Calmodulin Is Tightly Associated with Synaptic Vesicles Independent of Calcium*

Joan E. Hooper† and Regis B. Kelly

From the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

A protein in highly purified synaptic vesicles from elasmobranch electric organ is recognized by two specific antisera that recognize different determinants of calmodulin. The protein is indistinguishable from authentic calmodulin by migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of calcium. It is tightly associated with the intact synaptic vesicle membrane even in the absence of calcium. It is on vesicles rather than membrane contaminants and cytoplasmically oriented since membrane immunoprecipitates at least 86% of intact synaptic vesicles. Surprisingly, another calmodulin antiserum (rabbit anti-calmodulin serum) specifically precipitates less than 20% of the intact vesicles. This antiserae (rabbit anti-calmodulin serum) also detects 4-15 times less calmodulin immunoreactivity than sheep anti-calmodulin antibody by radioimmunoassay of vesicles solubilized with non-denaturing detergents. The difference essentially disappears if the vesicle calmodulin is solubilized in sodium dodecyl sulfate. We suggest that the antigenic determinant recognized by rabbit anti-calmodulin serum is concealed in vesicle-associated calmodulin and may be involved in binding calmodulin to the vesicle.

MATERIALS -Because of erratic availability, we have used electric organs from two species of elasmobranchs, Discopyge ornata and Narcine brasiliensis. We have detected no differences in the biochemical or immunological properties of these two species. Synaptic vesicles were purified from the electric organ using a modification of the procedure described by Miljanich et al. (20). SoCaM, an affinity-purified antibody raised in sheep against native rat testes calmodulin (21), was purchased from CAABCO, Inc. (lot 188-011281, Houston, TX). RoCaM, a serum (654) raised in rabbit against bovine brain calmodulin, was generously provided by Dr. L. Van Eldik (Vanderbilt University, Nashville, TN).

Calmodulin is a ubiquitous calcium-binding protein known to mediate the calcium dependency of a wide variety of calcium-regulated enzymes (for review see Ref. 1). Calmodulin interaction with secretory granule or plasma membrane components is especially intriguing. Its physicochemical properties closely resemble those predicted for a "calcium effector" by electrophysiological studies of transmitter release of the nerve terminal (2). Trifluoperazine, a calmodulin antagonist, blocks secretion at a step distal to calcium entry in sea urchin eggs (3), chromaffin cells (4), and synapticosomes (5). In all of these systems, the relevant calmodulin target remains to be identified and its role or mechanism is speculative. Calmodulin-activated protein kinases are found in synaptic vesicle preparations (6, 7) and chromaffin granule membrane preparations (8), but an involvement of the resultant phosphoproteins in exocytosis has not been shown.

Many calmodulin-regulated enzymes such as cyclic nucleotide phosphodiesterase (9) require the calcium complexed form of calmodulin for activation. The physical basis for the calcium requirement is illustrated by the case of troponin I, for which free calmodulin and calmodulin-Ca"" have a 3500-fold difference in affinity (70 μM and 20 nM, respectively (10)). These interactions between calmodulin-Ca"" and its targets are inhibited by phenothiazines, such as trifluoperazine, which compete with targets for binding to calmodulin-Ca"" (11). Calcium-dependent calmodulin binding to unidentified targets has been reported in several membrane preparations including synaptic vesicles (7, 12) and secretory granules (8, 13).

High affinity calmodulin-target interactions in the absence of calcium have been reported. In the absence of calcium (≤10^-5 M), the adenylate cyclase of Bordetella pertussis is stimulated by calmodulin with a K_a = 2.4 × 10^-6 M (14). While the sensitivity of this enzyme to calmodulin increases in the presence of calcium, its high affinity activity in the absence of calcium implies a physiological role for this calmodulin cyclase interaction. Calmodulin is a permanently associated subunit of phosphorylase kinase. Trifluoperazine has little inhibitory effect on the calcium-dependent kinase activity of this kinase (15, 16). Calmodulin is bound to a 110,000-dalton protein from intestinal cytoskeleton in the absence of calcium but is dissociated by phenothiazine (17). Calmodulin is associated with gap junctions in the absence of calcium (18). There are several reports of a minor calcium-independent component in studies of the binding of 125I-calmodulin to secretory membranes, but a specific interaction has been demonstrated in only one case. Geisow and Burgoyne (19) report a saturable, high affinity (K_D = 31 nM) calmodulin-binding site in chromatin granule membranes which is seen at less than 10^-8 M Ca"" but disappears when Ca"" is raised to 10^-4 M.

