Selective Ca^{2+} -dependent interaction of calmodulin with the head domain of synapsin 1

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The calcium-dependent regulatory protein calmodulin is a critical element in the machinery regulating exocytosis at nerve terminals. Okabe & Sobue [(1987) FEBS Lett. 213, 184–188] showed that calmodulin interacts with one of the proteins intimately connected with the neuronal exocytotic process, i.e. synapsin 1. We have investigated the site at which calmodulin interacts with synapsin 1. We find that it is possible to generate chemically cross-linked Ca^{2+} -dependent complexes between synapsin 1 and calmodulin *in vitro*, and have used covalent cross-linking in conjunction with calmodulin affinity chromatography to identify fragments of synapsin 1 that interact with calmodulin. Ca^{2+} -dependent calmodulin binding is restricted to the 'head' domain (residues 1–453 in bovine synapsin 1). Within this domain the binding site is located in a unique 11 kDa *Staphylococcus aureus* V8 proteinase generated fragment. This fragment does not contain the site for cyclic-AMP-dependent phosphorylation and therefore does not represent the *N*-terminus of the protein.

INTRODUCTION

The regulation of exocytosis by a calcium signal requires the specific binding of Ca^{2+} to effector molecules. In various experimental systems numerous sites of action have been proposed, one of which is calmodulin. Calmodulin is present in all eukaryotic cells where regulated exocytosis has been studied. Moreover, calmodulin inhibitors are general inhibitors of regulated exocytosis (e.g. Burgoyne *et al.*, 1982; Kenigsberg *et al.*, 1982; Douglas & Nemeth, 1982), and anti-calmodulin antibodies block exocytosis both after microinjection into intact cells and during cell-free exocytosis *in vitro* (e.g. Steinhart & Alderton, 1982; Kenigsberg & Trifaro, 1985; Momayez *et al.*, 1987).

The regulatory actions of calmodulin on exocytosis are exerted through its binding to several classes of protein, including protein kinases, protein phosphatases, cytoskeletal proteins and proteins associated with exocytotic vesicles and plasma membranes. Ca^{2+} /calmodulin-dependent protein kinases are activated upon depolarization of nerve terminals and their requirement in synaptic exocytosis has been shown in microinjection experiments (Llinas *et al.*, 1985; Nichols *et al.*, 1990). Microinjection also reveals the importance of calmodulin-binding cytoskeletal proteins; for example, antibodies to spectrin (fodrin) block exocytosis from adrenal chromaffin cells (Perrin *et al.*, 1987).

Calmodulin also interacts directly with many exocytotic vesicles, including synaptic vesicles (Hooper & Kelly, 1984). Several calmodulin-binding proteins are closely associated with synaptic vesicle membranes, e.g. Ca^{2+} /calmodulin-dependent protein kinase 2, p65 and synapsin 1 (Ouimet *et al.*, 1984; Okabe & Sobue, 1987; Trifaro *et al.*, 1989).

Synapsin 1 is of particular interest, since it appears to be a critical regulatory protein in the process of neuronal exocytosis. It is one of the principal substrates for the Ca^{2+} /calmodulin-dependent protein kinases which are activated on depolarization of nerve endings, and by providing modulated linkages between synaptic vesicles and the cytoskeleton it regulates the availability of vesicles for exocytosis (Baines, 1987; Linstedt & Kelly, 1987; Hirokawa *et al.*, 1989). Two polypeptides, synapsin 1a and synapsin 1b, are encoded by the same gene and are related by differential splicing of the same primary mRNA transcript

(Sudhof *et al.*, 1989). No clear difference has been discerned between their activities, and they both appear to bind calmodulin (Okabe & Sobue, 1987). The role of the direct binding of calmodulin to synapsin 1 has not yet been clarified. One mol of calmodulin binds 1 mol of synapsin 1 at saturation, with a K_d of 450 nM. This is in the mid-range of binding affinities of calmodulin; for comparison, the K_d of binding to Ca^{2+} /calmodulin-dependent protein kinase 2 is 1 nM (Bennett & Kennedy, 1987), whereas the K_d for human erythrocyte spectrin is 6 μM (Burns & Gratzer, 1985). Since both calmodulin and synapsin 1 are extremely concentrated in nerve terminals [synapsin 1 is 6% of total small synaptic vesicle protein (Huttner *et al.*, 1983)], it is reasonable to postulate that an interaction with such an affinity should occur *in vivo*.

