Function of Calmodulin in Postsynaptic Densities

III. Calmodulin-binding Proteins of the Postsynaptic Density

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ABSTRACT A method has been developed for binding calmodulin, radioiodinated by the lactoperoxidase method, to denaturing gels and has been used to attempt to identify the calmodulin-binding proteins of cerebral cortex postsynaptic densities (PSDs). Calmodulin primarily bound to the major 51,000 M_r protein in a saturatable manner; secondarily bound to the 60,000 M_r region, 140,000 M_r region, and 230,000 M_r protein; and bound in lesser amounts to a number of other proteins. The major 51,000 M_r calmodulin-binding protein is one of unknown identity. Binding of iodinated calmodulin to these proteins was blocked by EDTA, EGTA, chlorpromazine, and preincubation with unlabeled calmodulin. Calmodulin iodinated by the chloramine-T method, which inactivates calmodulin, did not bind to the PSD but bound nonspecifically to histone. Calmodulin did not bind to proteins from a variety of sources for which calmodulin interactions have not been found. Except for three proteins, all of the proteins of synaptic membranes that bind calmodulin could be accounted for by proteins of the PSD which are a part of the synaptic membrane fraction. The major 51,000 M_r protein and the corresponding iodinated calmodulin binding were greatly reduced in cerebellar PSDs and this difference between cerebral cortex and cerebellar PSDs is discussed in light of the possible function of calmodulin in synaptic excitatory responses.

Calmodulin has been identified biochemically as a major component of postsynaptic densities (PSDs) (20, 21) and its presence there has also been confirmed immunohistochemically (35, 52). The relatively large amounts of calmodulin in the postsynaptic density as well as the presence there of both a calmodulin-activated protein kinase (21, 23, 24) and a calmodulin activated cyclic nucleotide phosphodiesterase (4, 21, 22) suggest an important role for this molecule in the regulation of neurotransmission. Because of the many important functions in which calmodulin is involved (cf. reference 49), it was deemed important to try to examine the interactions of PSD calmodulin with other PSD proteins. We know that it must interact with the kinase and the phosphodiesterase (21, 23, 24), and it would be instructive to know if it interacts with or binds other PSD proteins. Because the identity and function of most of the PSD proteins are unknown, a calmodulin-binding study would also aid in the tentative identification of at least some of them. To undertake such a study, it was felt necessary to separate the proteins electrophoretically, and in the case of the PSD proteins, to do so under denaturing conditions, because many of them are insoluble in nondenaturing solutions. In this study, a technique has been developed based upon several methods (1, 6, 8, 43) with which ligands could be bound to proteins on SDS gels. We used this technique to try to identify the calmodulin-binding proteins of the PSD on SDS polyacrylamide gels using radioiodinated calmodulin. This technique was found to be specific and indicated the presence of at least five calmodulin-binding proteins in the PSD. The primary binding components were the major 51,000 M_r protein of the cerebral cortex PSD (15, 29), which is phosphorylated by the calmodulin-activatable protein kinase (21, 23, 24) and which is postulated to be a unique component of asymmetric synapses (10, 11, 21), and a band in the gel which co-migrated with purified brain calcineurin and cyclic nucleotide phosphodiesterase. Independently of our binding studies (9, 12, this paper), Glenney and Weber (19) used a similar technique to find two calmodulin-binding proteins (M_r = 47,000 and 56,000) in chick brain acetone powder.

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MATERIALS AND METHODS

General Methods and Materials

PSDs were isolated from canine cerebral cortex by the short method of the procedure of Cohen et al. (15). Electrophoresis of PSD proteins was performed on a linear 5–15% acrylamide gradient gel as described before (15). Protein concentration was measured by the method of Lowry et al. (36). Calcineurin was a gift of Dr. C. Klee, National Institutes of Health (NIH) and Dr. R. Wallace (St. Judes Childrens Hospital). cAMP phosphodiesterase was a gift of Dr. C. Klee (NIH). Spinach chloroplast membranes was a gift of Dr. G. Bellemare (The Rockefeller University). Troponin-I was a gift of Dr. L. Van Eldik (The Rockefeller University). Torpedo acetylcholine receptor was a gift of Mr. D. Anderson (The Rockefeller University). Black widow spider venom gland extract was a gift of Dr. P. Wade (The Rockefeller University). Lactoperoxidase was obtained from Sigma Chemical Co. (St. Louis, Mo.). ^{125}I (17 Ci/mg) was obtained from New England Nuclear (Boston, Mass.).

