Function of Calmodulin in Postsynaptic Densities

II. Presence of a Calmodulin-activatable Protein Kinase Activity

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ABSTRACT Because the calmodulin in postsynaptic densities (PSDs) activates a cyclic nucleotide phosphodiesterase, we decided to explore the possibility that the PSD also contains a calmodulin-activatable protein kinase activity. As seen by autoradiographic analysis of Coomassie Blue-stained SDS polyacrylamide gels, many proteins in a native PSD preparation were phosphorylated in the presence of [γ-^{32}P]ATP and Mg^{2+} alone. Addition of Ca^{2+} alone to the native PSD preparation had little or no effect on phosphorylation. However, upon addition of exogenous calmodulin there was a general increase in background phosphorylation with a statistically significant increase in the phosphorylation of two protein regions: 51,000 and 62,000 M_r. Similar results were also obtained in sonicated or freeze-thawed native PSD preparations by addition of Ca^{2+} alone without exogenous calmodulin, indicating that the calmodulin in the PSD can activate the kinase present under certain conditions. The calmodulin dependency of the reaction was further strengthened by the observed inhibition of the calmodulin-activatable phosphorylation, but not of the Mg^{2+}-dependent activity, by the Ca^{2+} chelator, EGTA, which also removes the calmodulin from the structure (26), and by the binding to calmodulin of the antipsychotic drug chlorpromazine in the presence of Ca^{2+}. In addition, when a calmodulin-deficient PSD preparation was prepared (26), sonicated, and incubated with [γ-^{32}P]ATP, Mg^{2+} and Ca^{2+}, one could not induce a Ca^{2+}-stimulation of protein kinase activity unless exogenous calmodulin was added back to the system, indicating a reconstitution of calmodulin into the PSD. We have also attempted to identify the two major phosphorylated proteins. Based on SDS polyacrylamide gel electrophoresis, it appears that the major 51,000 M_r PSD protein is the one that is phosphorylated and not the 51,000 M_r component of brain intermediate filaments, which is a known PSD contaminant. In addition, papain digestion of the 51,000 M_r protein revealed multiple phosphorylation sites different from those phosphorylated by the Mg^{2+}-dependent kinase(s). Finally, although the calmodulin-activatable protein kinase may phosphorylate proteins I_a and I_b, the cyclic AMP-dependent protein kinase, which definitely does phosphorylate proteins I_a and I_b and is present in the PSD, does not phosphorylate the 51,000 and 62,000 M_r proteins, because specific inhibition of this kinase has no effect on the levels of the phosphorylation of these latter two proteins.

In the previous paper we have shown that the postsynaptic density (PSD) contains a cryptic calmodulin-activatable cyclic nucleotide phosphodiesterase activity (29). A further examination of the possible function of the calmodulin in the PSD was prompted by the demonstration by several groups of the existence of calmodulin-dependent protein kinases. Calmodulin has been shown to be a subunit of the Ca^{2+}-dependent myosin light chain kinase (14, 15, 30, 41, 59, 60, 62, 64) as well as activating muscle (12, 53) and platelet (24) phosphorylase kinase, glycogen synthase kinase, (18, 56), and the phosphorylation of histones (58). It has also been implicated in the phosphorylation of brain cytosol (63) and of synaptic vesicle

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(13) proteins. More to the point, Schulman and Greengaard (50, 51) recently showed that calmodulin was involved in stimulating the Ca^{2+}-dependent phosphorylation of two proteins of 51,000 and 62,000 M_r in lysed synaptosomes and in a crude synaptic membrane fraction. We now describe the presence and properties of a calmodulin-activatable protein kinase activity in a highly purified Triton X-100-derived PSD preparation from canine cerebral cortex, a preparation derived from a synaptic membrane fraction.

MATERIALS AND METHODS

Materials

Biochemical compounds were obtained as follows; acrylamide, bisacrylamide, and *N,N,N',N'*-tetramethylenediamine were from Eastman Organic Chemicals Div. Eastman Kodak Co. (Rochester, N. Y.); Tris, Coomassie Brillant Blue R, 2-mercaptoethanol, TCA, SDS, EGTA, chlorpromazine-HCl, PIPES, phosvitin, calf thymus histone type II-AS, papain (EC 3.4.22.2), bovine heart protein kinase inhibitor, and rabbit muscle phosphorylase B (EC 2.4.1.1) were obtained from Sigma Chemical Co. (St. Louis, Mo.); adenosine 5'-[γ-^{32}P]-triphosphate (>2,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, Ill.); Triton X-100 from Packard Instrument Co., Inc. (Downers Grove, Ill.); sucrose (density gradient grade) was from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.); and 2.4 cm GF/C glass microfiber paper discs were from Whatman. All other chemicals were reagent grade. Highly purified calcineurin and calmodulin-activatable cyclic nucleotide-phosphodiesterase from brain were kind gifts of Dr. Claude Klee (National Institutes of Health [NIH]). Chick brain calmodulin was a gift of Dr. D. M. Watterson (The Rockefeller University).

General Methods

Samples to be used for gel electrophoresis were resuspended in a solution containing 0.08 M Tris-HCl, pH 6.7, 7% glycerol, and 3% 2-mercaptoethanol. The sample was briefly sonicated, and SDS was added to a final concentration of 2%. SDS polyacrylamide gel electrophoresis (SDS PAGE) was performed according to the discontinuous buffer system of Neville (42) except that a slab gel (2 mm thick) containing a linear 5–15% (wt/vol) acrylamide gradient was used (11). Electrophoresis was carried out until the tracking dye (0.001% bromphenol blue) reached the bottom of the gel. The gels were fixed and stained in a solution containing 0.25% (wt/vol) Coomassie Blue in 50% (vol/vol) methanol, 7% (vol/vol) acetic acid. 5 mM EDTA was routinely added to the upper reservior buffer. Protein concentration was determined by the method of Lowry et al. (37).

A PSD fraction was isolated from synaptosomes in a short procedure by treatment with 0.5% Triton X-100 as previously described (11). This preparation was purified by further treatment with 0.5% Triton X-100, 75 mM KCl to remove contaminating membrane proteins (11). Purified calmodulins from canine brain and bovine brain cortex were isolated as described by Watterson et al. (61) as modified by us (26). Canine brain tubulin was prepared by the assembly-disassembly method of Gaskin et al. (23), except that PIPES buffer was used in place of 2-(*N*-morpholino)ethane sulfonic acid. A canine brain intermediate filament protein preparation was isolated by exposure of axons to low ionic strength medium for 1 h as described by Liem et al. (36). Skeletal muscle myosin light chain was prepared from canine thigh muscle according to the method of Pires and Perry (47).

