et al., 2002) (note that this is different from mutations in R233 of the C_2A domain Ca^{2+} binding site which has an effect on both the isolated C_2A domain and the double C_2A/B domain fragment) (Fernández-Chacón et al., 2001). The contribution of C_2A domain Ca^{2+} binding to the double C_2 domain/phospholipid complex can be unmasked by raising the ionic strength of the buffer which causes the double C_2A/B domain with a mutation in the C_2A domain Ca^{2+} binding site to dissociate from the

Table 1. Parameters of Synaptic Responses Monitored in Ca^{2+}- or Sr^{2+}-Containing Media in Cultured Autaptic Hippocampal Neurons from Wild-Type and Synaptotagmin 1-Deficient Mice

	Synaptotagmin 1 Wild-Type		Synaptotagmin 1 Deficient	
	Ca^{2+}	Sr^{2+}	Ca^{2+}	Sr^{2+}
Relative amplitudes (as fraction of total)[a]				
Fast component	0.84 ± 0.03	0.49 ± 0.04	0.46 ± 0.03*	0.23 ± 0.03*
Slow component	0.16 ± 0.03	0.50 ± 0.05	0.53 ± 0.02*	0.76 ± 0.03*
Absolute amplitude (1 s integral), normalized to the response in Ca^{2+}	100%	90 ± 16%	100%	88 ± 16%
Time constants (in ms)[b]				
Fast component	7.7 ± 0.7	29 ± 2	30 ± 2*	58 ± 5*
Slow component	292 ± 67	353 ± 73	306 ± 11	337 ± 19

Data were derived from 7 wild-type and 36 synaptotagmin 1-deficient neurons. Asterisks (*) indicate statistical significance of <0.05 between wild type and knockout neurons. Note that the increase in the time constant for the fast component in the synaptotagmin 1 knockout, as determined here in a fit of to the total release to only two exponentials, may indicate that the fast component is composed of multiple subcomponents; only the fastest of which is impaired in the synaptotagmin 1 knockout cells.

[a] Expressed as the fraction of the total release measured over 1 s period following induction of an evoked response due to the two components fitted on the time/charge transfer relation.

[b] Measured by two exponential fits to individual response charge integrals as shown in Figure 1B.

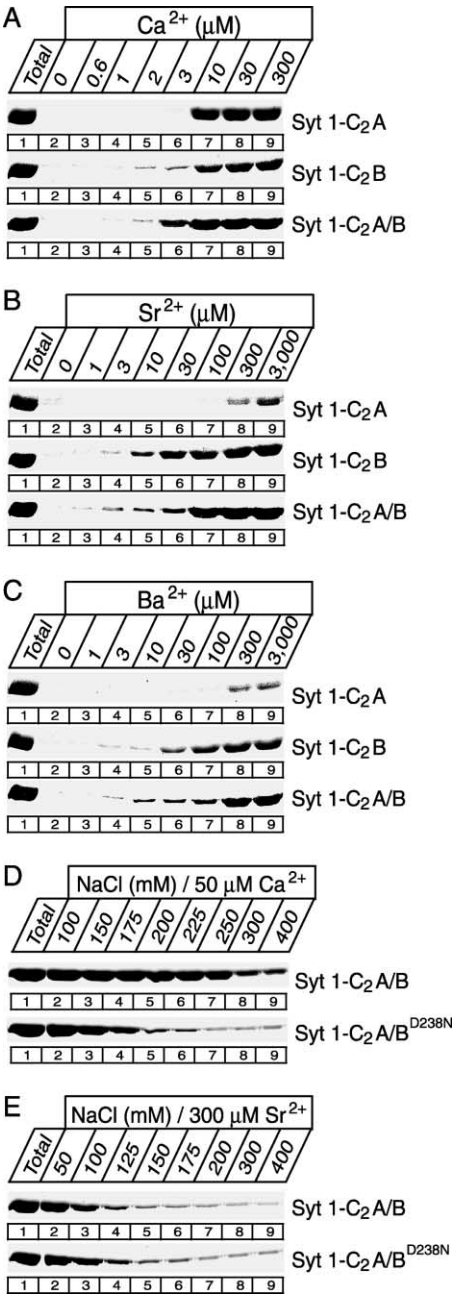

Figure 2. Ca^{2+}-, Sr^{2+}-, and Ba^{2+}-Dependent Binding of Synaptotagmin C_2 Domains to Phospholipids

(A–C) Liposomes composed of 25% phosphatidylserine and 75% phosphatidylcholine were incubated with purified C_2 domains in solutions containing Ca^{2+} (A), Sr^{2+} (B), and Ba^{2+} (C) buffered at the indicated free concentrations using EGTA buffers (see Experimental Procedures). Liposomes were pelleted by centrifugation, and bound proteins were examined by SDS-PAGE and Coomassie staining. The C_2A and C_2B domains and the double C_2A/B domain fragment of synaptotagmin 1 were employed as purified GST fusion proteins without the C_2B domain impurities (Ubach et al., 2001). Parallel experiments revealed that the isolated C_2A and C_2B domains of synaptotagmins 1, 3, and 7 exhibited the following apparent Ca^{2+} and Sr^{2+} affinities: synaptotagmin 1 C_2A = 10 μM Ca^{2+}/ >1 mM Sr^{2+}; C_2B = 10 μM Ca^{2+}/0.1 mM Sr^{2+}; synaptotagmin 3 C_2A = 2 μM Ca^{2+}/ >12 μM Sr^{2+}; C_2B = >10 mM Ca^{2+}/ >10 mM Sr^{2+}; and synaptotagmin 7 C_2A = 1 μM Ca^{2+}/15 μM Sr^{2+}; C_2B = 2 μM Ca^{2+}/50 μM Sr^{2+} (data not shown).

