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COMPARISON OF CALMODULIN BINDING TO BRAIN SYNAPTIC AND COATED VESICLES *

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Several characteristics of calmodulin association with brain synaptic and coated vesicles were analyzed and compared. Radioimmunoassay revealed that both classes of vesicles contain approx. 1 μg of calmodulin per mg of vesicle protein. Discontinuous sucrose gradients revealed that coated and synaptic vesicles preparations were homogeneous and had different sedimentation properties. Binding of ^{125}I-labeled calmodulin to synaptic and coated vesicles was Ca^{2+} dependent and displaced by unlabeled calmodulin but not by troponin-C. Scatchard analysis revealed the presence of two binding sites. In both vesicle types there was one high-affinity, low-binding-capacity site ($K_d = 1–39$ nM and $B_{max} = 4–16$ pmol/mg) and one low-affinity, high-binding-capacity site ($K_d = 102–177$ nM and $B_{max} = 151–202$ pmol/mg). ($Ca^{2+} + Mg^{2+}$)-ATPase activity was stimulated in both synaptic and coated vesicles by calmodulin. Thus synaptic and coated vesicles may possess similar calmodulin binding sites.

Introduction

Synaptic and coated vesicles are located in peripheral [1,2] and central [3] axon terminals. It has been proposed that upon an increase of axon terminal Ca^{2+} concentration, synaptic vesicles fuse with the presynaptic plasma membrane and release quantal packets of neurotransmitter [4–6]. It is hypothesized that synaptic vesicle membrane is selectively retrieved by coated pits which invaginate, pinch off and become coated vesicles which, in turn, may shed their coats to become synaptic vesicles [7]. In addition, both vesicle types express ($Ca^{2+} + Mg^{2+}$)-ATPase activity which functions in Ca^{2+} sequestration [8,9].

Calmodulin, a multifunctional Ca^{2+}-binding protein [10] is bound to synaptic and coated vesicles [11–13]. In synaptic vesicles, calmodulin activates protein phosphorylation concomitantly with neurotransmitter release [11]. Calmodulin has been detected in cultured β-lymphoblastoid coated vesicles and implicated in the recruitment of coat material to specific membrane receptors [14].

In view of the relationship between synaptic and coated vesicles in axon terminals, we characterized and compared aspects of their association with calmodulin employing radioimmunoassay, binding and enzymatic determinations. The data suggest that similar binding sites for calmodulin exist on both vesicle types. Calmodulin stimulation of ($Ca^{2+} + Mg^{2+}$)-ATPase in both

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate.

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vesicles suggests that this ATPase may represent one of the common calmodulin binding sites.

Experimental Procedures

Materials. [γ-^{32}P]ATP (spec. act. 8–10 mCi/μmol) was purchased from New England Nuclear, Massachusetts. ^{125}I-labeled calmodulin was a generous gift from D.M. Watterson of The Rockefeller University, New York. Sodium dodecyl sulfate and polyacrylamide were from Bio-Rad Lab., CA. All other reagents were of analytical grade I. Fresh bovine brain and chicken gizzard tissue were delivered in ice from a local slaughterhouse. Calmodulin was prepared from chicken gizzards as described by Jamieson and Vanaman [15].

Subcellular fractionation. The grey matter of fresh bovine brain was separated from white matter by suction. All subsequent steps were performed at 4°C. Highly purified coated vesicles were prepared as described by Keen et al. [16]. Synaptic vesicles were prepared adapting the technique described by DeLorenzo et al. [17]. Calmodulin-retaining vesicles were prepared in the absence of chelators. Calmodulin-depleted vesicles were prepared by suspending vesicles in 0.1 M Mes buffer, pH 6.5, containing 2 mM EGTA, 2 mM EDTA and 1 mM 2-mercaptoethanol (buffer A) and centrifuging at 150000 $\times g$ for 30 min. This step was repeated once. The final pellet was resuspended in 0.1 M Mes, 1 mM 2-mercaptoethanol, pH 6.5 (buffer B) and dialyzed against the same buffer for 16 h. Vesicles were viewed under a JEOL microscope. Their polypeptide composition was analyzed by SDS-polyacrylamide gel electrophoresis using the buffer system of Laemmli [18]; a 4% polyacrylamide stacking gel and a 5.0–15% linear gradient for the separating gel. The gel was run for 16 h at 7.0 mA.