Assay for Calcium-dependent Protein Kinase Activity

Calcium-dependent protein kinase activity of the PSD was performed according to the method of Schulman and Greengard (50, 51). The standard reaction mixture contained in a final volume of 0.1 ml: 50 mM PIPES buffer, pH 7.0; 10 mM Mg_2; 1 mM dithiothreitol; 0.2 mM EGTA; plus PSD protein. The plus calcium samples also contained 0.5 mM $CaCl_2$ with or without purified canine brain calmodulin. After a 1-min preincubation at 30°C, 5 μM [γ-^{32}P]ATP (1.6 Ci/mmol) was added and the mixture was incubated at 30°C. The reaction was usually terminated by the addition of 2% SDS, and SDS PAGE was performed as described in Materials and Methods. Autoradiography was performed on the dried gels using Cronex 2 DC medical film. On some occasions, the reaction was terminated with 5 ml ice-cold 5% TCA-1% pyrophosphate. 0.5 mg of carrier bovine serum albumin was added and the sample was transferred onto 2.4 cm GF/C glass microfiber paper discs using a Millipore sampling manifold (Millipore Corp., Bedford, Mass.). The samples were then washed three times with 5 ml TCA-pyrophosphate, once with 5 ml 100% methanol, once with 5 ml ethyl ether, and finally transfered to scintillation vials that contained 10 ml H_2O. Cerenkov radiation was monitored in the ^{3}H channel in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

RESULTS

We had previously described in the PSD the existence of a cyclic AMP-activatable protein kinase activity (2, 57) as well as its substrate proteins, proteins I_a and I_b. To assess whether or not our PSD preparation also had a calmodulin-activatable protein kinase activity, PSDs were incubated for various times with 5 μM [γ-^{32}P]ATP and Mg^{2+} in the absence and presence of Ca^{2+} and calmodulin. As seen in the results of a representative experiment (Fig. 1), incorporation of $^{32}PO_4$ into total PSD protein in the presence of Mg^{2+} reached a maximal level after 3 min of incubation at 30°C. In the presence of Ca^{2+} and calmodulin, the phosphorylation of total PSD protein was increased some 30% over the Mg^{2+} control. Identical results were obtained whether 5 μM or 1 mM ATP was used. Previously (11) it had been found that the PSD preparations contain no Ca^{2+}- or Mg^{2+}-ATPase activity. Although there was little apparent phosphatase activity in the Mg^{2+} controls up to 11 min of incubation (the longest time tested), some phosphatase activity was seen in the calmodulin-incubated PSD samples between 7 and 11 min. The 5 μM ATP concentration as well as the 3-min incubation time at 30°C was therefore used in all the subsequent studies.

Because the PSD fraction did appear to have a calmodulin-activatable protein kinase activity, the nature of the proteins phosphorylated were examined by subjecting the PSD after incubation to SDS PAGE. As seen in the autoradiograph (Fig. 2 *B*) of the Coomassie Blue-stained gel (Fig. 2 *A*), many proteins in the native PSD preparation were phosphorylated in the presence of Mg^{2+} alone (slot *1*). Upon the addition of Ca^{2+} to the PSD preparation, there was a slight increase in phosphorylation of the protein(s) in the major 51,000 M_r region (slot *2*). However, in the presence of both Ca^{2+} and exogenous calmodulin there was a marked increase in the phosphorylation of two major protein regions in the PSD: at M_r = 51,000 and at M_r = 62,000 (slot *3*). In this experiment an increase was also

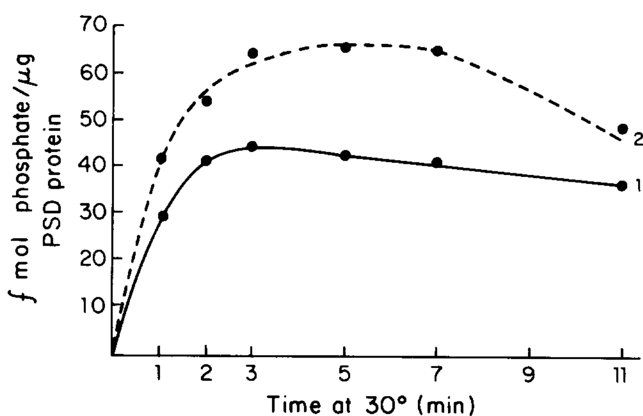


FIGURE 1 Incorporation of $^{32}PO_4$ into total PSD protein. Fresh PSDs (80 μg) were incubated for various times at 30°C with [γ-^{32}P]ATP and the reaction was stopped with 5 ml ice-cold 5% TCA-1% pyrophosphate as described in the text. The samples were then transfered onto paper dics and prepared for scintillation counting. The data are expressed as femtomoles phosphate incorporation per microgram total PSD protein. (*1*) PSDs incubated in the presence of 10 mM Mg^{2+} alone. (*2*) PSDs incubated as in *1* except that 0.5 mM Ca^{2+} and 4 μg chick brain calmodulin were also present.

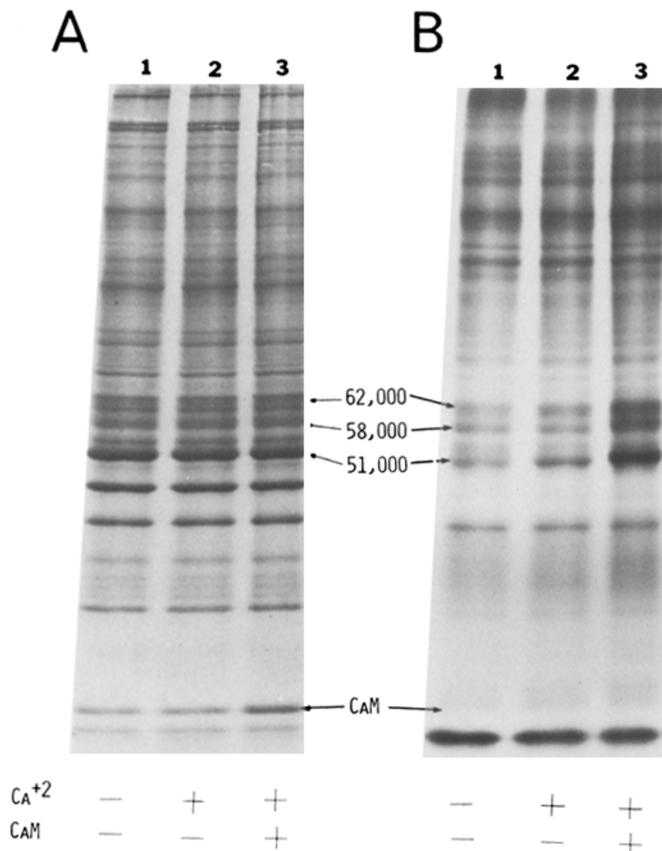
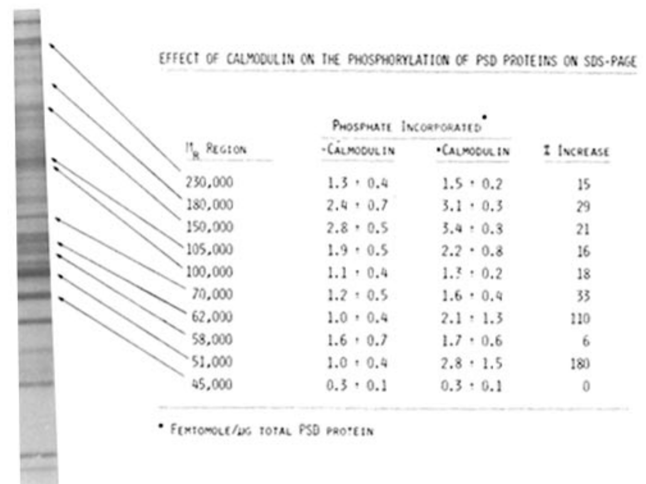