The present experiments demonstrate calmodulin on the outside surface of synaptic vesicles extensively purified in the presence of EGTA. Unlike the calmodulin-binding site described in the accompanying paper (12), this association of calmodulin with vesicles is entirely calcium-independent.

EXPERIMENTAL PROCEDURES

Materials -Because of erratic availability, we have used electric organs from two species of elasmobranchs, Discopyge ornata and Narcine brasiliensis. We have detected no differences in the biochemical or immunological properties of these two species. Synaptic vesicles were purified from the electric organ as described in the preceding paper (12). Synaptosomes were prepared from Discopyge electric organ using a modification of the procedure described by Miljanich et al. (20). SoCaM, an affinity-purified antibody raised in sheep against native rat testes calmodulin (21), was purchased from CAABCO, Inc. (lot 188-011281, Houston, TX). RoCaM, a serum (654) raised in rabbit against bovine brain calmodulin, was generously provided by Dr. L. Van Eldik (Vanderbilt University, Nashville, TN). The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, Na dodecyl SO_4; RoCaM, rabbit anti-calmodulin serum; SoCaM, sheep anti-calmodulin antibody; RoSV, rabbit anti-synaptic vesicle serum; RA, radioimmunoassay; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; BSA, bovine serum albumin.
Calcium-independent Vesicle Calmodulin

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Pig brain calmodulin was kindly provided by Dr. L. Kelly (University of California, Berkeley). The purification of calmodulin from Narcine electric organ was as described by Hooper and Kelly (12). Troponin C isolated from rabbit muscle was a generous gift of Dr. J. Potter (University of Cincinnati, Cincinnati, OH). Trifluoperazine HCl was a gift of Dr. Harry Green (Smith Kline & French Laboratories, Philadelphia, PA). RoSV, an antiserum that binds specifically to synaptic vesicles in crude homogenates of electric organ (22), was provided by Dr. S. Carlson (University of California, San Francisco).

Calmodulin Radioimmunoassay—Narcine calmodulin was iodinated to a specific activity of 825 Ci/mmol using chloramine-T as described by Van Eldik and Watterson (23). All of the trichloroacetic acid precipitable was incorporated into a polyacrylate that co-migrated with calmodulin by SDS-polyacrylamide gel electrophoresis and autoradiography. At least 95% of this \( ^{125}\)I-calmodulin was precipitable by ScCaM, while only 60% could be precipitated by RoCaM.

Competition radioimmunoassays of calmodulin used \( ^{125}\)I-labeled Narcine calmodulin (5000 cpm; 60 pg) and either RoCaM (0.05 M) or ScCaM (0.4 M). The amount of antiserum was chosen to give 50% precipitation of the iodinated calmodulin in the presence of competing antibodies. Experimental samples were prepared by diluting in RIA buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 0.2% BSA) without BSA, boiling for 5 min, and adding BSA to final concentrations of RoCaM and ScCaM. All of the trichloroacetic acid precipitable was removed by pelleting the vesicles and bound antibody was always less than 1%. A substantial ATPase activity present in the Pansorbin was not significant difference was seen between these and parallel samples

Unbound sera were removed by pelleting the vesicles and bound antibody was always less than 1%. ATPase activity in the supernatant was considered a more specific assay for calmodulin. This procedure was at least 95% effective in removing the bound antibodies while leaving the sample covalently bound to the paper. This procedure was at least 95% effective in removing the bound antibodies while leaving the sample covalently bound to the paper.

Immunoprecipitation of Intact Vesicles—One microgram of synaptic vesicle was mixed with the indicated dilution of antibody in 0.1 ml of iso-osmotic buffer (0.4 M NaCl, 10 mM Hepes, 1 mg/ml of BSA, pH 7.4), incubated overnight at 4 °C, and then precipitated by addition of 50 μl of washed Pansorbin for 20 min. Samples were layered with 0.1 ml of 0.6 M sucrose, 0.1 M NaCl, 10 mM Hepes, 1 mg/ml of BSA, pH 7.4, and pelleted at 12,500 × g for 10 min. Pellets and supernatants were assayed for free and membrane-bound ATP. A substantial ATPase activity present in the Pansorbin was not inactivated by the boiling that released the membrane-bound ATP, so ATP depletion from the supernatant was considered a more accurate reflection of vesicles precipitated. To check that sufficient Pansorbin was present to precipitate all bound antibody, excess unbound sera were removed by pelleting the vesicles and bound antibody at 100,000 × g for 30 min prior to Pansorbin addition. No significant difference was seen between these and parallel samples without excess antibody removed.