Sedimentation of vesicles in discontinuous sucrose gradients and Ca^{2+} dependence of calmodulin binding. Approx. 4–6 mg of vesicles were incubated for 2 h at 25°C with 17–20 μg ^{125}I-labeled calmodulin in the presence of 10^{-4} M $CaCl_2$, or 10^{-3} EGTA, and brought to a final volume of 3 ml with buffer A. The samples were layered on discontinuous sucrose gradients. Each layer had a volume of 5 ml and consisted of the following sucrose concentrations (w/v): 10%, 20%, 30%, 40%, 50% and 60% for coated vesicles, and 20%, 30%, 40%, 50%, 55%, 60% and 70% for synaptic vesicles. Samples prelayered with EGTA were layered on sucrose gradients containing 10^{-4} EGTA. Gradients were centrifuged at 80000 $\times g$ for 90 min in a SW-27 swinging bucket rotor. Aliquots of 1.5 ml were withdrawn sequentially and absorbance at 280 nm and radioactivity were determined.

Glass pore bead chromatography of synaptic vesicles. To further assess the purity of the synaptic vesicles approx. 2–3 mg of synaptic vesicles were resuspended in 2 ml 10 mM Mes, pH 6.5, containing 0.2 M sucrose and 0.19 M NaCl (buffer C). This was loaded onto a controlled pore glass column (1.5 $\times$ 90 cm) [19] and chromatographed at a rate of 14 ml/h with buffer C. Absorbance at 280 nm was determined for the fractions (2 ml). The polypeptide composition of the vesicles obtained were analyzed by SDS gel electrophoresis using a 4.5–15% exponential gradient for the separating gel and run at 6.5 mA.

Calmodulin quantitation. Competition radioimmunoassay for calmodulin in vesicles prior to calmodulin depletion was performed using chicken gizzard calmodulin, ^{125}I-labeled calmodulin antibodies prepared as described by Van Eldick et al. [20]. Binding of ^{125}I-labeled calmodulin was determined on calmodulin-depleted synaptic and coated vesicles.

Calmodulin binding affinity. A range of 0.1–22.0 μg ^{125}I-labeled calmodulin was incubated for 2 h at 25°C with 0.5 mg of synaptic or coated vesicles in the presence of 1 mM Ca^{2+} in a final volume of 1 ml buffer B. Maximum binding of calmodulin to vesicles occurred under these conditions. Samples were centrifuged at 150000 $\times g$ for 30 min at 4°C. Radioactivity in the pellets was determined.

^{125}I-labeled calmodulin binding competition. A series of 0.4 mg aliquots of vesicles were incubated for 2 h at 25°C with increasing amounts of unlabeled calmodulin or troponin-C (0–10000 ng range) in the presence of 1 mM Ca^{2+}. Radiolabeled calmodulin (10 ng) was added to all samples and incubated for an additional 2 h. Subsequent steps were identical to those described for calmodulin binding affinity. Troponin-C was isolated from rabbit striated muscle as described [21].

ATPase determinations. (Ca^{2+} + Mg^{2+})-ATPase

activity was determined by the release of $^{32}P_i$ from ATP as described [22]. Synaptic or coated vesicles containing 0.5 mg total protein were suspended in 40 mM Tris-HCl buffer, pH 7.4, containing 0.2 mM Ca^{2+}, 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM ouabain with or without 1.0 μg calmodulin. Samples were preincubated for 30 min at 37°C prior to addition of 0.4 mM ATP. ATP hydrolysis proceeded for 45 min at 37°C and samples were withdrawn at various time intervals.

Miscellaneous procedures. Protein concentrations were determined by the method of Lowry et al. [23] using bovine serum albumin as standard.

Results and Discussion

Synaptic vesicles viewed by electron microscopy were homogeneous and approx. 400 Å in diameter (Fig. 1a). Coated vesicles also were homogeneous with few membrane fragments, and their diameters measured between 500 and 700 Å (Fig. 1b).