FIGURE 2 SDS PAGE of phosphorylated PSD proteins. The incubation conditions (3 min at 30°C) as well as SDS PAGE and autoradiography was performed as described in the text. The Coomassie Blue-stained gels, 120 μg PSD protein, are shown in *A*, while the autoradiograms of the stained gels are shown in *B*. The arrows indicate the relative molecular masses of select regions on the gel. The absence or presence of either 0.5 mM Ca^{2+} and/or 4 μg canine brain calmodulin is shown at the bottom of the figure. The phosphorylated band below the calmodulin in *B* is the contaminanting myelin basic protein.

at M_r = 58,000, but see Fig. 3 for the average increases in six to eight experiments. No phosphorylation was seen in the total absence of Mg^{2+} under the conditions studied and it was, therefore, added in all the subsequent studies. The data indicate that the PSD does contain an endogenous calmodulin-activatable protein kinase as well as its substrate proteins, and this activity is most likely on the surface of the PSD, as it responds to added calmodulin.

Fig. 3 summarizes the data showing the effect of Ca^{2+} and calmodulin on the phosphorylation of individual PSD proteins, since there was variability among these proteins in this regard. Separate PSD preparations from the cortices of six to eight different animals were phosphorylated in the absence or presence of Ca^{2+} and calmodulin as described for Fig. 2. After SDS PAGE, protein bands were cut out of the wet gels and counted for radioactivity. The data are expressed as the mean (±SE) incorporation of phosphate into an individual protein region per total PSD protein incubated (fmol phosphate/μg total PSD protein per 3 min). As shown in the figure, although many protein regions showed average increases in phosphorylation of 6–33% over the Mg^{2+}-incubated control after calmodulin adition, by far the greatest average increase (180%) in phosphorylation was in the major 51,000 M_r region followed by the 62,000 M_r region (110%). With the exception of the myelin basic protein (M_r = 17,000) which is a major contaminant (3), proteins below M_r = 51,000 were phosphorylated either very poorly or not at all, nor was there an increased phosphorylation after calmodulin addition. Occasionally, however, there was a protein with M_r = 20,500 which could be phosphorylated with calmodulin, although no visible protein band could be seen on the stained gels. The phosphorylation in the 70,000 M_r region, known to also contain the substrate proteins (proteins I_a and I_b) for the endogenous cyclic AMP-dependent protein kinase (2, 57), was increased by 33% upon addition of Ca^{2+} and calmodulin, but there was a 183% increase (3.4 ± 1.1 fmol/μg total PSD protein) in this protein region when the PSD was incubated in the presence of 10 μM cyclic AMP instead of calmodulin (cf. Fig. 4*A*, slot *2*).

It appears from the above, that exogenous calmodulin induces a large increase in the phosphorylation of mainly two proteins in the isolated PSD, but the relatively low activity of the kinase in the presence of endogenous calmodulin in the native PSD preparation (only Ca^{2+} addition) was somewhat disturbing. However, under certain conditions, such as mild sonication for 10–15 s in either double-distilled H_2O or low ionic strength buffer, e.g., 6 mM Tris-HCl, pH 8.1, or by freezing the preparation at −80°C followed by thawing (again in H_2O or low ionic strength buffer), one can now induce a marked increase in phosphorylation in the 51,000 and 62,000 M_r regions by the addition of Ca^{2+} alone (Fig. 4*A*). For example, slot *4* shows the Coomassie Blue-stained gel of a PSD preparation (100 μg protein) which has been subjected to freeze-thawing. As seen in the autoradiograph in slot *1*, again many proteins are phosphorylated in the presence of Mg^{2+} alone, but with added Ca^{2+} there is a large increase in phosphorylation in the 51,000 and 62,000, and in this case in the 58,000, regions (slot *3*; compare to slot *6*, Fig. 4*B*). There is also a smaller increase in other protein regions on the gel (>M_r = 51,000); note especially the comparatively small increase in



EFFECT OF CALMODULIN ON THE PHOSPHORYLATION OF PSD PROTEINS ON SDS-PAGE

M_R Region	Phosphate Incorporated*		% Increase
	-Calmodulin	+Calmodulin	
230,000	1.3 ± 0.4	1.5 ± 0.2	15
180,000	2.4 ± 0.7	3.1 ± 0.3	29
150,000	2.8 ± 0.5	3.4 ± 0.3	21
105,000	1.9 ± 0.5	2.2 ± 0.8	16
100,000	1.1 ± 0.4	1.3 ± 0.2	18
70,000	1.2 ± 0.5	1.6 ± 0.4	33
62,000	1.0 ± 0.4	2.1 ± 1.3	110
58,000	1.6 ± 0.7	1.7 ± 0.6	6
51,000	1.0 ± 0.4	2.8 ± 1.5	180
45,000	0.3 ± 0.1	0.3 ± 0.1	0

* Femtomole/μg total PSD protein

FIGURE 3 Effect of Ca^{2+} and calmodulin on the phosphorylation of PSD proteins. Separate PSD preparations (100 μg protein) from six to eight different canine cerebral cortices were incubated with [γ-^{32}P]ATP for 3 min both in the absence or presence of 0.5 mM Ca^{2+} and 4 μg canine brain calmodulin as described in the text. Various protein regions were cut out of the wet gels and counted in 10 ml double-distilled H_2O. The data are expressed as the mean (±SE) incorporation of phosphate into an individual protein region per total PSD protein incubated (fmol/μg). When cyclic AMP was used in place of Ca^{2+} and calmodulin, the final concentration was 10 μM.

