

Function of Calmodulin in Postsynaptic Densities

I. Presence of a Calmodulin-activatable Cyclic Nucleotide Phosphodiesterase Activity

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ABSTRACT The postsynaptic density (PSD) fraction from canine cerebral cortex was found to contain an endogenous cyclic nucleotide-phosphodiesterase activity that was dependent on Mn^{2+} and/or Mg^{2+} but not on Ca^{2+} . Maximal activity was obtained at $1 \mu M Mn^{2+}$. This cyclic nucleotide phosphodiesterase activity was not decreased upon removal of the calmodulin from the PSD fraction, nor was it increased by the addition of calmodulin to a postsynaptic density fraction deficient in calmodulin. The enzymatic activity could be extracted by sonication, with the soluble enzyme having properties similar to those found in the native structure. Two peaks of cyclic nucleotide phosphodiesterase activities could be obtained after S-300 Sephacryl column chromatography of this soluble fraction: fraction I (excluded peak) and fraction II (215,000 mol wt). The fraction I activity preferred cyclic AMP over cyclic GMP and was not activated by calmodulin. The fraction II activity had an approximately fourfold lower K_m for cyclic GMP over cyclic AMP. This fraction II activity was activatable by calmodulin, which increased the V_{max} and decreased the K_m in the case of both cyclic nucleotides. We conclude that two activities are present in the PSD, one activatable, and one not activatable, by calmodulin.

The postsynaptic density (PSD) is a prominent structure, of unknown function, in central nervous system synapses (1). As observed by electron microscopy, the PSD appears as a densely staining thickening which lies along the cytoplasmic side of the postsynaptic membrane (1). Recently, we have isolated from canine cerebral cortex a PSD fraction that has been found to contain some 10 major and at least 20 minor proteins (10). Of the enzymatic activities tested, there are no Mg^{2+} or Ca^{2+} -ATPase (10) nor adenylate cyclase (18) activities, and very small amounts of cytochrome *c* oxidase (10) or 5'-nucleotidase (10) activities. However, two protein kinase activities are present, one activatable by cyclic AMP (4, 45) and one activatable by Ca^{2+} and calmodulin (7, 18, 20, 21); the protein substrates, proteins Ia and Ib, which are phosphorylated by the former kinase (4, 45) and the protein substrates, the major 51,000 M_r protein and a 62,000 M_r protein, which are phosphorylated by the latter kinase (7, 18, 20, 21) are also found in the PSD preparation.

Immunocytochemistry has been used to confirm the presence of proteins Ia and Ib in the PSD (6). Other proteins identified to be intrinsic constituents are actin (5, 27), the alpha and beta subunits of tubulin (5, 27, 35, 36, 48) and a major $M_r = 51,000$

protein of unknown identity (7, 20, 21, 27). The purity of this fraction has been attested to in previous publications (5, 10) by electron microscope examination, by assaying for marker enzymes from other subcellular organelles, and by mixing experiments using ^{125}I -labeled proteins of other subcellular organelles. Very little contamination was found; the only known major contaminating proteins are myelin basic protein (10) and neurofilament protein (7, 12, 17, 20, 30, 37).

Recently, this laboratory has reported the presence of a troponin C-like protein in PSDs (5) and has directly demonstrated (19) that this protein is actually calmodulin by the isolation and characterization of the PSD protein and by comparison to brain calmodulin. Although calmodulin can be removed from the PSD by EGTA pretreatment, and not by a wash with KCl or buffers alone, reconstitution and saturation studies suggest that the presence of calmodulin in the PSD is not artifactual (19); indeed the presence of calmodulin in the PSD has been confirmed by immunohistochemistry and by radioimmunoassay (31, 52). Because one of the functions of calmodulin in whole brain is to activate a specific cyclic nucleotide phosphodiesterase (9, 15, 26, 29, 47), the occurrence of calmodulin in the PSD preparation stimulated us to look for

a cyclic nucleotide phosphodiesterase in the same preparation. Many studies have shown that the cyclic nucleotide phosphodiesterases in brain appear to be localized at neuronal postsynaptic thickenings, as initially found histochemically by Florendo et al. (16) and confirmed by several others (cf. reference 13). Therien and Mushynski (43) found a synaptic junction-associated cyclic AMP-phosphodiesterase activity that was slightly decreased by the addition of EGTA, while no EGTA sensitivity was found to be associated with a synaptic membrane preparation. Recently, Ariano and Appleman (3) have histochemically shown the presence of a calmodulin-activatable cyclic nucleotide phosphodiesterase activity at postsynaptic sites in rat brain. Because procedures utilizing isolated synaptic junctions or histochemistry cannot distinguish localization between membrane and PSD, we looked for, found, and now describe some properties of a cyclic nucleotide-phosphodiesterase activity, particularly its sensitivity to calmodulin, found in our Triton X-100-derived PSD from canine cerebral cortex. A cyclic AMP-phosphodiesterase activity has also been previously noted by Cotman et al. (11) in a Sarkosyl-derived PSD preparation from rat brain, but none of its properties were given.