Immunoblotting Using Discarded Paper—SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (24) using 6% polyacrylamide in the stacking gel and 12 or 15% in the running gel. Following electrophoresis, proteins were electrophoretically transferred to activated 2-aminophenylthioether paper (Bio-Rad) and incubated with SaCaM (10 pg/ml) in antibody buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.25% gelatin, 0.05% Nonidet P-40, 1% BSA) for 60-90 min, and washed in antibody buffer. Immune complexes were detected with about 2 × 10\(^{-15}\) mg of antibody/vesicle (26), this corresponds to about one calmodulin/vesicle. These estimates of calmodulin immunoreactivity may be low as there is a limited density of activated groups on the filter and proteins with mobility on SDS gels similar to calmodulin might compete with calmodulin for coupling.

Results

Cholinergic synaptic vesicles were purified from electric organ of D. ommia, a marine ray, by a procedure that includes 10 mm EGTA in all buffer solutions (12). A protein whose association with synaptic vesicles is calcium-dependent should be removed during this purification. We have used two calmodulin-specific antisera to detect calmodulin in the synaptic vesicle preparation by immunoblotting (Fig. 1). As controls, we used intact synaptosomes from electric organ since these are known to be rich in calmodulin (25). Synaptic vesicle or synaptosome proteins separated by SDS-polyacrylamide gel electrophoresis were electrophoretically transferred and covalently coupled to filter paper and then probed with ScCaM, an affinity-purified antibody to calmodulin raised in sheep against the native protein (21). Only a single immunoreactive protein was detected in both synaptosomes and synaptic vesicles (lanes g and h). This protein exactly co-migrated with pig brain and electric organ calmodulin standards (lanes e and f). The pattern of immunoreactivity when the filter was reprobed with RoCaM was identical with that seen with ScCaM (lanes i-l). We conclude that there is a significant amount of calmodulin in this synaptic vesicle preparation even though it is purified in EGTA.

Estimates of the amount of calmodulin in synaptic vesicles and synaptosomes were made by densitometry of the immunoblot autoradiograms (Table 1) using the narcicin calmodulin as a standard. The calmodulin level in synaptic vesicles measured by ScCaM was 4.4 ± 0.2 and by RoCaM was 2.2 ± 0.01 μg of calmodulin/mg of protein. Assuming 8.7 × 10\(^{-15}\) mg of protein/vesicle (26), this corresponds to about one calmodulin/vesicle.

FIG. 1. Calmodulin in synaptic vesicles and synaptosomes detected by immunoblotting. 0.5 μg of pig brain calmodulin (lanes a–d), 0.5 μg of Narcine electric organ calmodulin (lanes h, j, and i), 5 μg of Discopyge synaptosomes (lanes c, g, and k), or 50 μg of Discopyge synaptic vesicles (lanes d, h, and l) was fractionated by SDS-polyacrylamide gel electrophoresis (12%) gel. One strip of the gel was stained with Coomassie blue (lanes a–d). The remainder was electroblotted to diazophenylthioether paper and detected first with SaCaM (lanes e–h) and then reprobed with RoCaM and \(^{125}\)I-gold anti-rabbit IgG (lanes i–l). 94 K, represents, for example, M, 94,000.
Calmodulin is a major protein in electric organ (27) and in synaptosomes from the electric organ (Tables I and 3 and Ref. 25). Calmodulin seen in vesicle preparations by immunoblotting could be associated with minor contaminants in the preparation rather than with synaptic vesicles. The apparent EGTA resistance of the calmodulin-membrane association could also be due to calmodulin trapped inside vesiculated membranes. We have taken advantage of the high ATP content of synaptic vesicles (0.17 mM inside (26)) to show that the calmodulin in the vesicle preparation is associated with vesicles rather than contaminants and is bound to the cytoplasmic face of ATP-containing synaptic vesicles (Fig. 2). Intact synaptic vesicles were immunoprecipitated using SoCaM, RoCaM, or RoSV, a synaptic vesicle-specific serum raised in rabbits (22). SoCaM and RoSV were capable of precipitating at least 86 and 99% of the vesicles are monitored by their ATP content. Curiously, RoCaM was little more effective than rabbit nonimmune serum at precipitating vesicles. The poor ability of RoCaM to precipitate intact vesicles could be explained if the domain recognized by RoCaM were inaccessible to the antibody when calmodulin was bound to the vesicle. Since at least 84% of the vesicles can be specifically precipitated by SoCaM, calmodulin must be present on at least 84% of vesicles. Since only intact vesicles contain ATP, the calmodulin must be present on the outside or cytoplasmic face of the vesicles. Therefore, synaptic vesicles have calmodulin tightly associated with their membrane and cytoplasmically oriented even in the absence of calcium.