The polypeptide composition of synaptic and coated vesicles as determined by 5–15% SDS-polyacrylamide gel electrophoresis reveals that clathrin, a 180 kDa polypeptide, was detected only in the coated vesicle preparation (Fig. 2).

The sedimentation profiles of synaptic and coated vesicles in discontinuous sucrose gradients is illustrated in Figs. 3a and 3b, respectively. Synaptic and coated vesicles equilibrated in the 60% and 30–40% sucrose concentration regions, respectively. The fact that both vesicle types sedimented in one major region along with the different sedimentation patterns observed for synaptic and coated vesicles implied that the vesicles were not substantially cross contaminated with each other and that they were relatively homogeneous. The sedimentation of ^{125}I-labeled calmodulin with both synaptic and coated vesicles (Fig. 3) only in the presence of Ca^{2+}, indicated that calmodulin bind-

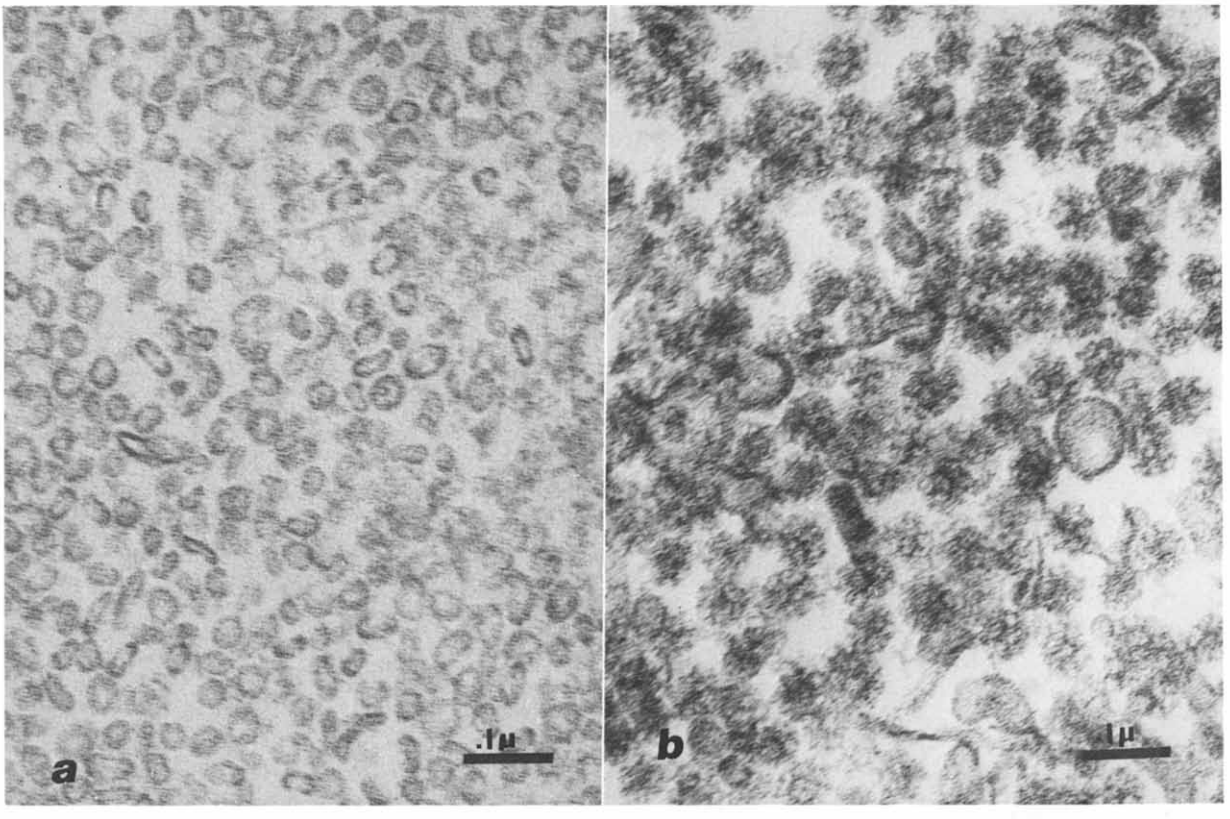

Fig. 1. Electron micrographs of synaptic (a) and coated (b) vesicles. Magnification ×120000.