MATERIALS AND METHODS

Biochemical compounds were obtained as follows: cyclic AMP, cyclic GMP, EDTA, EGTA, Trizma base, 5'-nucleotidase from *Crotalus adamantus* venom (EC 3.1.3.5), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. St. Louis, Mo.; Blue Dextran-2000, bovine thyroid thyroglobulin, horse spleen ferritin, bovine liver catalase (EC 1.11.1.6), rabbit muscle aldolase (EC 4.1.2.13), and S-300 Sephacryl were obtained from Pharmacia Fine Chemicals, Div. Pharmacia Inc. (Piscataway, N. J.); [^3H]cyclic AMP (25 Ci/mmol) and [^3H]cyclic GMP (15.8 Ci/mmol) were from Amersham Corp. (Arlington Heights, Ill.); Triton X-100 was from Packard Instrument Co., Inc. (Downer's Grove, Ill.); and sucrose (density gradient grade) was from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Bovine, chicken, and porcine brain calmodulin, which are virtually identical in physicochemical properties and phosphodiesterase activator activity (47) were a gift from Dr. D. M. Watterson (The Rockefeller University).

General Methods

A crude PSD fraction was isolated from synaptosomes in a short procedure by treatment with 0.5% Triton X-100 as previously described (10). This preparation was purified by further treatment with 0.5% Triton X-100-75 mM KCl to remove contaminating membrane proteins (10). Partially purified calmodulin-dependent cyclic nucleotide phosphodiesterase and purified calmodulin were isolated from canine cerebral cortex and from isolated PSD preparations using essentially the methods devised for bovine brain by Watterson et al. (51; cf. reference 19). Canine brain calmodulin, as well as PSD calmodulin, co-migrated with authentic porcine brain calmodulin and was found to be >95% pure by analysis on SDS polyacrylamide gel electrophoresis (SDS PAGE) (19). In addition, canine brain calmodulin and PSD calmodulin were further identified by their ability to activate a canine brain cyclic nucleotide phosphodiesterase preparation, by increased mobility on SDS PAGE in the presence of Ca^{2+} , and by amino acid analysis (19). Calmodulin-deficient PSDs were obtained by incubation in 50 mM EGTA-3 mM Tris-HCl, pH 8.1, for 1 h in ice to remove the calmodulin (cf. references 5 and 19). The calmodulin-deficient PSDs were then pelleted by centrifugation at 284,000 g through 1 M sucrose at 4°C in a Beckman SW-40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and the resultant pellets rinsed with double distilled H_2O . PSD protein concentration was determined by the method of Lowry et al. (33) and canine brain calmodulin concentration was determined from its absorbance at 276 nm (51).

ASSAY FOR CYCLIC AMP-PHOSPHODIESTERASE ACTIVITY: The standard reaction mixture contained in a final volume of 0.5 ml: 40 mM Tris-HCl, pH 7.9; 2 mM cyclic nucleotide; 0.4 mM MnCl_2 ; 0.11 U 5'-nucleotidase and enzyme. The PSD preparation was briefly sonicated (3-4 s, at amplitude 3 in a Branson sonifier [Branson Sonic Power Co., Danbury, Conn.]) before use. The reaction was terminated by the addition of 0.1 ml 60% (vol/vol) trichloroacetic acid on ice, centrifuged, and the phosphate concentration determined in the trichloroacetic acid supernate (2). Zero time values were subtracted to correct for nonspecific phosphate levels in the reaction. The 5'-nucleotidase had no detectable cyclic nucleotide phosphodiesterase activity.

SOLUBILIZATION OF THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY FROM THE PSD: The PSD preparation was suspended in either double-distilled H_2O (cf. Table II) or buffer (cf. Fig. 2 and Table III). The 3- to 5-ml sample was then sonicated in a 15-ml plastic conical centrifuge tube in an ice-NaCl bath with stirring for 3 min at amplitude 3 with a Bronson W-185 sonifier equipped with a prechilled tapered microtip. The power output was 33 W in air and 48 W in the sample. Depending on the volume of the sample, it was then centrifuged at 15,000 g for 30 min in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, N. Y.) or at 106,000 g for 30 minutes in the Ti-50 Spinco rotor at 4°C. Similar results in both cases were obtained using the supernate that contained the solubilized enzyme activity.