We have sought conditions that solubilize this membrane-associated calmodulin. Vesicles subjected to a variety of solubilization conditions were centrifuged for 30 min in a Beckman Airfuge. Calmodulin in the pellet and supernatant was measured by RIA with SaCaM (Table I). The calmodulin-vesicle association was calcium-independent under all conditions tested. It was not released by 100 pM trifluoperazine, which dissociates most calcium-dependent calmodulin interactions. It was only partially released by boiling even though boiling might be expected to break calmodulin-target interactions. 70% of the calmodulin was released by solubilization of the vesicle membrane in Nonidet P-40 and 80% was extracted from the vesicle membranes at pH 11. The calmodulin was not released from the membranes by five cycles of freeze-thaw, which is sufficient to release all acetylcholine and ATP from the vesicles. Therefore, the calmodulin seems to be tightly associated with the vesicle membrane.

**TABLE I**

*Calmodulin immunoreactivity in synaptic vesicles: densitometry of immunoblots*

| Solubilization Condition | Solubilized in SaCaM (μg/mg) | Solubilized in RoCaM (μg/mg) | Ratio SaCaM:RoCaM | Mean ± Range of Ratios |
|-------------------------|-------------------------------|-------------------------------|--------------------|------------------------|
| Electric organ calmodulin | 1000 ± 16| 1000 ± 56 | 1.00 ± 0.08 | Electric organ calmodulin |
| Pig brain calmodulin | 1600 ± 450 | 810 ± 270 | 1.12 ± 0.16 | Pig brain calmodulin |
| Electric organ synaptosomes | 112 ± 68 | 68 ± 6 | 1.65 ± 0.28 | Electric organ synaptosomes |
| Electric organ synaptic vesicles | 4.4 ± 0.2 | 2.2 ± 0.1 | 1.81 ± 0.03 | Electric organ synaptic vesicles |

*Mean ± range of ratios.

**TABLE II**

*Solubilization of synaptic vesicle calmodulin*

| Solubilization condition | Solubilized in Ca²⁺ (1 mM) | Solubilized in EGTA (10 mM) | Mean ± Range of Ratios |
|-------------------------|----------------------------|-----------------------------|------------------------|
| Freeze-thaw (five cycles) | 1 | <1 | Freeze-thaw (five cycles) |
| Trypsin (10 μg/ml) | 1 | | Trypsin (10 μg/ml) |
| Trifluoperazine (100 μM) | 8 | | Trifluoperazine (100 μM) |
| Boiling (5 min) | 24 | 20 | Boiling (5 min) |
| Nonidet P-40 (1%) | 67 | 70 | Nonidet P-40 (1%) |
| pH 11 | 96 | 79 | pH 11 |

*After treatment, vesicles were centrifuged at 100,000 × g in a Beckman Airfuge for 30 min. Solubilized and pelleted calmodulins were measured by radioimmunoassay with SaCaM. 80–90% of vesicle protein and calmodulin were recovered in the pellets in control samples. The ratio of calmodulin remaining in the supernatant of control samples and experimental samples was taken to arrive at per cent solubilized. Vesicles used in these experiments had from 7.8 to 14 μg of calmodulin/mg of protein.*

