Jeffrey, P. D., Milthorpe, B. K., & Nichol, L. W. (1976) *Biochemistry 15*, 4660–4665.
Keniry, M. A., & Smith, R. (1979) *Biochim. Biophys. Acta 578*, 381–391.
Krigbaum, W. R., & Hsu, T. S. (1975) *Biochemistry 14*, 2542–2546.
Lapanje, S., & Tanford, C. (1967) *J. Am. Chem. Soc. 89*, 5030–5033.
Liebes, L. F., Zand, R., & Phillips, W. D. (1975) *Biochim. Biophys. Acta 405*, 27–39.
Littlemore, L. A. T. (1978) *Aust. J. Chem. 31*, 2387–2398.
Milthorpe, B. K., Jeffrey, P. D., & Nichol, L. W. (1975) *Biophys. Chem. 3*, 169–176.
Moscarello, M. A., Katona, E., Neumann, A. W., & Epand, R. M. (1974) *Biophys. Chem. 2*, 290–295.
Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.
Rumsby, M. G. (1978) *Biochem. Soc. Trans. 6*, 448–462.
Smith, R. (1977a) *Biochim. Biophys. Acta 470*, 170–184.
Smith, R. (1977b) *Biochim. Biophys. Acta 491*, 581–590.
Smith, R., & McDonald, B. J. (1979) *Biochim. Biophys. Acta 554*, 133–147.

High Levels of a Heat-Labile Calmodulin-Binding Protein ($CaM\text{-}BP_{80}$) in Bovine Neostriatum†

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ABSTRACT: Bovine brain contains a heat-labile, 80 000-dalton calmodulin-binding protein ($CaM\text{-}BP_{80}$) which inhibits the calmodulin-dependent activities of cyclic 3′,5′-nucleotide phosphodiesterase, adenylate cyclase, and Ca^{2+}-ATPase in vitro. $CaM\text{-}BP_{80}$ is composed of two polypeptides (60 000 and 18 500 daltons) present in a 1:1 ratio. An antibody directed against $CaM\text{-}BP_{80}$ was raised in rabbits, and a radioimmunoassay was developed, having a sensitivity of 60 fmol of $CaM\text{-}BP_{80}$. Using the radioimmunoassay, we determined the levels of $CaM\text{-}BP_{80}$ in various bovine tissues. The protein was found primarily in the brain, present in particularly high levels in the neostriatum. These results, together with immunohistochemical localization of $CaM\text{-}BP_{80}$ at the postsynaptic densities and the microtubules of postsynaptic dendrites [Wood, J. G., Wallace, R., Whitaker, J., & Cheung, W. Y. (1980) *J. Cell Biol. 84*, 66–76], suggest that the protein may have a role in the cerebrum at the site of neurotransmitter action and at the level of microtubular function.

Adenosine 3′,5′-monophosphate (cAMP)[1] is a versatile regulator, modulating many cellular functions and processes. Regulation of the cellular level of the nucleotide involves two complex enzyme systems, the synthetic enzyme adenylate cyclase and the degradative enzyme phosphodiesterase. In mammalian brain, both enzymes require Ca^{2+} for maximal activity. Activation by Ca^{2+} is mediated through calmodulin, a Ca^{2+}-dependent modulator protein ubiquitous in eucaryotes (Cheung et al., 1975b; Waisman et al., 1975).

Regulation of Ca^{2+}-activatable adenylate cyclase (Brostrom et al., 1975, 1976, 1977; Cheung et al., 1975a, 1978; Lynch et al., 1977), phosphodiesterase (Cheung et al., 1975a, 1978; Kakiuchi & Yamazaki, 1970; Kakiuchi et al., 1975; Wolff & Brostrom, 1974; Brostrom & Wolff, 1974, 1976; Teo & Wang, 1973; Wang et al., 1975), and Ca^{2+}-Mg^{2+}-ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977, 1978; Hanahan et al., 1978; Lynch & Cheung, 1979) by calmodulin has been extensively studied. In the presence of Ca^{2+}, calmodulin undergoes a change toward a more helical conformation, which is the active species, and interacts with an apoenzyme to form the active holoenzyme (Liu & Cheung, 1976; Klee, 1977; Dedman et al., 1977). Calmodulin also regulates the activities of skeletal muscle phosphorylase kinase (Cohen et al., 1978), myosin light chain kinase (Waisman et al., 1978; Dabrowska & Hartshorne, 1978; Dabrowska et al., 1978; Sherry et al., 1978; Yagi et al., 1978), NAD kinase (Anderson & Cormier, 1978; D. Epel, R. W. Wallace, and W. Y. Cheung, unpublished experiments), phospholipase A_2 (Wong & Cheung, 1979), Ca^{2+} transport in erythrocytes (Hinds et al., 1978; Larsen & Vincenzi, 1979) and sarcoplasmic reticulum (Katz & Remtulla, 1978), phosphorylation of membranes (Schulman & Greengard, 1978a,b), and the disassembly of microtubules (Marcum et al., 1978). Thus, calmodulin appears to be a major receptor or mediator of Ca^{2+}, regulating many cellular functions [for reviews, see Wolff & Brostrom (1979), Cheung (1980), Wang & Waisman (1980), and Klee et al. (1980)].

