Synaptotagmins form a hierarchy of exocytotic Ca^{2+} sensors with distinct Ca^{2+} affinities

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Synaptotagmins constitute a large family of membrane proteins implicated in Ca^{2+} -triggered exocytosis. Structurally similar synaptotagmins are differentially localized either to secretory vesicles or to plasma membranes, suggesting distinct functions. Using measurements of the Ca^{2+} affinities of synaptotagmin C_2 -domains in a complex with phospholipids, we now show that different synaptotagmins exhibit distinct Ca^{2+} affinities, with plasma membrane synaptotagmins binding Ca^{2+} with a 5- to 10-fold higher affinity than vesicular synaptotagmins. To test whether these differences in Ca^{2+} affinities are functionally important, we examined the effects of synaptotagmin C_2 -domains on Ca^{2+} -triggered exocytosis in permeabilized PC12 cells. A precise correlation was observed between the apparent Ca^{2+} affinities of synaptotagmins in the presence of phospholipids and their action in PC12 cell exocytosis. This was extended to PC12 cell exocytosis triggered by Sr^{2+} , which was also selectively affected by high-affinity C_2 -domains of synaptotagmins. Together, our results suggest that Ca^{2+} triggering of exocytosis involves tandem Ca^{2+} sensors provided by distinct plasma membrane and vesicular synaptotagmins. According to this hypothesis, plasma membrane synaptotagmins represent high-affinity Ca^{2+} sensors involved in slow Ca^{2+} -dependent exocytosis, whereas vesicular synaptotagmins function as low-affinity Ca^{2+} sensors specialized for fast Ca^{2+} -dependent exocytosis.

Keywords: C_2 -domain/ Ca^{2+} binding protein/exocytosis/neurotransmitter release/synaptic plasticity

Introduction

When an action potential invades a presynaptic nerve terminal, Ca^{2+} influx triggers neurotransmitter release (Katz, 1969). Ca^{2+} activates two components of release: a fast synchronous component that accounts for >90% of total release, and a slow asynchronous component that mediates <10% of total release (Geppert *et al.*, 1994; Goda and Stevens, 1994; Atluri and Regehr, 1998). Both components exhibit a high degree of Ca^{2+} cooperativity but different apparent Ca^{2+} affinities (Dodge and

Rahamimoff, 1967; Goda and Stevens, 1994). In addition to stimulating release, Ca^{2+} modulates release during short-term synaptic plasticity (Katz and Miledi, 1968; Dittman *et al.*, 2000). Short-term synaptic plasticity is at least partly induced by accumulation of residual Ca^{2+} during repetitive stimulation (Delaney and Tank, 1994; Kamiya and Zucker, 1994) and is an important determinant of the properties of neuronal networks (Dobrunz and Stevens, 1999; Gil *et al.*, 1999).

Ca^{2+} is thought to trigger release and regulate synaptic plasticity by binding to Ca^{2+} sensors. Although many Ca^{2+} binding proteins have been identified that could potentially serve as synaptic Ca^{2+} sensors, the best current candidates at the synapse are synaptotagmins 1 and 2, abundant synaptic vesicle proteins that bind Ca^{2+} . Synaptotagmins 1 and 2 are highly homologous but differentially distributed, with synaptotagmin 1 primarily expressed in forebrain and synaptotagmin 2 in the brain stem and spinal cord (Ullrich *et al.*, 1994; Marqueze *et al.*, 1995). However, at least 11 other synaptotagmins are present in brain besides synaptotagmins 1 and 2. Of these ‘other’ synaptotagmins, synaptotagmins 3 and 7 are the most abundant (reviewed in Südhof, 2002). Different from synaptotagmins 1 and 2, synaptotagmins 3 and 7 are uniformly co-distributed throughout the brain (Ullrich and Südhof, 1995) and are localized to plasma membranes instead of to synaptic vesicles (Butz *et al.*, 1999; Sugita *et al.*, 2001).

All synaptotagmins are composed of an N-terminal transmembrane region, a linker sequence and two C-terminal C_2 -domains (referred to as the C_2A - and C_2B -domains). In synaptotagmin 1, the C_2A -domain binds three Ca^{2+} ions, while the C_2B -domain binds only two Ca^{2+} ions (Südhof and Rizo, 1996; Ubach *et al.*, 1998; Fernandez *et al.*, 2001). Both C_2 -domains bind Ca^{2+} ions with a low intrinsic affinity (>500 μM Ca^{2+}). The apparent Ca^{2+} affinity of the synaptotagmin 1 C_2 -domains is dramatically increased in the presence of phospholipid membranes (~5–50 μM Ca^{2+} depending on the lipid composition), presumably because the phospholipid headgroups provide additional coordination sites for the bound Ca^{2+} ions (Zhang *et al.*, 1998; Fernandez *et al.*, 2001; Fernandez-Chacon *et al.*, 2001). In the ternary C_2 -domain– Ca^{2+} –phospholipid complex, the C_2A -domain probably contains three Ca^{2+} ions, but the C_2B -domain contains only two Ca^{2+} ions. As a result, the C_2B -domain complex is more labile, and Ca^{2+} -dependent phospholipid binding to the synaptotagmin 1 C_2B -domain was only detected recently by methods in which binding was studied in solution (Fernandez *et al.*, 2001). The sequences of most C_2 -domains of other synaptotagmins are very similar to those of synaptotagmin 1, suggesting that most synaptotagmins also form Ca^{2+} -dependent phospholipid complexes via both C_2 -domains (Li *et al.*, 1995a,b). Ca^{2+} -dependent phospholipid interactions probably con-

Fig. 1. Phospholipid binding to the C₂A-domains of synaptotagmins 1, 2, 3, 5, 7 and 10 studied by Ca²⁺-dependent GST pull-downs of radiolabeled liposomes. GST fusion proteins containing the indicated C₂A-domains (Syt, synaptotagmin) were immobilized on glutathione-agarose and incubated at increasing concentrations of free Ca²⁺ with radiolabeled liposomes composed of 25% PS/75% PC. Ca²⁺ concentrations were clamped by Ca²⁺/EGTA buffers using the standard NaCl-based buffer (Gerber *et al.*, 2001). Agarose beads were washed three times in the respective incubation buffers and bound liposomes were quantified by liquid scintillation counting. Binding was normalized to 100% for the maximal point. Data shown are means $\pm$ SEMs from two experiments performed in triplicate. The binding curve for the synaptotagmin 1 C₂A-domain in (A) is repeated in open squares (B–F) as an internal reference point to facilitate comparisons. (C) Gray symbols display binding observed for the synaptotagmin 3 C₂A-domain (Syt 3-C₂A) containing a point mutation in a Ca²⁺ binding loop (D333N).

stitute an intrinsic component of synaptotagmin function since phospholipid binding is the only confirmed function of C₂-domains, as exemplified by enzymes such as phospholipase A2 or protein kinase C in which the C₂-domain mediates the Ca²⁺-dependent recruitment of the enzymes to the membrane (reviewed in Nalefski and Falke, 1996; Newton and Johnson, 1998).