(D and E) Contribution of the C_2A domain to the Ca^{2+}- and Sr^{2+}-dependent complex of the double C_2A/B domain fragment with phospholipids analyzed with a point mutant in the C_2A domain Ca^{2+} binding site. The double C_2A/B domain fragment of synaptotagmin 1 was produced as wild-type and D238N mutant GST fusion protein and bound to liposomes in the presence of 50 μM Ca^{2+} or 300 μM Sr^{2+} and of increasing NaCl concentrations as indicated.

phospholipids earlier than the wild-type C_2A/B domain fragment (Figure 2D) (Fernández-Chacón et al., 2002). We therefore tested the effect of the Ca^{2+} binding site mutation in the C_2A domain on the stability of the Sr^{2+}/phospholipid complex with the double C_2A/B domain fragment (Figure 2E). In contrast to the Ca^{2+}/phospholipid complex, no effect of the C_2A domain mutation on the Sr^{2+}/phospholipid complex was detected. Together these results indicate that even in the double C_2A/B domain fragment, Sr^{2+} exclusively binds to the C_2B domain.

Interactions of Synaptotagmin 1 with SNARE Proteins

A leading hypothesis for how Ca^{2+} binding to synaptotagmin 1 triggers release is by inducing an interaction of synaptotagmin 1 with SNAREs. Synaptotagmin 1 binds to two SNARE proteins, syntaxin and SNAP-25, in the absence and/or presence of Ca^{2+} (Bennett et al., 1992; Li et al., 1995b; Chapman et al., 1995; Kee and Scheller, 1996; Schiavo et al., 1997; Gerona et al., 2000). The idea that Ca^{2+}-dependent binding of synaptotagmin 1 to SNAREs may trigger release is attractive but difficult to test because SNARE proteins and synaptotagmins are biochemically sticky. Dominant-negative experiments in PC12 cells are not sufficient because SNAREs have a central role in exocytosis independent of synaptotagmins, and because such experiments do not alter the properties of endogenous proteins. However, the discrete binding of Sr^{2+} and Ba^{2+} to the C_2B domain of synaptotagmin 1 and the activity of Sr^{2+} as a low-affinity Ca^{2+} agonist in release raise the possibility that Sr^{2+} and Ba^{2+} could be used as tools to test the role of SNARE interactions with synaptotagmin 1.

We first bound rat brain proteins to immobilized GST fusion proteins of synaptotagmin 1 C_2 domains in the presence of EGTA, 1 mM Ca^{2+}, 10 mM Sr^{2+}, or 10 mM Ba^{2+}, eluted bound proteins with EGTA, and analyzed by immunoblotting the eluted proteins and the proteins remaining on the beads (Figure 3) As described previously (Li et al., 1995b; Kee and Scheller, 1996; Gerona et al., 2000), Ca^{2+} induced binding of syntaxin 1 and SNAP-25 only to the isolated C_2A domain, but not to the isolated C_2B domain of synaptotagmin 1. We observed significant Ca^{2+}-independent binding of SNAP-25 to the C_2B domain, possibly because the C_2B domain partly unfolds in the absence of divalent cations. The double C_2A/B domain fragment strongly bound SNAP-25 and syntaxin 1 as a function of Ca^{2+} as reported previously (Li et al., 1995b; Earles et al., 2001). Strikingly, Sr^{2+} and Ba^{2+} were ineffective even at high concentrations in promoting the binding of SNAP-25 or syntaxin 1 to either the isolated C_2A domain or to the double C_2 domain fragment (Figure 3). Constitutive binding was observed

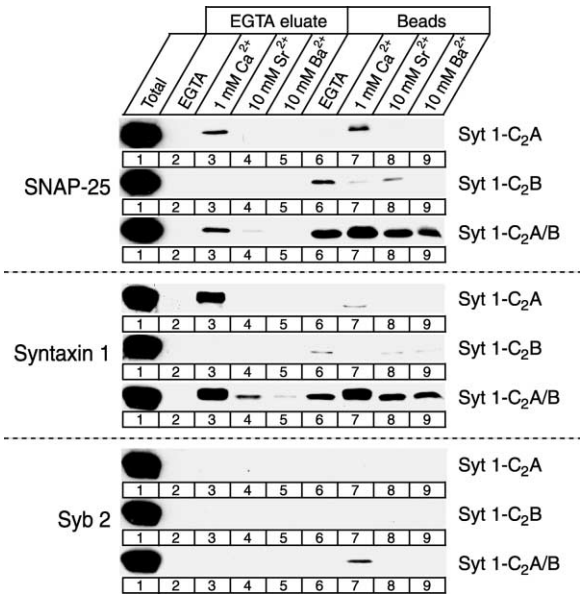

Figure 3. Ca^{2+}-, Sr^{2+}-, and Ba^{2+}-Dependent Binding of SNARE Proteins from Rat Brain Homogenates to Synaptotagmin 1 C_2 Domains