To clarify the role of the calmodulin–synapsin 1 interaction, we have investigated the site in synapsin 1 which binds calmodulin. Synapsin 1 has two principal domains which differ both structurally and functionally. The *N*-terminal 453 residues make up a globular 'head' domain, and the remaining residues comprise the fibrous 'tail' domain (Hirokawa *et al.*, 1989; Sudhof *et al.*, 1989). Each domain has distinct active sites, allowing the protein to bridge vesicles to the cytoskeleton (Bahler *et al.*, 1989; Benfenati *et al.*, 1989; Hirokawa *et al.*, 1989). To determine how the various activities of synapsin 1 are likely to be regulated by calmodulin, it is important to identify the site of binding. In this paper, we describe the interaction of synapsin 1 fragments with calmodulin. This allows positioning of the calmodulin binding site within a 11 kDa fragment generated from the head domain by *Staphylococcus aureus* V8 proteinase.

MATERIALS AND METHODS

Preparation of synapsin 1

Synapsin 1 was purified from bovine brain as previously described (Bennett *et al.*, 1985). Synapsin 1 was [^{32}P]phosphorylated using cyclic AMP-dependent protein kinase (Ueda & Greengard, 1977). This method specifically introduces a ^{32}P label at Ser-8 in the head domain (Czernic *et al.*, 1987). Synapsin 1 concentrations were estimated spectrophotometrically (Ueda & Greengard, 1977).

Abbreviations used: DTT, dithiothreitol; EGS, ethylene glycol bis(succinic acid *N*-hydroxysuccinimide ester).

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Preparation of calmodulin, ^{125}I -calmodulin and calmodulin affinity columns

Calmodulin was purified from bovine brain as described by Gopalakrishna & Anderson (1982), except that before phenyl-Sepharose chromatography the brain extract was extensively dialysed against 50 mM-Tris/HCl/1 mM- CaCl_2 /1 mM-dithiothreitol (DTT), pH 7.5. This step allows S100 protein (10 kDa) to diffuse out of the dialysis bag; if this is not done, S100 is present as a major contaminant in the calmodulin eluted from the phenyl-Sepharose column. Calmodulin concentrations were estimated spectrophotometrically (Jarrett & Penniston, 1977). ^{125}I -labelled calmodulin was prepared using ^{125}I -Bolton-Hunter reagent (Amersham), by the method of Agre *et al.* (1983). The specific radioactivity was 17 $\mu\text{Ci}/\text{mg}$. Azido- ^{125}I -calmodulin was prepared as described by Andreasen *et al.* (1981). Columns of calmodulin-Sepharose were prepared using activated CH-Sepharose (Pharmacia-LKB) according to the manufacturers' instructions, using between 6 and 7 mg calmodulin/g (dry wt.) of CH-Sepharose.

Preparation of synapsin 1 proteolytic fragments

Collagenase digestion was done as described by Ueda & Greengard (1977); in these experiments *Clostridium histolyticum* collagenase (Sigma), specificity -Pro-Xaa-Gly-Pro-, was used. For glutamate-specific cleavage, synapsin 1 in 100 mM- $(\text{NH}_4)\text{HCO}_3$ (pH 7.8)/1 mM-EDTA or in 100 mM-Na Hepes/1 mM-EDTA (pH 7.4) was digested for 45–90 min at 37 °C with *S. aureus* V8 proteinase at an enzyme/substrate ratio of 1:30 (Drapeau, 1976). Identical patterns of cleavage were obtained in each buffer. Digestion was stopped by addition of di-isopropyl fluorophosphate to a final concentration of 1 mM.

Affinity chromatography

Synapsin 1 or proteolytic fragments were dialysed into buffer A [500 mM-NaCl/20 mM-Hepes/0.2 mM- CaCl_2 /0.2 mM-DTT/0.02% (w/v) NaN_3 , pH 7.4]. These were applied to a 2 ml column of calmodulin-Sepharose (equilibrated in buffer A) and washed with 10 column vol. of buffer A. The column was then washed with 10–20 column vol. of buffer A. Bound protein was eluted from the column with buffer B (buffer A with 5 mM-EGTA but no CaCl_2). In initial experiments, 100 mM-NaCl was used in all buffers and washes).