Knockout mice revealed that, in hippocampal synapses, synaptotagmin 1 is essential for fast but not for slow Ca²⁺-stimulated neurotransmitter release, suggesting that synaptotagmin 1 is an essential Ca²⁺ sensor for fast exocytosis (Geppert *et al.*, 1994). This hypothesis is supported by mutant mice in which a point mutation, R233Q, was introduced into synaptotagmin 1 by homologous recombination (Fernandez-Chacon *et al.*, 2001). The R233Q mutation caused an ~2-fold reduction in the overall Ca²⁺ affinity of synaptotagmin 1, and a similar decrease in the apparent Ca²⁺ affinity of transmitter release, suggesting that the Ca²⁺ affinity of synaptotagmin 1 determines the Ca²⁺ affinity of synaptic

exocytosis (Fernandez-Chacon *et al.*, 2001). In contrast to synapses, however, deletion of synaptotagmin 1 in neuroendocrine cells had a surprisingly small effect on exocytosis. In chromaffin cells, the synaptotagmin 1 knockout caused only a minor decrease in fast exocytosis (Voets *et al.*, 2001). Similarly, in PC12 cells lacking synaptotagmins 1 and 2, Ca²⁺ still induced robust secretion (Shoji-Kasai *et al.*, 1992). The lack of a requirement for synaptotagmin 1 for most large dense-core vesicle exocytosis, but its necessity for synaptic vesicle exocytosis, suggested that other Ca²⁺ sensors may be more important for large dense-core vesicle exocytosis. Indeed, studies in permeabilized PC12 cells demonstrated that the C₂A- and C₂B-domains of synaptotagmin 7 potentially inhibited exocytosis, whereas the C₂A- and C₂B-domains of synaptotagmin 1 were without significant effect, indicating that synaptotagmin 7 may constitute a Ca²⁺ sensor for exocytosis in these cells (Sugita *et al.*, 2001). However, since synaptotagmin 7 is also present in synapses, this raises the question of why synaptotagmin 7 can substitute for synaptotagmin 1 in PC12 cells but not in synapses.

One possible explanation for this conundrum is that in synapses and endocrine cells, vesicular synaptotagmins may be responsible for fast exocytosis triggered at higher Ca²⁺ concentrations, and plasma membrane synaptotagmins for slower exocytosis stimulated at lower Ca²⁺ concentrations. The more severe phenotype of the synaptotagmin 1 knockout at synapses, and the less severe phenotype in endocrine cells, would then be due to the fact that most synaptic exocytosis, but only a small part of endocrine exocytosis, are mediated by the fast component. However, it is unclear whether plasma membrane and vesicular synaptotagmins have intrinsic functional differences as predicted by this hypothesis, and also whether these differences apply to other synaptotagmins, especially synaptotagmin 3, which is co-localized with synaptotagmin 7 on synaptic plasma membranes but whose location in neuroendocrine cells is unclear. In the present study, we set out to address these issues. Our results demonstrate an unsuspected functional specialization of synaptotagmins whereby plasma membrane synaptotagmins exhibit a higher Ca²⁺ affinity than vesicular synaptotagmins, and even vesicular synaptotagmins are heterogeneous with respect to Ca²⁺ affinity. These findings indicate that at central synapses, a series of Ca²⁺ sensors with distinct affinities may operate in triggering fast release. In large dense-core vesicle exocytosis, by contrast, fusion is probably largely driven by high-affinity synaptotagmins that operate more slowly but require lower Ca²⁺ levels.

Results

Relative Ca²⁺ affinities of the synaptotagmin C₂A-domains

We compared, in the same experiment, the apparent Ca²⁺ affinities of the C₂A-domains of synaptotagmins 1, 2, 3, 5, 7 and 10. These synaptotagmins were chosen because synaptotagmins 3 and 7 are the most abundant synaptotagmins after 1 and 2 (Butz *et al.*, 1999; Sugita *et al.*, 2001), and because synaptotagmins 3, 5, 6 and 10 form a class of closely related synaptotagmins (Fukuda *et al.*,

Fig. 2. Phospholipid binding by the C₂A-domains of synaptotagmins 1, 2, 3, 5, 7 and 10 studied by co-sedimentation with liposomes. Soluble purified GST fusion proteins containing the indicated C₂A-domains were incubated with liposomes composed of 25% PS/75% PC in the presence of free Ca²⁺ at the concentrations shown, clamped by Ca²⁺/EGTA buffers. Liposomes were centrifuged and washed, and bound proteins were estimated by SDS-PAGE. Data shown are Coomassie Blue-stained gels from a single representative experiment repeated multiple times. Numbers on the right indicate approximate Ca²⁺ concentrations required for half-maximal binding as estimated from multiple experiments.

1999). Synaptotagmin 6 was not studied because we were unable to produce soluble properly folded C₂-domains from this isoform. We estimated the apparent Ca²⁺ affinities of the C₂A-domains by Ca²⁺-dependent phospholipid binding, and compared two independent methods and three different buffers to control for potential artifacts. Ca²⁺-dependent phospholipid binding assays were chosen because synaptotagmins bind Ca²⁺ at physiological concentrations only in the presence of phospholipids and because Ca²⁺-dependent phospholipid binding most likely constitutes part of their physiological function (reviewed in Südhof, 2002).

We first measured the apparent Ca²⁺ affinity of the C₂A-domains with a standard resin-based assay in which immobilized glutathione *S*-transferase (GST) fusion proteins of the synaptotagmin C₂A-domains were incubated with radiolabeled liposomes at different Ca²⁺ concentrations (Davletov and Südhof, 1993). Figure 1 demonstrates that each synaptotagmin is characterized by a distinct Ca²⁺ affinity, as measured by this assay. The vesicular synaptotagmins 1 and 2 consistently had the lowest Ca²⁺ affinities [$EC_{50} \approx 10\text{--}20 \mu\text{M}$ Ca²⁺ with liposomes composed of 25% phosphatidylserine (PS)/75% phosphatidylcholine (PC); Figure 1A and B]. By contrast, the plasma membrane synaptotagmins 3 and 7 exhibited the highest Ca²⁺ affinities ($EC_{50} \approx 1\text{--}2 \mu\text{M}$ Ca²⁺; Figure 1C and E). Synaptotagmins 5 and 10 (whose localizations are unknown, but which are most closely related to the plasma membrane synaptotagmins 3 and 6) also displayed relatively high Ca²⁺ affinities ($EC_{50} \approx 3 \mu\text{M}$ Ca²⁺; Figure 1D and F). Moreover, we observed that synaptotagmins 1 and 2 (which are both localized to synaptic vesicles but differentially distributed in brain; Ullrich *et al.*, 1994; Marqueze *et al.*, 1995) also differ in Ca²⁺ affinity (Figure 1A and B), with synaptotagmin 2 reproducibly exhibiting an ~ 2 -fold lower Ca²⁺ affinity than

synaptotagmin 1. The specificity of the phospholipid binding reaction was confirmed by a point mutant in the predicted Ca²⁺ binding site of the synaptotagmin 3 C₂A-domain (D333N), which abolished Ca²⁺-dependent phospholipid binding (Figure 1C).