Radioiodination of Calmodulin

Calmodulin was purified from canine cerebral cortex by the method of Watterson et al. (48) with the following modifications (20): Gel filtration was performed using Sephacryl S-200 instead of Sephadex G-100. The calmodulin fraction after gel filtration was dialysed against 300 mM NaCl in buffer A (10 mM Tris-HCl, pH 7.6; 1 mM EDTA; 1 mM 2-mercaptoethanol) and then applied to a DEAE-Sephadex A-25 column equilibrated with 300 mM NaCl in buffer A. The sample was then eluted from the column using a 300–700 mM NaCl gradient in buffer A (five times the bed volume). The elution of calmodulin was monitored by SDS polyacrylamide gel electrophoresis (PAGE). Calmodulin was iodinated by the method of Richman and Klee (41) with the following slight modifications to increase the specific activity (~17-fold): Iodination was performed (25°C) in a reaction volume of 1 ml that contained 0.05 M sodium phosphate (pH 7.0), 1 mg calmodulin, 12 μg lactoperoxidase, and 0.4 mM $CaCl_2$. 2 mCi ^{125}I-Na was added to the reaction mixture and the reaction was initiated by the addition of 5 μl of H_2O_2 (diluted 1:500). After 7 min, 5 μl of H_2O_2 (1:500) was again added to the reaction mixture and the reaction was allowed to proceed for an additional 7 min. The iodinated calmodulin was isolated as described by Richman and Klee (41). The specific activity of the iodinated protein was ~1,000,000 cpm/μg, as determined by liquid scintillation counting. In some cases calmodulin was radioiodinated by the chloramine-T method (28). The properties of calmodulin seemed to be unaltered by its iodination under our conditions, based upon the following experiments and conclusions: In comparison to the Richman-Klee procedure (41), we added twice as much H_2O_2 and lactoperoxidase but no carrier KI. A calculation using the specific radioactivity of the iodinated calmodulin indicated that much less than 1.0% of the calmodulin molecules was iodinated. Also, under these conditions, only monoiodinated calmodulin was formed, which is known to be active (33, 41). Calmodulin iodinated by the chloramine-T method and by our modification of the Richman-Klee method co-migrate on SDS PAGE with purified unlabeled calmodulin, and there were no other radioactive bands on the gels, indicating no breakdown of either of these radioactive proteins. Finally, calmodulin iodinated by the Richman-Klee method, which is biologically active (41), gave the same pattern of binding to SDS gels as did the calmodulin iodinated by our modification of their method.

Binding of Radioiodinated Calmodulin Proteins on SDS Gels

The method of binding ^{125}I-calmodulin to proteins on SDS gels was developed from previous binding techniques (1, 6, 8, 43) and has been previously described by us (12). Proteins in 2% SDS were electrophoresed in linear 5–15% polyacrylamide slab gels (2 mm × 1 cm × 18 cm) containing 0.1% SDS. After electrophoresis the gels were fixed with 25% isopropanol–10% acetic acid for 12 h with at least four changes by shaking the gels in plastic trays. All washing and binding was done at 25°C. The gels were then washed for 5–10 min in distilled water and then washed with buffer A (50 mM Tris, pH 7.6; 0.2 M NaCl; 1 mM $CaCl_2$) for 12 h with at least four changes of buffer A. The gels were further washed for 2 h with buffer A containing 1 mg/ml bovine serum albumin (BSA). For incubation with iodinated calmodulin, the gel together with 10 ml of buffer A (no BSA) plus 10 μg iodinated calmodulin (the amounts were proportionally larger for large gels) were inserted into a Sears boilable cooking pouch which was then sealed with Sears Seal'N-Save. The pouches were shaken for 12 h on a rotary shaker. The gels, after removal from the pouches, were again placed in plastic trays and were washed for 36 h with buffer A with at least six changes. The gels were then stained for 2 h in 0.25% Coomassie Blue, 50% methanol, 7% acetic acid, and destained in 25% methanol, 7% acetic acid. The gels were dried and exposed at 25°C for 24–48 h on Dupont Cronex 2D X-ray film or at −90°C for 1–10 hours using a Dupont Cronex Lighning-Plus YH enhancing screen. It was found that the addition of BSA increased specific binding at least 10 times, and under these conditions a calmodulin-binding protein can be detected on the gel in the absence of a Coomassie Blue-stained band, the lower limit of detection of which is 100–300 ng.