Cross-linking

1. **EGS [ethylene glycol bis(succinic acid *N*-hydroxysuccinimide ester)]**. EGS (Sigma) was added from a 50 mM solution in dimethyl sulphoxide to calmodulin mixed with either synapsin 1 or its proteolytic fragments in 50 mM-Hepes (pH 7.4)/500 mM-NaCl with either 1 mM- CaCl_2 or 1 mM-EGTA as indicated in Figure legends. Incubations were at 23 °C for 1 h, and were stopped by the addition of a saturated glycine solution.

2. **Azido-calmodulin**. Azido- ^{125}I -calmodulin (4 $\mu\text{g}/\text{ml}$) was incubated with synapsin 1 (0.4 mg/ml) for 1 h at 4 °C in 50 mM-Hepes (pH 7.4)/500 mM-NaCl with either 1 mM- CaCl_2 or 1 mM-EGTA. To activate cross-linking, samples were irradiated with a Mineralight UVS-11 positioned 1 cm above the sample for 3 min. Samples were then immediately prepared for SDS/PAGE. Comparison of the two cross-linkers suggested that much higher sensitivity in generating cross-linked complexes could be obtained with EGS, which was used for the experiments reported here.

SDS/PAGE and autoradiography

SDS/PAGE (Laemmli, 1970) was carried out using 8% or 11–18% (w/v) linear gradient polyacrylamide minigels

(8 cm $\times$ 10 cm). For autoradiography, Coomassie Blue-stained gels were dried on to Whatman 3MM paper and exposed to X-ray film for up to 18 h. Autoradiographs and Coomassie Blue-stained gels were quantified by densitometry, ensuring linearity of film or dye response.

RESULTS

Binding of calmodulin to synapsin 1

Two methods for detecting interactions between calmodulin and synapsin 1 were used. Okabe & Sobue (1987) showed that synapsin 1 could bind to calmodulin affinity columns in the presence of Ca^{2+} , and that it could be recovered by elution with EGTA. We have confirmed this result (results not shown). We have also used chemical cross-linking to identify complexes. Cross-linking has been used by many investigators to identify complexes between calmodulin and its targets (e.g. Laporte *et al.*, 1979; Andreasen *et al.*, 1981; Agre *et al.*, 1983; Burns & Gratzner, 1985). In this study we used calmodulin labelled with ^{125}I to provide a sensitive probe for complexes which were generated either by using a photoactivatable cross-linker (Andreasen *et al.*, 1981) or by using the bifunctional reagent EGS, which cross-links amino groups. In both cases Ca^{2+} -dependent complexes were formed; these were detected by autoradiography as bands of 93 kDa on SDS/PAGE (Fig. 1). Note that calmodulin cross-linked alone generated no such complex; neither were cross-linked calmodulin oligomers seen. These results confirm that calmodulin binds synapsin 1. In all the cross-linking experiments we used 500 mM-NaCl to reduce binding in the absence of Ca^{2+} . In experiments using no added NaCl, some binding in EGTA occurred. This is unsurprising, since calmodulin (pI 4.3) and synapsin 1 (pI 10.7) are very strongly charged at pH 7.4. NaCl (500 mM) was also included in affinity chromatography experi-

Fig. 1. Ca^{2+} -dependent cross-linking between synapsin 1 and ^{125}I -calmodulin

^{125}I -Calmodulin (0.5 mg/ml) in the presence of 0.2 mM- CaCl_2 (lanes 1 and 2) or 1 mM-EGTA (lanes 3 and 4) was mixed with synapsin 1 (0.3 mg/ml; lanes 1 and 3) or buffer alone (lanes 2 and 4), as described in the Materials and methods section. EGS, a cross-linker, was added (0.1 mM final concentration) and cross-linking was allowed to proceed for 60 min at 23 °C. The reaction was stopped by the addition of a saturated glycine solution (10 μl added to 100 μl of reaction mixture), and samples were analysed by SDS/PAGE and autoradiography. Autoradiographs are shown. In lane 1, a band at 93 kDa shows ^{125}I associated with a cross-linked complex between synapsin 1 (73 kDa) and ^{125}I -calmodulin (17 kDa). In lane 3, no complex is found in the presence of EGTA. In the absence of added synapsin 1, no ^{125}I -calmodulin complexes are formed. Identical results were obtained with a photoaffinity-labelled calmodulin (results not shown).