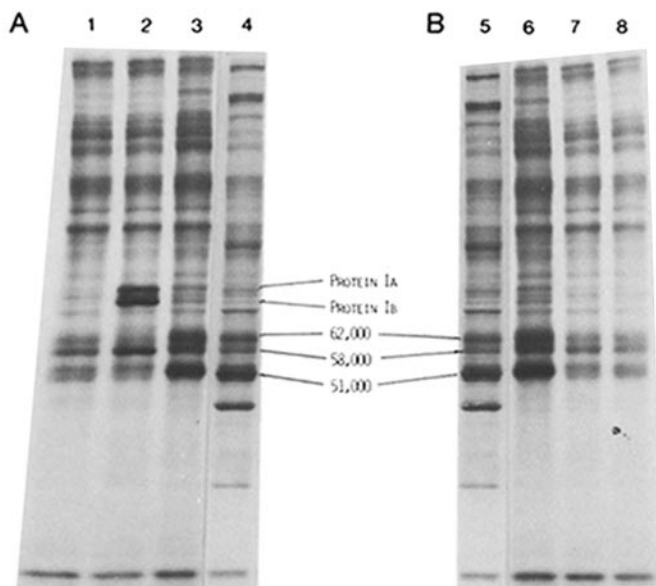


FIGURE 4 Effects of freeze-thawing, EGTA, and chlorpromazine on the calmodulin-activatable protein kinase activity in the PSD. PSDs were homogenized and frozen in double-distilled H_2O in a dry ice-methanol bath and stored overnight at −80°C. The samples were then thawed under warm running tap water and immediately put on ice before use. The PSDs were then incubated with [γ-^{32}P]ATP as described in the text. Slot *4* shows the Coomassie Blue-stained gel of this freeze-thawed PSD preparation (75 μg protein). The autoradiograms of the freeze-thawed PSDs incubated under varying conditions are shown in slots *1–3*. (*1*) PSDs incubated in the presence of 10 mM Mg^{2+} alone. (*2*) Same as *1* but with the addition of 10 μM cyclic AMP. (*3*) Same as *1* but with the addition of 0.5 mM Ca^{2+}. *B* shows the effects of both EGTA and chlorpromazine on calmodulin-dependent protein kinase activity in the PSD. Slot *5* again shows the Coomassie Blue-stained gel of the freeze-thawed PSD preparation (75 μg). Slots *6–8* show the autoradiograms of the freeze-thawed PSD preparation incubated in the presence of [γ-^{32}P]ATP and Ca^{2+} and 3 μg canine brain calmodulin with the following additions: (*6*) no additions (*7*) 1 mM EGTA, and (8) 1 mM chlorpromazine.

protein $I_a + I_b$. For comparison, the autoradiogram in slot *2* was made from PSDs incubated with Mg^{2+} and cyclic AMP, and as was shown in earlier papers (2, 57); there were two proteins phosphorylated in the 70,000 M_r region: protein I_a (M_r = 73,000) and protein I_b (M_r = 68,000). Clearly the two protein kinase systems in the PSD are different in both activator and substrate requirements and, as will be shown later (cf. Table I), in the nature of the two kinases.

The Ca^{2+} and calmodulin activatability of the PSD protein kinase activity was further tested and the data are summarized in Fig. 4*B*. Freeze-thawed PSDs that were used in the experiment shown in Fig. 4*A* were incubated with [γ-^{32}P]ATP in the presence of Mg^{2+}, Ca^{2+}, and calmodulin. The autoradiogram in slot *7* shows that calmodulin-stimulated phosphorylation was completely inhibited to the Mg^{2+} control level (see slot *1*, Fig. 4*A*) after Ca^{2+} chelation with EGTA, which will also remove calmodulin from the PSD (26). An identical effect was seen after the addition of 1 mM chlorpromazine (slot *8*), an antipsychotic agent known to bind to calmodulin in the presence of Ca^{2+} (35). Complete inhibition of the calmodulin-activatable kinase activity was also seen at 0.1 mM concentration of chlorpromazine (not shown).

To verify the calmodulin dependency of the increased phosphorylation, a calmodulin-deficient PSD preparation was prepared as described in a previous paper (26), sonicated, and incubated with [γ-^{32}P]ATP, Mg^{2+} and Ca^{2+}. If the PSD had a kinase dependent for Ca^{2+} alone one might have been able to expose the activity by sonication. However, one could not induce a Ca^{2+} stimulation of protein kinase activity unless exogenous calmodulin was added back to the system. As shown in Fig. 5*A*, the proteins whose phosphorylation was increased in this reconstituted system were similar to those observed when either exogenous calmodulin is added to a fresh, unaltered, PSD preparation (Fig. 2), or when Ca^{2+} is added to a freeze-thawed or sonicated PSD preparation (Fig. 4). In addition, the calmodulin-activatable protein kinase activity in the reconstituted system reached saturation levels, and the final level of total phosphate incorporated (65 fmol/μg total PSD protein) was similar to that found for the native PSD preparation (67 fmol/μg total PSD protein) when incubated with exogenous calmodulin (compare Fig. 5*B* to Fig. 1). It appears from these data that the PSD does indeed contain a calmodulin-activatable protein kinase system, and that the calmodulin which is reconstituted into the PSD is able to activate this kinase, and probably is reconstituted into its proper place in the structure.

What can we say about the possible identity of the PSD proteins whose phosphorylation is increased in the presence of calmodulin? One possibility is that the 51,000 M_r protein corresponds to one component of a brain intermediate filament protein preparation of the same approximate molecular weight, because the intermediate filament proteins are found in the PSD preparations, but are believed to be contaminants (6, 33, 39). We therefore compared the gel mobilities of a purified filament preparation with those of the phosphorylated PSD

TABLE I

Effect of the Inhibitor of the Cyclic AMP-dependent Protein Kinase on the Calmodulin-activatable Protein Kinase in the PSD

Additions*	Increase relative to the control		
	Protein I	62,000 M_r protein	51,000 M_r protein
Cyclic AMP	1.6	0	0
Calmodulin	1.2	4.0	5.1
Cyclic AMP + calmodulin	2.7	4.0	5.3
+40 μg Inhibitor	1.7	4.0	5.5
+80 μg Inhibitor	1.7	4.5	5.3
+120 μg Inhibitor	1.5	4.2	5.0
+120 μg Inhibitor + chlorpromazine	0	0	0
Cyclic AMP + chlorpromazine	1.4	0.4	0.1
Cyclic AMP + 120 μg inhibitor	0.1	0	0.1
Calmodulin + chlorpromazine	0.4	0	0
Calmodulin + 120 μg inhibitor	1.3	4.2	5.1

Fresh PSDs (50 μg) were phosphorylated in the presence of 50 mM PIPES (pH 7.0), 10 mM $MgCl_2$, 1 mM isobutylmethylxanthine, 0.2 mM EGTA, and 5 μM [γ-^{32}P]ATP for 3 min at 30°C under the conditions given below. SDS PAGE was performed as described in the text. After drying, the gel was subjected to autoradiography and the regions corresponding to protein I ($I_a + I_b$), the 62,000 M_r protein, and the major 51,000 M_r PSD protein, were scanned at 550 nm in a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). The data are expressed as the increase in peak intensity relative to the Mg^{2+}-incubated control.

* The concentrations of the added components are as follows: 10 μM cyclic AMP; 1 mM chlorpromazine; and 3 μg bovine brain calmodulin plus 0.5 mM $CaCl_2$.

