An unusual C₂-domain in the active-zone protein piccolo: implications for Ca²⁺ regulation of neurotransmitter release

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Ca²⁺ regulation of neurotransmitter release is thought to require multiple Ca²⁺ sensors with distinct affinities. However, no low-affinity Ca²⁺ sensor has been identified at the synapse. We now show that piccolo/aczonin, a recently described active-zone protein with C-terminal C₂A- and C₂B-domains, constitutes a pre-synaptic low-affinity Ca²⁺ sensor. Ca²⁺ binds to piccolo by virtue of its C₂A-domain via an unusual mechanism that involves a large conformational change. The distinct Ca²⁺-binding properties of the piccolo C₂A-domain are mediated by an evolutionarily conserved sequence at the bottom of the C₂A-domain, which may fold back towards the Ca²⁺-binding sites on the top. Point mutations in this bottom sequence inactivate it, transforming low-affinity Ca²⁺ binding (100–200 µM in the presence of phospholipids) into high-affinity Ca²⁺ binding (12–14 µM). The unusual Ca²⁺-binding mode of the piccolo C₂A-domain reveals that C₂-domains are mechanistically more versatile than previously envisaged. The low Ca²⁺ affinity of the piccolo C₂A-domain suggests that piccolo could function in short-term synaptic plasticity when Ca²⁺ concentrations accumulate during repetitive stimulation.

Keywords: C₂-domain/Ca²⁺-binding protein/exocytosis/neurotransmitter release/synaptic plasticity

Introduction

Synaptic transmission constitutes the major mechanism by which neurons communicate. When an action potential depolarizes a presynaptic nerve terminal, Ca²⁺ influx triggers neurotransmitter release, leading to a postsynaptic signal (Katz, 1969). Ca²⁺ probably stimulates release by binding to Ca²⁺ sensors of moderately high affinity (~10 µM at the calyx synapse; Bollmann *et al.*, 2000; Schneggenburger and Neher, 2000). Ca²⁺ stimulates two components of release: a fast synchronous component that accounts for the majority of release, and a slow asynchronous component that accounts for only a small percentage (Geppert *et al.*, 1994; Goda and Stevens, 1994; Atluri and Regehr, 1998). Both components exhibit a high degree of Ca²⁺ cooperativity (Dodge and Rahamimoff, 1967; Goda and Stevens, 1994). In addition to acutely triggering release, Ca²⁺ regulates release during

short- and long-term presynaptic plasticity (Katz and Miledi, 1968; Zucker, 1989; Dobrunz *et al.*, 1997; Dittman *et al.*, 2000). Short-term synaptic plasticity is at least partly induced by accumulation of residual Ca²⁺ after repeated stimulations of a synapse (Zucker, 1989; Delaney and Tank, 1994; Kamiya and Zucker, 1994). Short-term synaptic plasticity shapes the properties of neuronal networks, and is of major importance for understanding how the brain processes information (Dobrunz and Stevens, 1999; Gil *et al.*, 1999). Modeling of the synaptic responses during repetitive stimulation revealed that besides the Ca²⁺ sensors that trigger release, at least two additional Ca²⁺ sensors have to be invoked to explain the observed dynamics of short-term synaptic plasticity (Dittman *et al.*, 2000).

The various actions of Ca²⁺ at the synapse are probably mediated by separate Ca²⁺ sensors with distinct regulatory functions. Several Ca²⁺-binding proteins have been identified that could potentially serve as synaptic Ca²⁺ sensors, e.g. synaptotagmins I–XIII, rabphilin, Doc2s, synapsins and calmodulin. Interestingly, most candidate Ca²⁺ sensors at the synapse bind Ca²⁺ via a widespread Ca²⁺-binding module, the C₂-domain (Brose *et al.*, 1992; Davletov and Südhof, 1993; Shirataki *et al.*, 1993; Li *et al.*, 1994, 1995; Südhof and Rizo, 1996). C₂-domains are often found in membrane trafficking and signal transduction proteins where they mediate the Ca²⁺-dependent recruitment of proteins to phospholipid membranes (reviewed in Nalefski and Falke, 1996; Rizo and Südhof, 1998). All C₂-domains are composed of a stable β-sandwich with flexible loops on top and at the bottom. Most (but not all) C₂-domains bind Ca²⁺. Usually 2–3 Ca²⁺ ions are coordinated at closely spaced sites, which are formed by the flexible top loops (Sutton *et al.*, 1995; Shao *et al.*, 1996; Essen *et al.*, 1997; Perisic *et al.*, 1998; Rizo and Südhof, 1998; Sutton and Sprang, 1998; Ubach *et al.*, 1998, 1999). Intrinsic Ca²⁺ binding tends to be non-cooperative and of low affinity, probably because the bound Ca²⁺ ions contain empty coordination sites. For example, in the synaptotagmin I C₂A-domain, the intrinsic Ca²⁺-binding affinities for the first, second and third Ca²⁺ ions are ~50 µM, ~500 µM and >10 mM, respectively (Ubach *et al.*, 1998; Fernández-Chacón *et al.*, 2001). The majority of C₂-domains that bind to Ca²⁺ also bind to phospholipid membranes and form a Ca²⁺–C₂-domain–phospholipid complex (Davletov and Südhof, 1993; Shao *et al.*, 1996; Uellner *et al.*, 1997; Lomasney *et al.*, 1999; Perisic *et al.*, 1999). Strikingly, phospholipid membranes increase the overall Ca²⁺ affinity of all C₂-domains tested (2–20 µM), and convert non-cooperative Ca²⁺ binding into cooperative Ca²⁺ binding (Davletov and Südhof, 1993; Li *et al.*, 1995; Shao *et al.*, 1996; Hixon *et al.*, 1998; Nalefski *et al.*, 1998; Perisic *et al.*, 1998). Phospholipids probably achieve this effect by providing additional coordination sites for the

incompletely ligated Ca^{2+} ions at the top of the C_2 -domains (Zhang *et al.*, 1998; Verdaguer *et al.*, 1999). The Ca^{2+} affinity of C_2 -domains in the presence of phospholipids is thought to be their most physiologically relevant Ca^{2+} affinity because their intrinsic Ca^{2+} affinity is too low to be involved in intracellular signaling. This conclusion is supported by the fact that Ca^{2+} -dependent phospholipid binding is the normal function for C_2 -domains, at least in signal transduction proteins such as protein kinase C (PKC) and phospholipases.

Functionally, the best characterized putative synaptic Ca^{2+} sensor is the synaptic vesicle protein synaptotagmin I (Perin *et al.*, 1990; Geppert *et al.*, 1991; Brose *et al.*, 1992; reviewed in Südhof and Rizo, 1996). Using knockout mice, synaptotagmin I was shown to be essential for fast Ca^{2+} -triggered release in the hippocampus, but not for release stimulated by Ca^{2+} -independent agonists such as α -latrotoxin and hypertonic sucrose (Geppert *et al.*, 1994). In knockin mice containing a point mutation in the C_2A -domain of synaptotagmin I, the overall Ca^{2+} affinity of synaptotagmin I correlated with the apparent Ca^{2+} affinity of transmitter release (Fernández-Chacón *et al.*, 2001), suggesting that synaptotagmin I is a major Ca^{2+} sensor for fast transmitter release. However, all of the currently characterized synaptic Ca^{2+} -binding proteins, including synaptotagmin I, have relatively high Ca^{2+} affinities ($<20 \mu\text{M}$). Although many synaptic proteins could potentially serve as Ca^{2+} sensors (e.g. >10 synaptotagmins, calmodulin, rabphilin, synapsins), no synaptic Ca^{2+} -binding protein has yet been identified that has a lower Ca^{2+} affinity consistent with selective activation by the increased levels of residual Ca^{2+} that accumulate during repetitive stimulation. In the current study, we have examined the Ca^{2+} -binding properties of a recently discovered active-zone protein called aczonin (Wang *et al.*, 1999) or piccolo (Fenster *et al.*, 2000). Our studies reveal that by virtue of its C-terminal C_2A -domain, piccolo/aczonin exhibits unusual Ca^{2+} -binding properties that differ from those of any other C_2 -domain containing synaptic Ca^{2+} sensors. These results uncover a novel mechanism of Ca^{2+} binding by a C_2 -domain in which Ca^{2+} binds cooperatively to the C_2 -domain and induces a conformational change in the entire domain. The relatively low apparent Ca^{2+} affinity of piccolo even in the presence of phospholipids is consistent with a potential role as a Ca^{2+} sensor in short-term synaptic plasticity when high Ca^{2+} concentrations accumulate during repetitive stimulation.