GST fusion proteins of the C_2A domain, the C_2B domain, and the double C_2A/B domain fragment from synaptotagmin 1 were immobilized on glutathione agarose and used for affinity chromatography of total rat brain proteins solubilized in Triton X-100. Proteins were bound to the beads in the presence of EGTA, 1 mM Ca^{2+}, 10 mM Sr^{2+}, or 10 mM Ba^{2+}. After extensive washing, bound proteins were eluted with EGTA, and proteins in the eluates and remaining on the beads after EGTA elution were analyzed by immunoblotting for the synaptic SNARE proteins syntaxin 1, SNAP-25, and synaptobrevin/VAMP 2 (Syb 2) as indicated.

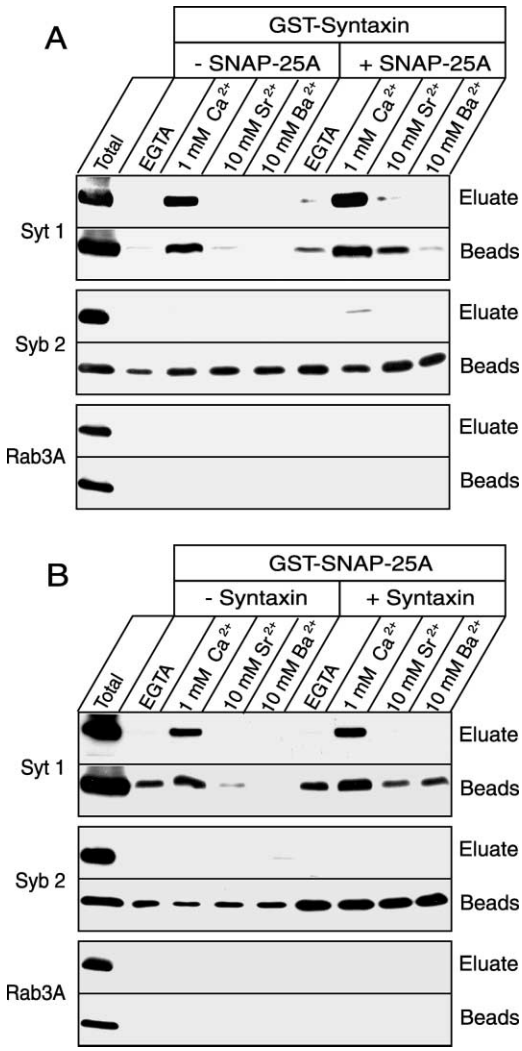

Figure 4. Ca^{2+}-, Sr^{2+}-, and Ba^{2+}-Dependent Binding of Brain Synaptotagmin 1 to Immobilized SNARE Complexes

GST fusion protein of syntaxin 1 alone or complexed with recombinant SNAP-25 or GST fusion protein of SNAP-25 alone or complexed with recombinant syntaxin 1 were immobilized on glutathione agarose and used for affinity chromatography experiments as described in Figure 3. EGTA eluates and proteins remaining on the beads after EGTA elutions were studied by immunoblotting for synaptotagmin 1 (Syt 1), synaptobrevin 2 (Syb 2), and Rab3A (as a negative control).

to the beads in the case of the C_2A/B domain fragment, suggesting that the double C_2A/B domain fragment may bind constitutively to SNAREs and that Ca^{2+} induces additional binding of SNAREs, but that Sr^{2+} and Ba^{2+} are unable to trigger further binding of synaptotagmin 1 to SNAREs even at high concentrations.

To confirm these results, we used immobilized GST-syntaxin 1 either alone or in a complex with recombinant SNAP-25 (Figure 4) for affinity chromatography of rat brain proteins in the absence or presence of 1 mM Ca^{2+}, 10 mM Sr^{2+}, or 10 mM Ba^{2+}. Bound proteins were again eluted with EGTA to uncover divalent cation-dependent interactions, and proteins in the EGTA eluates and on the beads after EGTA elution were analyzed by immunoblotting. Application of brain extracts to GST-syntaxin alone or to the GST-syntaxin/SNAP-25 complex led to the formation of trimeric core complexes, as synaptobrevin 2 was efficiently captured in the presence or absence of Ca^{2+} (Figure 4A). We detected Ca^{2+}-dependent and Ca^{2+}-independent binding of native synaptotagmin 1 to SNARE complexes (Figure 4A) as described previously (Chapman et al., 1995; Li et al., 1995b; Earles et al., 2001; Kee and Scheller, 1996). Ca^{2+}-dependent binding was not completely reversible since the amount of synaptotagmin 1 remaining on the beads after EGTA elution was higher when the beads were first reacted with brain extracts in the presence of Ca^{2+} than in the presence of EGTA. Sr^{2+} or Ba^{2+}, however, did not increase the amount of synaptotagmin 1 either in the EGTA eluates or on the remaining beads compared to EGTA alone, suggesting that Sr^{2+} and Ba^{2+} cannot stimulate binding of native synaptotagmin 1 to GST-syntaxin or to the syntaxin/SNAP-25 complex (Figure 4A).