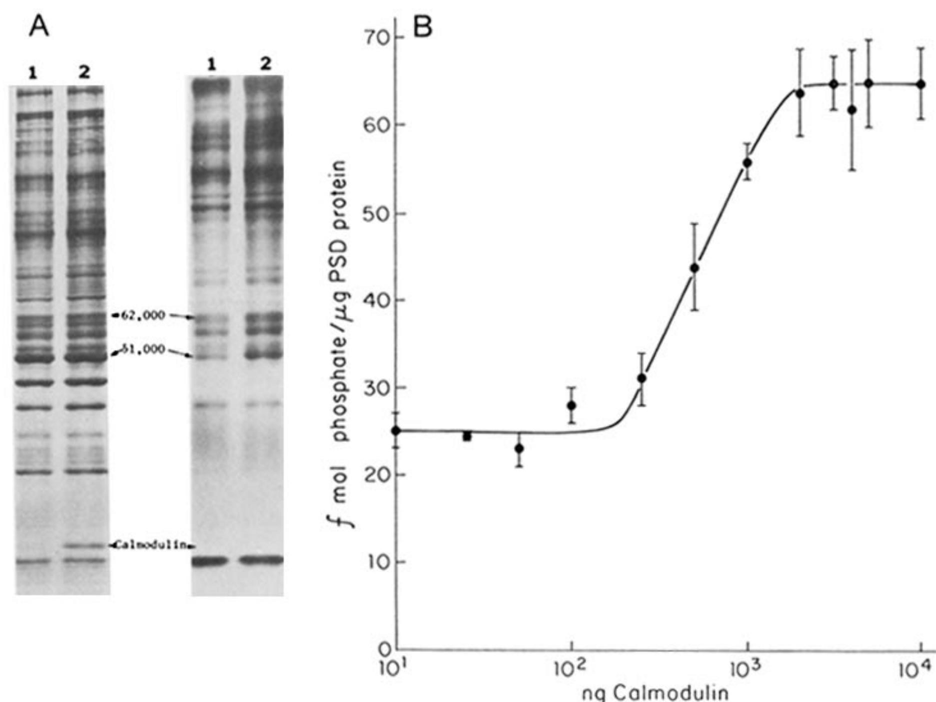


FIGURE 5 Effect of calmodulin on phosphorylation of calmodulin-deficient PSDs. PSDs made deficient in calmodulin (26) were incubated in the standard phosphorylation reaction mixture, as given in the text. In *A*, 100 μg calmodulin-deficient PSDs were sonicated and then incubated in the presence of 0.5 mM Ca^{2+} alone (slot *1*) or with 4 μg canine brain calmodulin (slot *2*). The Coomassie Blue-stained gels are shown on the left and the corresponding autoradiograms are shown on the right. In *B*, 25 μg calmodulin-deficient PSDs were incubated in the presence of Ca^{2+} and increasing amounts (10^1-10^4 ng) of canine brain calmodulin. The reaction was stopped with 5% TCA-1% pyrophosphate and the samples counted for radioactivity as described in the text. The data are expressed as femtomoles phosphate incorporated per microgram total PSD protein and the curve represents the mean (±SE) of triplicate determinations.

proteins. Firstly, we found that the brain intermediate filament preparation has an endogenous protein kinase activity, but this activity was not activatable by calmodulin (data not shown). Secondly, as seen in Fig. 6, the 51,000 M_r component of the brain intermediate filament preparation moves sometimes slightly ahead of (Fig. 6*A*) or sometimes behind (Fig. 6*B*) the major 51,000 M_r phosphorylated band (cf. reference 19 for variable electrophoretic behavior of filament proteins on SDS gels). In addition, when PSDs are co-incubated with a brain intermediate filament preparation, no increase in phosphorylation of the 51,000 M_r region was observed (data not shown). It therefore appears that the 51,000 M_r region phosphorylated by the endogenous calmodulin-protein kinase in the PSD contains in fact the unique major cerebral cortex PSD protein (6, 27, 33). An added proof is the observation that [^{125}I]calmodulin was found to bind strongly to the 51,000 M_r region and not to any of the brain filament proteins on SDS PAGE gels (5, 7, 8). We are still not certain that there is only one protein in the 51,000 M_r region, since the protein is highly insoluble and resists purification. However, the available evidence (6) strongly suggests that there is only one protein that is phosphorylated and that binds calmodulin.

We would now like to present some evidence that the calmodulin-activatable protein kinase phosphorylates domains in this 51,000 M_r PSD protein which are different from those phosphorylated by the general kinase activity, rather than increasing the phosphorylation of the same domains. PSDs were phosphorylated in the absence or presence of calmodulin and subjected to SDS PAGE as described in the text. The 51,000 M_r PSD protein, which was well separated on the gel from the 51,000 M_r brain intermediate filament protein, and assumed to be pure, was digested with papain and the phosphorylated polypeptide fragments analyzed by autoradiography (cf. Fig. 7). In addition to the quantitative differences (3.3 times more phosphate incorporation in the presence of calmodulin), there was a significant qualitative difference in phosphate incorporation into the various papain-produced peptide fragments. As seen in Fig. 7, papain proteolysis of the 51,000 M_r protein phosphorylated in the presence of Mg^{2+} alone yielded three discernible radioactive peptide fragments: M_r's = 14,800 (1), 12,300 (2), and 10,950 (3). Approximately 50% of the phosphate incorporated into the entire 51,000 M_r protein occurred in the 12,300 M_r peptide fragment, while 25 and 10% of the total phosphate was incorporated into the 10,950 M_r and 14,800 M_r peptide fragments respectively. In contradistinction, the overall autoradiographic pattern of the proteolysis products of the 51,000 M_r protein after calmodulin-activatable phosphorylation was significantly different, yielding two additional radioactive peptide fragments: M_r's = 9,350 (4) and 8,150 (5). It should also be noted that phosphate incorporation into the smaller M_r peptides (2 to 5) were similar: 20–25% of the total incorporated phosphate. So it appears that not only is there an overall increase in the amount of phosphate incorporated into the 51,000 M_r protein catalyzed by the PSD calmodulin-activatable protein kinase, but the kinase acts to phosphorylate sites in this protein different from, and significantly apart, those acted upon by the more general kinase activity(s) present in the PSD. We assume that there is only one major phosphorylated protein in this region (6). However, it is possible that two populations of the 51,000 M_r protein exist in the cerebral

cortex PSD, one phosphorylated by the Mg^{2+}-protein kinase, and the other by the calmodulin-activatable protein kinase which binds calmodulin (8).