Fig. 2. Interaction of the 48 kDa head domain of synapsin 1 with a calmodulin-Sepharose column

Synapsin 1 (0.3 mg/ml) was digested with collagenase (100 units/ml) for 30 min at 23 °C; the reaction was stopped by the addition of diisopropyl fluorophosphate and dialysis of the mixture against buffer A and 1 mM-DTT. Non-diffusible material was chromatographed on calmodulin-Sepharose. Column fractions were analysed by SDS/PAGE. (a) Polypeptides flowing through the column in Ca^{2+} ; (b) polypeptides recovered from the column after extensive washing in Ca^{2+} and elution in EGTA. Note that the 48 kDa head domain of synapsin 1 (marked H) is recovered in the EGTA eluate. Collagenase (marked C) in the digest mixture flows through the column unretarded.

ments, since otherwise yields were extremely poor (cf. Okabe & Sobue, 1987). The use of high salt concentrations is widely accepted in investigating calmodulin complexes, but the observation of interactions between calmodulin and synapsin 1 in the absence of Ca^{2+} raises the possibility of interactions *in vivo* which do not require raised cytosolic Ca^{2+} .

Interaction of the collagenase head fragment with calmodulin

A 48 kDa synapsin 1 fragment representing the synapsin 1 head domain can conveniently be generated by collagenase (Ueda & Greengard, 1977). Since there appears only to be a single site for Ca^{2+} -dependent calmodulin binding in synapsin 1, if the 48 kDa collagenase fragment interacts with calmodulin, the site must be in the head. Affinity chromatography of the 48 kDa fragment on calmodulin-Sepharose showed a Ca^{2+} -dependent interaction (Fig. 2). We conclude that the head contains the Ca^{2+} -dependent calmodulin-binding site. The affinity step yielded a highly purified 48 kDa fragment, free of residual collagenase. This is a convenient and quick method for purification of the head domain.

Interaction of *S. aureus* V8 proteinase-generated fragments of synapsin 1 with calmodulin

Limited digestion of native synapsin 1 with V8 proteinase yields major fragments of 27 kDa and a 23 kDa, several fragments of 15–17 kDa and several fragments of 10–13 kDa (Fig. 3, lane 1). One 11 kDa fragment contains the site for cyclic AMP-dependent protein kinase (Fig. 3, lane 3). The 27 kDa fragment is collagenase-sensitive and represents the entire tail of synapsin

1b (residues 409–670) and most of the tail of synapsin 1a (residues 409–675) (results not shown). The other fragments arise from the head, since they are not collagenase-sensitive and can also be derived by digestion of purified head with V8 (results not shown). To determine further where the calmodulin site is located, the interactions of V8 fragments with calmodulin were tested by affinity chromatography and by cross-linking.

Affinity chromatography. A 1 ml sample of V8 digest (0.4 mg of starting synapsin 1/ml) was chromatographed on calmodulin-Sepharose. Initially we used buffers containing 100 mM-NaCl throughout the affinity chromatography. The eluates contained 65 kDa, 27 kDa, 23 kDa and 11 kDa fragments (results not shown). To improve the specificity of the column, we included 500 mM-NaCl in all buffers. In this case fewer fragments were found to interact in the presence of Ca^{2+} . The 27 kDa fragment interacted weakly with the column, but was eluted slowly and in a large volume of Ca^{2+} -containing buffer. One major fragment, of 11 kDa, showed an interaction that was dependent on Ca^{2+} (Fig. 3); a 65 kDa fragment also bound to the column. The 11 kDa fragment did not contain the *N*-terminal portion of the protein, since fragments which could be phosphorylated in the presence of cyclic AMP-dependent protein kinase (and thus contain Ser-8) did not bind to the column. The bound 11 kDa calmodulin-binding fragment could not be phosphorylated in the presence of cyclic AMP-dependent protein kinase (Fig. 3).