The distinct Ca²⁺ affinities of synaptotagmins could have important implications for the Ca²⁺-dependent regulation of neurotransmitter release. This is illustrated by the R233Q point mutation in synaptotagmin 1, which causes an ~ 2 -fold decrease in the Ca²⁺ affinity of synaptotagmin 1 and a similar decrease in the Ca²⁺ responsiveness of synapses (Fernandez-Chacon *et al.*, 2001), suggesting that the Ca²⁺ affinity of vesicular synaptotagmins controls synaptic responses. In view of the potential importance of differences in Ca²⁺ affinity, we sought to confirm the measured apparent Ca²⁺ affinities of synaptotagmins with an independent assay. For this purpose we incubated soluble C₂A-domain–GST fusion proteins with liposomes at different Ca²⁺ concentrations, isolated the liposomes by centrifugation, and determined the amount of bound synaptotagmin C₂A-domains by Coomassie Blue staining of SDS gels. This assay, referred to as the liposome centrifugation assay, utilizes C₂A-domains in solution and thus avoids possible immobilization artifacts of the standard resin-based assay. Figure 2 shows that the liposome centrifugation assay produced results similar to the resin-based assay, revealing an ~ 2 -fold higher Ca²⁺ affinity of synaptotagmin 1 than of synaptotagmin 2, and an ~ 5 - to ~ 10 -fold higher affinity of the plasma membrane synaptotagmins than of vesicular synaptotagmins. A major difference between the two assays was that in the resin-based assay, a bell-shaped Ca²⁺ concentration dependence was observed for synaptotagmins 3 and 7 (Figure 1), whereas the liposome centrifugation assay exhibited no decrease in phospholipid binding at higher Ca²⁺ concentrations (Figure 2). This difference may reflect the more stringent washes used in the resin-based assay, which would also explain the inability of the resin-based assay to detect Ca²⁺ binding to the C₂B-domain (see below; Fernandez *et al.*, 2001). The concurrence of the apparent Ca²⁺ affinities determined with the two assays confirms that the Ca²⁺ affinity is not dependent on whether the C₂A-domains are immobilized or in solution.

Synaptotagmin C₂B-domains exhibit similar differences in Ca²⁺ affinity

Synaptotagmins have two Ca²⁺ binding domains: the C₂A- and C₂B-domains. Initially the C₂B-domains were thought to be unable to bind to phospholipids as a function of Ca²⁺ because the resin-based assay did not detect such binding (reviewed in Südhof and Rizo, 1996). Recently, however, less stringent assays revealed that the C₂B-domain of synaptotagmin 1 specifically binds to phospholipids in a Ca²⁺-dependent manner with an apparent Ca²⁺ affinity that resembles that of the C₂A-domain (Fernandez *et al.*, 2001). The C₂B-domain probably forms a less tight Ca²⁺–phospholipid complex than the C₂A-domain because the C₂B-domain contains only two Ca²⁺ binding sites as opposed to the three Ca²⁺ binding sites of the C₂A-domain. To test whether the C₂B-domains of synaptotagmins 3 and 7 also bind phospholipids in response to Ca²⁺ and whether the various C₂B-domains exhibit similar differences in Ca²⁺ affinity as the C₂A-domains, we examined the

Fig. 3. Phospholipid binding by the C₂B-domains of synaptotagmins 1, 3 and 7 studied by co-sedimentation with liposomes. Purified GST–C₂B-domain fusion proteins were incubated in solution with liposomes composed of 25% PS/75% PC in the presence of the indicated concentrations of free Ca²⁺ and binding was measured as described in Figure 2. Data shown are from a single representative experiment repeated multiple times; numbers on the right indicate approximate Ca²⁺ concentrations required for half-maximal binding as estimated from multiple experiments.

C₂B-domains using the liposome centrifugation assay (Figure 2). Figure 3 shows that the synaptotagmin 1 and 7 C₂B-domains, but not the synaptotagmin 3 C₂B-domain, bind phospholipids as a function of Ca²⁺. The apparent Ca²⁺ affinity of the synaptotagmin 7 C₂B-domain was ~10-fold higher than that of the synaptotagmin 1 C₂B-domain (Figure 3), demonstrating that the C₂A- and C₂B-domains of these synaptotagmins exhibit the same difference in Ca²⁺ affinity. The lack of Ca²⁺-dependent phospholipid binding by the synaptotagmin 3 C₂B-domain, as judged by this assay, is somewhat surprising considering its sequence similarity to other C₂B-domains. It may be due either to a lack of Ca²⁺ binding by this domain (as indicated by the crystal structure; Sutton *et al.*, 1999) or to a selective inability to bind phospholipids as a function of Ca²⁺.

The relative Ca²⁺ affinities of synaptotagmin C₂A-domains are independent of Ca²⁺ buffers

The apparent Ca²⁺ affinities for synaptotagmin C₂A-domains determined above and in previous studies (Fernandez-Chacon *et al.*, 2001) were determined with the use of Ca²⁺/EGTA buffers, which may introduce systematic errors. Furthermore, although Ca²⁺ binding assays and secretion measurements in permeabilized PC12 cells both employ Ca²⁺/EGTA buffers, these buffers have distinct compositions (Gerber *et al.*, 2001; Sugita *et al.*, 2001). To better relate Ca²⁺-dependent phospholipid binding to Ca²⁺-induced secretion in PC12 cells and to evaluate the accuracy of the Ca²⁺/EGTA buffers, we directly compared three different Ca²⁺ buffers: the traditional NaCl-based Ca²⁺/EGTA buffer (Gerber *et al.*, 2001); the K-glutamate Ca²⁺/EGTA buffer containing 2 mM Mg²⁺, 2 mM ATP and 0.1% bovine serum albumin (BSA), which is used for permeabilized PC12 cell experiments (Sugita *et al.*, 2001); and a NaCl-based buffer, which contained only Ca²⁺ but no EGTA (see Materials and methods). All three buffer systems generally gave similar results, suggesting that the Ca²⁺/EGTA buffers are reliable (data not shown). The only major difference noted was that the synaptotagmin 1 C₂A-domain displayed a lower apparent Ca²⁺ affinity in the K-glutamate Ca²⁺/EGTA buffer than in the traditional Ca²⁺/EGTA buffer or the Ca²⁺-only buffer, probably because