Competition radioimmunoassay is a highly sensitive and specific method for measuring the level of antigen in a complex mixture. Antigenic determinants recognized by the antibody must also be present on the purified radiolabeled probe to be detected. Highly sensitive RIAs for solubilized calmodulin have been reported (21, 23). Table III shows the results of calmodulin competition radioimmunoassay with the two different antisera using purified electric organ calmodulin as standard and as radiolabeled probe. The assay is more than 2000-fold specific for calmodulin over the closely related calcium-binding protein troponin C using either antisera. Pig brain calmodulin is 24% more effective than electric organ calmodulin in binding RoCaM and 85% more effective in binding SoCaM. This may reflect subtle species differences between the two calmodulins as well as different determinants of calmodulin recognized by the two antisera.
Accurate quantitation by competition RIA requires that antibodies have free access to the antigen. Sample preparation methods which disrupt reversible calmodulin-target interactions such as boiling (21) might not free calmodulin from antibodies have free access to the antigen. Sample preparation to solubilize the vesicle calmodulin (Table II), we chose boiling membranes or large aggregates (14). As Nonidet P-40 seemed to solubilize the vesicle calmodulin (Table II), we chose boiling as the sample preparation method for the RIA. As a control, calmodulin was measured in synaptosomes by RIA using S0CaM or RaCaM (Table IV). The levels were within the range of the levels measured by immunoblot with these two sera, although the mean values were somewhat higher. The level of calmodulin measured in synaptic vesicles by RIA with S0CaM was 6.1 pg/mg, in reasonable agreement with the level measured by immunoblot. In two other vesicle preparations, levels of 7.3 and 12 pg/mg were detected by RIA with S0CaM. A surprising difference between quantitation of calmodulin by RIA and immunoblot emerged when vesicles were assayed by RIA with RaCaM. Instead of being 30-60% higher, the levels of calmodulin detected in synaptic vesicles by RIA with RaCaM were lower than by immunoblot. In three different vesicle preparations, the calmodulin levels measured by RIA with RaCaM ranged from 0.6 to 1.5 pg/mg. The ratio between the calmodulin levels detected by the two antibodies in vesicles ranged from 4 to 15 with an average of 7.4 (Table III).

The difference in calmodulin levels in vesicles measured by RIA with the two antibodies (Table III) is consistent with the different efficiency with which the two antibodies immunoprecipitate intact synaptic vesicles (Fig. 2). Such a difference was not seen by immunoblot of vesicles (Table I), suggesting that SDS either destroyed a S0CaM antigenic determinant or exposed one recognized by RaCaM. If RaCaM immunoreactivity in vesicles were anomalously low by RIA because antigenic sites were masked in Nonidet P-40, the difference seen by the two antisera might be eliminated by SDS denaturation before RIA. Vesicles were extracted by pH 11 to remove the majority of the phospholipids, then boiled in SDS and assayed with RaCaM and S0CaM in the radioimmunoassay (Table III). Only 34% of the antigen recognized by S0CaM survived this procedure. In contrast, this treatment doubled the amount of calmodulin seen by RaCaM and reduced the ratio of the calmodulin seen by the two sera from 13 to 1.7. This ratio for vesicle calmodulin was very close to that seen by the immunoblot technique (Table I). Apparently, SDS treatment, like SDS-polyacrylamide gel electrophoresis (Fig. 1), removed the difference seen by the two sera, at least partly by increasing the number of sites available for RaCaM binding.

**Table III**

|                | S0CaM* µg/mg | RaCaM µg/mg | Ratio S0CaM:RaCaM* |
|----------------|--------------|--------------|---------------------|
| Electric organ calmodulin | 1850 ± 110 (3) | 1240 ± 120 (3) | 1.49 ± 0.17        |
| Pig brain calmodulin    | <0.4         | <0.5 (3)     |                     |
| Rabbit troponin C       | 149 ± 46 (3) | 112 ± 42 (3) | 1.33 ± 0.65        |
| Electric organ synaptosomes | 7.3 ± 1.4 (7) | 0.6 (1) | 12                |
| Preparation A           | 12 ± 1.5 (3) | 0.79 ± 0.6 (4) | 15 ± 1.5          |
| Preparation B           | 6.1 ± 0.7 (5) | 1.5 ± 0.1 (2) | 4.0 ± 1.0         |
| Preparation C           | 8.5 ± 3.1 (17) | 0.96 ± 0.47 (7) | 8.9 ± 5.4        |

* Calmodulin values are expressed as micrograms/mg of protein relative to Narcine electric organ calmodulin. Results are mean ± S.D. (number of determinations).

* Ratio of means.

![Fig. 3. Tryptic fragments of calmodulin recognized by two different antisera. Electric organ calmodulin (1 mg/ml) was digested with trypsin (2 µg/ml in 20 mM NaHCO3, pH 8.2, 1 mM EGTA or 16.7 µg/ml in 20 mM NaHCO3, pH 8.2, 0.1 mM CaCl2) at 20 °C for the indicated times. Digestion was stopped by addition of a 10-fold excess (w/w) of soybean trypsin inhibitor. The peptides were separated by SDS-polyacrylamide gel electrophoresis (15% gel) in 1 mM EGTA, electroblotted onto diazophenylthioether paper, and detected with the indicated antibodies. A shows digestion in EGTA; B shows digestion in calcium. Protein, Coomassie blue staining; Ab 1, S0CaM; Ab 2, RaCaM. Digestion times, in minutes, are indicated under each lane. E1, E2, E3, and CII indicate the major large tryptic fragments, using the notation of Walsh et al. (28). 20 K represents, for example, Mr = 20,000.](http://www.jbc.org/)