To exclude artifacts due to a particular design of the affinity matrix, we also tested GST-SNAP-25 alone or in a complex with recombinant syntaxin 1. We obtained essentially the same results, indicating that Ca^{2+} effectively and selectively promoted interactions with synaptotagmin , whereas Sr^{2+} and Ba^{2+} did not (Figure 4B). Again synaptobrevin 2 was efficiently captured at all conditions, suggesting that full SNARE complexes were being formed. With GST-SNAP-25 as the immobilizing anchor, we detected more constitutive binding of native synaptotagmin 1 to SNAREs in the presence of EGTA,

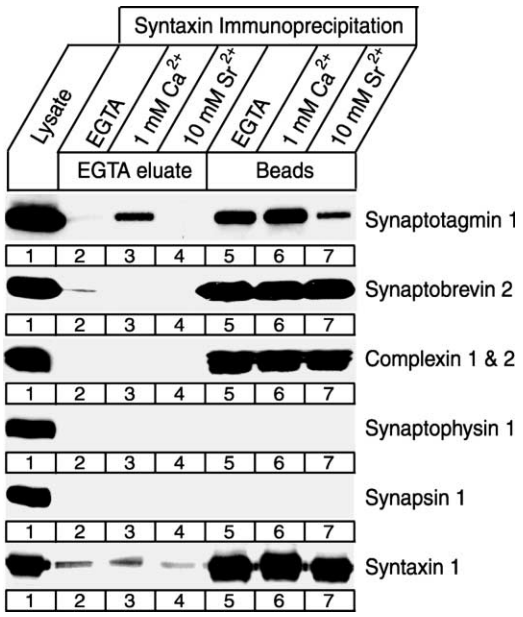

Figure 5. Effect of Ca^{2+} or Sr^{2+} on Synaptotagmin 1 Coimmunoprecipitation with Syntaxin

Total rat brain proteins were solublized in Triton X-100, and immunoprecipitations were performed in the presence of EGTA, 1 mM Ca^{2+}, or 10 mM Sr^{2+} using a monoclonal antibody to syntaxin 1 (HPC-1) immobilized on protein G-Sepharose. EGTA eluates and proteins remaining on the beads after EGTA elutions were studied by immunoblotting with antibodies to the proteins indicated on the right.

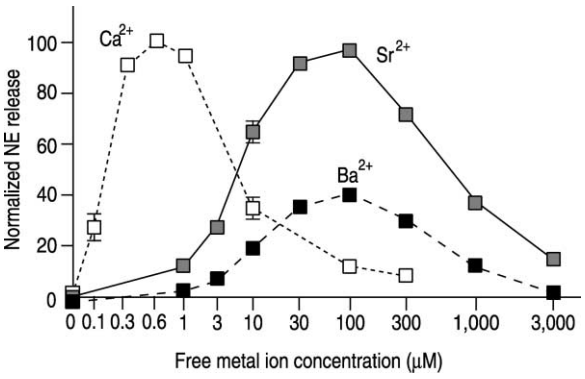

Figure 6. Ca^{2+}, Sr^{2+}, and Ba^{2+} Dependence of Exocytosis in Permeabilized PC12 Cells

PC12 cells were loaded with ^{3}H-labeled norepinephrine, cracked, and release induced over 30 min by Ca^{2+}, Sr^{2+}, and Ba^{2+} at the indicated concentrations was determined. All release was normalized to the maximum observed with Ca^{2+}.

but found no increase in synaptotagmin 1 binding to SNAREs above this background by either Sr^{2+} or Ba^{2+} (Figure 4B). In all of these experiments, Rab3A was examined as a negative control for the specificity of binding.