Fig. 4. Localization of synaptotagmins 1, 3 and 7 in PC12 cells analyzed with transfected EYFP fusion proteins. PC12 cells were transfected with expression vectors encoding synaptotagmins 1, 3 or 7 as indicated; all synaptotagmins are expressed as C-terminal fusion proteins with EYFP. Unfixed PC12 cells were incubated with the fluorescent dye FM4-64 to stain the plasma membrane and viewed in a confocal microscope. Synaptotagmin 3 is shown in two examples to document reproducibility. Transfected cells are highlighted by a white arrow. The scale bar (2 μm) applies to all panels.

the ATP in the K-glutamate buffer inhibits phospholipid binding to the C₂A-domain of synaptotagmin 1 but not to that of the other synaptotagmins (data not shown).

Synaptotagmins in PC12 cells

As a first approach to testing the functional consequences of the different Ca²⁺ affinities of synaptotagmins, we employed neuroendocrine PC12 cells as a model system. These cells were chosen because they have been productively used in studying Ca²⁺-triggered exocytosis (Ahnert-Hilger *et al.*, 1987; Walent *et al.*, 1992; McFerran *et al.*, 1998; Avery *et al.*, 2000) and are known to express at least synaptotagmins 1, 3 and 7 (Shoji-Kasai *et al.*, 1992; Mizuta *et al.*, 1994; Sugita *et al.*, 2001). Previous studies have shown that synaptotagmin 1 is present on secretory vesicles in neuroendocrine cells, while synaptotagmin 7 is localized to plasma membranes (Perin *et al.*, 1991; Sugita *et al.*, 2001). The localization of synaptotagmin 3, however, is unclear. Some studies in pancreatic β-cells (the only endocrine cell where it has been studied) reported synaptotagmin 3 on secretory vesicles (Mizuta *et al.*, 1997; Brown *et al.*, 2000; Gao *et al.*, 2000), although a more recent study detected it on the plasma membrane (Gut *et al.*, 2001), similar to its localization in brain (Butz *et al.*, 1999). To address this discrepancy with an independent approach, we examined in PC12 cells the localization of transfected synaptotagmin 3–EYFP fusion protein in

Fig. 5. Inhibition of Ca^{2+} -triggered exocytosis in permeabilized PC12 cells by the C_2A -domains of synaptotagmins 1, 2, 3, 5 and 10. PC12 cells were loaded with ^3H -labeled norepinephrine, permeabilized and pre-incubated with the indicated purified GST fusion proteins of wild-type synaptotagmin C_2A -domains (A–C, E and F) or mutant synaptotagmin 3 C_2A -domain containing a single amino acid substitution in the Ca^{2+} binding site (D333N) (D). Exocytosis was triggered by addition of Ca^{2+} clamped at the concentrations shown with Ca^{2+} /EGTA buffers. Norepinephrine release was normalized to 100% for the maximal release observed under control conditions run in parallel for each experiment with GST (gray symbols). Synaptotagmin C_2A -domains and GST were added at 6 and 9 μM , respectively. Data are means $\pm$ SEMs from three experiments performed in duplicate and repeated multiple times. Note that the control traces in each graph (amount of secretion in the same assays observed with addition of 9 μM GST alone) differ slightly between graphs because each assay was performed with its own separate controls.

comparison with other synaptotagmins. This approach was employed because the low abundance of synaptotagmin 3 made detection of the endogenous protein difficult, and because the direct fluorescence of EYFP avoids possible artifacts due to indirect immunofluorescence procedures. Figure 4 shows that synaptotagmin 3 is quantitatively deposited into the plasma membrane, which is labeled with the fluorescent dye FM4-64. The localization of synaptotagmin 3 is identical to that of synaptotagmin 7–EYFP, whereas synaptotagmin 1–EYFP is exclusively present in intracellular vesicles (Figure 4). These findings suggest that synaptotagmins 3 and 7 are general plasma membrane proteins in neurons and endocrine cells.

Inhibition of Ca^{2+} -dependent norepinephrine secretion from permeabilized PC12 cells by synaptotagmin C_2A -domains

We loaded PC12 cells with radioactive norepinephrine, permeabilized the cells by freeze–thawing and triggered exocytosis by addition of Ca^{2+} at increasing concentrations. In the absence of protein additions or in the presence

of only GST, we observed a bell-shaped Ca^{2+} response curve for exocytosis from the permeabilized PC12 cells (control in Figure 5A). This curve was similar to the Ca^{2+} -concentration dependence of phospholipid binding by synaptotagmins 3 and 7 as measured by the resin assay (Figure 1; Sugita *et al.*, 2001). Addition of the synaptotagmin 1 GST– C_2A -domain fusion protein to the PC12 cells caused a small but significant inhibition of exocytosis at high Ca^{2+} concentrations (Figure 5A). A similar but lower amount of inhibition was also observed for the C_2A -domain of synaptotagmin 2 (Figure 5B), consistent with its lower apparent Ca^{2+} affinity (Figures 1 and 2). The C_2A -domain of synaptotagmin 3, however, almost abolished Ca^{2+} -triggered exocytosis at all Ca^{2+} concentrations (Figure 5C). This inhibition is similar to the effect we previously observed with the C_2A -domain of synaptotagmin 7 (Sugita *et al.*, 2001). Inhibition by the C_2A -domain of synaptotagmin 3 required Ca^{2+} binding to the C_2A -domain because the Ca^{2+} binding site mutant of the C_2A -domain (D333N), which is unable to mediate Ca^{2+} -dependent phospholipid binding (Figure 1C), had no effect on exocytosis (Figure 5D). Finally, the C_2A -domains of synaptotagmins 5 and 10 had an intermediate effect and significantly inhibited Ca^{2+} -triggered exocytosis only at higher Ca^{2+} concentrations (Figure 5E and F).