Results

Structure of the C_2 -domains of piccolo

Databank searches identified an anonymous human cDNA sequence (KIAA0559; DDBJ/EMBL/GenBank accession No. AB011131) that exhibits a high degree of homology to the presynaptic active-zone protein *bassoon* (tom Diek *et al.*, 1998). The homology extends over the entire KIAA0559 coding region except for the C-terminus, where KIAA0559 contains PDZ- and C_2 -domains that are absent from *bassoon*. Indeed, recently published studies of the mouse, chicken and rat homologs of KIAA0559, named *aczonin* (Wang *et al.*, 1999) or *piccolo* (Fenster *et al.*, 2000), revealed that KIAA0559 encodes a *bassoon*-

like protein that is highly enriched in the active zone. Both *bassoon* and *piccolo* are large proteins ($>400 \text{ kDa}$) primarily composed of zinc finger sequences and of sequences with a high propensity for coiled-coil formation. *Piccolo* and *bassoon* are synthesized in multiple alternatively spliced forms. Of these, alternative splicing of the C-terminus of *piccolo* is particularly noteworthy. It generates a less-abundant 'long' form that includes two C_2 -domains (referred to as C_2A - and C_2B -domains), and a more abundant 'short' form with only the single C_2A -domain (Figure 1A; Wang *et al.*, 1999). In independently isolated overlapping cDNA clones encoding the C-terminal half of rat *piccolo*, we generally confirmed the previously published sequences except that additional alternative splicing of *piccolo* N-terminal to the PDZ-domain was observed (data not shown).

The presence of C_2 -domains in *piccolo/aczonin* suggests that it is a potential Ca^{2+} sensor at the active zone. The combination of the PDZ- and the C_2A -domains in *piccolo* is attractive because it indicates a possible mechanism by which *piccolo* could be bound to a Ca^{2+} -independent target via its PDZ-domain, and recruit additional Ca^{2+} -dependent targets via the C_2A -domain. As a first step towards understanding the function of *piccolo*, we analyzed the sequences of its C_2 -domains. Databank searches revealed that the *piccolo* C_2A -domain is closely related to the C_2A -domains of synaptotagmins and rabphilin (Figure 1B). The major difference between the C_2A -domains of synaptotagmins and *piccolo* is that *piccolo* contains a unique bottom sequence of 11 residues between β -strands 3 and 4, which is absent from synaptotagmins. In the top loops, the *piccolo* C_2A -domain includes all of the residues that are involved in Ca^{2+} binding in the synaptotagmin I C_2A -domain (Ubach *et al.*, 1998). This allows formulation of a model for potential Ca^{2+} -binding sites in the *piccolo* C_2A -domain (Figure 1C), which suggests that the Ca^{2+} -binding properties of the *piccolo* C_2A -domain should be similar to those of the synaptotagmin C_2A -domains.

In contrast to the similarity between the C_2A -domains of *piccolo* and synaptotagmin, the alternatively spliced C_2B -domain of *piccolo* is quite different from other C_2 -domains in the databanks (Figure 1B). The *piccolo* C_2B -domain lacks four of the five canonical aspartate residues involved in Ca^{2+} binding in C_2 -domains, suggesting that the *piccolo* C_2B -domain does not function as a Ca^{2+} -binding domain. In addition, the *piccolo* C_2B -domain does not contain the bottom α -helix between β -strands 7 and 8 that is characteristic for C_2B -domains in synaptotagmins or rabphilin (highlighted in red in the alignment of Figure 1B; Ubach *et al.*, 1999), or the atypical sequences that characterize the RIM C_2 -domains (Wang *et al.*, 1997, 2000). Other C_2 -domains lacking Ca^{2+} -binding sites have been described previously, e.g. in PKC δ and synaptotagmins IV and XI (von Poser *et al.*, 1997; Pappa *et al.*, 1998). However, the *piccolo* C_2B -domain is equally dissimilar to these C_2 -domains and to classical C_2 -domains, suggesting that the *piccolo* C_2B -domain does not belong to a general class of atypical C_2 -domains (data not shown). Since the C_2B -domain is present only in a minority of the *piccolo* transcripts and is unlikely to bind Ca^{2+} , it was not investigated further in the present study.

Fig. 1. Structure of piccolo/aczonin. (A) Domain structures of the short and long splice variants of piccolo/aczonin, and design of the recombinant GST fusion proteins used for the current experiments. Only the C-termini of two alternatively spliced variants of piccolo are shown, with residue numbers (corresponding to the rat short and the mouse long form; Wang *et al.*, 1999; Fenster *et al.*, 2000) indicated above the domains. (B) Sequence alignments of the C₂-domains of mouse piccolo (mPic.), rat synaptotagmins I (rSI) and VII (rSVII) and rat rabphilin (rRb). On the left, 'A' and 'B' denote C₂A- and C₂B-domains, respectively. Residues present in at least 50% of the sequences are highlighted with a structure-based color code: yellow, top loops; blue, β -strands; green, bottom loops; red, bottom α -helix specific for C₂B-domains of synaptotagmins and rabphilin. Aspartates and serines in the top loops involved in Ca²⁺ binding are highlighted in black and marked by arrowheads. (C) Structure of the Ca²⁺-binding sites of the synaptotagmin I C₂A-domain (Ubach *et al.*, 1998), and model for the Ca²⁺-binding sites of the piccolo C₂A-domain based on the sequence homology between these domains (see B). Only the Ca²⁺ ligands are drawn. The positions of other residues are indicated by small solid circles along the curves that symbolize the two sequence loops that form the Ca²⁺-binding region (loops 1 and 3). The three Ca²⁺-binding sites are indicated as solid circles labeled Ca1, Ca2 and Ca3.

Piccolo exhibits unusual Ca²⁺-binding properties

To test whether the C₂A-domain of piccolo binds Ca²⁺ and to characterize its Ca²⁺-binding properties, we used a combination of circular dichroism (CD) and NMR spectroscopy. The CD spectrum of the Ca²⁺-free piccolo C₂A-domain is characteristic for a β -sheet protein, and exhibits only slight changes upon addition of 30 mM Ca²⁺ (data not shown). A similar behavior was previously

observed for the synaptotagmin I C₂A-domain (Shao *et al.*, 1997). NMR spectroscopy, however, revealed that Ca²⁺ induces major alterations in the ¹H-¹⁵N heteronuclear single quantum correlation (HSQC) spectrum of the piccolo C₂A-domain, demonstrating that Ca²⁺ binds to this domain (Figure 2A). These shifts were qualitatively different from those found in the synaptotagmin C₂A-domain, in which Ca²⁺ binding selectively changes

Fig. 2. Analysis of the Ca^{2+} -binding properties of the piccolo C_2A -domain by NMR spectroscopy. (A) ^1H - ^{15}N HSQC spectra of the isolated C_2A -domain from piccolo recorded in the absence (black contours) and presence (red contours) of 30 mM Ca^{2+} . Spectra are superimposed to demonstrate widespread Ca^{2+} -dependent cross-peak shifts. (B) Expansions illustrating the progressive changes in the cross-peaks caused by increasing concentrations of Ca^{2+} . The cross-peaks shown are identified in (A) by arrows in the ^1H - ^{15}N HSQC spectra. Cross-peak positions are displayed at 0.0, 1.0, 2.5, 4.0 and 30.0 mM Ca^{2+} from top to bottom. Note that in contrast to the gradual cross-peak movements observed for the synaptotagmin C_2A -domain (Shao *et al.*, 1996), the Ca^{2+} -free cross-peaks disappear concomitant with the appearance of Ca^{2+} -bound cross-peaks. (C) ^1H - ^{15}N HSQC spectra of the mutant C_2A -domain from piccolo with the double aspartate-to-alanine substitutions in the Ca^{2+} -binding loops (D4668A/D4674A; Figure 1) in the absence (black contours) or presence (red contours) of 30 mM Ca^{2+} . Note the lack of Ca^{2+} -dependent cross-peak shifts in the mutant.

only ^1H - ^{15}N HSQC cross-peaks from the Ca^{2+} -binding region but not from any other part of the C_2A -domain (Shao *et al.*, 1996, 1998). In contrast, significant Ca^{2+} -dependent shifts were observed for the majority of cross-peaks of the piccolo C_2A -domain (Figure 2A), which suggests that Ca^{2+} binding to this domain results in a change of the entire piccolo C_2A -domain. In addition to the widespread shifts in ^1H - ^{15}N HSQC cross-peaks, Ca^{2+} also induced substantial resonance broadening due to dimerization (see below).