RESULTS

Previous work (25, 33, 41) had shown that calmodulin labeled with ^{125}I by the lactoperoxidase method could still activate a cyclic nucleotide phosphodiesterase but with an affinity lower than that of the native molecule, and that it could bind to the phosphodiesterase (33, 41) and to calcineurin (41). A next step was to show that it could interact with proteins of the PSD in a fashion similar to that of the unmodified molecule. Grab et al. (20) had previously shown that calmodulin can be removed from the PSD with EGTA and that it can be reconstituted back again into the PSD by the addition of calcium. Table I shows the ability of the iodinated calmodulin to be reconstituted into the PSD and the inhibition of this reconstitution by both EDTA and EGTA. Also, unlabeled calmodulin was able to compete with the radioactive calmodulin in this reconstitution experiment, whereas BSA had no effect. Even increasing 20-fold the amount of albumin had no competing effect on the amount of radioactive calmodulin bound to PSDs. Chlorpromazine, a drug that blocks the action of calmodulin (34), also reduced the reconstitution of iodinated calmodulin. If the iodinated calmodulin is iodinated with chloroamine-T (28), a compound that inactivates calmodulin (25, 45, 47), then no reconstitution is observed. These results were confirmed by running the pellets on SDS PAGE, performing autoradiography within the limits that the intensity of the film darkening is proportional to the amount of radioactivity, and scanning the autoradiographs. We interpret these results as indicating that the iodinated calmodulin is specifically interacting with its natural binding proteins in the PSD, since these results parallel those using unlabeled calmodulin in reconstitution experiments (20, 23).

Using the procedure in Materials and Methods, we then inquired into the nature of the PSD proteins which bind calmodulin, and the results of binding iodinated calmodulin to an SDS PAGE profile of PSD proteins is shown in Fig. 1. Radioiodinated calmodulin is found to primarily bind the major 51,000 M_r protein of the PSD, with intermediate binding to the proteins at the 60,000, 140,000 230,000, and in this experiment, the 165,000 M_r regions, and with lesser binding to

TABLE I

Binding of ^{125}I-Calmodulin to Calmodulin-Depleted PSDs

Treatment	^{125}I-Calmodulin reconstituted
	%
Control	100
+ Chlorpromazine, *1 mM*	40
+ EDTA, *1 mM*	0
+ EGTA, *1 mM*	0
+ Unlabeled CaM, *5 µg*	7
+ BSA, *5 µg*	100
Chloramine-T calmodulin	0

Cerebral cortex PSDs (150 μg) were depleted of calmodulin as described before (20). 1 μg iodinated calmodulin (1 × 10^6 cpm) was incubated with PSDs at 37°C for 10 min in the presence of 0.5 mM $CaCl_2$. After incubation the PSDs were spun down in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.), washed once with the incubation mixture, and the pellets were counted with a Geiger counter. The 100% value indicates that all the added radioactivity was recovered in the pelleted PSDs. Chloramine-T calmodulin indicates that calmodulin was iodinated using chloramine-T (28) instead of our modification of the lactoperoxidase method (41).

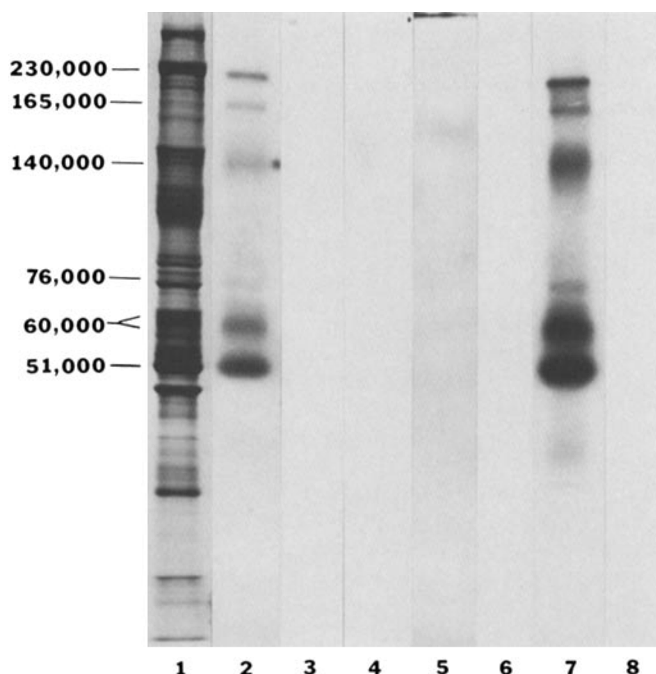


FIGURE 1 Binding of radioiodinated calmodulin to proteins from cerebral cortex PSDs separated on SDS polyacrylamide gels. The method of binding is given in Materials and Methods with 175 μg PSD proteins being used in all slots. The M_r of the major calmodulin-binding proteins are shown on the left. Slot *1* shows the Coomassie Blue staining of the PSD preparation. Slot *2* shows the autoradiograph of the gel after treatment with radioiodinated calmodulin. Slots *3–8* show autoradiography of the requisite controls. The binding of calmodulin was performed in the presence of: slot *3*, 2 μM EGTA; slot *4*, 0.1 mM EDTA; slot *5*, 1 mM chloropromazine. Slot *6* shows the effect of preincubation with 1 mg unlabeled calmodulin for 2 h, while slot *7* shows the effect of preincubation with 5 mg BSA for 2 h. Slot *8*: the gel was washed with 1 mM EGTA for 12 h after binding of radioiodinated calmodulin and then the autoradiograph was made. Autoradiographs were exposed at −90°C for 6 h using a Dupont Cronex Lightning-Plus YH enhancing screen.