Native Synaptotagmin/SNARE Complexes in Brain

The GST pulldown experiments provide insight into the binding of native SNAREs to recombinant synaptotagmin 1 C_2 domains, or conversely of native synaptotagmin 1 to recombinant SNAREs, but in both experiments at least one of the participating membrane proteins misses its transmembrane region which may be important for binding (Chapman et al., 1995). To test whether Ca^{2+}-dependent synaptotagmin/SNARE complexes exist in brain, we immunoprecipitated SNARE complexes from solubilized brain proteins in the presence of EGTA, 1 mM Ca^{2+}, or 10 mM Sr^{2+} using antibodies to syntaxin 1. Proteins that were bound to the immunoprecipitates as a function of Ca^{2+} or Sr^{2+} were eluted with EGTA, and the eluted proteins and remaining immunoprecipitates were analyzed by immunoblotting. As shown in Figure 5, synaptobrevin and complexins were quantitatively coimmunoprecipitated with syntaxin 1 under all conditions (SNAP-25 could not be analyzed because its size is similar to that of the immunoglobulin light chain), suggesting that a complete SNARE complex was immunoprecipitated because complexins only bind to fully assembled SNARE complexes (McMahon et al., 1995; Chen et al., 2002). Synapsin 1 and synaptophysin 1 were excluded, demonstrating specificity of the immunoprecipitations (Figure 5). Synaptotagmin 1 was coimmunoprecipitated with the SNARE complex in the presence and absence of Ca^{2+}, and an additional significant amount of synaptotagmin 1 was attached to the immunoprecipitated SNARE complex in a Ca^{2+}-dependent manner. Thus, at least in detergent-solubilized brain extracts, SNARE/synaptotagmin 1 complexes are constitutively present, and Ca^{2+} induces additional synaptotagmin 1 binding to SNARE complexes consistent with the GST pulldowns (Figures 3 and 4). In the presence of Sr^{2+}, however, no EGTA-elutable synaptotagmin 1 was associated with the SNARE complexes, and the constitutive association of synaptotagmin 1 to SNARE complexes was decreased (Figure 5). These data independently confirm the conclusions from the GST pulldowns that different from Ca^{2+}, Sr^{2+} is unable to induce binding of synaptotagmin 1 to SNARE complexes.

Role of Synaptotagmin 1 Binding to SNAREs in PC12 Cell Exocytosis

Synaptic and large dense-core vesicle exocytosis share many characteristics (for example, in both types of exocytosis the fast component depends on synaptotagmin 1) (Geppert et al., 1994; Voets et al., 2001), but differ fundamentally in the spatial organization of release sites. It is thus possible that a synaptotagmin 1/SNARE interaction may be important for large dense-core vesicle exocytosis as suggested (Zhang et al., 2002), even if it is not essential for synaptic vesicle exocytosis. To test this possibility, we examined the role of Sr^{2+} and Ba^{2+} in PC12 cell exocytosis.

Stimulation of permeabilized PC12 cells, preloaded with labeled norepinephrine, by addition of Sr^{2+} and Ba^{2+} revealed that both divalent cations triggered exocytosis much less efficiently than Ca^{2+} (Figure 6). At high concentrations, Sr^{2+} was as active as Ca^{2+}, whereas Ba^{2+} never elicited the same amount of exocytosis as Ca^{2+}, consistent with electrophysiological studies at a much better temporal resolution (Kishimoto et al., 2001). Thus, similar to synaptic vesicle exocytosis, the exocy-

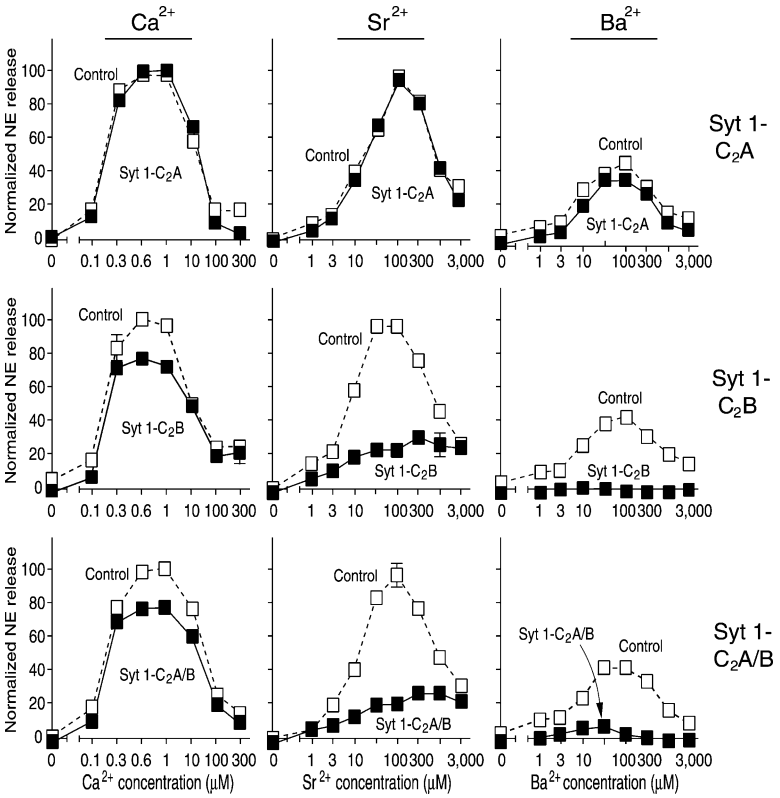

Figure 7. Effect of Synaptotagmin 1 C_2 Domains on Ca^{2+}-, Sr^{2+}-, and Ba^{2+}-Dependent Exocytosis in Cracked PC12 Cells

Cracked PC12 cells, prelabeled with ^{3}H-norepinephrine, were preincubated with purified GST fusion proteins (6 μM) of the C_2A or C_2B domain or the double C_2A/B domain fragment of synaptotagmin 1. Norepinephrine release was triggered by the addition of Ca^{2+}, Sr^{2+}, or Ba^{2+} at the indicated concentrations. Each series of experiments included a control in which the effect of the same concentration of GST alone was assayed ("control").

totic Ca^{2+} sensors in large dense-core vesicle exocytosis appear to bind Sr^{2+} and Ba^{2+} only at higher concentrations. These concentrations correlate well with the apparent Sr^{2+} and Ba^{2+} affinities of the C_2 domain/phospholipid complexes of synaptotagmin 1 and 7 (Figure 2).