The two most likely causes for the widespread Ca^{2+} -dependent shifts in ^1H - ^{15}N HSQC cross-peaks of the piccolo C_2A -domain are: (i) an overall conformational change, or (ii) a global instability of the Ca^{2+} -free C_2A -domain with stabilization of the domain by Ca^{2+} binding. To distinguish between these possibilities, we performed temperature-denaturation experiments monitored by CD. These demonstrated that the Ca^{2+} -free piccolo C_2A -domain is remarkably stable, with a denaturation temperature of 70°C, and thus is not an intrinsically unstable domain. Addition of 30 mM Ca^{2+} shifted the denaturation temperature to 80°C, similar to other C_2A -domains (data not shown). Together, the CD and NMR data indicate that Ca^{2+} binding to the piccolo C_2A -domain causes a general conformational change, suggesting a mechanism for Ca^{2+} -dependent interactions that is quite distinct from the C_2A -domain of synaptotagmin.

The large difference between the effects of Ca^{2+} on the C_2A -domain of piccolo versus that of synaptotagmin is unexpected in view of the similarity between their presumptive Ca^{2+} -binding sites (see Figure 1C). This difference was confirmed, however, by Ca^{2+} titrations monitored with ^1H - ^{15}N HSQC spectra (Figure 2B). For the synaptotagmin I C_2A -domain, incremental increases in Ca^{2+} cause progressive movements of cross-peaks from the Ca^{2+} -free to the Ca^{2+} -bound positions (Shao *et al.*,

1996). The observation of three components along these titration curves demonstrated binding of three Ca^{2+} ions with intrinsic affinities of $\sim 50 \mu\text{M}$ to $>10 \text{ mM}$ Ca^{2+} , and no binding cooperativity (Ubach *et al.*, 1998; Fernández-Chacón *et al.*, 2001). The progressive Ca^{2+} -dependent movements of cross-peaks in the synaptotagmin I C_2A -domain reflect a fast exchange between Ca^{2+} -free and -bound states on the NMR time scale, as expected for a low-affinity Ca^{2+} sensor that does not exhibit significant conformational changes. A very different behavior was observed for the cross-peaks of the piccolo C_2A -domain: cross-peaks characteristic for the Ca^{2+} -free form disappeared with increasing Ca^{2+} , with the concomitant appearance of the corresponding cross-peaks from the Ca^{2+} -bound form. Thus, instead of a gradual progression, there was a digital switch (exemplified for the cross-peaks in the expansions of Figure 2B), reflecting a slow exchange between the Ca^{2+} -free and -bound states. The slow exchange between Ca^{2+} -free and -bound states implies that the on-rate(s) for Ca^{2+} binding to the piccolo C_2A -domain is well below the diffusion limit, in support of the notion that Ca^{2+} binding involves a significant conformational change. Ca^{2+} titrations demonstrated that the piccolo C_2A -domain exhibits an intrinsic Ca^{2+} affinity of $\sim 1.5 \text{ mM}$, and that binding is cooperative with an apparent Hill coefficient of >2 (Figure 8, Table I and data not shown). Although the slow exchange, low affinity and line broadening in the ^1H - ^{15}N HSQC cross-peaks of the Ca^{2+} -bound piccolo C_2A -domain hinder determination of the exact number of Ca^{2+} -binding sites, the Hill coefficient confirms binding of multiple Ca^{2+} ions, as predicted by the sequence alignment (Figure 1).

Ca²⁺ binds to the top loops of the C₂A-domain of piccolo and causes dimerization

In view of the sequence similarity between the piccolo C_2A -domain and other C_2 -domains, especially in the

Table 1. Ca²⁺ affinity and Ca²⁺ cooperativity of wild-type and mutant piccolo C₂A-domains in isolation (intrinsic Ca²⁺ affinity) and in the presence of phospholipids (apparent Ca²⁺ affinity)

1. Intrinsic Ca ²⁺ affinities ^{a,b}						
Protein domains	<i>K</i> _d		Hill _{coeff.}	<i>N</i>		
Piccolo C ₂ A-domain	1.52 ± 0.18 mM		2.1 ± 0.2	4		
Piccolo C ₂ A-domain V4688S/M4689S	0.80 ± 0.05 mM		2.3 ± 0.1	4		
Piccolo C ₂ A-domain V4690S/V4691S	0.82 ± 0.08 mM		2.2 ± 0.1	4		
2. Apparent Ca ²⁺ affinities with phospholipid membranes ^{b,c}						
Protein domains	30% PS/70% PC liposomes			50% PI/50% PC liposomes		
	EC ₅₀ (μM)	Hill _{coeff.}	<i>N</i>	EC ₅₀ (μM)	Hill _{coeff.}	<i>N</i>
Piccolo C ₂ A-domain	164 ± 13	1.9 ± 0.1	16	127 ± 16	1.8 ± 0.2	5
Piccolo C ₂ A-domain V4688S/M4689S	14 ± 1	8.4 ± 2.8	3	not determined		
Piccolo C ₂ A-domain V4688S	97 ± 5	1.7 ± 0.2	3	not determined		
Piccolo C ₂ A-domain M4689S	38 ± 4	1.7 ± 0.3	3	not determined		
Piccolo C ₂ A-domain V4690S/V4691S	12 ± 1	11.0 ± 1.0	3	not determined		
Piccolo C ₂ A-domain Q4692A/N4693A	103 ± 16	1.9 ± 0.5	3	not determined		
Piccolo C ₂ A-domain A4694S	187 ± 28	2.3 ± 0.8	3	not determined		
Piccolo PDZ/C ₂ A-domain	75 ± 8	1.4 ± 0.5	3	49 ± 1	1.2 ± 0.1	2
Synaptotagmin I C ₂ A-domain	12 ± 1	5.9 ± 0.5	3	10 ± 1	4.7 ± 0.3	2

^aIntrinsic Ca²⁺-binding affinities were determined by titrations of purified recombinant proteins monitored with HSQC spectra as described in Figure 8.

^bData shown are mean ± SEM from the number of experiments indicated (N).

^cAffinities were determined using Ca²⁺-dependent binding of radiolabeled liposomes with the indicated compositions, performed in triplicate as described in Figure 6.

presumptive Ca²⁺-binding sites (Figure 1), it is surprising that the piccolo C₂A-domain has such unusual Ca²⁺-binding properties. These properties raise the possibility that Ca²⁺ binds to different sites in the piccolo C₂A-domain than in other C₂-domains. To test this directly, we mutated two aspartate residues in the presumptive Ca²⁺-binding sites of the piccolo C₂A-domain and substituted them for alanines. We then examined Ca²⁺ binding to the mutant C₂A-domain by NMR spectroscopy (Figure 2C). Superposition of the ¹H-¹⁵N HSQC spectra of the mutant in the absence and presence of 30 mM Ca²⁺ showed that the mutation abolished all Ca²⁺-dependent chemical shift changes, and thus rendered the domain unable to bind Ca²⁺. Nevertheless, the overall spectrum of the mutant domain was similar to that of the Ca²⁺-free wild-type domain, demonstrating that the mutant domain was still folded normally. These data confirm that Ca²⁺ binds similarly to the top loops of the synaptotagmin and piccolo C₂A-domains, in spite of the different structural consequences of Ca²⁺ binding to these C₂-domains.

The cross-peak broadening observed for the Ca²⁺-bound form of the piccolo C₂A-domain suggests that Ca²⁺ binding leads to oligomerization. To explore this hypothesis, we analyzed the piccolo C₂A-domain by equilibrium ultracentrifugation (data not shown). In the absence of Ca²⁺, the C₂A-domain was monomeric, in agreement with the narrow line widths observed by NMR spectroscopy. In the presence of 15 mM Ca²⁺, however, the C₂A-domain migrated at an apparent mol. wt of 30 588, and the sedimentation data are best fit to a monomer/dimer equilibrium with a dissociation constant of 4.3 μM. Similar data were obtained in the presence of 30 mM Ca²⁺, which yielded a slight increase in the calculated mol. wt (32 362) and a decrease in the dimer dissociation constant (1.7 μM; data not shown). The results suggest that

Ca²⁺ binding to the piccolo C₂A-domain leads to dimerization, with a more complete formation of the dimer at 30 mM Ca²⁺, as expected from the low intrinsic Ca²⁺ affinity.