S-300 SEPHACRYL CHROMATOGRAPHY OF THE SONICATED SUPERNATANT FRACTION FROM THE PSD: Approximately 13 mg PSD was sonicated in 5 ml column buffer (10 mM PIPES; 100 mM NaCl; 3.75 mM 2-mercaptoethanol; 0.5 mM MnCl_2 , pH 7.0) as described above. After centrifugation, 3.5 ml of supernate was applied to a 1.5×8.25 cm S-300 Sephacryl column equilibrated in column buffer. The sample was then eluted with column buffer at a flow rate of 48 ml/h. Proteins were monitored at 280 nm and cyclic nucleotide phosphodiesterase activity determined as described in the legend to Fig. 2 or Table III. Column calibrations were run using Blue Dextran 2000, thyroglobulin, ferritin, catalase, and aldolase.

RESULTS AND DISCUSSION

Table I shows the presence of a cyclic nucleotide phosphodiesterase in our PSD preparation. This activity was found to be three times more active when cyclic AMP was the substrate than when cyclic GMP was the substrate at a concentration of 2 mM for each substrate (data not shown). The specific activity was some five to sixfold less than in the parent synaptosome fraction. However, there are some reports in the literature (3, 8, 13, 15, 38, 44, 46) describing the existence of multiple forms of the cyclic nucleotide-phosphodiesterase in brain depending on specific cation activation and on substrate specificity, so one cannot make a valid comparison between the specific activities in the synaptosome and PSD until the exact nature of the brain phosphodiesterase activities is known. Because the PSD preparation contains both a cyclic nucleotide phosphodiesterase activity and calmodulin, it was assumed that this preparation contained a calmodulin-activatable cyclic nucleotide phosphodiesterase activity. Thus, the PSD preparation was incubated in the absence or presence of exogenous calmodulin. In neither case was there an activation of the cyclic AMP-phosphodiesterase.

TABLE I

Cyclic AMP-Phosphodiesterase Activity of Native and Calmodulin-deficient PSD Preparations from Canine Cerebral Cortex in the Presence of Exogenous Calmodulin

Sample*	Additions	Cyclic AMP-phosphodiesterase activity†	
		exp A	exp B
Native PSD	None	9.8	13.3
Native PSD	Canine brain calmodulin	8.7	14.3
Native PSD	Porcine brain calmodulin	—	14.7
Calmodulin-deficient PSD	None	10.9	13.8
Calmodulin-deficient PSD	Canine brain calmodulin	10.3	14.1
Calmodulin-deficient PSD	Porcine brain calmodulin	—	14.1

25 μg PSD protein was incubated with either 1.2 or 1.5 μg calmodulin in exp A and exp B, respectively. The enzyme activity was measured as given in Materials and Methods. The calmodulin-deficient PSD preparations were obtained as given in Materials and Methods.

* Calmodulin-deficient PSDs were prepared from the corresponding native PSD fraction.

† nmol Inorganic phosphate produced/min per mg PSD protein at 30°C.

ase activity (Table I). The addition of EGTA as a control was avoided in the above study, because EGTA concentration sufficient to remove Ca^{2+} from the incubation media could also remove calmodulin from the PSD preparation (personal observations). Because it was now possible that the PSD enzyme(s) was already saturated by the calmodulin present in the isolated PSD preparation, a calmodulin-deficient PSD preparation was also incubated as above. As seen in Table I, there was no change in the cyclic AMP-phosphodiesterase activity, relative to the control, whether exogenous calmodulin was added or not. If a calmodulin-dependent cyclic nucleotide-phosphodiesterase activity was present in the PSD preparation, one would have expected it to decrease upon removal of calmodulin or to be stimulated by addition of exogenous calmodulin and Ca^{2+} . It should be noted that EGTA was not able to remove the enzyme activity present in the preparation. A similar lack of effect of EGTA and of calmodulin on a gross particulate phosphodiesterase preparation from rat brain was noted previously (34).

Because the PSD fraction did not appear to have a calmodulin-dependent cyclic AMP-phosphodiesterase, the nature of the enzymatic activity present was examined by assaying it in the presence of various divalent cations. The PSD preparation in absence of additional cation had some basal cyclic AMP-phosphodiesterase activity (3.3 U)¹ which could be completely inhibited with 1 mM EDTA, with EGTA having a less pronounced effect (1.5 U). In the presence of 1 mM Mn^{2+} or 1 mM Mg^{2+} the basal activity increased approximately threefold, with Mn^{2+} being the more effective cation. The addition of 1 mM Ca^{2+} was found to decrease the basal activity (1.1 U). When the cations were incubated in various combinations, maximal cyclic AMP-phosphodiesterase activity was obtained only when Mn^{2+} was present; Ca^{2+} , at 1 mM, had little or no effect.