The PC12 cell experiments suggest that the Ca^{2+} sensor responsible for triggering PC12 cell exocytosis is activated by low micromolar Ca^{2+} concentrations, which precisely match the apparent Ca^{2+} affinities of synaptotagmins 3 and 7, and is inhibited by the C_2A -domains from these synaptotagmins. The Ca^{2+} binding measurements described above showed that the C_2B -domains of synaptotagmins 1 and 7 exhibited similar differences in Ca^{2+} affinities, raising the question of whether this also applies to the PC12 cell inhibitions. To test this, we measured the effects of the synaptotagmin 1, 3 and 7 C_2B -domains on PC12 cell exocytosis. Figure 6 shows that the synaptotagmin 1 and 3 C_2B -domains had little effect on Ca^{2+} -triggered exocytosis, whereas the synaptotagmin 7 C_2B -domain effectively inhibited exocytosis at all Ca^{2+} concentrations. These results again agree well with the Ca^{2+} -dependent phospholipid binding measurements (Figure 3), indicating that there is an overall correlation for synaptotagmins in their apparent Ca^{2+} affinities as measured by Ca^{2+} -dependent phospholipid binding and their effects in PC12 cells.

We had previously found that the synaptotagmin 7 C_2A -domain inhibits Ca^{2+} -triggered exocytosis at all Ca^{2+} concentrations except very low ones (Sugita *et al.*, 2001), and a similar trend was observed for the synaptotagmin 3 C_2A -domain (Figure 5C). Interestingly, the synaptotagmin 7 C_2B -domain exhibited the opposite behavior, in that inhibition of exocytosis was most effective at low Ca^{2+} concentrations (Figure 6C), suggesting that exocytosis at very low Ca^{2+} concentrations is primarily mediated by this C_2 -domain, and is not due to calmodulin. This suggestion is also supported by the finding that Sr^{2+} , a divalent cation that is unable to activate calmodulin (Chao *et al.*, 1984) but does bind to synaptotagmins, strongly activates exocytosis in PC12 cells (see below) and that calmodulin antagonists had no effect on exocytosis under our assay conditions (data not shown).

Fig. 6. Inhibition of Ca^{2+} -triggered exocytosis in permeabilized PC12 cells by the C2B-domains of synaptotagmins 1 (A), 3 (B) and 7 (C). PC12 cells were loaded with ^3H -labeled norepinephrine, permeabilized, pre-incubated with the indicated purified GST fusion proteins of synaptotagmin C2B-domains or GST alone, and stimulated for exocytosis as described in Figure 5. Data are means $\pm$ SEMs from two experiments performed in duplicate.

Sr²⁺ as a Ca²⁺ mimetic for synaptotagmins and PC12 cell exocytosis

As an independent approach to test the potential Ca^{2+} sensor function of synaptotagmins in exocytosis, we examined the ability of Sr^{2+} to substitute for Ca^{2+} . Differently to previous studies (Li *et al.*, 1995b), the current experiments were performed using $\text{Sr}^{2+}/\text{EGTA}$ buffers to control the free Sr^{2+} concentration more precisely, to eliminate potential interference by contaminating Ca^{2+} and to allow a direct comparison of Ca^{2+} binding and of Ca^{2+} triggered exocytosis in PC12 cells. Figure 7 shows that at all Sr^{2+} concentrations examined, the C2A-domains of vesicular synaptotagmins 1 and 2 did not bind to phospholipids, whereas synaptotagmins 3 and 7 exhibited similar apparent Sr^{2+} affinities in both buffers ($\text{EC}_{50} \approx 30\text{--}50 \mu\text{M}$). The resin-based and the liposome centrifugation assays for measuring the apparent Sr^{2+} affinity gave identical results (Figure 7). The specificity of the reactions was confirmed with the synaptotagmin 3 mutant D333N, which, similarly to Ca^{2+} -dependent phospholipid binding (Figure 1C), was unable to support Sr^{2+} -dependent phospholipid binding (data not shown).

We next measured the response of permeabilized PC12 cells to increasing Sr^{2+} concentrations and examined the effects of recombinant synaptotagmin C2A-domains on this response. As before, GST alone was employed in all experiments as a negative control analyzed in parallel with the test proteins. Under control conditions, Sr^{2+} was as effective as Ca^{2+} in triggering exocytosis (Figure 8). A

Fig. 7. Sr^{2+} -dependent phospholipid binding by synaptotagmin C2A-domains measured with the resin-based assay (A) or the liposome centrifugation assay (B). (A) Liposome binding to immobilized GST fusion proteins containing the C2A-domains shown was measured as described in Figure 1, except that binding was performed at the indicated concentrations of free Sr^{2+} , which were clamped by $\text{Sr}^{2+}/\text{EGTA}$ buffers. Data shown are means $\pm$ SEMs from two representative experiments performed in triplicate. (B) Liposome binding of GST fusion proteins containing the indicated C2A-domains was measured as described in Figure 2, except that binding was performed at the indicated concentrations of free Sr^{2+} clamped by $\text{Sr}^{2+}/\text{EGTA}$ buffers.

bell-shaped Sr^{2+} concentration curve was measured similarly to the Ca^{2+} concentration curve, although much higher Sr^{2+} concentrations (100–300 μM) were required for maximal effect. Similarly to Ca^{2+} -triggered exocytosis, the synaptotagmin 1 C2A-domain had only a limited effect on Sr^{2+} -triggered exocytosis, with a moderate inhibition at high Sr^{2+} concentrations (Figure 8A). In contrast, the wild-type synaptotagmin 3 C2A-domain completely abolished Sr^{2+} -triggered exocytosis (Figure 8B), but the mutant synaptotagmin 3 C2A-domain (which is unable to form Sr^{2+} -dependent phospholipid complexes; see Figure 7) had no effect (Figure 8C). The synaptotagmin 7 C2A-domain was even more effective in inhibiting exocytosis than the synaptotagmin 3 C2A-domain, and depressed release below non-stimulated control levels (Figure 8D).

Discussion

Synaptotagmins constitute a large family of membrane proteins that are preferentially expressed in brain and are composed of an N-terminal transmembrane region, a linker sequence and two C-terminal C2-domains. Synaptotagmin 1, the first synaptotagmin studied, is an abundant synaptic vesicle protein that binds Ca^{2+} and phospholipids via both of its C2-domains and is essential for fast Ca^{2+} -triggered neurotransmitter release (reviewed in Südhof and Rizo, 1996). A large body of evidence supports a role for synaptotagmin 1 and its close homolog synaptotagmin 2 as a Ca^{2+} sensor in exocytosis, especially the observation that changes in the Ca^{2+} affinity of synaptotagmin 1 produce a coordinate change in the

Fig. 8. Inhibition of Sr^{2+} -triggered exocytosis in permeabilized PC12 cells by the C_2A -domains of synaptotagmins 1 (A), 3 (B and C) and 7 (D). PC12 cells were loaded with ^3H -labeled norepinephrine, permeabilized and pre-incubated with the indicated purified GST fusion proteins of synaptotagmin C_2A -domains. In the case of the synaptotagmin 3 C_2A -domain, a wild-type (B) and a mutant C_2A -domain (C) containing a single amino acid substitution in the Ca^{2+} binding site (D333N) were tested. Exocytosis was triggered by addition of the indicated concentrations of Sr^{2+} clamped with $\text{Sr}^{2+}/\text{EGTA}$ buffers, and normalized to 100% for the maximal release observed under control conditions run in parallel (gray symbols). Data are means $\pm$ SEMs from two experiments performed in duplicate.