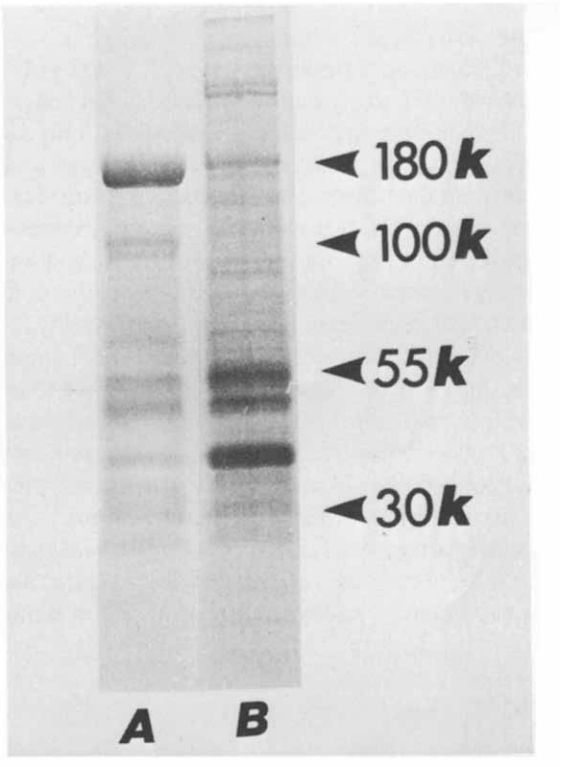

Fig. 2. Polypeptide composition of coated vesicles (A) and synaptic vesicles (B) as determined by 5–15% SDS linear polyacrylamide gel electrophoresis.

ing to the vesicles was Ca^{2+} dependent.

The elution profile of the synaptic vesicle preparation on a controlled pore glass bead column is illustrated in Fig. 4a. It is apparent that the vesicles separated into two peaks. SDS-polyacrylamide gel electrophoresis analysis (Fig. 4b) revealed that the polypeptide composition of the synaptic vesicles prior to chromatography and the vesicles obtained in both peaks were virtually identical. A small amount of a protein with M_r of approx. 180000 seemed to be more concentrated in the second peak. Electron microscopy revealed that the vesicles were separated by virtue of their state of aggregation with no observable differences in degree of membrane contamination (data not shown). The vesicles in the first peak were more aggregated than the vesicles in the second peak. Because a fraction of the vesicles was partly aggregated it is likely that the entire vesicle population cosedimented in the discontinuous sucrose gradient to the 60% sucrose concentration region.