One could argue that unlike Mg^{2+} , the Ca^{2+} and Mn^{2+} concentrations (1 mM) used in the above experiments were probably much higher than the physiological concentrations in the brain. To circumvent this argument, cyclic AMP-phosphodiesterase in the PSD was assayed in the presence of 0.25–10 μM cation. To remove possibly activating cations, the preparation was initially treated with 6 μM EDTA (cf. Fig. 1), resulting in a decrease of the activity to basal levels; at this point increasing amounts of cations were then added. As can be seen from the data in Fig. 1, Mn^{2+} was the most effective cation in stimulating cyclic AMP-phosphodiesterase activity at concentrations <1 μM . At all the concentrations tested, Ca^{2+} was again ineffective, while Mg^{2+} , at 10 μM , was able to reach some 40% of the maximal enzyme activity. For comparison, the K_m for Mg^{2+} was found by Cheung (8) to be 13 μM in a brain 30,000 g supernatant fraction. Yet the activation of the PSD enzyme by Mn^{2+} was evident at a concentration as low as 0.25 μM even though the stability constant of EDTA for this cation is some 10^3 – 10^5 times greater than for Ca^{2+} or Mg^{2+} , respectively (40). These data suggest that the initial lowering of the enzymatic activity by EDTA may be caused by the chelation of trace amounts of Mn^{2+} in the PSD preparation. It should be noted that the effect of Mn^{2+} is on the phosphodiesterase and not on the 5'-nucleotidase used in the coupled assay, because the latter enzyme is insensitive to Mn^{2+} addition (24).

¹ One unit = nanomoles inorganic phosphate produced per minute per milligram protein at 30°C.

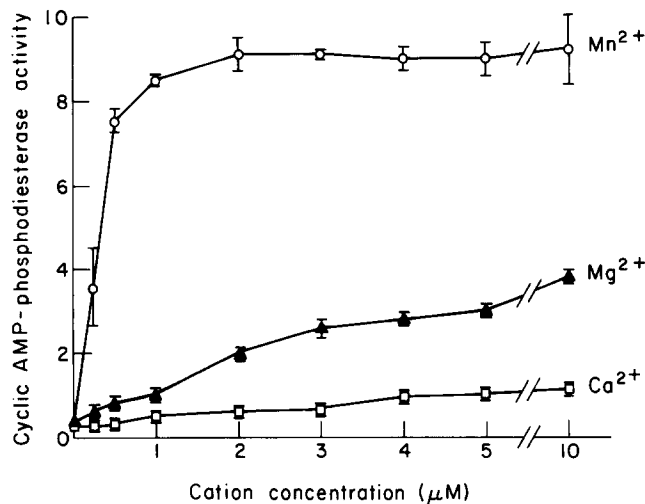


FIGURE 1 Activation of cyclic nucleotide phosphodiesterase activity of PSD with different divalent cations. 25 μg PSD protein was incubated for 30 min at 30°C. The reaction mixture (0.5 ml) contained 40 mM Tris-HCl (pH 7.9), 2 mM cyclic nucleotide phosphodiesterase, and 0.11 U 5'-nucleotidase. Increasing amounts of EDTA were added until the activity was reduced to basal level; this required 6 μM EDTA. Increasing amounts of divalent cation were then added, and these are the values shown on the abscissa. Each point represents the mean and range of duplicate samples. Activity is expressed in units, with 1 U of activity being nmol inorganic phosphate produced/30 min at 30°C.

To try to further elucidate the properties of the PSD enzyme, attempts were made to solubilize the enzyme, and it was found that the PSD phosphodiesterase could be solubilized by sonication (Table II). Approximately 60% of the Mn^{2+} -requiring enzyme activity could be solubilized by sonication (PSD concentration was 250 $\mu\text{g}/\text{ml}$) with an overall recovery of activity, in both supernatant and pellet forms, of ~70%. The response of the solubilized enzyme to the various cations, and to calmodulin, was the same as the response of the PSD-bound enzyme: compare in Table II the supernate of the sonicated preparation with the pellet of the native PSD preparation. As a control, only little of the enzyme in the unsonicated PSD preparation was found in the supernate after resuspension and centrifugation. Thus, the solubilized enzyme behaved as in the case of the PSD enzyme, like an $\text{Mn}^{2+}/\text{Mg}^{2+}$ -requiring one and not like a Ca^{2+} /calmodulin-activatable one. The enzyme activities of both the whole PSD preparation and of the solubilized preparation behaved somewhat differently from that of a whole brain enzyme, partially purified as described in Materials and Methods. This latter preparation was activated more by Mn^{2+} than by either Mg^{2+} or Ca^{2+} , similar to the PSD preparation, but the Mn^{2+} activity was further increased some twofold by the addition of Ca^{2+} and calmodulin, in contradistinction to the PSD preparations which showed no such increase.