Ca^{2+} affinity of neurotransmitter release (Fernandez-Chacon *et al.*, 2001). Nevertheless, multiple observations indicate that neurotransmitter release is not triggered by synaptotagmins 1 and 2 alone. These include the findings that some Ca^{2+} -triggered release remains in synapses lacking synaptotagmins 1 and 2 (Geppert *et al.*, 1994), chromaffin cells deficient in synaptotagmin 1 exhibit a selective impairment in the minor fast component of Ca^{2+} -triggered exocytosis (Voets *et al.*, 2001) and PC12 cells without synaptotagmins 1 or 2 are capable of relatively normal Ca^{2+} -stimulated exocytosis (Shoji-Kasai *et al.*, 1992). Although other synaptotagmins, such as synaptotagmins 3 and 7, are present at synapses and in neuroendocrine cells in addition to synaptotagmin 1, these synaptotagmins are localized to the plasma membrane instead of to synaptic vesicles (Butz *et al.*, 1999; Sugita *et al.*, 2001; Figure 4). At synapses, the plasma membrane synaptotagmins are clearly not redundant with synaptotagmin 1 because the synaptotagmin 1 knockout has a strong synaptic phenotype, but the other synaptotagmins may mediate Ca^{2+} -triggered exocytosis in endocrine cells that do not require synaptotagmin 1 (Shoji-Kasai *et al.*, 1992; Sugita *et al.*, 2001). Viewed together, these findings raised the possibility that different synaptotagmins are specialized for distinct Ca^{2+} -regulated functions in exocytosis that correlate with their subcellular localizations. We have tested this hypothesis by examining the apparent Ca^{2+} affinities of the most abundant synaptotagmins and by relating these affinities to Ca^{2+} -triggered exocytosis in neuroendocrine PC12 cells.

Our major finding is that different synaptotagmins exhibit distinct apparent Ca^{2+} affinities (Figures 1–3). Affinities were measured by Ca^{2+} -dependent phospholipid binding, which most likely represents the physiological Ca^{2+} binding activity of C_2 -domains, because phospholipid binding is the best functionally validated general property of C_2 -domains and because the apparent Ca^{2+} affinity of the C_2 -domains of synaptotagmins in the presence of phospholipids ($<0.05 \text{ mM Ca}^{2+}$), but not in their absence ($>0.4 \text{ mM Ca}^{2+}$), is in the physiological range (Ubach *et al.*, 1998; Zhang *et al.*, 1998; Fernandez *et al.*, 2001; Fernandez-Chacon *et al.*, 2001). We have examined both C_2 -domains of the major synaptotagmins. To rule out potential artifacts created, for example, by the use of $\text{Ca}^{2+}/\text{EGTA}$ buffers or of immobilized GST fusion proteins, we have confirmed the results by independent methods in multiple buffer systems. Our measurements showed that synaptotagmin 2 has an ~ 2 -fold lower Ca^{2+} affinity than synaptotagmin 1, providing the first evidence for a functional divergence between these two differentially distributed isoforms of vesicular synaptotagmins. More importantly, our data demonstrated that synaptotagmins 3 and 7 have an ~ 5 - to 20 -fold higher Ca^{2+} affinity than synaptotagmins 1 and 2, suggesting that the plasma membrane synaptotagmins are activated at lower Ca^{2+} concentrations than the vesicular synaptotagmins. Moreover, synaptotagmins 5 and 10 also exhibited relatively high Ca^{2+} affinities, whose significance is difficult to evaluate because the localizations of these synaptotagmins are unknown. It should be noted that the Ca^{2+} affinities described here are not absolute but depend on the composition of the phospholipid membranes and on the ionic strength (Zhang *et al.*, 1998; Fernandez-Chacon *et al.*, 2001). Our numbers therefore provide only general figures for the relative Ca^{2+} affinities of the various synaptotagmins. The ‘true’ affinities may vary by a factor of 2–4, depending on the precise lipid composition of the sites of exocytosis, which is unknown. Furthermore, double C_2 -domain fragments probably have higher apparent Ca^{2+} affinities than individual C_2 -domains.

Our second major finding is that the apparent Ca^{2+} affinities of synaptotagmin C_2 -domains correlate with their ability to inhibit Ca^{2+} -stimulated exocytosis in PC12 cells (Figures 5 and 6). Among others, we show that both of the currently known synaptic plasma membrane synaptotagmins, synaptotagmins 3 and 7, are also present on plasma membranes in PC12 cells (Figure 4) and that interference with their functions severely impairs exocytosis, suggesting that these two synaptotagmins together serve as plasma membrane Ca^{2+} sensors for exocytosis (Figure 5). The mechanism by which the C_2 -domains of these synaptotagmins block exocytosis is unknown, but the inhibition is clearly specific, as shown by the differential effects observed with various phospholipid-binding C_2 -domains (Figure 5) and confirmed in control experiments with C_2 -domains from another synaptic protein, rabphilin (data not shown). The functional analysis of C_2 -domains was extended to PC12 cells where exocytosis was stimulated with Sr^{2+} instead of Ca^{2+} : Sr^{2+} -triggered exocytosis was also abolished by the C_2A -domains of synaptotagmins 3 and 7, but not by synaptotagmin 1 (Figure 8). Based on these observations, we propose an overall model whereby two classes of

Fig. 9. Diagram of the symmetrical localizations, but asymmetrical Ca^{2+} affinities, of synaptotagmins on secretory vesicles and plasma membranes. The structures of synaptotagmins are schematically indicated with extracytoplasmic sequences (yellow; note that vesicular synaptotagmins are glycosylated, as indicated by 'CHO'), transmembrane regions (white), linker sequences (blue; break in the plasma membrane synaptotagmins indicates alternative splicing in synaptotagmin 7), C_2 -domains (red) and C-terminal sequences (green). The C_2 -domains of synaptotagmins are thought to bind to phospholipids as a function of Ca^{2+} and possibly also to SNARE proteins. The apparent affinities of the C_2 -domains in the presence of negatively charged phospholipids are described next to the C_2 -domains. Ca^{2+} affinities shown apply to single C_2 -domains in the presence of negatively charged phospholipids. Because of cooperativity between C_2 -domains, the double C_2 -domain fragments have an ~ 3 -fold higher Ca^{2+} affinity.