We next tested the ability of synaptotagmin 1 C_2 domains to inhibit Sr^{2+}- or Ba^{2+}-induced exocytosis in PC12 cells (Figure 7). Previous studies demonstrated that synaptotagmin C_2 domains can serve as dominant-negative inhibitors of Ca^{2+}-dependent exocytosis in cracked PC12 cells if their apparent Ca^{2+} affinity in phospholipid complexes corresponds to that of exocytosis (Sugita et al., 2001; Shin et al., 2002). Thus synaptotagmin 1 C_2 domains have only small effects in cracked PC12 cells because their relative Ca^{2+} affinities are too low, whereas synaptotagmin 7 C_2 domains completely inhibit exocytosis because their Ca^{2+} affinities are high (Sugita et al., 2002). We now find that different from Ca^{2+}-induced exocytosis, Sr^{2+}- and Ba^{2+}-evoked exocytosis was inhibited by the C_2B domain, but not the C_2A domain, from synaptotagmin 1 (Figure 7). This result is consistent with the relatively high Sr^{2+} and Ba^{2+} affinity that we observed for the C_2B domain, but not the C_2A domain, of synaptotagmin 1 (Figure 2). Notably, the Sr^{2+} and Ba^{2+} concentrations that mediate exocytosis correspond to the apparent affinities of the respective C_2 domain/phospholipid complexes, but do not stimulate SNARE binding (see Figures 3 and 4), effectively ruling out Sr^{2+}- or Ba^{2+}-induced SNARE binding to synaptotagmin 1 as a mechanism of triggering exocytosis in PC12 cells.

Discussion

In a long-standing tradition, Sr^{2+} has been used as a tool to examine neurotransmitter release (Miledi, 1966; Dodge et al., 1969; Goda and Stevens, 1994; Xu-Friedman and Regehr, 2000). These studies revealed that Sr^{2+}, when applied to the extracellular medium, supports neurotransmitter release similar to Ca^{2+}, but that Sr^{2+}-induced release differs from Ca^{2+}-induced release in two important properties. First, Sr^{2+} stimulates release less effectively than Ca^{2+}, as documented by a decrease in Sr^{2+}-induced synaptic amplitudes (Goda and Stevens, 1994; Xu-Friedman and Regehr, 2000). Second, Sr^{2+} buffering and clearance are less effective than Ca^{2+} buffering and clearance, resulting in increased residual Sr^{2+} concentrations and a potentiation of delayed release (Rumpel and Behrends, 1999; Xu-Friedman and Regehr, 1999, 2000). This implies that the Sr^{2+} binds to the Ca^{2+} sensor which triggers the fast component of release, but activates it less efficiently than Ca^{2+}. Therefore the Ca^{2+} sensor for fast release must either have a lower apparent affinity for Sr^{2+} than for Ca^{2+}, and/or Sr^{2+} binding must be an inefficient activator of the Ca^{2+} sensor. We have used these predictions as the starting point to test whether synaptotagmin 1, the primary candidate for the exocytotic Ca^{2+}-dependent catalyst for fast neurotransmitter release (Perin et al., 1990; Geppert et al., 1994; Fernández-Chacón et al., 2001), is involved in the Sr^{2+} response, and have employed the differential properties of Ca^{2+} versus Sr^{2+} binding to synaptotagmin 1 to probe the mechanism of action of synaptotagmin 1. Our data show that Sr^{2+} binding to the C_2B domain of

synaptotagmin 1 is sufficient to trigger exocytosis, and that this triggering reaction does not require a Sr^{2+}-induced complex of the synaptotagmin C_2 domains with SNAREs, while phospholipid binding appears to be essential. This conclusion, which rules out an essential role for the Ca^{2+}-dependent synaptotagmin 1/SNARE complex in stimulating fast release, but not in other stages of exocytosis (e.g., vesicle recruitment), is based on the following evidence.

First, we showed that synaptotagmin 1 is required for the fast component of Sr^{2+}-induced synaptic responses in hippocampal neurons. Comparison of Sr^{2+}-induced release in wild-type and synaptotagmin 1 knockout neurons documented that the Sr^{2+} response is impaired in the knockouts, and thus synaptotagmin 1 is required (Figure 1; Table 1).

Second, we demonstrated that Sr^{2+} and Ba^{2+} act as low-affinity Ca^{2+} agonists for the C_2B domain and the double C_2A/B domain fragment of synaptotagmin 1, but do not activate the C_2A domain (Figure 2). The apparent Sr^{2+} and Ba^{2+} affinities of synaptotagmin 1 are significantly lower than the apparent Ca^{2+} affinity, consistent with the relative efficacies of these divalent cations in release (Miledi, 1966; Silinsky, 1978; Goda and Stevens, 1994; Xu-Friedman and Regehr, 2000). The Sr^{2+}-dependent complex of the double C_2A/B domain fragment with phospholipids is not as tight as the corresponding Ca^{2+}-dependent complex and does not involve secondary Sr^{2+} binding to the C_2A domain after the C_2B domain has been bound, suggesting that Sr^{2+} exclusively binds to the C_2B domain even in the double C_2 domain fragment (Figures 2D and 2E).