Because several multiple forms of the phosphodiesterase have been described in brain (3, 8, 15, 38, 44, 46) it was of interest to initially characterize by gel filtration the enzymatic activity in the sonicated supernatant fraction from the PSD. To obtain enough activity for gel filtration it was necessary to use a higher concentration (13 mg PSD protein/5 ml) for the sonication; under these conditions only 20–46% of the initial activity was solubilized. Initial gel filtration studies on Sepharose-6B under varying conditions (Mn^{2+} , or EDTA, and Aprotinin, glycerine or BSA in the running buffer) indicated that the solubilized activity could be resolved into two Mn^{2+} -de-

TABLE II
Solubilization by Sonication of the Cyclic AMP-
Phosphodiesterase Activity of Native PSD

Sample	Additions*	Cyclic AMP-phosphodiesterase activity \ddagger			
		Pellet		Supernate	
		exp A	exp B	exp A	exp B
Native PSD	None	152	93	72	64
	Mn ²⁺	316	343§	55	34
	Mg ²⁺	196	260	39	62
	Ca ²⁺	80	70	0	9
	Ca ²⁺ + calmodulin	ND	80	0	19
	Ca ²⁺ + Mg ²⁺ + calmodulin	ND	243	ND	19
	Ca ²⁺ + Mn ²⁺ + calmodulin	ND	330	ND	48
Sonicated PSD	None	37	0	39	0
	Mn ²⁺	76	13	212	227
	Mg ²⁺	14	0	170	174
	Ca ²⁺	62	0	44	44
	Ca ²⁺ + calmodulin	0	0	69	37
	Ca ²⁺ + Mg ²⁺ + calmodulin	ND	0	ND	153
	Ca ²⁺ + Mn ²⁺ + calmodulin	ND	9	ND	256

The PSD preparation was resuspended in double-distilled H₂O to a final protein concentration of 250 μ g/ml. A 3-ml aliquot was then sonicated as described in Materials and Methods. The sample was then centrifuged at 15,000 *g* for 30 min. A 3-ml aliquot of untreated PSD was also centrifuged as above. The supernates were saved and the pellets were resuspended in 3 ml of double-distilled H₂O. 100- μ l aliquots of pellets and supernate were assayed in duplicate as described in Materials and Methods under the conditions shown. ND, not determined.

* Each cation was at 1 mM. Where added, 2 μ g chick brain calmodulin was used.

\ddagger pmol inorganic phosphate produced/min at 30°C.

§ 13.7 nmol inorganic phosphate produced/min per mg PSD protein at 30°C.

pendent fractions: one higher molecular weight species that elutes with the void volume and another fraction with an apparent molecular weight of 215,000 \pm 15,000. Neither fraction could be activated with calmodulin. However, our column runs usually took 10–12 h to complete, and it was found that the eluted activities were very labile, losing 80–90% of their activities when stored overnight at –80°C. Thompson and Appleman (44) have also described this instability of whole brain cyclic nucleotide phosphodiesterase after elution on Agarose columns. Although they could stabilize their enzyme by rapidly concentrating it by ultrafiltration, this method was not practical in our case. However, rapid elution of the two PSD activities could be accomplished using S-300 Sephacryl columns. Under the conditions described in Fig. 2 and Materials and Methods, the run could be completed in 1.5–2 h. As seen in Fig. 2B, the solubilized PSD enzyme can still be resolved into high (I) and low (II) molecular weight species. Moreover, fraction II (215,000 mol wt) can now be shown to be activated by calmodulin, unlike the activity of fraction I. This has been repeated in five different experiments. Although the experiment shown was done in the presence of Mn²⁺ in the column buffer, the addition of EDTA or EGTA in the presence of phenylmethylsulfonyl fluoride (PMSF) in the buffer did not change the total profiles. Preparation of PSD in the presence