Ca²⁺-dependent phospholipid binding to the C₂A-domain of piccolo

The best characterized and most widely shared functional property of C₂-domains is Ca²⁺-dependent phospholipid binding. To test whether Ca²⁺ also induces phospholipid binding to the piccolo C₂A-domain, we used a standard assay in which immobilized glutathione *S*-transferase (GST) fusion proteins are used as an affinity matrix for Ca²⁺-dependent interactions with radioactively labeled liposomes (Davletov and Südhof, 1993). The immobilized GST fusion proteins are incubated with liposomes of defined phospholipid composition in buffers containing various concentrations of divalent cations and NaCl. Bound liposomes are then separated from free liposomes by low-speed centrifugation and washing of the beads, with the specificity of binding estimated by use of control GST fusion proteins.

In a first set of experiments, we employed liposomes with four different phospholipid compositions: 100% phosphatidylcholine (PC); 30% phosphatidylserine (PS) with 70% PC; 50% phosphatidylinositol (PI) with 50% PC; and 50% phosphatidylethanolamine (PE) with 50% PC (Figure 3). The GST fusion protein of the piccolo C₂A-domain bound selectively to negatively charged phospholipids (PS and PI) in the presence of Ca²⁺, while a control GST fusion protein containing the PDZ-domain of piccolo did not interact with any liposomes in the absence or presence of Ca²⁺. Furthermore, the aspartate-to-alanine substitutions in the Ca²⁺-binding loops of the piccolo C₂A-domain that ablate Ca²⁺ binding (see

Fig. 3. Ca²⁺-dependent phospholipid binding by the C₂A-domain of piccolo. Purified GST fusion proteins from rat piccolo containing the PDZ-domain (GST-Pic-PDZ) (A), the C₂A-domain (GST-Pic-C2A) (B) or the mutant C₂A-domain (GST-Pic-C2A^{D4668A/D4674A} substituting two Ca²⁺-binding aspartates for alanines) (C) were immobilized on glutathione beads. ³H-labeled liposomes composed of 100% PC, 30% PS/70% PC, 50% PI/50% PC, or 50% PE/50% PC were used in binding experiments with the three GST fusion proteins in the presence or absence of 1 mM free Ca²⁺ (see Materials and methods). Note the expanded scale of the graphs of the GST-PDZ domain and the mutant C₂A-domain to illustrate the small amount of background binding. Data are mean ± SEM from a representative experiment performed in triplicate.

Figure 2C) completely abolish Ca²⁺-dependent phospholipid binding (Figure 3). These data demonstrate that Ca²⁺ binding to the piccolo C₂A-domain triggers phospholipid binding in a specific reaction.

We next tested whether Ca²⁺-dependent phospholipid binding is specific for Ca²⁺, or can also be induced by other divalent cations. In these experiments we directly compared the C₂A-domains of piccolo and synaptotagmin I to evaluate their relative Ca²⁺ specificity. Since some of the cation concentrations used were high and might cause liposome precipitation, we included GST as a negative control for the binding proteins and 100% PC liposomes as a negative control for the phospholipids. For both the piccolo and the synaptotagmin C₂A-domain, no difference in binding between 1 and 10 mM Ca²⁺ was observed, indicating that these Ca²⁺ concentrations were saturating for phospholipid binding. Since no binding was obtained with 10 mM Mg²⁺, binding was specific for Ca²⁺ versus Mg²⁺. At least with PI-containing liposomes, the piccolo C₂A-domain exhibited a higher Ca²⁺ specificity than the synaptotagmin C₂A-domain (Figure 4). Sr²⁺ and Ba²⁺ partly substituted for Ca²⁺ in the synaptotagmin C₂A-domain, whereas they were much less effective for the piccolo C₂A-domain. GST alone did not bind any liposomes under any condition, nor did PC interact with

either C₂A-domain, demonstrating that binding is specific (Figure 4).

Electrostatic interactions in Ca²⁺-dependent phospholipid binding to the C₂A-domain of piccolo

We next examined the role of electrostatic interactions in Ca²⁺-dependent phospholipid binding to the piccolo C₂A-domain by testing the effect of increasing NaCl concentrations (and therefore increasing ionic strength) on binding. A series of liposomes with different phospholipid compositions was employed to control for the effects of ionic surface density on the liposomes, and to exclude artifacts due to particular phospholipid headgroups (Figure 5). At low ionic strength, the overall phospholipid-binding specificities of the synaptotagmin and piccolo C₂A-domains were very similar. As observed above, both domains bind to all negatively charged phospholipids [PS, PI, phosphatidylinositolphosphate (PIP) and phosphatidylinositol biphosphate (PIP₂)] as a function of Ca²⁺, but not to neutral phospholipids (PE and PC; Figure 5). The piccolo C₂A-domain binds relatively fewer liposomes than the synaptotagmin C₂A-domain, especially liposomes containing PI and PIP, possibly because binding is less tight. NaCl blocked phospholipid binding to the piccolo C₂A-domain at low concentrations (<0.3 M NaCl). In contrast, higher NaCl concentrations (>0.3 M NaCl) were required to inhibit phospholipid binding to the synaptotagmin I C₂A-domain, which is known to require electrostatic interaction for phospholipid binding (Davletov *et al.*, 1998; Zhang *et al.*, 1998; however, for a divergent view see Bai *et al.*, 2000). Only for PIP₂ was the synaptotagmin C₂A-domain as sensitive to ionic strength as the piccolo C₂A-domain. These results show that similarly to the synaptotagmin I C₂A-domain, the piccolo C₂A-domain requires electrostatic interactions for phospholipid binding. However, the interpretation of these results is more complicated for the piccolo C₂A-domain than for the synaptotagmin C₂A-domain because a conformational change is required for Ca²⁺ binding in the piccolo C₂A-domain. Thus, high salt could inhibit phospholipid binding either by interfering with electrostatic interactions between the phospholipid headgroups and the top of the C₂A-domain, as in the synaptotagmin C₂A-domain, or by blocking the Ca²⁺-dependent conformational change of the piccolo C₂A-domain (see below).

Ca²⁺ affinity for phospholipid binding

We next determined the apparent Ca²⁺ affinity of the piccolo C₂A-domain in the presence of phospholipids, again in direct comparison with the synaptotagmin I C₂A-domain (Figure 6). For this purpose we measured the Ca²⁺ concentration dependence of phospholipid binding to the C₂A-domain, using Ca²⁺/EGTA buffers to clamp the free Ca²⁺ concentration and liposomes with either PS or PI in order to avoid phospholipid-specific artifacts. Our results reveal an apparent Ca²⁺ affinity of the piccolo C₂A-domain in the presence of phospholipids of ~0.16 and ~0.13 mM for PS- and PI-containing liposomes, respectively (Figure 6). Thus, the apparent Ca²⁺ affinity of the piccolo C₂A-domain is considerably higher in the presence of phospholipids than its intrinsic Ca²⁺ affinity in the absence of phospholipids (~1.5 mM; see above), probably

Fig. 4. Divalent cation specificity of phospholipid binding to the C₂A-domains of synaptotagmin I and piccolo. GST alone (as a control), and the GST fusion proteins of the C₂A-domain of synaptotagmin I (GST-SytI-C₂A) and of piccolo (GST-Pic-C₂A) were used in phospholipid-binding assays in the presence of the indicated concentrations of Ca²⁺, Ba²⁺, Sr²⁺ and Mg²⁺ using liposomes composed of 50% PI/50% PC or 100% PC. Data are mean $\pm$ SEM from a representative experiment performed in triplicate.

because the phospholipids provide additional coordination sites for the bound Ca²⁺ ions and thereby increase the apparent Ca²⁺ affinity (Zhang *et al.*, 1998). The apparent Ca²⁺ affinity of the piccolo C₂A-domain during phospholipid binding was, however, 10 times lower than that of the synaptotagmin C₂A-domain measured in the same experiments, and exhibited a lower Ca²⁺ cooperativity, as manifested in the Hill coefficient (Hill_{coeff}) (Figure 6). These results were reproducibly obtained in multiple experiments (summarized in Table I).