Third, although we confirm that Ca^{2+} induced binding of synaptotagmin 1 to SNARE complexes, we show that Sr^{2+} and Ba^{2+} do not stimulate SNARE binding by synaptotagmin 1 even at high concentrations. Binding was tested with native and recombinant synaptotagmin 1, SNAREs, and SNARE complexes to control for possible contributions of posttranslational modifications and/or transmembrane regions. Our data effectively rule out a selective Sr^{2+}- or Ba^{2+}-induced interaction of synaptotagmin 1 only with SNARE complexes, as has been described for complexins (McMahon et al., 1995), and confirm that native synaptotagmin 1 and SNAREs form Ca^{2+}-independent constitutive complexes as best revealed in the immunoprecipitations (Figure 5).

Viewed together, these results suggest that Sr^{2+} acts as an agonist for exocytosis at the Ca^{2+} binding sites of synaptotagmin 1 in vitro and in vivo by a mechanism that does not involve SNARE binding, and that Sr^{2+} binding to the C_2B domain is sufficient for triggering fast exocytosis. Similar biochemical properties were observed for Ba^{2+}, which also functions as a partial agonist in release (Miledi, 1966; Silinsky, 1978). These experiments complement the results of Mackler et al. (2002) who showed that in *Drosophila*, mutations in the Ca^{2+} binding sites of the C_2B domain of synaptotagmin 1 abolish exocytosis. The fact that Sr^{2+} only binds to the C_2B, but not the C_2A, domain agrees well with the observation that the apparent cooperativity of Sr^{2+}-triggered release is lower than that of Ca^{2+}-triggered release (Xu-Friedman and Regehr, 2000). In spite of the similar architecture of Ca^{2+} binding sites revealed in C_2 domain structures with and without bound Ca^{2+} ions (reviewed in Rizo and Südhof, 1998), we still cannot predict the properties of C_2 domains. Among others, our measurements reveal that the Ca^{2+} and Sr^{2+} binding properties of synaptotagmin C_2 domains vary independent of each other and do not correlate (Figure 2). This enhances the usefulness of Sr^{2+} and Ba^{2+} as tools in differentiating between different candidate Ca^{2+} sensors in exocytosis, and emphasizes the need for direct measurements of individual C_2 domains and double C_2A/B domain fragments in order to make conclusions about their behavior.

Our results do not address the possibility that Ca^{2+}-dependent interactions of synaptotagmin 1 with SNAREs have important roles in other stages of the vesicle cycle, or that Ca^{2+}-independent interactions of synaptotagmin 1 with SNAREs play a major role. Furthermore, although our data show that activation of the C_2B domain of synaptotagmin 1 is sufficient for inducing exocytosis, this result does not test or exclude an important role of Ca^{2+} binding to the C_2A domain in regulating release. Previous studies using Ca^{2+} binding site mutants in the C_2A domain of synaptotagmin 1 introduced by homologous recombination into mice (Fernández-Chacón et al., 2002) and by transgenic rescue into *Drosophila* (Robinson et al., 2002) demonstrated that full Ca^{2+} binding to the C_2A domain is not required for exocytosis. However, it is unclear if these studies mutated all of the Ca^{2+} binding sites, and Ca^{2+} binding may still operate in the double C_2 domain fragments containing the C_2A-domain mutations. Furthermore, although the *Drosophila* study concluded that the mutation in the Ca^{2+} binding site had an effect on the double C_2 domain fragment (Robinson et al., 2002), the effect of the mutation was measured by a resin-based assay that does not adequately monitor the activity of the C_2B domain (Fernandez et al., 2001). Thus it remains a key question whether Ca^{2+} binding to the C_2A domain contributes to the regulatory properties of synaptotagmin 1, or whether only Ca^{2+}-independent interactions by the C_2A domain (for example, interactions mediated by R233 as revealed in the R233Q mutant mouse [Fernández-Chacón et al., 2001]) are important in modulating Ca^{2+} binding by the C_2B domain.

Finally, in an attempt to reconcile our results—which apply primarily to synapses—with previous studies postulating an essential role for Ca^{2+}-induced SNARE binding in neuroendocrine PC12 cells (Zhang et al., 2002), we examined the action of Sr^{2+} and Ba^{2+} in permeabilized PC12 cells. Our data show that Sr^{2+} can serve as a fully active low-affinity Ca^{2+} agonist for exocytosis in PC12 cells, and Ba^{2+} as a partially active agonist (Figure 6), consistent with electrophysiological studies (Kishimoto et al., 2001). Furthermore, we found that Sr^{2+} and Ba^{2+} action in PC12 cells (different from Ca^{2+} action under the same conditions which measure primarily slow release [see Shoji-Kasai et al., 1992]) may involve synaptotagmin 1 (Figure 7). However, again at the Sr^{2+} and Ba^{2+} concentrations used, no interaction of synaptotagmin 1 with SNAREs was observed, effectively ruling out an essential role for such interactions in Ca^{2+}-triggered PC12 cell exocytosis.