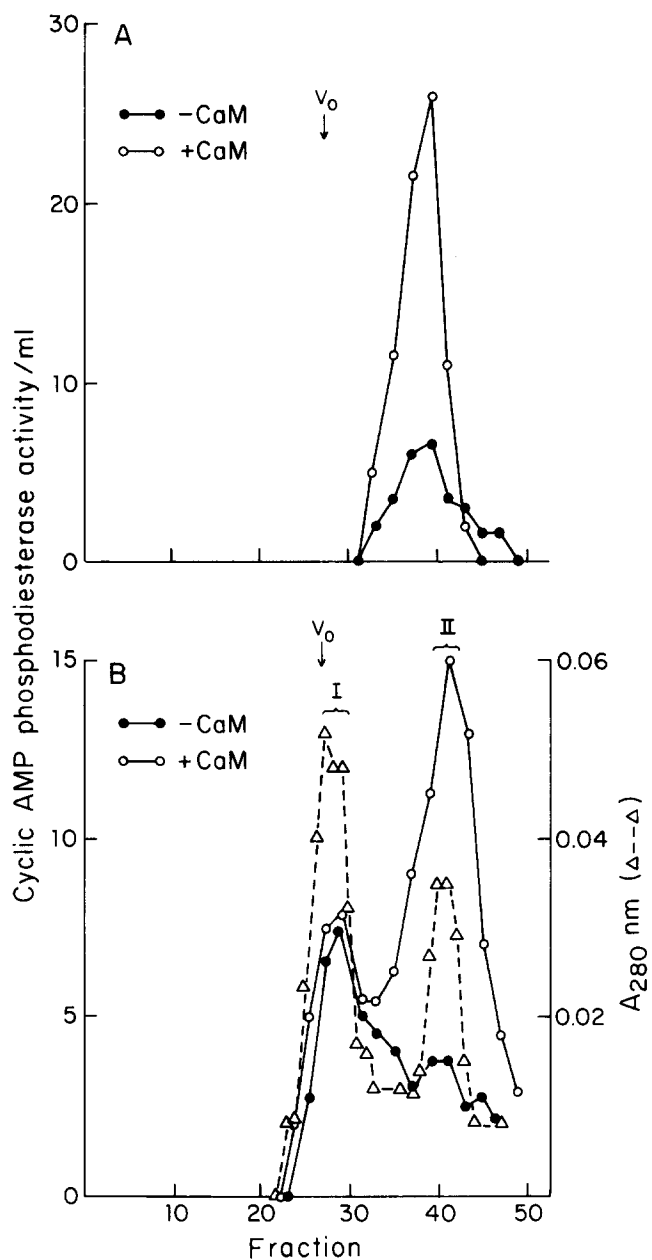


FIGURE 2 Column chromatography of the sonicated supernatant fraction from the PSD. The PSDs were sonicated and the supernatant fraction was subjected to S-300 Sephacryl column chromatography as described in Materials and Methods. Approximately 20% of the activity in the PSD was solubilized under these conditions in this experiment. 2-ml fractions were collected and 0.2-ml aliquots were assayed for cyclic nucleotide phosphodiesterase activity. The reaction contained in 0.25 ml: 40 mM Tris-HCl, pH 7.9; 2 mM cyclic nucleotide; 0.4 mM MnCl₂; 0.055 unit 5'-nucleotidase, and 20 μ g carrier bovine serum albumin. Semipurified cerebral cortex cyclic nucleotide phosphodiesterase was prepared as described earlier (51, cf. reference 19). Phosphodiesterase activity is as given in the legend to Fig. 1. (A) Elution profile of whole cerebral cortex semipurified cyclic nucleotide-phosphodiesterase activity, in the absence or presence of 1 mM CaCl₂ and 1.5 μ g bovine calmodulin. Approximately 0.7 mg enzyme protein in a volume of 2 ml was applied. (B) Elution profile of the cyclic nucleotide phosphodiesterase activity extracted from PSDs by sonication, in the presence or absence of 1 mM CaCl₂ and 1.5 μ g bovine calmodulin. Although in this figure the protein and enzymatic activity peaks coincide, in other experiments the enzymatic activity peak was displaced slightly from the protein peak.

of PMSF in the isolation buffers also had no effect. The total activities recovered varied between 16–46% when based on the initial activities in the presence of Mn^{2+} which were placed on the column. The crude calmodulin-dependent cyclic nucleotide-phosphodiesterase preparation from whole canine cerebral cortex was also found to coelute as one peak, with an apparent molecular weight of 215,000 (Fig. 2A). Therefore, it appears that fraction II from PSD contains a calmodulin-activatable phosphodiesterase, and that this activity resides in a complex similar to that which can be obtained from the entire cerebral cortex. When the cyclic nucleotide concentrations were 2 mM, cyclic AMP was preferred over cyclic GMP as substrate in both fractions. A similar separation of the activatable and nonactivatable PDEs by Sephadex G-200 chromatography of a sonic extract of total membranes from rat cerebrum was noted in a footnote (14).

To obtain kinetic data on the fraction II activity, it was assayed against a similar range of substrate concentrations, and Table III summarizes the results. The enzyme was found to be active against both substrates, with a much higher affinity for cyclic GMP but with a higher V_{max} for cyclic AMP. These differences in kinetic parameters between the two substrates are very similar to the basal enzyme activity observed by others (15). When Ca^{2+} plus calmodulin was added to the protein II peak, the V_{max} was increased with both substrates, still being higher when cyclic AMP was the substrate. The addition of calmodulin also seemed to decrease the K_m for both substrates. The K_m values were similar to those reported for a brain extract (15) and for a highly purified calmodulin-activatable phosphodiesterase from brain (29). On the other hand, the fraction I enzyme is not calmodulin-activatable and displayed different apparent K_m values for both substrates from those obtained for the fraction II enzyme: the K_m 's for cyclic AMP and cyclic GMP were 1.1 and 2.3 mM, respectively. Thus, the fraction I enzyme appears to be identical to the enzyme in the intact PSD and to the enzyme which is solubilized from it by sonication.