the proteins at lesser molecular weights. A more comprehensive map of the binding is shown later in Fig. 6. Various controls indicate that the radioiodinated calmodulin is binding specifically. All binding activity is removed by the addition of either EGTA (slot *3*), EDTA (slot *4*), or chlorpromazine (slot *5*), or by preincubation of the gel with unlabeled calmodulin (1 mg in 10 ml) (slot *6*). Also if the gel is first labeled, washing the gel subsequently in EGTA removes all binding (slot *8*). The addition of a randomly chosen protein, BSA, did not only block binding but actually enhanced the binding of calmodulin to the gel (compare slots *2* and *7*). This enhancement of binding by preincubation of the gel in 1 mg/ml BSA before incubation with radioiodinated calmodulin was >10 times the control in various experiments. This increase in binding seems to be specific because it occurs only with proteins on the gel that bind calmodulin in the absence of BSA. We do not know why this occurs, but some possible reasons could include (*a*) neutralizing static charges which are possibly generated on the surface of the gel during washing, (*b*) the removal by BSA of residual SDS, acetic acid, or isopropanol, or (*c*) the blocking by BSA of nonspecific sites allowing a greater relative concentration of calmodulin to stain the specific sites. This effect of BSA has been taken advantage of in all subsequent experiments.

A second set of controls is shown in Fig. 2, which compares the binding of radioiodinated calmodulin to PSD proteins and to histone. While histones were found to be capable of binding calmodulin, we believe this is caused by nonspecific interactions because calmodulin is acidic and histones are basic. To test this hypothesis, gels having either PSD proteins or histone were both incubated with either calmodulin labeled by the lactoperoxidase method or with calmodulin labeled by the chloramine-T method. While both these preparations were capable of binding to histones (slots *4* and *6*), only the lactoperoxidase-iodinated calmodulin was found to bind to PSD proteins (compare slots *3* and *5*). Because the chloramine-T method inactivates calmodulin (25, 45, 47), we conclude that the interaction of the lactoperoxidase-iodinated calmodulin with PSD proteins is specific and is not caused by simple ionic interactions.

Further added support that this binding is specific is shown in Fig. 3. Proteins from a number of sources do not bind radioiodinated calmodulin labeled by the lactoperoxidase method. Proteins from both *E. coli* (slot *1*) and spinach chloroplast membranes (slot *2*) do not bind calmodulin. Both pyruvate kinase (slot *3*), an enzyme that is inhibited by calcium, and the inhibitory subunit of troponin (slot *4*), which binds calmodulin in some in vitro assays (2) do not bind radioiodinated calmodulin. Because the electroplax of *Electrophorus electricus* has been reported to contain calmodulin (14), the purified acetylcholine receptor from Torpedo, a related organism, was tested and found not to bind calmodulin (slot *5*).

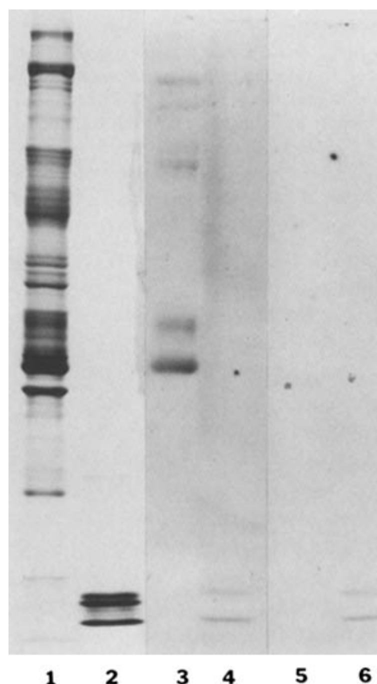


FIGURE 2 Comparison of the binding of calmodulin radioiodinated by lactoperoxidase with the binding of calmodulin radioiodinated by chloramine-T to proteins from cerebral cortex PSDs and to histones. Slot *1*, Coomassie Blue staining of PSD proteins (175 μg); slot *2*, Coomassie Blue staining pattern of histones (15 μg); slot *3*, binding of lactoperoxidase-iodinated calmodulin to PSD proteins; slot *4*, binding of lactoperoxidase-iodinated calmodulin to histones; slot *5*, binding of chloramine-T-iodinated calmodulin to PSD proteins; slot *6*, binding of chloramine-T iodinated-calmodulin to histones. Autoradiographs were exposed at −90°C for 6 h using a Dupont Cronex Lightning-Plus TH enhancing screen.