Cross-linking. EGS cross-linking of ^{125}I -calmodulin to a V8 proteinase digest of synapsin 1 gave a very simple pattern (Fig. 4). Only one band was found in samples cross-linked in the presence of EGTA: this was uncross-linked calmodulin (Fig. 4, lane 3). However, in the presence of Ca^{2+} , an additional band of 28 kDa was found (Fig. 4, lane 1). Taking the molecular mass of calmodulin to be 17 kDa, the fragment cross-linked to calmodulin should be 11 kDa. Note too that in the absence of synapsin 1 fragments no complexes were formed, showing that the 28 kDa band is not a calmodulin dimer (predicted molecular mass approx. 33 kDa). Therefore the two separate methods of cross-linking and affinity chromatography show that the calmodulin-binding site is within a 11 kDa V8 proteinase-generated fragment. Note too that the 27 kDa tail fragment shows no specific binding to calmodulin by either method, in good agreement with initial localization of the binding site to the head. To confirm that the 11 kDa calmodulin-binding fragment cannot be phosphorylated using cyclic AMP-dependent protein kinase, we produced ^{32}P -synapsin 1 by phosphorylation in the presence of cyclic AMP-dependent protein kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Mild V8 digestion of this preparation yielded two major ^{32}P -labelled fragments of 11 kDa and 44 kDa. The ^{32}P -labelled 11 kDa fragment represents the *N*-terminal 121 residues. We deduce that the 44 kDa fragment represents head domain residues 1–Glu-408. The predicted relative molecular mass of residues 1–408 is 44075 kDa. The 27 kDa tail fragment contains two sites for Ca^{2+} /calmodulin-dependent protein kinase 2, and is not phosphorylated under these conditions. In cross-linking with a range of EGS concentrations, we were unable to obtain cross-linking of the 11 kDa ^{32}P -labelled fragment to calmodulin (Fig. 5, lanes 1–4). However, the 44 kDa fragment was efficiently cross-linked in the presence of Ca^{2+} (Fig. 5) to give an approx. 60 kDa product. This result confirms that the calmodulin-binding site is in the head domain, but shows that the 11 kDa calmodulin-binding fragment is not the *N*-terminal 121 residues, identified as the 11 kDa ^{32}P -labelled fragment. Note too that the 44 kDa fragment is also partially cross-linked to produce the 60 kDa product in the presence of EGTA. Part of the interaction of calmodulin with the head domain detected by the

Fig. 3. Interaction of an 11 kDa fragment in a V8 proteinase digest of synapsin 1 with a calmodulin-Sepharose column

Synapsin 1 was digested with V8 proteinase (see the Materials and methods section) for 90 min and chromatographed on calmodulin-Sepharose. Column fractions were analysed by SDS/PAGE. Lane 1 shows SDS/PAGE of polypeptides in the initial digest. Lane 2 shows polypeptides recovered from the column after extensive washing in Ca^{2+} and elution in EGTA. One 11 kDa fragment is recovered in the EGTA eluate with smaller amounts of a 65 kDa fragment. Lanes 3–5: fragments in a V8 digest and in column fractions were phosphorylated using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and cyclic AMP-dependent protein kinase. Lane 3, whole V8 digest; lane 4, pooled breakthrough fractions; lane 5, fragments specifically eluted with EGTA. Autoradiographs are shown. Note that fragments which can be phosphorylated are only recovered in the breakthrough fractions, not in the bound fractions.

Fig. 4. Ca^{2+} -dependent cross-linking between fragments in a V8 proteinase digest of synapsin 1 and ^{125}I -calmodulin

^{125}I -calmodulin (0.5 mg/ml final concentration) in the presence of 0.2 mM- CaCl_2 (lanes 1 and 2) or 1 mM-EGTA (lanes 3 and 4) was mixed with a V8 digest of synapsin 1 (0.3 mg/ml final concentration; lanes 1 and 3) or buffer only (lanes 2 and 4), as described in the Materials and methods section. EGS was added (0.5 mM) and cross-linking was allowed to proceed for 60 min. The reaction was stopped by the addition of a saturated glycine solution (10 μl added to 100 μl of reaction mixture), and samples were analysed by SDS/PAGE and autoradiography. The Figure shows an autoradiograph. In lane 1, a band at 28 kDa shows ^{125}I associated with a cross-linked complex between ^{125}I -calmodulin (17 kDa) and, by inference, an 11 kDa fragment of synapsin 1. In lane 3 no complex is found in the presence of EGTA, and in lanes 2 and 4 no complexes are found in the absence of added fragments.

cross-linking method seems to be Ca^{2+} -independent, even in the presence of 500 mM- NaCl .