In piccolo, the C₂A-domain is expressed at the C-terminus of a long polypeptide chain, and is N-terminally preceded by a PDZ-domain and a linker sequence. To study whether the PDZ-domain and linker influence the Ca²⁺-binding properties of the piccolo C₂A-domain, we examined a recombinant protein of piccolo that includes both the PDZ- and the C₂A-domain (Figure 1A) by NMR spectroscopy and phospholipid-binding measurements (Figure 6, Table I and data not shown). Superposition of the ¹H-¹⁵N spectra of the PDZ/C₂A-domain fragment with those of the isolated C₂A-domain did not reveal significant differences in the C₂A-domain cross-peaks between the isolated C₂A-domain and the C₂A-domain coupled to the PDZ-domain (data not shown). The Ca²⁺-induced changes in the ¹H-¹⁵N HSQC spectra of the PDZ/C₂A-domain fragment were similar to those observed in the isolated C₂A-domain. A Ca²⁺ titration of these changes also yielded comparable results to those obtained in the absence of the PDZ-domain. Together these results suggest that the overall conformation and intrinsic Ca²⁺-binding ability of the C₂A-domain are similar in isolation and in the context of

the PDZ-domain. Furthermore, the overall phospholipid-binding properties of the PDZ/C₂A-double domain protein were similar to those of the C₂A-domain alone, suggesting that the C₂A-domain is also active in the context of multiple domains. However, the apparent Ca²⁺ affinity of the PDZ/C₂A-domain protein was significantly higher than that of the C₂A-domain alone (Figure 6; Table I). This difference was found for both PI- and PS-containing liposomes, indicating that they are not a phospholipid-specific artifact.

The low Ca²⁺ affinity of the piccolo C₂A-domain depends on a unique bottom sequence

To identify the basis for the unusual structural and functional properties of the piccolo C₂A-domain—its low Ca²⁺ affinity and large Ca²⁺-dependent conformational change—we analyzed its sequence. We observed a single striking feature in the piccolo C₂-domain that is absent from other C₂-domains: an 11 residue sequence inserted between β -strands 3 and 4 (Figure 1B). This sequence is characterized by four central hydrophobic residues flanked by glutamines (QVMVVQ); its potential importance is highlighted by the fact that it is completely conserved between rat, mouse, human and chicken piccolo (Figure 7A). Since this sequence is inserted in a region that in other C₂-domains forms a loop on the bottom surface (which does not bind Ca²⁺), the possibility arises that the unique bottom sequence in the piccolo C₂A-domain confers onto the bottom surface the ability to bind Ca²⁺. This would effectively create independent Ca²⁺-binding sites on the top and bottom of the domain. However, two findings rule out this possibility. First, as

Fig. 5. Effect of phospholipid composition and NaCl concentration on Ca^{2+} -dependent liposome binding to the C_2A -domains of synaptotagmin I and piccolo. Immobilized GST fusion proteins of the C_2A -domains of synaptotagmin I (GST-SytI- C_2A) and piccolo (GST-Pic- C_2A) were used in phospholipid-binding assays in the presence of increasing concentrations of NaCl as indicated, with or without 1 mM Ca^{2+} . Liposomes were composed of 100% PC, 30% PS/70% PC, 50% PE/50% PC, 50% PI/50% PC, 15% PIP/85% PC, or 10% PIP₂/90% PC. Note differences in scale between graphs. Data are mean $\pm$ SEM from a representative experiment performed in triplicate.

described above (Figures 2 and 3), mutations in the top loops of the C_2A -domain completely abolish Ca^{2+} binding; thus, the domain does not contain a second independent Ca^{2+} -binding region on the bottom. Secondly, the unique bottom sequence does not contain hydrophilic residues that are essential for forming Ca^{2+} -binding sites, but is instead characterized by conserved hydrophobic residues (Figure 7A).

Since the bottom sequence does not form an independent Ca^{2+} -binding site on the Ca^{2+} -independent lower surface of the C_2A -domain, it would have to modulate the top Ca^{2+} -binding loops if it was indeed responsible for the unusual Ca^{2+} -binding properties of

the piccolo C_2A -domain. To test the importance of the unique 11 residue sequence at the bottom of the piccolo C_2A -domain for the unusual Ca^{2+} -binding properties of the C_2A -domain, we introduced a series of point mutations into this sequence. We then examined the effects of these mutations using Ca^{2+} -dependent phospholipid binding to measure the apparent Ca^{2+} affinity of the C_2A -domain (Table I), and NMR spectroscopy to study changes in the structure and the intrinsic Ca^{2+} affinity of the C_2A -domain (Figure 8). To evaluate the effects of these mutations, we propose a working model (Figure 7B). This model suggests that the bottom sequence folds back towards

Fig. 6. Ca²⁺ concentration dependence of phospholipid binding to the C₂A-domain of synaptotagmin I (SytI), piccolo (Pic) and the combined PDZ- and C₂A-domains of piccolo. Phospholipid-binding reactions with the immobilized GST fusion proteins of the indicated C₂-domains were carried out in Ca²⁺/EGTA buffers to clamp the free Ca²⁺ concentration. For each experiment, a GST-only control was performed for high Ca²⁺ concentrations to exclude artifactual Ca²⁺-dependent lipid precipitation (open symbol in each panel). Titrations were carried out with liposomes composed of 30% PC/70% PC (PS; top panels), or 50% PI/50% PC (PI; bottom panels). The Ca²⁺ concentration corresponding to half-maximal binding (EC₅₀) and the apparent calculated cooperativity (Hill_{coeff}) are shown for each experiment in the corresponding panels. Data are mean ± SEM from a representative experiment performed in triplicate; results from multiple experiments are summarized in Table I.

the top Ca²⁺-binding loops in the Ca²⁺-free state of the piccolo C₂A-domain, and either directly affects the top Ca²⁺-binding loops or indirectly stabilizes the Ca²⁺-free state of the C₂A-domain. According to this model, Ca²⁺ would then trigger an overall conformational change in the C₂A-domain because the folded-back sequence has to be released in order for Ca²⁺ to bind.

We first replaced the four central hydrophobic amino acids of the unique bottom sequence with serines, changing two residues at a time (mutations V4688S/M4689S and V4690S/V4691S). Both substitutions caused a dramatic, >10-fold increase in the apparent Ca²⁺ affinity of the piccolo C₂A-domain in the presence of phospholipids (Table I). These mutant piccolo C₂A-domains exhibited a Ca²⁺ affinity that resembles that of other phospholipid-binding C₂-domains, e.g. those of synaptotagmin and PKC, suggesting that the hydrophobic residues in the unique bottom sequence are responsible for lowering the apparent Ca²⁺ affinity of the C₂A-domain. In addition to increasing the apparent Ca²⁺ affinity, the double amino acid substitutions in the bottom sequence augmented the apparent cooperativity of Ca²⁺ binding dramatically (Table I). The observed Hill coefficients suggest a hypercooperativity that may be due to the fact that the bound phospholipid liposomes effectively represent a solid phase that couples multiple C₂A-domains into a single binding reaction. We next explored the effects of

single amino acid substitutions in the central hydrophobic sequence on the apparent Ca²⁺ affinity in the presence of phospholipids. Replacing valine, the first hydrophobic residue in the unique sequence, with serine (V4688S) had a small effect on Ca²⁺ affinity, but replacing the following methionine (M4689S) increased the apparent Ca²⁺ affinity almost as much as the double amino acid substitutions (Table I). We also tested mutations in the adjacent QN sequence (Q4692A/N4693A) and the subsequent alanine residue (A4694S). The QN substitution had only a moderate effect on Ca²⁺ affinity, while the following single amino acid substitution had no effect. Together, these data show that the central hydrophobic residues in the unique 11-amino-acid insertion at the bottom of the piccolo C₂A-domain are essential for the low apparent Ca²⁺ affinity of the piccolo C₂A-domain.