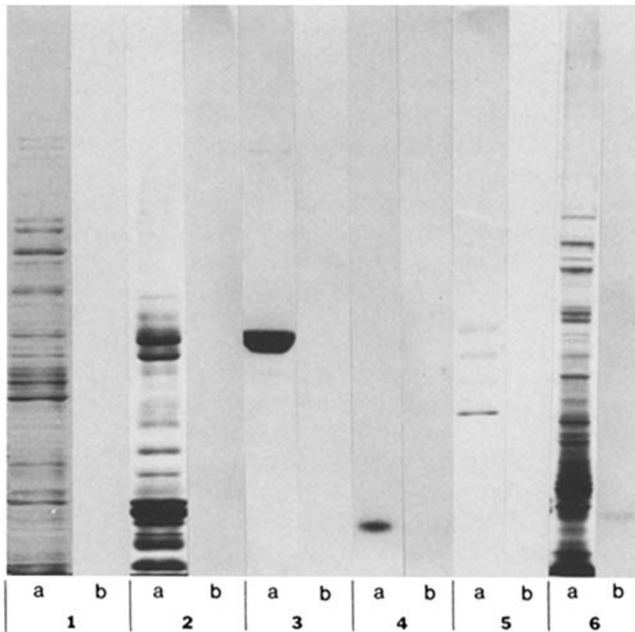


FIGURE 3 Lack of binding of calmodulin to proteins from various sources on SDS gels. All samples were treated identically as described in Materials and Methods and are directly comparable to Figs. 1 and 2. In all cases, slot *a* shows the Coomassie Blue-staining patterns of proteins on SDS gels, while slot *b* shows the autoradiograms of such preparations after pretreatment with radioiodinated calmodulin as given in Materials and Methods. The following are the various protein preparations: *1*, 75 μg *E. coli*; *2*, 125 μg spinach chloroplast membranes; *3*, 40 μg pyruvate kinase; *4*, 10 μg of the inhibitory subunit of troponin; *5*, 20 μg purified acetylcholine receptor from Torpedo; *6*, 125 μg crude extract from the venom glands of the black widow spider. Autoradiographs were exposed as described in Fig. 1.

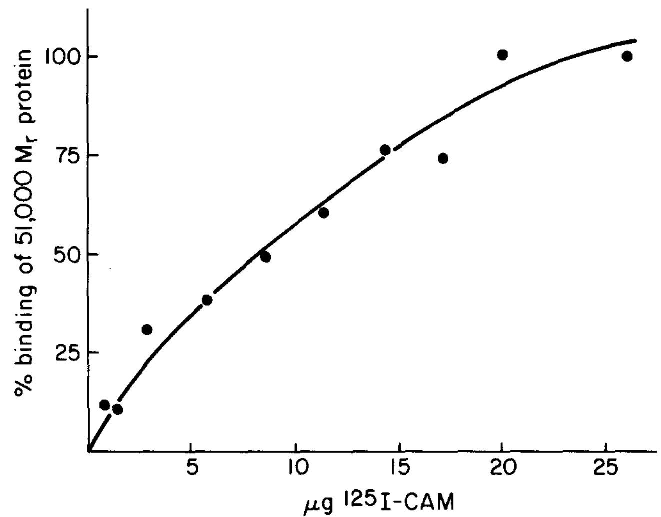


FIGURE 4 Saturation curve of calmodulin binding to the major 51,000 M_r protein. Binding to the major 51,000 M_r protein was determined by scanning the autoradiogram after binding to 175 μg PSD proteins run on the gel, cutting out the 51,000 M_r peak from the scan, and weighing it. The highest value of binding was arbitrarily set at 100%, determined from densitometry tracings of the autoradiograms, exposed within the linear range of radioactivity vs. autoradiograph density. The background is a clear autoradiogram.

However, when radioiodinated calmodulin is added to a denaturing gel containing a crude extract from black widow spider venom glands, a single binding protein is found (slot *6*). All these experiments show that out of some 150 protein bands found in these six preparations, only one band binds calmodulin, and this one protein may or may not be a true binding protein. All of the above control results indicate that the binding of radioiodinated calmodulin to proteins on denaturing gels is indeed specific. On the other hand some proteins that have been found to interact specifically with calmodulin in other systems do not bind radioiodinated calmodulin on gels. Thus a positive binding reaction in the gel system would indicate specific binding, but a negative reaction would indicate either that the protein does not bind calmodulin, or that it does, but has not renatured correctly in the assay and has lost its capacity to bind calmodulin.

Fig. 4 shows a saturation curve of the binding of radioiodinated calmodulin to a gel containing 175 μg of PSD proteins. The amount of label incorporated into the major 51,000 M_r protein is compared with the amount of calmodulin added. The amount of calmodulin binding comes close to saturation but not completely. This is caused most likely by calmodulin only binding to the surface of the gel (cf. Fig. 5), and increasing the amount of calmodulin to very high levels will slowly continue to push the equilibrium of the binding towards the interior of the gel. However, the near saturation again demonstrates the specific binding in this system.