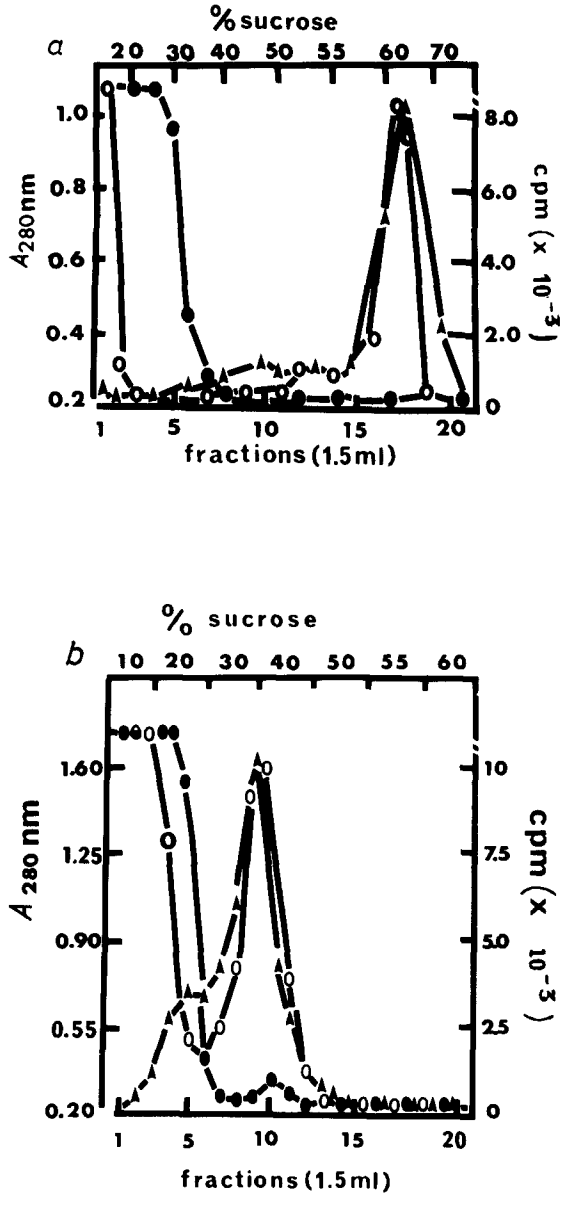

Fig. 3. (a) Sucrose gradient profile of synaptic vesicles. Calmodulin-depleted synaptic vesicles (6.0 mg of total proteins) were incubated with 25 μg ^{125}I-labeled calmodulin (total cpm = 100262) in the presence of 10^{-4} M $CaCl_2$ or 10^{-3} M EGTA and centrifuged at 80000 × *g*. Aliquots of 1.5 ml were withdrawn and the values measured are plotted as a function of absorbance at 280 nm (▲) and cpm (×10^{-3}). ○, cpm in the presence of 10^{-4} M Ca^{2+}; ●, cpm in the presence of 10^{-3} M EGTA. (b) Sucrose gradient profile of coated vesicles. Calmodulin-coated vesicles (4.5 mg total protein) were incubated with ^{125}I-labeled calmodulin and treated as described in legend for Fig. 3a. ▲, absorbance at 280 nm; ○, cpm in the presence of 10^{-4} M Ca^{2+}; ●, cpm in the presence of 10^{-3} EGTA.

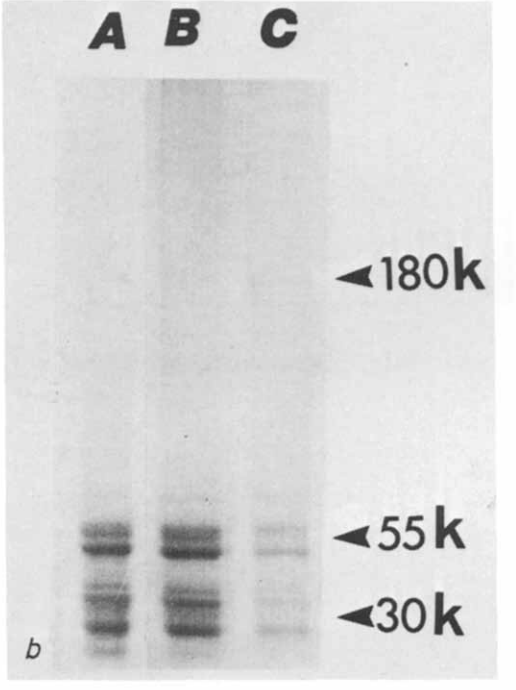

Fig. 4. (a) Elution profile of synaptic vesicle preparation by controlled pore glass bead chromatography. Absorbance at 280 nm was read in every fraction collected. (b) Polypeptide composition of synaptic vesicles prior to glass pore bead chromatography (A) and synaptic vesicles obtained after chromatography; peak I (B) and II (C) as determined by 4.5–15% SDS exponential polyacrylamide gel electrophoresis.

Based on the criteria we employed, both synaptic and coated vesicle preparations appeared relatively pure. We cannot, however, rule out the possibility of minor degrees of cross-vesicle contamination and/or contamination with other membrane systems.

Calmodulin radioimmunoassay competition curves for chicken gizzard calmodulin, and synaptic and coated vesicles are displayed in Fig. 5. As determined by this procedure synaptic and coated vesicles contain 0.98 and 1.05 μg immunoreactive calmodulin/mg protein, respectively. When synaptic and coated vesicles were treated to remove their endogenous calmodulin, calmodulin content decreased below the limit of detection.