Because tubulin was known to be an intrinsic part of the PSD (3, 20, 33, 40), although the actual amounts appear to be questionable (9), we decided to determine whether the 62,000 M_r region whose phosphorylation is increased by calmodulin, as well as the 58,000 M_r region whose phosphorylation was also occasionally increased by calmodulin, were the alpha or beta subunits of tubulin respectively. Again, a comparison was made of the gel motilities of the tubulin preparation and the phosphorylated PSD proteins. Again we found that canine brain tubulin prepared by the assembly-disassembly procedure of Gaskin et al. (23), had an endogenous protein kinase activity that was independent of added calmodulin (data not shown). Although only the tubulin-associated proteins were phosphorylated, none of these phosphorylations were calmodulin-activatable. On a 5–15% SDS PAGE gel the beta subunit of tubulin migrated behind the major 51,000 M_r phosphorylated band, but the alpha subunit did co-migrate on this gel with the 58,000 M_r protein whose phosphorylation was occasionally increased by the addition of calmodulin (data not shown). However, on a 5–10% SDS PAGE gel the alpha subunit of tubulin moved between the 62,000 and 58,000 M_r phosphorylated proteins, while the beta-subunit moved between the 58,000 and 51,000 M_r bands (27). In addition, co-incubation of tubulin with the PSD did not increase the phosphorylation of the 58,000 M_r region (not shown), nor were the tubulin subunits found to bind calmodulin on gels (5, 7, 8). Thus, it appears that none of the phosphorylated proteins are identifiable as tubulin proteins.

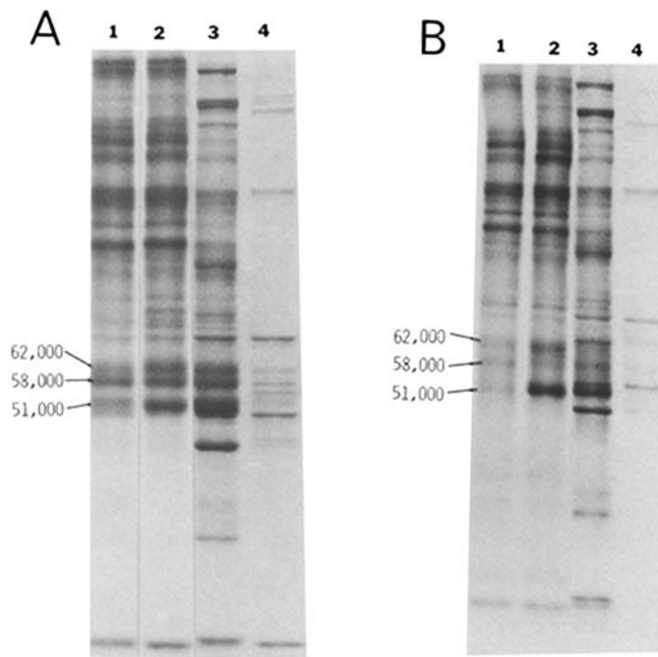


FIGURE 6 Comparison of the 51,000 M_r PSD protein to the 51,000 M_r component of the brain intermediate filament protein preparation. PSDs (80 μg) were incubated with [γ-^{32}P]ATP and SDS PAGE performed as described in the text. *A* and *B* show the data from two identical experiments using different PSD preparations, but incubated and run on SDS PAGE under similar conditions. (*1*) Autoradiogram of the PSD incubated in the presence of 10 mM Mg^{2+} alone. (*2*) Autoradiogram of the PSD incubated as in *1* but with the addition of 0.5 mM Ca^{2+} and 3 μg canine brain calmodulin. (*3*) Coomassie Blue-stained gel of the PSD preparation (80 μg protein). (*4*) Coomassie Blue-stained gel of the brain intermediate filament preparation (10 μg).

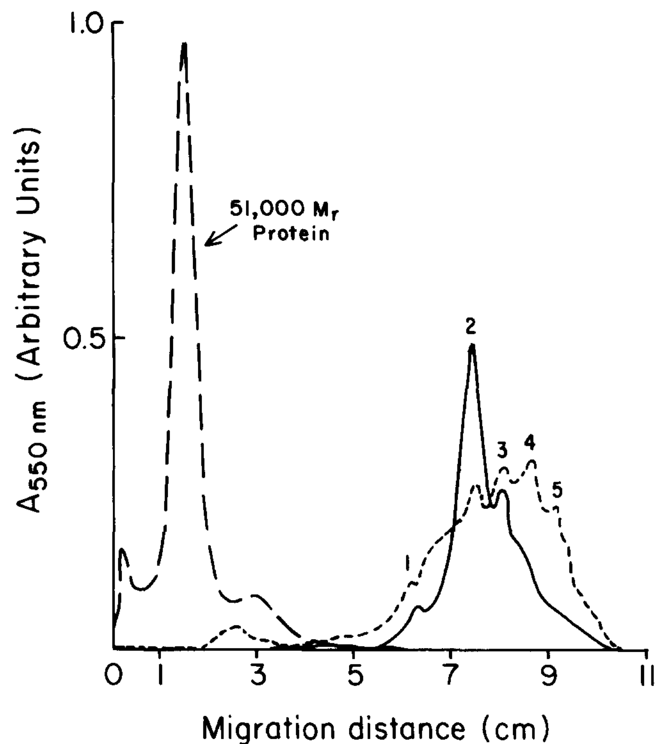


FIGURE 7 A scan of the autoradiogram after limited proteolysis by papain of the phosphorylated 51,000 M_r PSD protein. A PSD fraction (100 μg) was phosphorylated in the absence or presence of Ca^{2+} plus 3 μg bovine brain calmodulin, and then subjected to SDS PAGE as described in the text. After electrophoresis the gel was briefly stained and destained, and the portion of the gel containing the major 51,000 M_r PSD protein band, which was easily separated from the marker 51,000 M_r intermediate brain filament protein, was cut out and soaked in 54 mM Tris-sulfate (pH 6.1) for 1 h. In this experiment 3.3 times more $^{32}PO_4$ was incorporated into the 51,000 M_r protein in the presence of Ca^{2+} plus calmodulin than in the Mg^{2+}-incubated control (3 fmol vs. 0.7 fmol phosphate/μg total PSD protein respectively). After soaking in buffer, the protein band was subjected to a second SDS gel electrophoresis (15% separating gel with a 3% stacking gel) in the absence or presence of 1 μg papain according to the method of Cleveland et al. (10), except that the Neville buffer system (42) was used. After drying, the gel was subjected to autoradiography and the exposure time normalized so that the intensities of the 51,000 M_r regions in both the Mg^{2+} and Ca^{2+}/calmodulin samples were identical. Scanning of the autoradiogram was performed at 550 nm using the Zeiss Spektraphotometer PM6. The figure is a composite of the scan of three slots, the untreated 51,000 M_r protein, and the two papain-treated samples. The phosphorylated band seen above the 51,000 M_r protein region is probably an aggregated form of the 51,000 M_r protein that failed to enter the separating gel. The estimated M_r's of phospho-peptide peaks *1–5*, as compared to standards run at the same time are: (*1*) 14,800; (*2*) 12,300; (*3*) 10,950; (*4*) 9,350; and (*5*) 8,150.