synaptotagmins, vesicular synaptotagmins 1 and 2 and plasma membrane synaptotagmins 3 and 7, execute distinct functions in exocytosis (Figure 9). The central tenet of this working model is that the two classes of synaptotagmins cooperate in exocytosis, with the high-affinity plasma membrane synaptotagmins mediating Ca^{2+} -triggered exocytosis in neuroendocrine cells and the slow component of synaptic vesicle exocytosis, whereas the low-affinity vesicular synaptotagmins are largely dispensable for slow neuroendocrine exocytosis but are required for fast synaptic vesicle exocytosis. It has been suggested that calmodulin may be a major Ca^{2+} sensor for exocytosis (Chen *et al.*, 1999; Quetglas *et al.*, 2000) instead of modulating release as originally proposed (Chamberlain *et al.*, 1995; Kibble and Burgoyne, 1996). Our data demonstrating that Sr^{2+} is fully capable of triggering release rule out an essential role for calmodulin as a Ca^{2+} trigger for exocytosis in PC12 cells.

Our findings are at odds with some of the observations on synaptotagmins reported in the literature. In pancreatic β -cells, synaptotagmin 3 was found on secretory vesicles instead of the plasma membrane (Mizuta *et al.*, 1997; Brown *et al.*, 2000; Gao *et al.*, 2000). However, these studies used antibodies that recognized either no synaptotagmin 3 protein in brain, or a protein of different size. Since synaptotagmin 3 is primarily expressed in brain and not subject to alternative splicing (Li *et al.*, 1995a; Südhof, 2002), this result raises the possibility that the antibodies did not react with synaptotagmin 3 with high

affinity. Indeed, this possibility was confirmed by Gut *et al.* (2001), who showed with affinity-purified antibodies that synaptotagmin 3 is on the plasma membrane in pancreatic β -cells. Another discrepancy with published data (Desai *et al.*, 2000) is the fact that we observe no significant inhibition of exocytosis by the C_2A - or C_2B -domain of synaptotagmin 1 at Ca^{2+} concentrations $< 10 \mu\text{M}$, but do see inhibition at concentrations $> 0.1 \text{ mM}$ Ca^{2+} . However, our results demonstrate that the overall Ca^{2+} affinity of synaptotagmin 1, both of the C_2A - and the C_2B -domain, is much lower than the Ca^{2+} response curve of exocytosis (compare Figure 1A with 6A). These data are in perfect agreement with previous analyses of cells lacking synaptotagmin 1, showing that this synaptotagmin is not required for the vast majority of neuroendocrine exocytosis (Shoji-Kasai *et al.*, 1992; Voets *et al.*, 2001).

The finding that different synaptotagmins exhibit distinct apparent Ca^{2+} affinities and subcellular localizations may have significant functional implications. The difference in Ca^{2+} affinities between synaptotagmins 1 and 2 suggests that these highly homologous synaptotagmins, which are differentially distributed but otherwise similar in subcellular localization (Geppert *et al.*, 1991; Ullrich *et al.*, 1994), could contribute to the Ca^{2+} responsiveness of a synapse. Our data thus predict that synapses expressing synaptotagmin 2 require higher Ca^{2+} concentrations for release than those expressing synaptotagmin 1. Furthermore, vesicular and plasma membrane synaptotagmins with distinct Ca^{2+} affinities

appear to be co-expressed on opposing membranes in synapses. Since Ca^{2+} influx during an action potential causes a rapid increase, but slower decline in the synaptic Ca^{2+} concentration, plasma membrane and vesicular synaptotagmins are presumably activated in parallel. However, as the Ca^{2+} concentration declines, the plasma membrane synaptotagmins may remain active, whereas vesicular synaptotagmins are shut off. This would suggest that the remaining slow component in synaptotagmin 1 knockout mice is at least partly mediated by plasma membrane synaptotagmins. Moreover, if the relative abundance of various synaptotagmins differed between synapses, this could potentially explain the differences in release probabilities between nerve terminals. For example, layer 2/3 pyramidal cells in the cortex form synapses with a high probability of release with bitufted interneurons, and synapses with a low probability of release with multipolar interneurons (Rozov *et al.*, 2001). Other examples are the parallel fiber and the climbing fiber synapses in cerebellum, which have similar sizes and numbers of docked vesicles, but which exhibit an up to 10-fold difference in release probability (Xu-Friedman *et al.*, 2001). One factor that might contribute to these differences is the mix of synaptotagmins that is expressed at these synapses. Again, future experiments will have to test whether such differences are, at least in part, caused by variations in synaptotagmins.

Materials and methods

Expression and purification of recombinant proteins

All recombinant proteins were expressed as bacterial GST fusion proteins in pGEX-KG (Guan and Dixon, 1991). Most vectors have been described previously (Sugita *et al.*, 2001) except for the synaptotagmin 3 plasmids, which were constructed by PCR using standard methods (Sambrook *et al.*, 1989). GST fusion proteins were purified essentially as described (Guan and Dixon, 1991; Sugita and Südhof, 2000; Sugita *et al.*, 2001) and C_2B -domain proteins were additionally treated to remove the bacterial contaminants that stick to these domains (Ubach *et al.*, 2001). For this purpose, extracted proteins were incubated with 1500 U/l benzamide (Novagen Inc.) for 1 h at room temperature before binding to glutathione-agarose. The agarose was packed into a polypropylene column with a paper disc column (Quick-Sep, Isolab) and additionally washed five times with 5 ml of phosphate-buffered saline (PBS), 1 M NaCl, 1% Triton X-100, 1 mM EDTA and 0.1 g/l phenylmethylsulfonyl fluoride (PMSF), followed by six washes with 5 ml of PBS, 1 M NaCl, 1 mM EDTA and 0.1 g/l PMSF.