The effects of increasing concentrations of troponin-C and unlabeled calmodulin on the binding of ^{125}I-labeled calmodulin to synaptic and coated vesicles is illustrated in Figs. 6a and 6b, respectively. Troponin-C, a homologous Ca^{2+}-binding protein found in striated muscle [24], inhibited 12% and 2% of ^{125}I-labeled calmodulin binding at the maximum concentrations used in synaptic and coated vesicles, respectively. Un-

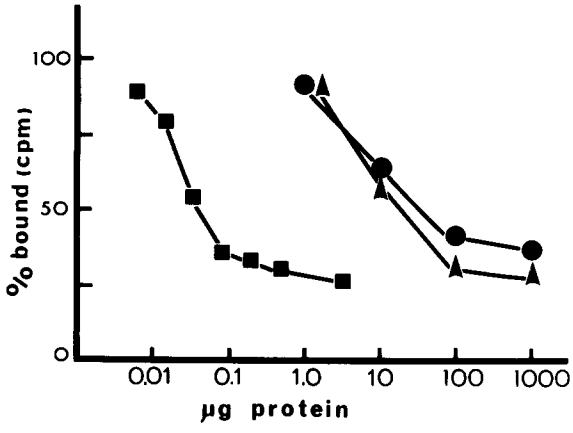

Fig. 5. Competition radioimmunoassay curves for chicken gizzard calmodulin (■), synaptic (●) and coated vesicles (▲). Reaction mixtures containing a limiting dilution of anti-calmodulin sera and varying concentrations of unlabeled calmodulin, synaptic or coated vesicles were incubated at 4°C for 8 h. ^{125}I-labeled calmodulin was added and the solution incubated at 4°C for an additional 8 h. Goat anti-rabbit serum was then added and the mixtures incubated at 4°C for an additional 8–24 h. The mixtures were washed and then centrifuged at 900× *g* for 10 min. This procedure was repeated once. Radioactivity in the pellets was determined. Calmodulin concentration in synaptic and coated vesicles was quantitated by comparing the amounts of vesicle protein and unlabeled calmodulin protein that inhibited 50% of ^{125}I-labeled calmodulin antibody binding.

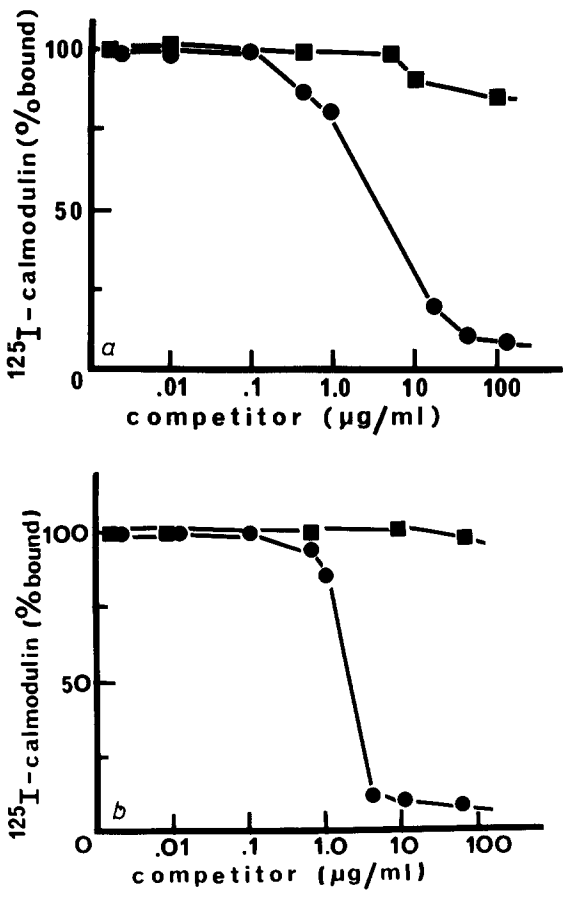

Fig. 6. Competition of unlabeled calmodulin and troponin-C with ^{125}I-labeled calmodulin in synaptic (a) and coated (b) vesicles. A series of 0.4 mg total protein of synaptic or coated vesicles were incubated with a range of 0–100 μg of troponin-C or unlabeled calmodulin for 2 h at 25°C. ^{125}I-labeled calmodulin (10 ng) was added and the suspension incubated for an additional 2 h in the presence of Ca^{2+}. The samples were centrifuged at 150000×*g* for 30 min and radioactivity in the pellet measured. ^{125}I-labeled calmodulin (% bound) is plotted as a function of competing concentrations of calmodulin (●) and troponin-C (■).