Structural consequences of the loop mutations

To examine how the bottom sequence influences Ca²⁺ binding by the top loops of the piccolo C₂A-domain, we studied the two mutants with the largest increase in apparent Ca²⁺ affinity, V4688S/M4689S and V4690S/V4691S (Table I), by NMR spectroscopy. Comparison of the ¹H-¹⁵N HSQC spectra of both mutants with and without Ca²⁺ showed that Ca²⁺ still caused changes in most cross-peaks in both mutants (Figure 8 and data not shown); therefore, the mutants still experienced an overall

Fig. 7. Evolutionary conservation of the unique bottom sequence in the piccolo C₂A-domain, and localization of the sequence in a model of the C₂A-domain. **(A)** Alignment of the primary structures of mouse, rat, human and chicken piccolo C₂A-domains in the region containing the piccolo-specific bottom sequence (bold type) that was mutated in the experiments described in Figures 8 and 9 and Table I. Amino acid substitutions are underlined and shown in italic. **(B)** Model of the piccolo C₂A-domain with three bound Ca²⁺ ions on top (orange spheres) and the unusual sequence that is specific for the C₂A-domain on the bottom (shown in yellow). The model proposes that the unique bottom sequence folds back towards the top Ca²⁺-binding loops of the C₂A-domain. The bottom sequence could either directly influence Ca²⁺ binding to the top loops, or stabilize the Ca²⁺-free conformation of the overall C₂A-domain and thereby indirectly influence Ca²⁺ binding. The model is based on the structure of the synaptotagmin C₂A-domain where the 11 residue loop has been manually inserted in an arbitrary conformation (prepared with the MOLSCRIPT program; Kraulis, 1991).

conformational change upon Ca²⁺ binding. Furthermore, Ca²⁺-dependent line broadening was observed for both mutant C₂A-domains, indicating that they still dimerize. However, superposition of the ¹H-¹⁵N HSQC spectra of the wild-type and the V4690S/V4691S mutant C₂A-domains revealed that the spectra were very different for the Ca²⁺-free forms of the C₂A-domain, but nearly identical for the Ca²⁺-bound forms (Figure 8). Similar results were obtained for the V4688S/M4689S mutant C₂A-domain (data not shown), which indicates that the loop mutations selectively perturb the conformation of the Ca²⁺-free but not the Ca²⁺-bound form of the C₂A-domain.

We also performed Ca²⁺ titrations of the two mutant and the wild-type C₂A-domains to compare their intrinsic Ca²⁺ affinities. Both mutants exhibited a nearly 2-fold higher Ca²⁺ affinity than the wild-type C₂A-domain, but a comparable Ca²⁺ cooperativity (Figure 8C and D; Table I). Together, these results strongly suggest that the bottom sequence stabilizes a conformation of the wild-type piccolo C₂A-domain that is unable to bind Ca²⁺, and that the hydrophobic residues in the sequence are essential for this stabilization. Elucidation of the precise mechanism by which the bottom sequence achieves this effect on Ca²⁺ binding will require an atomic structure of the domain.

Fig. 8. NMR spectra of wild-type and mutant piccolo C₂A-domains. (A and B) Superpositions of ¹H-¹⁵N HSQC spectra of the wild-type piccolo C₂A-domain (black contours) and the V4690S/V4691S mutant (red contours) in the absence (A) and presence (B) of saturating Ca²⁺ concentrations (30 mM for wild type and 10 mM for the mutant). (C) Expansions of ¹H-¹⁵N HSQC spectra of wild-type piccolo C₂A-domain, the V4690S/V4691S mutant (VV) and the V4688S/M4689S mutant (VM) acquired at the Ca²⁺ concentrations described on the left (in millimolar). The expansions illustrate the disappearance of a cross-peak from the Ca²⁺-free form and the concomitant appearance of the cross-peak from the Ca²⁺-bound form (identified by arrows in A and B) with increasing Ca²⁺ concentrations. (D) Ca²⁺ dependence of the intensity of the cross-peak from the Ca²⁺-bound form appearing in (C). The intensities were normalized to the intensity of the cross-peak at Ca²⁺ saturation. Solid lines represent curve fitting of the data to a standard Hill equation; the resulting Ca²⁺-binding parameters are summarized in Table I. For clarity, the plot only shows data obtained up to 5 mM Ca²⁺, but the fits were obtained using data up to 30 mM Ca²⁺ for wild type and up to 10 mM Ca²⁺ for the mutants (see Table I).

Independent of the mechanism by which the bottom sequence stabilizes the Ca²⁺-free conformation of the C₂A-domain, the fact that the hydrophobic residues of this sequence are required suggests that these residues participate in tertiary hydrophobic contacts in the Ca²⁺-free but not the Ca²⁺-bound form. This hypothesis predicts that the NaCl sensitivity of Ca²⁺-dependent phospholipid binding to the piccolo C₂A-domain (Figure 5) may be due, at least in part, to these hydrophobic contacts, since such contacts (which need to be broken for Ca²⁺ binding) would be strengthened by increasing NaCl concentrations. To test this hypothesis, we examined whether the mutant C₂A-domains exhibit a change in the NaCl sensitivity of Ca²⁺-dependent phospholipid binding (Figure 9). Gratifyingly, Ca²⁺-dependent phospholipid binding to the mutant piccolo C₂A-domains was much more resistant to increasing ionic strength than that to the wild-type C₂A-domain. This effect was particularly pronounced for the V4690S/V4691S mutant C₂A-domain, which exhibited no decrease in phospholipid binding at 0.3 M NaCl, whereas the wild-type C₂A-domain was almost completely inhibited at this NaCl concentration (Figure 9).

Discussion

Piccolo/aczonin is a recently described presynaptic protein that is selectively localized to the active zone of synapses (Cases-Langhoff *et al.*, 1996; Wang *et al.*, 1999; Fenster *et al.*, 2000; reviewed in Garner *et al.*, 2000). Piccolo is a very large protein (~500 kDa) containing multiple domains, many of which are alternatively spliced. The C-terminus of piccolo is composed of a PDZ- and a C₂A-domain that are present in all known splice variants, and a C₂B-domain that is present in a subset of splice

variants (Wang *et al.*, 1999). The piccolo C₂A-domain is similar to those of synaptotagmins and other proteins, and includes all of the residues in the top Ca²⁺-binding loops that coordinate the three Ca²⁺ ions in the synaptotagmin C₂A-domain (Figure 1; Ubach *et al.*, 1998). Since piccolo is one of the few proteins known to be specifically localized to the presynaptic active zone (Garner *et al.*, 2000), a localization that would be ideally suited for regulating neurotransmitter release, we set out to examine the possibility that piccolo constitutes a presynaptic Ca²⁺ sensor due to the presence of its C₂A-domain. Our results demonstrate that piccolo is a presynaptic Ca²⁺-binding protein by virtue of its C₂A-domain, which exhibits unexpected Ca²⁺-binding properties that differ from those of other C₂-domain proteins. Our data show that Ca²⁺ binds to the piccolo C₂A-domain with low affinity but high specificity, and causes a major conformational change in the overall domain. Surprisingly, mutagenesis revealed that the low Ca²⁺ affinity of the piccolo C₂A-domain depends on a unique sequence predicted to be located at the bottom of the C₂A-domain. This result suggests that the unique bottom sequence influences Ca²⁺ binding to the top loops of the C₂A-domain, indicating that the bottom sequence may fold back towards the top loops to modulate Ca²⁺ binding. The structural studies on the mutant C₂A-domains showed that the bottom sequence stabilizes the Ca²⁺-free domain and thus regulates Ca²⁺ binding indirectly by hindering the conformational change that is required for Ca²⁺ binding. Based on the low affinity but high specificity of Ca²⁺ binding to piccolo, we propose that piccolo plays a selective regulatory role at the synapse when Ca²⁺ accumulates in nerve terminals after repetitive stimulation, such as during processes of short-term synaptic plasticity.

Fig. 9. Effect of increasing NaCl concentrations on Ca^{2+} -dependent liposome binding to wild-type (WT) and mutant piccolo C_2A -domains. Ca^{2+} -dependent phospholipid-binding measurements were carried out with immobilized GST fusion proteins of the wild-type and mutant piccolo C_2A -domains as described for Figures 3 and 5 using liposomes composed of 30% PS/70% PC. Ca^{2+} -binding affinities of these mutants determined in multiple independent experiments are summarized in Table I. To facilitate comparisons, results were normalized for the amount of binding observed in 0.1 M NaCl. Data are mean $\pm$ SEM from a representative experiment performed in triplicate.