We do have tentative evidence that the increased phosphorylation in the 62,000 M_r region may be on the calmodulin-activatable cyclic nucleotide-phosphodiesterase which is found in the PSD (29). Both the calmodulin-binding proteins, calcineurin A subunit and the catalytic subunit of the calmodulin-dependent cyclic nucleotide-phosphodiesterase, both obtained through the kind courtesy of Dr. Claude Klee at the NIH, migrate to this 62,000 M_r region, but can be separated on our gel system. However, the calmodulin-increased phosphorylation of the PSD proteins in this region appears to occur on the

phosphodiesterase, rather than on the calcineurin A, for the increased phosphorylation occurs at the protein band migrating similarly to the phosphodiesterase rather than to the calcineurin A (27). Relevant to these findings, we found that ^{125}I-calmodulin binds to the calcineurin/phosphodiesterase protein region on gels (5, 7, 8).

We have been unable to purify and positively identify the protein kinase responsible, since it seems to resist extensive solubilization. Co-incubation of the PSD preparation with phosphorylase *b*, glycogen synthase, as well as with myosin light chains from canine skeletal muscle, substrates known to require a calmodulin-dependent protein kinase (12, 14, 15, 18, 24, 30, 41, 53, 56, 59, 60, 62, 64), did not induce any phosphorylation in the substrate proteins used. Histone II-AS and phosvitin were also not phosphorylated by the PSD kinase. It may well be that the substrates tested here, as well as earlier (brain filaments or tubulin), were unavailable to bind to the catalytic site of the calmodulin-activatable protein kinase present in the PSD as isolated.

As described earlier with regard to the data shown in Figs. 3 and 4*A*, the PSD calmodulin-activatable protein kinase can phosphorylate the protein substrates for the endogenous cyclic AMP-dependent protein kinase, proteins I_a and I_b, although not as intensely as it does the 51,000 and 62,000 M_r regions. In view of this observation, experiments were done to decipher what interrelationships, if any, exist between these two endogenous PSD protein kinase systems. A fresh PSD preparation was phosphorylated in the presence of Mg^{2+} and with cyclic AMP or calmodulin alone or in combination, together with the specific inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinases (1) or with the calmodulin inhibitor chlorpromazine (35). As seen in Table I, the phosphorylation of protein I (I_a and I_b) increased to a similar degree when PSDs were incubated in either the presence of cyclic AMP (160%) or calmodulin (129%). When both activators were incubated together, the effect on the increase of protein I phosphorylation was almost additive (270%). In contrast to this result, while calmodulin caused a dramatic increase in phosphate incorporation into the 51,000 and 62,000 M_r protein regions, cyclic AMP failed to have any significant effect on the phosphorylations of these two proteins. As can also be seen in the table, the cyclic AMP-dependent protein kinase inhibitor almost completely blocked the cyclic AMP activation of protein I phosphorylation, yet it had no effect on preventing the calmodulin-induced phosphorylation of both 51,000 and 62,000 M_r proteins. If calmodulin was also present in the incubation media together with cyclic AMP, the inhibitor protein was only able to reduce the total additive activation of protein I phosphorylation from 270 to 150%, while virtually having no effect on the phosphorylation of the substrate proteins for the calmodulin protein kinase. When the PSDs were incubated with calmodulin plus chlorpromazine, the phosphorylation of the 51,000 and 62,000 M_r proteins was completely blocked, while protein I phosphorylation was inhibited some 67%. Chlorpromazine had little effect on cyclic AMP induced phosphorylation of protein I. Finally, the presence of both the inhibitor and chlorpromazine effectively blocked the activation of the phosphorylation of all three substances when both calmodulin and cyclic AMP were added together. The above data taken together indicate that although the calmodulin-activatable protein kinase may in fact phosphorylate protein I at a site different from the cyclic AMP-dependent protein kinase in the PSD, as suggested by recent work with synaptosomes (31, 34), the reverse situation, however, is unlikely. That is, the cyclic AMP protein kinase does not interact with the substrates for the calmodulin-activatable kinase system. Perhaps the calmodulin system is a more general one, one preferring the 51,000 and 62,000 M_r proteins not because of any strict substrate specificity, but because of some strict geometry inherent in the PSD structure.

DISCUSSION

In the present study, we describe the presence of a calmodulin-activatable protein kinase activity in our Triton X-100 derived PSD preparation which can stimulate the phosphorylation of mainly two endogenous PSD proteins of ~51,000 and 62,000 M_r. This activity appears to be located on the surface of the native structure, since it is accessible to the addition of exogenous calmodulin. This accessibility is in contrast to the calmodulin-activatable cyclic nucleotide phosphodiesterase activity present in the PSD (29) which cannot be activated by the addition of exogenous calmodulin. Another interesting difference between the kinase and phosphodiesterase activities in the PSD is that the former enzyme can be activated by the endogenous-calmodulin in the presence of Ca^{2+} after mild sonication or freeze-thawing, whereas similar treatment or even extensive sonication has little or no effect on the latter enzyme. The possible reasons for this lack of activation of the phosphodiesterase have been pointed out in the previous paper (29).

Schulman and Greengard (50, 51) recently showed that added calmodulin stimulated the phosphorylation of two proteins of 51,000 and 62,000 M_r in lysed synaptosomes and in a crude synaptic membrane (SM) preparation. Because the PSD is part of the synaptosome and synaptic membrane fractions, we compared the level of phosphorylations between them. Using millimolar concentrations of ATP to prevent substrate depletion (43, 44), and using a similar SM preparation, we find a twofold increase by calmodulin in the phosphorylation of these two protein regions in the PSD isolated from this SM preparation (data not shown), suggesting that the proteins phosphorylated in the SM preparation are probably PSD in origin. Although they did not look for a calmodulin-activatable protein kinase activity, Ng and Matus (44), using millimolar ATP concentrations, also find that the specific activity of general phosphoprotein labeling is some twofold higher in the PSD than in the parent SM preparation, again suggesting that the majority of the phosphorylation reactions observed in the SM are caused by the PSD which is a part of it. If exogenous calmodulin can stimulate a protein kinase activity in SM preparations, presumably of PSD origin, it is not unreasonable to assume that the protein kinase in the PSD is located on the surface of the structure facing the cell cytosol, because if it were present on the PSD surface attached to the membrane facing the synaptic cleft, protein kinase activation might be blocked by the presence of the synaptic membrane.

Besides possessing an intrinsic calmodulin-activatable protein kinase system, the PSD has previously been shown to contain a cyclic AMP-dependent protein kinase as well as its substrates: proteins I_a and I_b (2, 57). We have shown here that these two kinase systems are different: while the calmodulin system can somewhat phosphorylate protein I, it seems to prefer the 51,000 and 62,000 M_r proteins as substrates; in addition, the cyclic AMP system cannot phosphorylate the 51,000 and 62,000 M_r proteins; and finally, the specific inhibitor of the cyclic AMP-dependent protein kinase has no effect on the calmodulin-activatable kinase.