The different abilities of the two antisera to recognize synaptic vesicle calmodulin predict that they recognize different determinants on the calmodulin molecule. To verify this prediction, we examined the antigenicity of peptide fragments...
of calmodulin. Limited digestion of calmodulin with trypsin gives three major fragments when digested in ECTA (E1, 1-107; E2, 1-90; and E3, 108-148) and two when digested in Ca²⁺ (Cl, 1-77; and CII, 78-148) (28). Partial trypptic digests of calmodulin in the presence or absence of calcium were immunoblotted and probed with RoCaM and SoCaM (Fig. 3). The sets of tryptic peptides recognized by the two antisera were completely different. The peptides E1, E2, E3, and CII are tentatively identified on the basis of their staining intensity, kinetics of appearance, and calcium-dependent mobility shift. RoCaM recognized the COOH-terminal peptides CII and E3 but not E1 and E2, consistent with published results (23). SoCaM recognized E1 but not E2, E3, or CII. Thus, the determinants recognized by the two antisera are different and in different domains of the calmodulin molecule.

**DISCUSSION**

Calmodulin, as detected by specific antibodies, is on the cytoplasmic surface of cholinergic synaptic vesicles even in the absence of calcium. This protein behaves like purified calmodulin in the following ways: 1) It is recognized by SoCaM, a highly specific affinity-purified antiserum to calmodulin. 2) It co-migrates with authentic calmodulin by SDS-polyacrylamide gel electrophoresis in the absence of calcium. 3) It shows the same calcium-dependent mobility shift as authentic calmodulin by SDS-polyacrylamide gel electrophoresis in the absence of calcium. 4) It is membrane-bound even after boiling in EDTA. 2) It does not activate vesicle fraction had 7.3 pg of calmodulin/mg of protein using RaCaM and 0.6 pg of calmodulin/mg of protein using RaCaM (Table III). Such a dramatic difference was not seen with synapticosome calmodulin (Table II) nor when vesicles were assayed following SDS treatment by RIA (Table IV) or by immunoblot (Table I). The ratio of the calmodulin levels detected by the two sera was less than 2 for all samples in all assays, except when synaptic vesicles were assayed under nondenaturing conditions.

The difference between synaptic vesicle calmodulin and other calmodulins in terms of its recognition by RoCaM under nondenaturing conditions is best explained if the determinant recognized by RoCaM is masked in vesicles but unmasked by SDS denaturation. It is unlikely that the unique feature of vesicles recognized by SoCaM is not calmodulin, since the antibody is affinity-purified and no other antibody-binding protein is seen in immunoblots. It is also unlikely that vesicle calmodulin is modified such that SoCaM (raised against authentic calmodulin) binds better to the modified calmodulin than to synapticosomal or purified calmodulin.

The calmodulin measured in the synapticosome preparation used here was high, ranging from 7 to 15% of the total protein. By phosphodiesterase activation, this preparation has 36 µg of calmodulin/mg of protein. Another synapticosome preparation gave 47-54 µg of calmodulin/mg of protein when assayed by radioimmunoassay with SoCaM (data not shown). When the calmodulin in a thin synaptosome preparation was measured by densitometry of Coomassie-stained gels (25), 48 µg of calmodulin/mg of protein were detected in the synaptosome peak. While the concentration of nerve terminal calmodulin given by these three different methods is remarkably high, it must be remembered that at least 1% of total electric organ protein is calmodulin (27).

Assuming that the values measured by SoCaM in Table III are a true reflection of the calmodulin in synaptic vesicles and using 8.7 × 10⁻¹⁵ mg of protein/vesicle (26), there are two to four calmodulin molecules/vesicle. The immunoprecipitation experiment in Fig. 2 gives an independent estimate of calmodulins/vesicle. At least 84% of the vesicles are specifically precipitated by SoCaM. Assuming one calmodulin/vesicle is sufficient to immunoprecipitate the whole vesicle and that the calmodulin has a Poisson distribution, this gives a lower limit of 3 µg of calmodulin/mg of vesicle protein. Since this is close to the value measured by RIA, the majority of the calmodulin is presumably on the cytoplasmic surface.