The process by which iodinated calmodulin binds to proteins on SDS gels is indicated by the curves in Fig. 5. In panel *A* is a plot of the amount of radioactivity incorporated into the major 51,000 M_r protein as a function of increasing amounts of PSD. The resulting curve is not linear but logarithmic, indicating the technique is not quantitative except possibly on a logarithmic scale. If the amount of bound radioiodinated calmodulin is instead plotted as a function of the width of the major 51,000 M_r protein band, a linear relationship is observed. These data indicate calmodulin is binding only to the surface of the gel. Thus the thickness of the gel has no strong influence on the binding of calmodulin, except that for a given amount of protein a thinner gel will result in a wider band. Because the binding of calmodulin is dependent on the width of a band and not on the total amount of protein, the technique is very sensitive. Using the preincubation with BSA (cf. above), we routinely are able to detect less than the 100–300 ng of a calmodulin-binding protein which are detectable by Coomassie Blue staining.

A comparison of the binding of radioiodinated calmodulin to synaptic membranes, cerebral cortex PSDs, and cerebellar PSDs is shown in Fig. 6. The pattern of binding to a preparation of cerebral cortex PSDs (slot *5*) is very similar to that of a synaptic membrane preparation from which they are derived (slot *4*), suggesting that the majority of the calmodulin-binding proteins in synaptic membranes are caused by the presence of PSDs in this fraction. This finding agrees well with the immunohistochemistry results showing the presence of calmodulin at the postsynaptic density and not at other synaptic membrane sites (35, 52). However, there are three binding proteins at 29,000, 125,000, and 130,000 M_r that are found in the synaptic membrane preparation that are not found in the PSD preparation. These proteins might possibly be either adenyl cyclase and/or the calcium-activatable ATPase as both these enzyme activities have been found in synaptic membranes, but have not been found in the PSD (15). A comparison of the patterns of calmodulin binding on gels to proteins from cerebral cortex (slot *5*) and cerebellar (slot *6*) PSDs shows the cerebellar

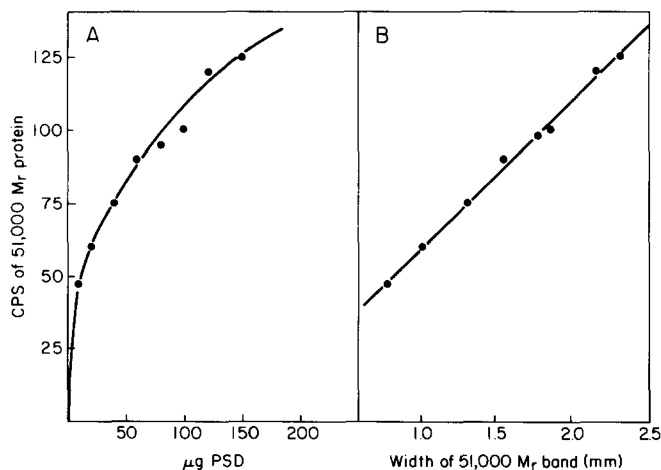
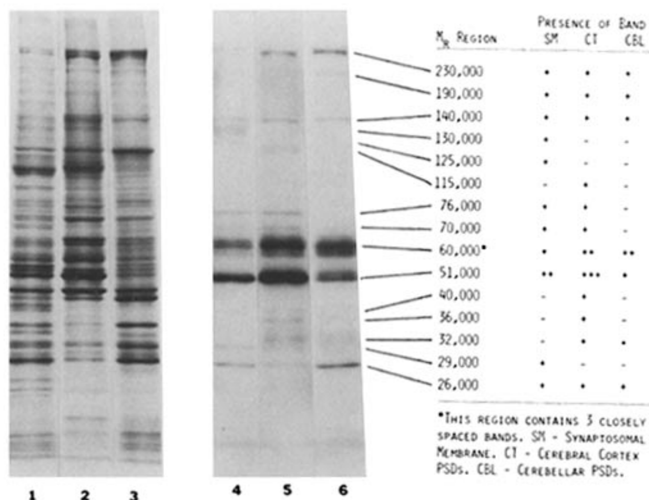


FIGURE 5 Amount of calmodulin binding to the major 51,000 M_r protein as a function of total amount of 51,000 M_r protein (*A*) and as a function of the width of the 51,000 M_r protein band (*B*). Both panels *A* and *B* were derived from the same gels. The amount of calmodulin bound to the major 51,000 M_r protein was determined by placing a Geiger counter over a piece of lead which had a 0.4 × 1.5 mm slot cut into it through which the radioactivity of the major 51,000 M_r protein was measured. Results were confirmed by performing autoradiography within the limits that the intensity of the film darkening is proportional to the amount of radioactivity, and scanning the autoradiograph. The width of the 51,000 M_r protein was determined by placing negative pictures of the Coomassie Blue-stained gels from various amounts of PSDs in a slide projector, and measuring the width of the projected 51,000 M_r band.