labeled calmodulin blocked 89% and 88% of ^{125}I-labeled calmodulin binding at the highest concentrations used in synaptic and coated vesicles, respectively. The concentration of unlabeled calmodulin which inhibited 50% of ^{125}I-labeled calmodulin binding (IC_{50}) was 400 and 300 nM for synaptic and coated vesicles, respectively. The relatively ineffective competition of troponin-C for radio-labeled binding sites implied that the sites were specific for calmodulin.

A Scatchard plot of specific ^{125}I-labeled calmodulin binding to synaptic and coated vesicles is illustrated in Figs. 7a and 7b, respectively. Levels of non-specific binding determined from the aforementioned competition studies were subtracted from the total binding to calculate specific binding. Scatchard plots for both synaptic and coated vesicles displayed curves with downward slopes not corresponding to straight lines, suggesting more than one binding site. Affinity values obtained,

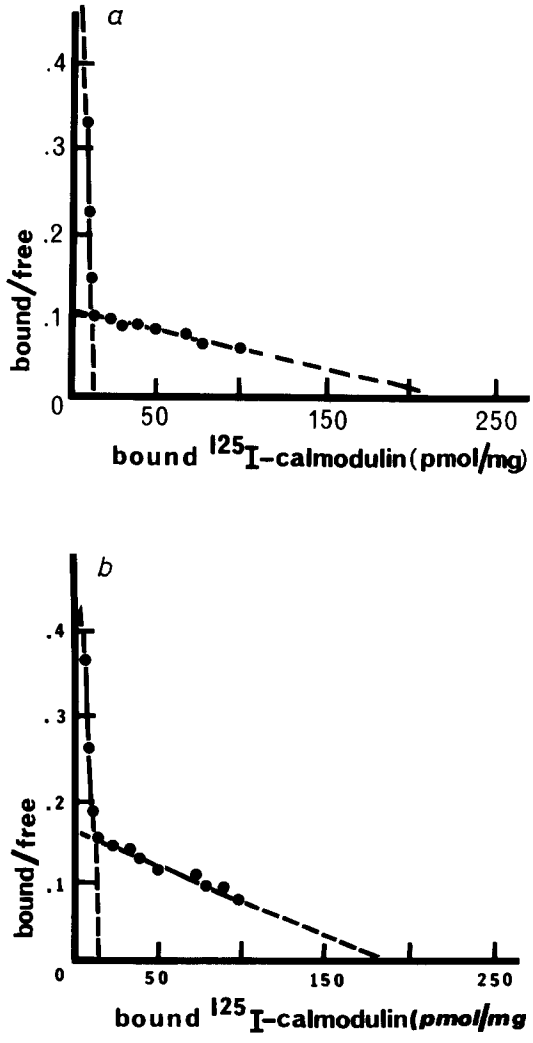

Fig. 7. Scatchard analysis of specific ^{125}I-labeled calmodulin binding to synaptic (a) and coated (b) vesicles. Data points fit two straight lines indicating more than one binding site. Dashed lines are extrapolations dederived from the solid lines constructed between the experimental data points.

based on the assumption of two existing binding sites, are presented in Table I. The K_d values for both sites are similar for the two types of vesicle. The B_{max} values obtained for each site and the B_{max} total for both vesicle types also are comparable. There appeared to be one high-affinity, low-binding-capacity site, and one low-affinity, high-binding-capacity site. It has been demonstrated that calmodulin binds to several coated vesicle proteins [12]. Thus far, however, only one functional binding site, a protein kinase, has been reported in synaptic vesicles [11].

The similar nature of calmodulin binding to both vesicle types, particularly similar K_d values, implied that calmodulin bound to similar sites in both vesicles. Results from our laboratory indicate that calmodulin did not bind to clathrin, the major coat protein in coated vesicles [25].