Phospholipid binding assays

These were performed essentially as described using GST fusion proteins either immobilized on glutathione-agarose in a resin assay (Davletov and Südhof, 1993; Li *et al.*, 1995a,b) or in solution with a centrifugation assay (Fernandez *et al.*, 2001) with three different buffers. Buffer A: NaCl-based Ca^{2+} /EGTA buffer (50 mM HEPES–NaOH pH 6.8, 0.1 M NaCl, 4 mM Na_2EGTA , and total Ca^{2+} or Sr^{2+} to produce the indicated concentrations of free Ca^{2+} or Sr^{2+}); buffer B: K-glutamate-based Ca^{2+} /EGTA buffer (120 mM K-glutamate, 20 mM K-acetate, 2 mM EGTA, 20 mM HEPES–NaOH pH 7.2, and total Ca^{2+} or Sr^{2+} to produce the indicated concentrations of free Ca^{2+} or Sr^{2+}); buffer C: NaCl-based Ca^{2+} -only buffer [50 mM HEPES–NaOH pH 6.8; 0.1 M NaCl passed through AG MP-50 resin (Bio-Rad) to eliminate Ca^{2+} contamination after resin ion conversion to Na^+ from H^+ , with Ca^{2+} or Sr^{2+} added directly to the buffer from 1 M stock solution (Fluka Chemical Corp., NY) to achieve the indicated total Ca^{2+} or Sr^{2+} concentration]. For composition of Ca^{2+} /EGTA and Sr^{2+} /EGTA buffers, the respective amounts of total Ca^{2+} or Sr^{2+} needed to achieve a defined free Ca^{2+} or Sr^{2+} concentration were calculated with EqCal software (Biosoft, Ferguson, MI) using buffer constants given by the program or published by Martell and Smith (1974). All buffers were prepared in high-resistance MilliQ water using a 1 M Ca^{2+} standard solution (Fluka Chemical Corp.).

Resin assay. PS and PC (1.75 mg total; Avanti Polar Lipids, AL) were dissolved in chloroform, mixed in a 1:3 weight ratio including <0.01% ^3H -PC (Amersham Pharmacia Biotech, NJ) and dried under a stream of nitrogen. Dried lipids were resuspended in Ca^{2+} -free buffer A or B (10 ml) by vigorous vortexing for 1 min, sonicated for 5 min in a waterbath sonicator (model G112PIG; Laboratory Supply Co. Inc., NJ; output: 80 kc, 80 W) and centrifuged (15 min at ~5000 g) to remove aggregates. Binding assays contained ~25 μg of recombinant protein, with 1 g protein/l wet glutathione beads. Beads were equilibrated in 0.1 ml of the respective binding buffers (buffer A or B with 8.75 μg of phospholipids and 0.025 μCi of ^3H -PC). Binding reactions were incubated for 10 min at 30°C with vigorous shaking, briefly centrifuged, washed three times with 0.8 ml of the respective binding buffers and phospholipid binding quantified by scintillation counting of the beads (Beckman LS6000SC; Beckman Instruments Inc., CA).

Centrifugation assay. Phospholipids (PS/PC = 25/75, w/w) in chloroform were dried as a thin layer under a stream of nitrogen. Buffers A, B or C containing 0.5 M sucrose were added to the dried phospholipid layer, vortexed for 20 min and sonicated for 5 min in a bath sonicator (model G112SPIG; Laboratory Supply Co. Inc.). After liposome formation, 4 vols of each buffer without sucrose were added and centrifuged to separate heavy liposome from free phospholipids (100 000 g for 30 min). Heavy liposomes were washed once and re-pelleted (13 000 r.p.m. for 10 min). Liposomes were resuspended in each buffer with various concentrations of free Ca^{2+} and used within 1 h. Purified soluble recombinant GST-synaptotagmin C_2 -domains (6 μg) and liposomes (100 μg of phospholipids; total volume = 1 ml) were incubated for 10 min on an Eppendorf thermal mixer at 30°C and 1400 r.p.m., liposomes were re-isolated by centrifugation (20 800 g for 10 min) and washed three times with 0.5 ml of the corresponding buffers. Chloroform:methanol (1:2, v/v) solution was added into the pelleted liposomes to denature protein and dissolve phospholipids. After centrifugation (20 800 g for 15 min), the protein precipitate was resuspended in 30 μl of 2× SDS sample buffer and analyzed by SDS-PAGE and Coomassie Blue staining.

Permeabilized PC12 cell secretion assay

Secretion assays were conducted using freeze–thaw permeabilization of PC12 cells (Klenchin *et al.*, 1998; Sugita *et al.*, 2001). PC12 cells were maintained in 75 cm^2 flasks (uncoated) in RPMI1640 with 5% fetal bovine serum (heat inactivated), 10% horse serum (heat inactivated), penicillin (50 U/ml) and streptomycin (50 U/ml) at 37°C in 5% CO_2 . For each assay, PC12 cells were plated in 10 cm^2 dishes (Costar) at 40–50% confluency. Two to three days after plating (70% confluency), PC12 cells fresh medium (total 10 ml) containing 4 μl of [^3H]norepinephrine (NEN; 1 $\mu\text{Ci}/\mu\text{l}$ in stock) and 0.5 mM ascorbic acid was added. After 24 h, cells were washed with physiological saline solution (145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM glucose, 15 mM HEPES–NaOH pH 7.4), detached from the wells by pipetting a stream of Ca^{2+} -free ice-cold buffer B, washed twice with 6 ml of ice-cold buffer B and resuspended in 6 ml of buffer B in a 15 ml cone tube. For permeabilization, cells were frozen overnight at –80°C, thawed by warming the tube at room temperature, 10 mM EGTA was added and the thawed cells were left on ice for 1–5 h to allow efficient extraction of soluble proteins. Resulting cell ghosts were washed three times with 3 ml of ice-cold Ca^{2+} -free buffer B containing 1 g/l BSA, with centrifugations at 1000 g for 5 min. About 18–36 secretion reactions were conducted with each preparation with standard reaction mixes containing in 1.5 ml microcentrifuge tubes (total volume 0.1 ml): washed cell ghosts, 2 mM ATP, 2 mM MgCl_2 , 10 μl rat brain cytosol (10 mg/ml) in buffer B with various concentrations of CaCl_2 and recombinant proteins. Reactions were incubated for 30 min at 30°C, terminated by chilling to 0°C and samples were centrifuged at 4°C for 3 min at 20 800 g. Supernatants and the pellets solubilized in 1% Triton X-100 were analyzed by liquid scintillation counting.

PC12 cell transfections

Vectors encoding EYFP fusion proteins were constructed in a CMV-based expression plasmid using standard procedures (Sugita *et al.*, 2001). For transfection, PC12 cells were plated on polylysine-coated coverslips in RPMI medium supplemented with 10% fetal bovine serum and 5% horse serum. After 1 day in culture, cells were transfected with appropriate vectors using Tfx-50 (Promega). Forty-eight to 72 h after transfection, culture medium was replaced with 0.2 ml of minimal essential medium containing 10 μM FM4-64 (Molecular Probes Inc.) and cells were incubated at 37°C for 5–10 min. FM4-64-labeled coverslips

were mounted on slides and imaged using a Bio-Rad MRC-1024 confocal microscope with a 100× (NA1.3) objective.

Miscellaneous procedures

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

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