The above results indicate that one of the possible functions of the calmodulin that has been localized in the PSD (19) is to activate a cyclic nucleotide phosphodiesterase which is also found there. This enzyme is probably intrinsic to the PSD, as the basal activity (16) and the calmodulin-activatable activity (3) were found there by histochemical methods. It is present in PSDs obtained by the ionic detergent Sarkosyl (11), and it is not removed by EGTA treatment, but requires vigorous sonication to remove part of the activity.

However, although one can detect in the solubilized enzyme preparation after gel filtration a portion which is calmodulin-activatable, this activation is obscured in both intact PSDs and the sonicated, solubilized fraction before gel filtration. There are several possible explanations for this result: (a) in the solubilized fraction there are two activities, one sensitive and one insensitive to calmodulin, but the former is such a small fraction of the whole that the identification of the former is masked; (b) there is only an insensitive form of the enzyme in the solubilized and in the whole PSD fraction, but during gel filtration this activity becomes calmodulin-sensitive, e.g., there is only a calmodulin-sensitive activity in the PSD, but this activatable activity is inhibited by some factor in the preparation; (c) there is a calmodulin-sensitive enzyme in the PSD, but during isolation of the PSD this activity becomes fully activated by calmodulin; and (d) a calmodulin-activatable form of phosphodiesterase exists in the PSD *in vivo* but is converted into a calmodulin-insensitive form by post-translation events occur-

TABLE III
Kinetics of Cyclic Nucleotide Phosphodiesterase Activity in the S-300 Sephacryl Column Fraction II

Substrate	K_m^*		V_{max}^\ddagger	
	-Calmodulin	+Calmodulin	-Calmodulin	+Calmodulin
Cyclic AMP	152	122	7.7	15.1
Cyclic GMP	19	9	2.1	4.1

PSDs were sonicated in the column buffer, and the supernatant fraction was subjected to S-300 Sephacryl chromatography as described in Materials and Methods. Fraction II was collected and assayed in a volume of 0.125 ml containing 40 mM Tris-HCl, pH 7.9, either cyclic AMP (25–1,000 μ M) or cyclic GMP (2–100 μ M) containing 10 μ Ci of the appropriate 3H -labeled cyclic nucleotide; 0.4 mM $MnCl_2$, 10 μ g carrier BSA, and 0.1 ml enzyme fraction II. The plus calmodulin samples also contained 1 mM $CaCl_2$ and 1.5 μ g bovine brain calmodulin. The reaction was incubated for 30 min at 30°C and terminated by boiling for 5 min. Boiled enzyme blanks containing substrate were also incubated. The samples were then centrifuged for 5 min in an Eppendorf microfuge. The nucleotides were separated using the high-performance liquid chromatography method of Watterson et al. (50). The AMP or GMP fractions were collected with an LKB redi-rac (LKB Instruments, Inc., Rockville, Md.) and then counted in Aquasol using a Beckman liquid scintillation counter. The data obtained were based on a plot of V/S vs. S using eight to nine substrate concentrations, and the method of least squares was used to plot the experimental data ($r^2 \geq 0.97$).

* μ M.

† nmol $5'$ -Mononucleotide produced/min per A_{280} unit.

ing *in vivo* or during sacrifice of the animal and subsequent isolation of PSDs (e.g., limited proteolysis, phosphorylation, methylation, extreme sensitivity of calmodulin-active phosphodiesterase to drug/sacrifice induced inactivation).

Of the above explanations, we can rule out *c*, because removal of calmodulin from the PSD by EGTA did not lower the cyclic nucleotide-phosphodiesterase activity. As for explanation *b*, two inhibitor proteins have been described in brain, one being heat labile (28, 49) and the other heat stable (39), both being capable of suppressing the calmodulin activation of the phosphodiesterase. Using immunocytochemical techniques, Wood et al. (52) found a heat-labile inhibitory protein to be localized in the PSD region in rat basal ganglia. Thus, the simplest explanation is that in the PSD and in the sonicate, the calmodulin activation of the enzyme is suppressed by the binding of the heat-labile binding protein to calmodulin. Using a radioimmunoassay, a marginal amount (80 ng/ml) of the heat-labile inhibitory protein was found in our S-300 Sephacryl fraction I, and the amount detected in fraction II (25 ng/ml) was found to be at the limit of detection (Dr. Robert Wallace, University of Tennessee, personal communication). However, if the suppression is caused by the presence of this inhibitor, then the addition of excess exogenous calmodulin should have overcome this inhibition, but this was not the case. Furthermore, the addition of exogenous calmodulin did cause an increase in the activity of another PSD enzyme, a protein kinase (7, 18, 20, 21). Thus, it would appear that the lack of calmodulin effect in intact PSDs may not be caused by a calmodulin bound to the inhibitor protein, but rather by the spatial localizations of the cyclic nucleotide-phosphodiesterase and calmodulin within the PSD structure.