$(Ca^{2+} + Mg^{2+})$-ATPase activity for both types of vesicles is illustrated in Fig. 8. The effect of calmodulin on synaptic and coated vesicle ATPase was always stimulatory. At 45 min, ATP hydrolysis was stimulated 50% by calmodulin in both vesicle types. Levels of stimulation varied within a certain range with different preparations. On a mg-protein basis, and under the conditions tested, coated vesicles always exhibited a higher level of ATP hydrolysis than synaptic vesicles. This may indicate a more important role performed by coated vesicles in calcium ion sequestration. Our results [25] do not confirm the observation that coated vesicles lack $(Ca^{2+} + Mg^{2+})$-ATPase activity [26]. Linearity was observed up to 10 min of enzymatic incubation. Beyond this point, the rate of ATP hydrolysis decreased. The moderate level

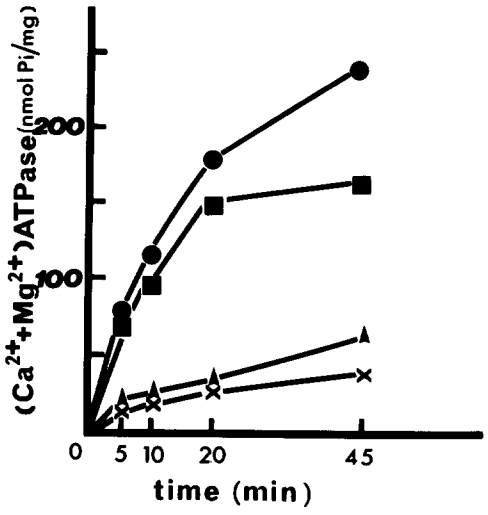

Fig. 8. $(Ca^{2+} + Mg^{2+})$-ATPase activity. ATP hydrolysis was measured by the release of $^{32}P_i$ from ATP. Denoted are synaptic vesicles incubated without calmodulin (×), synaptic vesicles incubated with calmodulin (▲), coated vesicles incubated without calmodulin (■), and coated vesicles incubated with calmodulin (●).

of calmodulin activation by calmodulin of this ATPase is comparable to that found in synaptic plasma membrane [27]. Similar levels of calmodulin activation of erythrocyte Ca^{2+} uptake were found by Sarkedi et al. [28]. A time lag in calmodulin stimulation of the ATPase in both brain vesicle types was observed. This delayed activation is similar to that reported for erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase [29]. It is possible therefore that calmodulin bound to and activated a $(Ca^{2+} + Mg^{2+})$-ATPase in both vesicle types. The fact that there was a time lag in calmodulin-

TABLE 1

AFFINITY VALUES OF SPECIFIC ^{125}I-LABELED CALMODULIN BINDING TO SYNAPTIC AND COATED VESICLES

Comparison of K_d and B_{max} values obtained from Scatchard analysis of specific ^{125}I-labeled calmodulin binding to synaptic and coated vesicles shown in Figs. 2a and 2b.

Vesicle type	Site I		Site II		B_{max} total (pmol/mg)
	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	
Synaptic	1.0	4.1	177	202	206
Coated	3.9	16.3	102	151	167

ATPase activation in conjunction with a moderate level of stimulation suggested that the (Ca^{2+} + Mg^{2+})-ATPase may represent the low-affinity, high-binding-capacity site, site II.

Site I in synaptic vesicles may represent the calmodulin-dependent protein kinase previously implicated in neurotransmitter release [11]. This is strengthened by the recent finding that a 30 kDa protein common to both synaptic and coated vesicles binds to calmodulin and is a protein kinase [30]. Recently it was demonstrated that synaptic vesicles manifest calmodulin-dependent phospholipase A_2 activity which was implicated in in vitro synaptic vesicle interactions [31]. It is possible that synaptic vesicle phospholipase A_2 may present one of the synaptic vesicle calmodulin binding sites. Calmodulin binding to site I in coated vesicles may be related to endocytosis, recycling of membrane or recruitment of clathrin to form coated pits and/or vesicles. The latter function has been implicated by Salisbury et al. [14]. Thus calmodulin may be involved in the modulation of brain synaptic and coated vesicle function.

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