The C_2A -domain of piccolo exhibits the following similarities to other Ca^{2+} -binding C_2 -domains. (i) Its sequence is very similar to that of other C_2 -domains. Especially the top loops of the piccolo C_2A -domain are almost identical to those of other Ca^{2+} -binding C_2 -domains, with full conservation of all residues involved in Ca^{2+} binding (Figure 1). (ii) Ca^{2+} appears to bind to the top loops of the piccolo C_2A -domain since Ca^{2+} binding was abolished when two of the five aspartates in the loops were substituted for alanines (Figures 2 and 3). (iii) Ca^{2+} triggers phospholipid binding to the piccolo C_2A -domain in a promiscuous reaction that requires negatively charged phospholipids (Figures 3–5). (iv) Similar to other C_2 -domains, the piccolo C_2A -domain is specific for Ca^{2+} over Mg^{2+} , and slightly more selective than other C_2 -domains for Ca^{2+} over Ba^{2+} and Sr^{2+} (Figure 4). (v) The apparent Ca^{2+} affinity of the piccolo C_2A -domain in the presence of phospholipids is 10-fold higher than its intrinsic Ca^{2+} affinity. This suggests that similarly to the C_2A -domain of synaptotagmin I (Zhang *et al.*, 1998) and the C_2 -domain of $\text{PKC}\alpha$ (Verdaguer *et al.*, 1999), bound phospholipids provide additional Ca^{2+} coordination sites and thereby increase the apparent Ca^{2+} affinity.

In spite of these similarities, however, the properties of the C_2A -domain of piccolo are strikingly different from those of any other currently characterized C_2 -domain, suggesting that it falls into a novel class. (i) Ca^{2+} binding causes chemical shift changes in virtually all cross-peaks in the HSQC spectrum of the piccolo C_2A -domain. Thus, Ca^{2+} binding triggers a global conformational change in the C_2A -domain of piccolo (Figure 2). By contrast, in other C_2 -domains Ca^{2+} only has a local effect at the binding region, as exemplified by the Ca^{2+} -free and -bound structures of the synaptotagmin I and phospholipase

$\text{C}\delta$ C_2 -domains (Essen *et al.*, 1997; Shao *et al.*, 1998). (ii) Consistent with a conformational change, intrinsic Ca^{2+} binding to the piccolo C_2A -domain is cooperative (Figures 2 and 8). This result suggests that the Ca^{2+} -binding sites on top of the C_2A -domain are not filled sequentially but simultaneously, in parallel with the conformational switch. (iii) Ca^{2+} binding to the piccolo C_2A -domain induces dimerization, which may be the consequence of the overall conformational change. (iv) The piccolo C_2A -domain exhibits a lower apparent Ca^{2+} affinity in the presence of phospholipids (130–160 μM) than other phospholipid-binding C_2 -domains (2–20 μM ; Figure 7; Table I; Davletov and Südhof, 1993; Li *et al.*, 1995; Shao *et al.*, 1996; Essen *et al.*, 1997; Nalefski *et al.*, 1998). In the more natural context of the PDZ/ C_2A -domain tandem, this affinity increased to 50–70 μM (Table I). Although apparent Ca^{2+} affinities measured for ternary complexes such as C_2 -domain– Ca^{2+} –phospholipids need to be interpreted with caution since they depend on the concentrations of the components, our data directly compared the properties of the piccolo and the synaptotagmin C_2A -domains, suggesting that the relative Ca^{2+} affinities of these C_2 -domains are likely to be correct.

What is the structural basis for the unique Ca^{2+} -binding properties of the piccolo C_2A -domain? We identified a unique 11 residue sequence in the piccolo C_2A -domain that is absent from other C_2 -domains, and connects β -strands 3 and 4 at the bottom of the piccolo C_2A -domain. Point mutations in hydrophobic residues located in the center of the 11 residue sequence increased the apparent Ca^{2+} affinity of the piccolo C_2A -domain 10-fold, although the mutant C_2A -domains still exhibited a Ca^{2+} -dependent conformational change (Figure 8; Table I).

¹H-¹⁵N HSQC spectra of the Ca²⁺-free and -bound wild-type and mutant C₂A-domains showed that these mutations perturb the Ca²⁺-free form of the C₂A-domain but have little effect on the Ca²⁺-bound form. This result suggests that a major determinant of the low Ca²⁺-binding affinity of the C₂A-domain is the stability of the Ca²⁺-free form, which is mediated by hydrophobic interactions of the unique bottom sequence. Consistent with this conclusion, Ca²⁺-dependent phospholipid binding to the bottom sequence mutants was less sensitive to high ionic strength than Ca²⁺-dependent phospholipid binding to the wild-type piccolo C₂A-domain (Figure 9). Overall, these results demonstrate that Ca²⁺ binding to the piccolo C₂A-domain is governed by a new principle for a C₂-domain, a Ca²⁺-dependent conformational switch that affects the entire C₂A-domain even if Ca²⁺ binding is restricted to the top of the domain. High-resolution structures of the Ca²⁺-free and -bound forms of the piccolo C₂A-domain will be required to determine the mechanisms underlying these fascinating and unusual Ca²⁺-binding properties.

Ca²⁺ triggers synaptic vesicle exocytosis in a tightly regulated manner, and additionally regulates short- and long-term synaptic plasticity during repetitive stimulation. Synapses probably contain a multitude of Ca²⁺ sensors that are activated by different Ca²⁺ concentrations and that contribute differentially to the many actions of Ca²⁺ in the nerve terminal. Several Ca²⁺-binding proteins at the synapse have been identified, which could potentially serve as presynaptic Ca²⁺ sensors. However, none of these candidate Ca²⁺ sensors is selectively activated at the higher Ca²⁺ concentrations that are built up during repetitive stimulation (Kamiya and Zucker, 1994; Dittman *et al.*, 2000). Our results show that piccolo constitutes an unusual synaptic Ca²⁺ sensor whose Ca²⁺ affinity is too low to serve as a Ca²⁺-buffer or as a Ca²⁺ sensor for release, but appears to be ideally suited for a role during Ca²⁺-build-up after repetitive stimulation. The slow conformational switch in the piccolo C₂A-domain, in contrast to the fast electrostatic switch in the synaptotagmin C₂A-domain (Shao *et al.*, 1996, 1997, 1998), also agrees well with this possibility since short-term plasticity has a relatively slow time constant. Studies with genetically altered mice will be required to test this hypothesis.

Materials and methods

Cloning of rat piccolo/aczonin

GenBank searches identified piccolo/aczonin as a potentially novel C₂-domain protein in an anonymous human brain cDNA sequence (KIAA0559, accession No. AB011131) and a human expressed sequence tag (EST) clone (accession No. AA319026). Using the EST clone (obtained from the American Type Culture Collection, Manassas, VA) and fragments of subsequently isolated cDNA clones, we screened a rat brain λZAPII cDNA library (Stratagene, La Jolla, CA) by standard procedures (Südhof *et al.*, 1987; Sambrook *et al.*, 1989). We isolated 17 overlapping cDNA clones that cover the entire C-terminal half of the short form of piccolo as revealed by Wang *et al.* (1999) and Fenster *et al.* (2000) and published during the course of this study. The sequences of overlapping cDNA clones corresponding to the entire C-terminal half of piccolo were obtained on both strands and were largely identical to the piccolo sequences in the databanks with minor changes and additional splice variants. Of the cDNA clones isolated, clone pBSPic-C51 contained the entire C-terminal coding region of the short form of rat piccolo (residues 4296–4880; see Figure 1A) and was used for construction of all vectors used in the current study.