The calcium-dependent calmodulin-binding site on the outside of the vesicles (12) differs from the calcium-independent calmodulin association with vesicles described here by three additional criteria. These are 0.45 calcium-dependent calmodulin-binding sites/vesicle and two to four calcium-independent calmodulin-binding sites per vesicle. No additional calmodulin as detected by RIA is found associated with vesicles when exogenous unlabeled calmodulin is added in the presence of calcium. This confirms that only a small fraction of the calmodulin-binding sites is calcium-dependent. The kinetics of the two types of binding is quite different. The half-time of dissociation of calmodulin at the calcium-dependent site is 54 s, while at the calcium-independent site the half-time of dissociation must be days. Finally, the calcium-dependent binding is inhibited by trifluoperazine, while the calcium-independent association is trifluoperazine-resistant.

In the few known cases of calcium-independent calmodulin binding, the data suggest that the binding allows calcium regulation. The sensitivity of B. pertussis adenylate cyclase to calmodulin stimulation increases 200-fold in the presence of calcium (14). In phosphorylase kinase, the calmodulin subunit contributes to the calcium activation of the catalytic subunit (29). Gap junction permeability is regulated by calcium, and

| Vesicle treatment* | SoCaM | Roy | RaCaM | SoCaM:RaCaM |
|-------------------|-------|-----|-------|-------------|
| Boiled in Nonide P-40 | 7.8 | 0.6 | 13 |       |
| pH 11 supernatant | 5.4 | 1.0 | 5.7 |       |
| pH 11 supernatant boiled in SDS | 2.1 | 1.2 | 1.7 |       |

* Samples were prepared by boiling for 5 min in 1% SDS, 1 mM EDTA, and then diluting 10-fold into RIA buffer with 0.5% Nonidet P-40. These samples (final detergent concentration 0.5% Nonidet P-40, 0.1% SDS) were assayed as described under "Experimental Procedures." No difference was seen in standard curves with Narcine calmodulin between assay done in 0.1% Nonidet P-40 and in 0.5% Nonidet P-40, 0.1% SDS. Micrograms of calmodulin values are expressed as immunoreactive equivalents/mg of vesicle protein (before pH 11 extraction and SDS treatment).

Calmodulin

**TABLE IV**

SDS unmasking RoCaM-binding sites in synaptic vesicles by radioimmunoassay

| Vesicle treatment* | SoCaM | Roy | RaCaM | SoCaM:RaCaM |
|-------------------|-------|-----|-------|-------------|
| Boiled in Nonide P-40 | 7.8 | 0.6 | 13 |       |
| pH 11 supernatant | 5.4 | 1.0 | 5.7 |       |
| pH 11 supernatant boiled in SDS | 2.1 | 1.2 | 1.7 |       |

* Samples were prepared by boiling for 5 min in 1% SDS, 1 mM EDTA, and then diluting 10-fold into RIA buffer with 0.5% Nonidet P-40. These samples (final detergent concentration 0.5% Nonidet P-40, 0.1% SDS) were assayed as described under "Experimental Procedures." No difference was seen in standard curves with Narcine calmodulin between assay done in 0.1% Nonidet P-40 and in 0.5% Nonidet P-40, 0.1% SDS. Micrograms of calmodulin values are expressed as immunoreactive equivalents/mg of vesicle protein (before pH 11 extraction and SDS treatment).

Calmodulin

* B. B. Olwin, personal communication.
the calmodulin bound to gap junction in a calcium-independent manner (18) may well be the effector. In synaptic vesicles, the endogenous calmodulin could be a regulator of a calcium-dependent process. Both the protein kinase and the calcium transport in synaptic vesicles (6) require exogenous calmodulin for activation, as does in vitro transmitter release from crude brain vesicles, attributed to a tubulin kinase (30). The function of the endogenous calmodulin described here is more likely to be one that does not require exogenous calmodulin for activation. Calcium buffering has been proposed for the calmodulin of intestinal brush-borders (31) and that might also be a major role for the high levels of calmodulin in the synaptosomes and nerve terminals. However, such a function would not require a tight, calcium-independent binding to vesicle membranes.