Experimental Procedures

Neuronal Culture and Electrophysiology

The preparation of primary autaptic cultures from newborn wild-type or synaptotagmin 1 knockout mice and electrophysiological

measurements from excitatory neurons were performed at 25°C as described (Pyott and Rosenmund, 2002; Rosenmund et al., 2002). The number of vesicles released by an action potential or included in the readily releasable vesicle pool was calculated by dividing the charge of the evoked EPSC or the transient component in the synaptic response to 500 mOsm sucrose, respectively, by the average mEPSC charge (wild-type: 109 fC, n = 26; synaptotagmin 1 knockout: 113 fC, n = 28). Measurements are presented as means ± SEMs. Statistical significance between two groups was estimated using the paired and unpaired nonparametric Student's t test.

Expression and Purification of Recombinant Proteins

All recombinant proteins were produced as bacterial GST fusion proteins in pGEX-KG and purified essentially as described (Guan and Dixon, 1991; Sugita et al., 2001). C_2B domain proteins were additionally treated to remove the bacterial contaminants that stick to these domains (Ubach et al., 2001).

Centrifugation Phospholipid Binding Assays

Centrifugation phospholipid binding assays were carried out with purified soluble GST fusion proteins in buffer A (50 mM HEPES-NaOH, pH 6.8, 0.1 M NaCl, 4 mM Na_2EGTA) using a centrifugation assay in which the C_2 domain proteins are bound in solution to heavy liposomes at different concentrations of free Ca^{2+} essentially as described (Fernandez et al., 2001; Shin et al., 2002). After binding, liposomes are reisolated by centrifugation through a sucrose cushion, and bound C_2 domain proteins are precipitated, resuspended in 30 μl of 2×SDS sample buffer, and analyzed by SDS-PAGE and Coomassie blue staining.

GST Pulldowns

Unstripped rat brains (~1.5 g/brain; Pel-Freez Biologicals, Rogers, AR) were homogenized with a tissue homogenizer (Thomas Scientific, Philadelphia, PA) in buffer A (10 ml/brain) containing protease inhibitor cocktail (Roche, Indianapolis, IN) and 1 mM DTT. 1% Triton X-100 was added, proteins were extracted for 1 hr at 4°C with rocking, insoluble proteins were removed by centrifugation (100,000 × g for 1 hr), and the supernatant brain lysate was used for all experiments. Pulldown reactions contained 250 μl brain lysate, ~30 μg GST fusion proteins attached to 30 μl of glutathion agarose beads, and 250 μl of 2 × 1 mM Ca^{2+}/EGTA, 10 mM Sr^{2+}/EGTA, or 10 mM Ba^{2+}/EGTA buffers (all in buffer A). Binding reactions were incubated at 4°C for 1 hr with rocking, and beads were washed six times with 1 ml of the corresponding buffers containing 0.5% Triton X-100. Bound proteins were eluted three times by 100 μl of buffer A containing 20 mM EGTA and 0.5% Triton X-100, precipitated, and resuspended in 50 μl SDS sample buffer, and aliquots (30 μl) were analyzed by SDS-PAGE and immunoblotting. Beads were further washed five times using 1 ml buffer A containing 20 mM EGTA and 0.5% Triton X-100, 50 μl SDS sample buffer was added, and aliquots (30 μl) were analyzed by SDS-PAGE and immunoblotting.

Syntaxin Immunoprecipitations

Syntaxin immunoprecipitations were performed with monoclonal syntaxin 1 antibody HPC-1 (15 μl ascites) attached to protein G-Sepharose (10 μl; Pharmacia) and 500 μl brain lysate preparared as described above in 1 ml volume of buffer A with the indicated additions of 1 mM Ca^{2+}/EGTA or 10 mM Sr^{2+}/EGTA buffers. The binding reactions were incubated at 4°C for 2 hr with rocking, Sepharose beads were washed six times with 1 ml of the corresponding buffers containing 0.5% Triton X-100, and bound proteins were eluted two times with 0.1 ml buffer A containing 20 mM EGTA and 0.5% Triton X-100. TCA precipitated proteins in the eluate and proteins bound to the beads were resuspended in 50 μl SDS sample buffer, and 30 μl aliquots were analyzed by SDS-PAGE and immunoblotting.

Cracked PC12 Cell Secretion Assays

Cracked PC12 cell secretion assays were carried out essentially as described previously (Sugita et al., 2001; Shin et al., 2002). Free concentrations of Ca^{2+}, Sr^{2+}, or Ba^{2+} were calculated by EqCal (Biosoft) (Sugita et al., 2002).

Miscellaneous Procedure

SDS-PAGE and immunoblotting were performed using standard procedure (Laemmli, 1970; Johnston et al., 1989). Immunoblots were developed by enhanced chemiluminescence (Amersham).

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