M_R Region	Presence of Band SM	CT	CBL
230,000	•	•	•
190,000	•	•	•
140,000	•	•	•
130,000	•	-	-
125,000	•	-	-
115,000	-	•	-
76,000	•	•	-
70,000	•	•	-
60,000*	•	••	••
51,000	••	•••	•
40,000	-	•	-
36,000	-	•	-
32,000	-	•	•
29,000	•	-	-
26,000	•	•	•

*This region contains 3 closely spaced bands. SM - Synaptosomal Membrane. CT - Cerebral Cortex PSDs. CBL - Cerebellar PSDs.

FIGURE 6 Binding of iodinated calmodulin to different brain parts. Slots *1–3* show the Coomassie Blue-staining patterns of 175 µg each of synaptosomal membrane, cerebral cortex PSD, and cerebellar PSD proteins, respectively. Slots *4–6* show the corresponding autoradiograph obtained after binding iodinated calmodulin to the gels as described in Materials and Methods. The table lists the presence and approximate molecular weights of the various reproducibly identified calmodulin-binding proteins in each fraction. Autoradiographs were exposed at 25°C for 24 h without a screen.

PSDs lacking the 115,000, 76,000, 70,000, 40,000, 36,000, and 29,000 M_r proteins, while the 26,000 M_r protein is greatly enhanced in the cerebellar PSDs. These differences may be important in the function of the PSD (see discussion). The major 51,000 M_r calmodulin-binding protein is present in much less quantities in cerebellar than in cerebral cortex PSDs (compare slots *2* and *3*, and slots *5* and *6*). This result agrees with our previous characterization of PSDs from different brain parts (10, 11, 21) in which the cerebellar PSDs had much less of the 51,000 M_r protein and of calmodulin than did the cerebral cortex PSDs. A second minor protein is also seen at ~51,000 M_r which is present in the three preparations in Fig. 6 and is noted to distinguish it from the major 51,000 M_r protein.

Because biochemical immunohistochemical studies indicate that the PSD contains calcineurin (52) and a calmodulin-activatable cyclic nucleotide phosphodiesterase (4, 22), we compared the binding of calmodulin to these proteins with that of the PSD proteins. Both purified calcineurin (30) and purified cAMP phosphodiesterase (30) were found to co-migrate with two bands in the 60,000 M_r region of the PSD (data not shown). We know calcineurin is probably present and binding calmodulin in the PSD because (*a*) calcineurin has been identified in the PSD using immunohistochemistry (52); (*b*) Dr. R. W. Wallace, University of Tennessee (personal communication) has found calcineurin in our PSD preparations using radioimmunoassay (46); and (*c*) purified calcineurin binds calmodulin strongly on our gel system (data not shown). However, because the initial breakdown product of calcineurin co-migrates with the phosphodiesterase band, we do not know if the cAMP phosphodiesterase or the breakdown product is binding calmodulin in our assay.

DISCUSSION

We have described an assay for the detection of calmodulin-binding proteins on denaturing gels which has some advantages over the use of calmodulin affinity columns. It is possible that a calmodulin-binding protein is part of an extractable protein complex, and because this entire complex may bind to the affinity column, it is impossible to identify which protein in this complex is really binding to calmodulin. A second advantage of this assay is that calmodulin-binding proteins can be identified even in structures that are insoluble, such as the PSD, except under extreme denaturing conditions. The controls that were run during this study indicate calmodulin interacts with its true binding proteins. The binding in the 60,000 M_r region is probably to one or both, of the two proteins there, calcineurin A and the cyclic nucleotide phosphodiesterase. Also, Dr. C. Klee, National Institutes of Health (personal communication) has found that a doublet brain protein of M_r 230,000, which we found to co-migrate with our PSD M_r 230,000 calmodulin-binding protein, binds to a calmodulin affinity column. There is one disadvantage of the binding assay in that calmodulin is not able to bind to some proteins that interact with calmodulin in other assays, for these proteins (such as troponin-I) may not have renatured correctly after removal of SDS and are unable to interact with calmodulin; thus a negative reaction does not necessarily mean the lack of calmodulin-binding capacity for that protein.

The obvious goal in the use of the calmodulin-binding technique reported here lies in the initial steps in identifying the various calmodulin-binding proteins in the PSD in order to finally elucidate the function of calmodulin in the PSD. As discussed before, the PSD lacks a calcium-calmodulin-stimulated ATPase and adenyl cyclase although they have been described in synaptic membranes (7, 13, 32, 44). We have identified in the PSD a calcium-calmodulin-activatable protein kinase (23, 24) which might be one of the high M_r calmodulin-binding proteins. Also a calcium-calmodulin-dependent cyclic

nucleotide phosphodiesterase has also been described by us in the PSD (22), while others have described the presence of calcineurin there (52), both of these being in the 60,000 M_r region of the gel which binds calmodulin.