The most dramatic calcium-regulated function of the synaptic vesicle is exocytosis. Influenza virus hemagglutinin catalyzes low pH-triggered fusion of the viral envelope with intracellular membranes by exposing a hydrophobic domain (32). By analogy, a nerve terminal fusion protein might be expected to expose a hydrophobic region on binding calcium. In fact, calmodulin exposes a hydrophobic domain in response to calcium (33). Calmodulin associated with the vesicle membrane in the absence of calcium is in an ideal position to react quickly to the changes in calcium which trigger exocytosis. The vesicle calmodulin retains the capacity to bind calcium, as indicated by its mobility on SDS-polyacrylamide gel electrophoresis, but we do not know if it can undergo a conformational change upon binding calcium. It is unlikely that calmodulin alone catalyzes vesicle exocytosis, since vesicle fusion has not been reported in nerve terminals. The plasma membrane presumably carries vital components of the exocytotic machinery.

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REFERENCES

1. Klee, C. B. & Vanaman, T. C. (1982) Adv. Protein Chem. 35, 213-322
2. Reichardt, L. F. & Kelly, R. B. (1983) Annu. Rev. Biochem. 52, 871-926
3. Baker, P. F. & Whittaker, M. J. (1980) J. Physiol. (Lond.) 298, 55P
4. Baker, P. F. & Knight, D. E. (1981) Philos. Trans. R. Soc. Lond. B Biol. Sci. 296, 83-103
5. DeLorenzo, R. J. (1981) Cell Calcium 2, 365-385
6. Rephaeli, A. & Parsons, S. M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5783-5787
7. DeLorenzo, R. J., Freedman, S. D., Yobe, W. B. & Maurer, S. C. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1838-1842
8. Burgoyne, R. D. & Geisow, M. J. (1981) FEBS Lett. 131, 127-131
9. Teshima, Y. & Kakiuchi, S. (1978) J. Cyclic Nucleotide Res. 4, 219-231
10. Olwin, B. B., Keller, C. H. & Storm, D. S. (1982) Biochemistry 21, 5669-5675
11. Weiss, B. & Levin, R. (1978) Adv. Cyclic Nucleotide Res. 9, 285-303
12. Hooper, J. E. & Kelly, R. B. (1984) J. Biol. Chem. 259, 141-147
13. Grinstein, S. & Furuya, W. (1982) FEBS Lett. 140, 49-52
14. Greenlee, D. V., Andresen, T. J. & Storm, D. S. (1982) Biochemistry 21, 2769-2774
15. Shenolikar, S., Cohen, P. T. W., Cohen, P., Nairn, A. C. & Perry, S. V. (1979) Eur. J. Biochem. 100, 329-337
16. Walsh, K. X., Miliken, D. M., Schlenker, K. K. & Reimann, E. M. (1980) J. Biol. Chem. 255, 5036-5042
17. Glenney, J. R., Bretscher, A. & Weber, K. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6458-6462
18. Walsh, M. J., Asher, J. C., Ireland, M., Alcala, J. & Maisel, H. (1982) Science (Wash. D. C.) 216, 642-643
19. Geisow, M. J. & Burgoyne, R. D. (1983) Nature (Lond.) 301, 432-435
20. Miljanich, G. P., Brasier, A. R. & Kelly, R. B. (1982) J. Cell Biol. 94, 88-96
21. Chafouleas, J. G., Dedman, J. R., Munjaal, R. P. & Means, A. R. (1979) J. Biol. Chem. 254, 10262-10267
22. Carlson, S. S. & Kelly, R. B. (1980) J. Cell Biol. 87, 98-103
23. Van Eldik, L. J. & Watterson, D. M. (1981) J. Biol. Chem. 256, 4205-4210
24. Laemml, U. K. (1970) Nature (Lond.) 227, 680-685
25. Hooper, J. E., Deutsch, J. W., Miljanich, G. P., Brasier, A. R. & Kelly, R. B. (1983) J. Physiol. (Paris) 78, 443-453
26. Wagner, J. A., Carlson, S. S. & Kelly, R. B. (1978) Biochemistry 17, 1199-1206
27. Childers, S. R. & Siegel, F. L. (1975) Biochim. Biophys. Acta 405, 99-108
28. Walsh, M., Stevens, F. C., Kuznicki, J. & Drabikowski, W. (1977) J. Biol. Chem. 252, 7440-7443
29. Chan, K.-F. J. & Graves, D. J. (1982) J. Biol. Chem. 257, 5966-5961
30. Burke, B. E. & DeLorenzo, R. J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 991-995
31. Glenney, J. R., Jr. & Weber, K. (1980) J. Biol. Chem. 255, 10551-10554
32. Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A. & Wiley, D. C. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 968-972
33. LaPorte, D. C., Wiermas, H. M. & Storm, D. R. (1980) Biochemistry 19, 3814-3819
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