Construction of expression vectors and protein expression

For production of GST fusion proteins, sequences encoding the C₂A-domain (residues 4635–4776), the tandem PDZ- and C₂A-domains (residues 4439–4776) and the PDZ domain (residues 4439–4554) of rat piccolo were amplified by PCR using the cDNA clone pBSPic-C51 as template and primers A versus C, B versus C and D versus E (A = CCGGAATTCTAGCCTCTACCCCAATTACAGGAG; B = CCGGAATTCCTCATGCAAGGATAAAAAATCACC; C = CCCAAGCTTTAGCTCTCAGTTTGTCTTTTTCAG; D = CGCGGATCCCCTCATGCAAGGATAAAAAATCACC; E = CCCAAGCTTTTATGGCGGTTTCATGAAGTTCCAG). The PCR products were cloned into the *EcoRI*–*HindIII* and *Bam*HI–*HindIII* sites, respectively, of pGEX-KG (Guan and Dixon, 1991) to yield pGEXPic-C2A, pGEXPicPDZ/C2A and pGEXPicPDZ (Figure 1A). Mutagenesis of D⁴⁶⁶⁸ and D⁴⁶⁷⁴ into alanines in pGEXPic-C2A was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and primers F versus G and H versus I (F = GCAAGAAACCTAGTCCCCAGAGCCAAACATGGCTACTCTGACCCG; G = CGGGTCAGAGTAGCCATTGTTGGCTCTGGGGAC-TAGGTTTCTTGC; H = CCAACAATGGCTACTCTGCCCGTTTGT-TTAAGGTGTAC; I = GTACACCTTAACAAACGGGGCAGAGTA-GCCATTGTTGG). Mutations in the bottom loop of the piccolo C₂A-domain were made by introducing a *Sac*II site 5' and a *Bsi*WI site 3' of the bottom loop using silent site-directed mutagenesis and primers J versus K and L versus M (J = GTGTACTACTTCCAGGCCGCGGT-CAAGTCATGGTTGTC; K = GACAACCATGACTTGACCGCGGC-CTGGAAGTAGGTACAC; L = GCAAGTGCTGAGTACAAGAGAC-GTACGAAATATGTCCAGAAAAGTC; M = GACTTTCTGGACA-TATTTCTGACGTCTCTTGTACTCAGCACTTGC). Bottom loop mutants V4688S/M4689S, V4688S, M4689S, V4690S/V4691S, Q4692A/N4693A and A4694S were then introduced by subcloning annealed oligonucleotides N versus O, P versus Q, R versus S, T versus U, V versus W, X versus Y into the *Sac*II and *Bsi*WI sites, respectively (N = GGTCAAAGCTCAGTTGTCCAGAAATGCTAGCGCTGAGTACAAG-AGAC; O = GTACGTCTCTTGTACTCAGCGCTAGCATTCTGG-ACAACTGAGCTTTGACCGC; P = GGTCAAAGCATGGTTGTCCAGAAATGCTAGCGCTGAGTACAAGAGAC; Q = GTACGTCTCTTGTACTCAGCGCTAGCATTCTGGACAACCATGCTTTGACCGC; R = GGTCAAGTCTCAGTTGTCCAGAAATGCTAGCGCTGAGTACAAGAGAC; S = GTACGTCTCTTGTACTCAGCGCTAGCATTCTGGACAACCTGAGACTTGACCGC; T = GGTCAAGTC-ATGAGCTCAGCAATGCTAGCGCTGAGTACAAGAGAC; U = GTACGTCTCTTGTACTCAGCGCTAGCATTCTGTGAGCTCATG-ACCTTGACCGC; V = GGTCAAGTCTCAGTTGTCCAGAAATGCTAGCGCTGAGTACAAGAGAC; W = GTACGTCTCTTGTACTC-AGCGCTAGCTGCTGCGACAACCATGACTTGACCGC; X = GGT-CAAGTCATGGTTGTCCAGAAATCAAGCGCTGAGTACAAG-AGAC; Y = GTACGTCTCTTGTACTCAGCGCTTGAATTGTG-ACAACCATGACTTGACCGC). All plasmids were verified by sequencing. Recombinant GST fusion proteins were purified on glutathione-agarose by standard procedures (Guan and Dixon, 1991), and the amounts and purity of the recombinant proteins were standardized on Coomassie Blue-stained SDS-polyacrylamide gels (Laemmli, 1970).

Phospholipid-binding assays

Phospholipid-binding assays were performed essentially as described (Davletov and Südhof, 1993; Li *et al.*, 1995) with GST fusion proteins immobilized on glutathione-agarose. Phospholipids (1.75 mg total; Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, mixed in the indicated weight ratios with a trace amount (<0.01% of total) of ³H-labeled PC (Amersham Pharmacia Biotech, Piscataway, NJ) and dried under a stream of nitrogen. Dried lipids were resuspended in 10 ml of 50 mM HEPES–NaOH pH 7.4, 0.1 M NaCl by vigorous vortexing for 1 min, sonicated for 5 min in a waterbath sonicator (model G112PIG; Laboratory Supply Co. Inc., Hicksville, NJ; output: 80 kc, 80 W), and centrifuged for 15 min at ~5000 g to remove aggregates. Binding assays contained ~25 µg of recombinant protein, with 1 µg of protein/µl of wet glutathione beads. Beads were equilibrated in 0.1 ml of the respective binding buffers [50 mM HEPES–NaOH pH 6.8, 0.1 M NaCl (if not indicated differently), 4 mM Na₂EGTA, 8.75 µg of phospholipids with 0.025 µCi of [³H]PC-labeled PC] and either no additions or the respective amounts of divalent cations to produce the free concentrations stated in the figure legends. The mixture was incubated for 10 min at room temperature with vigorous shaking in an Eppendorf shaker, briefly centrifuged, and washed three times with 800 µl of the respective binding buffers. Phospholipid binding was quantified by scintillation counting of the beads (Beckman LS6000SC; Beckman Instruments, Inc., Fullerton,

CA). For the composition of Ca^{2+} /EGTA buffers, the respective amount of total Ca^{2+} needed to achieve a defined free Ca^{2+} concentration was calculated with commercial software (EqCal for Windows; Biosoft, Ferguson, MO). All buffers were prepared in high-resistance MilliQ water using a 1 M Ca^{2+} standard solution (Fluka Chemical Corp., Rankonkoma, NY). Binding data were analyzed with the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA) for EC₅₀ and Hill_{coeff}.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed in absorbance mode at 25°C on a Beckman XL-I analytical ultracentrifuge at rotor speeds of 18 000, 20 000 and 22 000 r.p.m. using samples of 5.3, 7.9 and 10.7 μM piccolo C₂A-domain dissolved in 20 mM acetate pH 6.0, 150 mM NaCl, and 0, 15 or 30 mM CaCl_2 . The data were analyzed using MICROCAL ORIGIN software (Beckman) assuming a partial specific volume of 0.735 ml/g and a solvent density of 1 g/ml.

NMR spectroscopy

Recombinant ^{15}N -labeled samples of the isolated C₂A-domain from rat piccolo (residues 4635–4776) and of the PDZ/C₂A-domain fragment from rat piccolo (residues 4439–4776) were expressed as GST fusion proteins in *Escherichia coli* BL21(DE3) cells using the same protocols as used previously for the synaptotagmin I C₂A-domain (Shao et al., 1997). Uniform ^{15}N -labeling was achieved by growing the bacteria in M9 minimal medium containing $^{15}\text{NH}_4\text{Cl}$ (Isotec) as the sole nitrogen source. The fusion proteins were isolated by affinity chromatography on glutathione–agarose (Sigma), cleaved with thrombin, and purified by gel filtration on a Superdex 75 column (Pharmacia) (C₂A-domain) or cation exchange chromatography on a MonoS column (Pharmacia) (PDZ/C₂A-domain fragment). ^1H - ^{15}N HSQC spectra were acquired using a sensitivity enhanced pulse sequence (Zhang et al., 1994) at 25°C on a Varian INOVA 500 spectrometer. Samples contained 100 μM C₂A-domain or 120 μM PDZ/C₂A-domain fragment dissolved in 20 mM acetate pH 6.0, 150 mM NaCl, with the specified Ca^{2+} concentrations. Ca^{2+} titrations of the wild-type and mutant C₂A-domains were performed with 145–155 μM samples dissolved in the same buffer. ^1H - ^{15}N HSQC spectra were acquired at 0, 0.25, 0.5, 0.75, 0.85, 1, 1.25, 1.75, 2.5, 4, 5 and 10 mM Ca^{2+} concentrations for the mutant C₂A-domains. For wild-type C₂A-domain, ^1H - ^{15}N HSQC were acquired at these Ca^{2+} concentrations and also at 3.25, 20 and 30 mM Ca^{2+} because of the lower affinity. Ca^{2+} affinities and cooperativities were obtained by curve fitting the dependence of the intensities of selected cross-peaks on the Ca^{2+} concentration to a standard Hill equation using Sigma Plot. Cross-peaks from the Ca^{2+} -free form that disappear with addition of Ca^{2+} , and cross-peaks that appear as a function of Ca^{2+} , were used in the curve fittings, and the affinities and cooperativities obtained were averaged to yield the values described in Table I. Spectral widths of 7600 and 1163 Hz were used in the ^1H and ^{15}N dimensions, respectively. Data sets consisted of 2×100 free induction decays (FIDs) of 768 complex points, and were zero filled to obtain matrices of 512×512 real points after Fourier transformation and removal of the aliphatic part of the spectra. Between 16 and 224 scans per FID were acquired, resulting in total acquisition times of 1–14 h.

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