Recently Grand and Perry (22) described the presence of a 22,000 mol wt calmodulin-binding protein in rabbit brain which was shown to inhibit the calmodulin activation of brain cyclic nucleotide-phosphodiesterase, with little or no effect on the activation of myosin light chain kinase. This low molecular weight calmodulin-binding protein was found to be the myelin basic protein (23). A confirmation of the binding to myelin

basic protein has been recently reported (25). Because our PSD preparation does contain myelin basic protein as a contaminant (10), it was possible that this protein could be involved in masking any calmodulin-activatable cyclic nucleotide-phosphodiesterase in the intact and sonicated preparations. This idea was substantiated by the finding that the PSD does contain a protein kinase activity that can be activated by the addition of exogenous calmodulin (7, 18, 20, 21). So our observations are in agreement somewhat with those of Grand and Perry (22); that is, addition of exogenous calmodulin to a PSD preparation will activate a protein kinase activity but not a cyclic nucleotide-phosphodiesterase activity. To test whether any contaminating myelin basic protein may, in part, be involved in masking any endogenous calmodulin-activatable cyclic nucleotide phosphodiesterase in the PSD, the intact PSD fraction, the sonicated fraction, as well as gel fraction I were preincubated with dialyzed rabbit anti-guinea pig myelin basic protein serum (provided by S. Chou, F. Chou, and R. F. Kibler, Emory University). However, the results were highly variable; although negative results were obtained using either PSDs or the sonicated fraction, in some cases there was a stimulation of the activity in fraction I, while in other cases there was no stimulation. In addition, when fraction I was co-incubated with a partially purified calmodulin-dependent cyclic nucleotide phosphodiesterase from canine cerebral cortex, the results were additive in both the absence and the presence of exogenous calmodulin, indicating that fraction I did not contain an unbound inhibitor (data not shown). However, we still cannot rule out the role of myelin or other bound inhibitor in the possible inhibition of calmodulin-activatable cyclic nucleotide-phosphodiesterase in the PSD, in the sonicated extract, or in fraction I.

On the basis of the above, we tentatively assume that in the PSD there are present two cyclic nucleotide-phosphodiesterases, one activatable, and one not, by calmodulin. We further assume that in the PSD the predominant form is the latter one, and that the activation by calmodulin of the former enzyme is not easily seen in the intact or sonicated PSD, but only when those two activities are separated (fractions I and II) can it be ascertained. In addition, there does exist in the PSD a calmodulin-binding protein (52) whose function seems to be to regulate calmodulin activity.

In conclusion, our finding that there exists in the PSD an enzyme that is cryptic and that may be activated by calmodulin under certain conditions makes it possible that during changing neurophysiological states the calmodulin present in the PSD differentially modulates the activity of that cyclic nucleotide phosphodiesterase present there. These postulated physiological changes occurring during synaptic function could result either in a loosening of the inhibitor protein(s) from the PSD or in a change in proximity of calmodulin to the enzyme, either or both resulting in an increased susceptibility of the enzyme to calmodulin activation. The resultant decrease in localized cyclic nucleotide concentration would affect the cyclic nucleotide-dependent protein kinase which was found to be present in the PSD (4, 45). Other recent evidence relevant to the PSD cyclic nucleotide phosphodiesterase is the finding that in some tissues the sensitivity of cyclic nucleotide phosphodiesterases to calmodulin regulation may change during differentiation (32, 41, 42). For example, brain homogenates appear to have a calmodulin-activatable cyclic nucleotide phosphodiesterase activity during all stages of fetal development (41), and this activity is decreased during retinal development (32). Finally, it should be added that another possible function of the PSD

calmodulin is to activate a protein kinase in the PSD that phosphorylates certain PSD proteins (7, 18, 20, 21).

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NOTE ADDED IN PROOF: It is possible that the lack of activation of the PSD-PDE by calmodulin is attributable to small amounts of Triton X-100 adsorbed onto the PSD, because a recent abstract (Sharma, R. K., 1981, *Fed. Proc.* 40:1739) noted that 15 μ M Triton X-100 caused a 50% inhibition of the calmodulin activatability of a soluble PDE preparation.

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