We have attempted to identify the 11 kDa fragment by sequencing from an Immobilon blot of peptides in the calmodulin eluate separated by SDS/PAGE (Matsudaira, 1989). On four separate occasions we have been unable to obtain any sequence,

Fig. 5. Ca^{2+} -dependent cross-linking between ^{32}P -labelled fragments in a V8 proteinase digest of synapsin 1 and calmodulin

Synapsin 1 was ^{32}P -phosphorylated using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and cyclic AMP-dependent protein kinase. Mixtures of a 45 min V8 digest of ^{32}P -synapsin 1 (0.3 mg/ml) and calmodulin (0.5 mg/ml) were prepared in the presence of 0.2 mM- CaCl_2 (lanes 1–4) or 1 mM-EGTA (lanes 5–8), as described in the Materials and methods section. EGS was added (lanes 1 and 5, 0.035 mM; lanes 2 and 6, 0.07 mM; lanes 3 and 7, 0.14 mM; lanes 4 and 8, 0.28 mM) and cross-linking was allowed to proceed for 60 min. The reaction was stopped by the addition of a saturated glycine solution (10 μl added to 100 μl of reaction mixture), and samples were analysed by SDS/PAGE and autoradiography. Autoradiographs are shown. Lanes 1–4 show that with increasing concentrations of cross-linker the 44 kDa ^{32}P -labelled fragment becomes incorporated into a 60 kDa complex with calmodulin. The 11 kDa ^{32}P -labelled fragment does not become cross-linked. Cross-linking is apparent at 0.07 mM-EGS. Lanes 5–8 show that the 44 kDa ^{32}P -labelled fragment is incorporated in to the 60 kDa complex much less efficiently in EGTA than in Ca^{2+} , although some cross-linking occurs at higher EGS concentrations. Note that in this case, too, the 11 kDa ^{32}P -labelled fragment is not cross-linked.

probably because the amount obtained on the blot (approx. 10 pmol) was below the sensitivity of the sequencer.

DISCUSSION

In examining the interactions of calmodulin there is a requirement to show the specificity of any interaction. Calmodulin is a 'sticky' hydrophobic and acidic molecule, especially in the presence of Ca^{2+} (Hussain *et al.*, 1985). The evaluation of its interaction with synapsin 1 is difficult, in that synapsin 1 is basic and tends to be hydrophobic. The stickiness of calmodulin is exemplified by the interaction of calmodulin with the tail region of synapsin 1, which does not seem to be Ca^{2+} -dependent, as well as a proportion of the interaction with the head. However, the studies of Okabe & Sobue (1987) showed a 1:1 stoichiometry of binding to intact synapsin 1 and a K_d of 450 nM, which suggests that the interaction may occur *in vivo*, especially considering that synapsin 1 and calmodulin are highly concentrated in nerve terminals. In this paper we have shown that Ca^{2+} -dependent calmodulin binding is restricted to a small (11 kDa) fragment of the head region of synapsin 1. This lends further weight to arguments that the interaction is specific, and not simply a random interaction between two highly charged and hydrophobic molecules. As yet we have been unable to identify the location of the 11 kDa fragment within the head domain by sequencing. However, since calmodulin recognition sequences are only 15–30 residues in length (O'Neill & DeGrado, 1990), the identification of the actual site of binding will require the production of much smaller peptides.

What aspect of synapsin 1 activity is calmodulin likely to

control? The interactions of two cytoskeletal filament systems, microfilaments and microtubules, with synapsin 1 appear to occur in both the head and tail regions (Bahler *et al.*, 1989; Hirokawa *et al.*, 1989; Aubert-Foucher & Font, 1990); these interactions are candidates for regulation by calmodulin. However, it is also of interest that the head can interact with phospholipids (Benfenati *et al.*, 1989). Several stretches of sequence within the head are predicted to form 'surface-seeking' amphipathic α -helices which may interact with phospholipids (Sudhof *et al.*, 1989). Such helices, if they were strongly basic, might well be able to interact with calmodulin. It will be of great interest to establish whether the interaction of synapsin 1 with the lipids of synaptic vesicle membranes is under regulation by direct interaction with calmodulin.

We and Okabe & Sobue (1987) have used 500 mM-NaCl throughout as a method of decreasing Ca^{2+} -independent interactions of synapsin 1 with calmodulin. We have noted that the 27 kDa tail fragment shows weak binding to calmodulin affinity columns; we suggest that this represents a charge-charge interaction, since it can mostly be eliminated by high concentrations of NaCl. Several investigations of the interactions of brain proteins with calmodulin have noted some that are Ca^{2+} -independent (e.g. Grand & Perry, 1979). Hooper & Kelly (1984) found that calmodulin was tightly associated with synaptic vesicles independently of Ca^{2+} . Although calmodulin binding to the tail does not satisfy normal criteria for specific interaction, in a highly condensed system such as a nerve terminal it is conceivable that binding occurs *in vivo*.

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