Although we do not know the identity of the major 51,000 M_r protein, some previous studies have given some clues as to its likely function. In a previous study in which two types of PSDs were isolated (10, 11), those isolated from cerebral cortex were found to be an enriched population of PSDs from asymmetric or Gray type I synapses while those isolated from cerebellum were found to be an enriched fraction of PSDs from symmetric or Gray type II synapses. At present we have little idea as to why we have an enrichment of these presumably type I PSDs from cerebral cortex and an enrichment of these presumably type II PSDs from cerebellum, because both types of synapses are found in both these brain areas. An extensive review of the literature led Eccles (17) to propose that type I synapses mediate excitation responses and type II synapses mediate inhibitory responses. The major differences in the protein composition between these two preparations is that the cerebellar PSDs have much smaller amounts of the major 51,000 M_r protein than do the cerebral cortex PSDs, and they contain 50% less calmodulin than do the cerebral cortex PSDs. Minor differences also exist in that the cerebellar PSDs lack the minor calmodulin-binding proteins at 115,000, 76,000, 70,000, 40,000, and 36,000 M_r which are found in the cerebral cortex PSDs. This finding suggests that the major 51,000 M_r protein, calmodulin, and possibly the other listed calmodulin-binding proteins are intimately involved in the excitation process at the synapses. The process by which the 51,000 M_r protein, which is the major protein of the PSD, may control excitation is by way of both (*a*) modulation of the action of the protein by direct interaction with calmodulin and (*b*) modulation of the action of the protein by the calcium-calmodulin-dependent phosphorylation (23). How these two systems interact is unknown, but we do know that phosphorylation of the 51,000 M_r protein by the calcium-calmodulin-dependent protein kinase did not release bound calmodulin from PSDs as indicated by SDS PAGE, indicating that calmodulin could be bound to the phosphorylated protein.

Although we are only now beginning to obtain identification of a few calmodulin-binding proteins, a question can be raised at this time as to the role, if any, of calmodulin in postsynaptic function. The large number of calmodulin-binding proteins at the postsynaptic site plus numerous recent reports in the literature on calmodulin indicate that calmodulin may be important in a large variety of synaptic functions. Firstly, calmodulin plays a role in calcium flux between the intracellular and extracellular environment. Phospholipid metabolism has been implicated as a controlling factor in calcium entry (26), and a number of the enzymes involved in the metabolism of phospholipids require calcium, and one of the primary enzymes, phospholipase A_2, has been shown to be activated by calmodulin (51). Also, it may be that it is Ca^{2+}-calmodulin (32, 44) and not just calcium that activates the ATPase which catalyses the removal of calcium from inside the cells. Secondly, calmodulin is intimately involved in the regulation of cAMP metabolism, because calmodulin activates synaptic membrane adenyl cyclase (7, 13) and the PSD phosphodiesterase (4, 22), thus controlling both the synthesis and degradation of cAMP. In this context, calmodulin may also modulate the role of neurotransmitter receptors in nerve signal conduction, as many neurotransmitters exert their effect through a coupled adenyl cyclase. Receptors that are not coupled to cAMP may also be under the control of calcium-calmodulin, as the binding of glutamic acid to its receptor is enhanced by calcium (5). Thirdly, calmodulin may be involved in excitatory responses by way of mediating the effects of calcium. Chlorpromazine, a drug that binds and inactivates calmodulin (34), caused a reduction in miniature endplate potential amplitudes at the neuromuscular junction (3). Also Mn^{2+}, a divalent cation which can partially bind calmodulin (20), partially inactivated the excitatory postsynaptic potential in cat spinal cord (31). Also, calcium has been shown to be required for desensitization or receptor inactivation in the neuromuscular junction (37, 40). If hippocampal CA1 neurons are repetitively fired they develop a long-lasting after-hyperpolarization as a result of a calcium-activated potassium current (27), and the long-term potentiation in the hippocampus, which may involve memory, has an absolute requirement for calcium (16). Finally, tubulin has been identified as part of the PSD (15, 18, 29, 39) and in a few cases microtubules have been seen extending to the PSD (50). As calmodulin has been found to possibly control microtubule polymerization-depolymerization (38), calmodulin may also be controlling the function of tubulin in the PSD.

We thus propose as a working hypothesis that (*a*) calmodulin and the major 51,000 M_r protein are involved in the modulation and possible generation of postsynaptic excitatory responses, (*b*) the interaction of calmodulin with other postsynaptic proteins mediates some of the above-described calcium-requiring events, and (*c*) these calcium-requiring postsynaptic responses occur in the PSD.

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