Other proteins associated with the PSD may also have

protein kinase activities and be responsible for the general phosphorylation activity obtained by the addition of only ATP and Mg^{2+}. For example, canine brain tubulin prepared by the assembly-disassembly procedure of Gaskin et al. (23) has an endogenous protein kinase activity that phosphorylates many tubulin-associated proteins and is not dependent on calmodulin for activation. There are several reports in the literature describing a tubulin-associated protein kinase activity (17, 21, 25, 32, 48, 55); however, the literature is inconsistent as to the substrate proteins which are phosphorylated, and as to the source of the kinase itself. Nonetheless, because tubulin is intrinsic to the PSD (3, 20, 33, 40), although the actual amounts are rather questionable (9), it is possible that tubulin-associated kinase(s) contribute to the general PSD phosphorylation observed in the presence of Mg^{2+}. Also a canine brain neurofilament (intermediate) protein preparation was found to have an endogenous protein kinase associated with it, but again, it was not dependent on added calmodulin. This finding is in comformity with a recent report (16) that a kinase co-purifies with brain neurofilaments and that this kinase activity is not cyclic AMP nor Ca^{2+} dependent. Also, Pant et al. (46) have reported that neurofilament protein from squid giant axon is phosphorylated, and this appears to be a general property of neurofilaments from different sources (52). Phosphorylation is also found for intermediate filaments from non-neuronal sources (4, 45), and may be a general property of intermediate filaments (45). Whether or not neurofilaments have an associated specific kinase is a point of question, but it is possible that a kinase that phosphorylates neurofilament protein is not intrinsic to the PSD because neurofilaments seem to be a contaminant in the PSD preparation (6, 33, 39); however, such kinase activity can contribute to the overall background phosphorylation seen in the presence of only Mg^{2+}. Singh and Spritz (54) reported that highly purified myelin from peripheral nerve contains at least two endogenous protein kinases. One kinase is membrane bound and can phosphorylate endogenous substrate proteins as well as phosvitin, and is not inhibited by an inhibitor of the cyclic AMP-dependent protein kinase. The other kinase is readily washed out by KCl, utilizes histone, and is inhibited by the kinase inhibitor. Because our PSD preparation has been washed with KCl during the isolation procedure, it is posible that if any contaminating kinase of myelin origin were present in the PSD it would be related to the KCl-insoluble myelin protein kinase species. It is thus possible that the isolated PSD preparation has at least two contaminating protein kinase systems, so one has to be careful to dissect the intrinsic from contaminating protein kinase systems not only in the PSD, but for other subcellular organelles as well. However, we believe that the calmodulin-activatable protein kinase is intrinsic to the PSD, because it has proved difficult to solubilize preparatory to purification, either with extensive sonication, or with 150 mM KCl, or with 100 mM EGTA which does remove several proteins (3); it seems to be concentrated in the PSD over a synaptic membrane fraction; and its major substrate, the 51,000 M_r protein, is a highly insoluble PSD protein. The problems of interpreting phosphorylation data have also been addressed recently by Matus et al. (38).

Calcium and cyclic nucleotides as secondary messengers have been implicated to play interrelated roles in modulating information transfer within the cell (49). What connection, if any, exists between Ca^{2+} and cyclic AMP within the PSD, and does this connection have any influence upon synaptic transmission? The PSD does have an endogenous calmodulin-activatable protein kinase activity and at least two specific substrate proteins: 51,000 and 62,000 M_r. The PSD also contains a cyclic AMP-dependent protein kinase activity and two other specific substrates: proteins I_a and I_b (2, 57). In addition, the PSD contains at least two cyclic nucleotide phosphodiesterases one of which is calmodulin-activatable (29). Thus, at least at the level of the PSD, there are two areas of interrelationship between cyclic AMP and calmodulin. Firstly, cyclic AMP levels can be directly altered by Ca^{2+} activation of the calmodulin-stimulatable phosphodiesterase present, thereby altering the effects of cyclic AMP-dependent protein kinase in the PSD. Because the endogenous calmodulin-activatable protein kinase may possibly phosphorylate the catalytic subunit of the calmodulin-dependent phosphodiesterase, another control may be imposed on the system; however, the effect of phosphorylation is, as yet, speculative.

Although we would like to define the exact nature of the interrelationships between the calmodulin and the cyclic AMP systems present in the PSD with regard to synaptic transmission, we can only, at best, make preliminary speculations. Firstly, we assume that both systems are in the same PSD structure. However, because our PSD preparation is undoubtedly a mixture of PSDs from different types of synapses, with regard to the neurotransmitters involved, we cannot be sure of this assumption. However, we do feel that these two systems are somehow involved in the modulation of the propagation of postsynaptic potentials. Recently, we have isolated PSDs from both cerebral cortex and cerebellum (6, 27) and find that calmodulin, as well as the calmodulin- and cyclic AMP-activatable protein kinase activities, and as well as the major 51,000 M_r protein which is phosphorylated by the calmodulin-activatable protein kinase, are much enriched in PSDs isolated from cerebral cortex as compared to PSDs isolated from the cerebellum. Because the PSDs isolated from the cerebral cortex may have been derived from excitatory synapses, whereas those isolated from the cerebellum appear to be derived from inhibitory synapses (6, 27), it is not unreasaonable to assume that both the calmodulin and cyclic AMP protein kinase systems together play a significant role in the excitation response.

Another possibility for the physiological role of the protein kinase arises from the results from DeLorenzo's laboratory (cf. reference 13). They found that calmodulin stimulates the phosphorylation of two synaptic vesicle-associated proteins of M_r = 51,000–54,000 and M_r = 62,000–63,000. Whether these two proteins are the same as the PSD 51,000 and 62,000 M_r proteins is not known at present, but it is intriguing that two proteins of very similar M_r values, located at both sides of the synapse, are phosphorylated by a calmodulin-activatable protein kinase. If these proteins are the same, or similar, it could signify a Ca^{2+}/calmodulin-mediated postsynaptic regulation of presynaptic neurotransmitter release via the synaptic vesicles, for the release mechanism seems to involve a calmodulin-activatable phosphorylation of the synaptic vesicle proteins (13). This idea of a relationship between the PSD and presynaptic vesicles is strengthened by the finding (2, 57) that a cyclic AMP-dependent protein kinase increases the phosphorylation of two proteins, proteins I_a and I_b, both in PSD and in synaptic vesicles; again at both sides of the synapse. Depolarizing conditions, which increase Ca^{2+} transport across synaptosomal membranes, also increase the phosphorylation of these same two proteins in synaptosomes (34) and in cerebral cortex slices (22).

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