In bovine brain, there are several proteins that bind calmodulin in a Ca^{2+}-dependent manner; the biological activities of these proteins have yet to be identified (Cheung et al., 1978; Wang & Desai, 1976, 1977; Klee & Krinks, 1978; Wallace et al., 1978a, 1979; LaPorte & Storm, 1978; Sharma et al., 1978, 1979). One of these proteins is heat labile and has a molecular weight of 80 000 ($CaM\text{-}BP_{80}$) (Cheung et al., 1978; Klee & Krinks, 1978; Wallace et al., 1978a, 1979; Wang & Desai, 1976). $CaM\text{-}BP_{80}$ contains two subunits, 60 000 and 18 500 daltons, in equivalent molar ratios (Wallace et al., 1979). However, Sharma et al. (1979) reported the molar ratio of the large and small subunits to be 1:2. The protein

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[1] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid; NaCl–P_i, phosphate-buffered saline [0.15 M NaCl and 20 mM sodium phosphate (pH 7.2)]; $CaM\text{-}BP_{80}$, heat-labile calmodulin-binding protein.

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suppresses the calmodulin-supported activities of adenylate cyclase, phosphodiesterase (Cheung et al., 1978; Wallace et al., 1978a, 1979), and Ca^{2+}-ATPase (Lynch & Cheung, 1979) but not their basal activities. Inhibition appears to result from the binding of calmodulin by CaM-BP_{80}, making it unavailable to the apoenzymes. This protein, previously designated as an inhibitor protein, may be a true inhibitor of calmodulin or another calmodulin-regulated protein whose biologic activity remains to be identified.

One of the difficulties in working with CaM-BP_{80} has been the lack of a suitable assay. CaM-BP_{80} has been measured by its ability to suppress calmodulin-supported phosphodiesterase activity in vitro. All tissues contain calmodulin and most likely phosphodiesterase; their presence interferes with the assay, making it difficult to determine CaM-BP_{80} in most tissue extracts. We felt that it was important to produce an antibody for CaM-BP_{80} which would allow development of a radioimmunoassay for the quantitative measurement of CaM-BP_{80} in tissue extracts and its immunocytochemical localization. Information on its tissue and subcellular localization may provide a clue to its physiological role.

This communication consists of two sections. The first part deals with the development of a radioimmunoassay for CaM-BP_{80}, establishing its specificity and validity. The second part summarizes the levels of CaM-BP_{80} in various bovine tissues and shows that CaM-BP_{80} is primarily a brain protein, present in particularly high levels in the neostriatum. Finally the significance of this finding is discussed.

Experimental Procedure

Chemicals. [^{3}H]cAMP (20 Ci/mmol) and ^{125}I (16 Ci/mmol) were purchased from Schwarz/Mann. Affi-Gel Blue (agarose beads covalently linked to Cibacron Blue F36A, 100–200 mesh), Affi-Gel 10 (a *N*-hydroxysuccinimide-activated agarose with a 10-Å spacer arm), acrylamide, *N*,*N*′-methylenebis(acrylamide) (Bis), sodium dodecyl sulfate ($NaDodSO_4$), Coomassie brilliant blue R250, and hydroxylapatite were supplied by Bio-Rad. Triton X-100 was from Sigma Chemical Co. Goat antirabbit IgG serum was a gift from Dr. William Walker, St. Jude Children's Research Hospital. Freund's complete adjuvant was obtained from Miles Laboratory. Rabbit skeletal muscle troponin and its individual subunits were a gift from Dr. Thomas C. Vanaman, Duke University. All other reagents were of the highest analytical grades.

Preparation and Enzyme Assay of CaM-BP_{80}. CaM-BP_{80} was purified to apparent homogeneity from bovine cerebra (Wallace et al., 1979). Briefly, 250 g of cerebra was homogenized in 750 mL of buffer A [50 mM Tris-HCl (pH 7.8), 3 mM $MgSO_4$, and 1 mM dithiothreitol] containing 1 mM EGTA. The extract was centrifuged at 10000*g* for 60 min. The supernatant fluid was filtered through glass wool, and the filtrate was loaded directly onto an Affi-Gel Blue column (3.2 × 46 cm). CaM-BP_{80}, eluted from the column with buffer A containing 0.2 M NaCl, was further purified on a calmodulin–agarose affinity column. The peak tubes containing CaM-BP_{80} were combined, concentrated with an Amicon UM-2 ultrafiltration membrane, and stored in small aliquots at −90 °C. CaM-BP_{80} thus purified appeared homogeneous by the criteria of analytical polyacrylamide gel electrophoresis and filtration chromatography on Sephacryl S-200.

CaM-BP_{80} was assayed by its ability to suppress calmodulin-supported bovine brain phosphodiesterase activity. One unit of CaM-BP_{80} is that amount of protein causing 50% inhibition of calmodulin-supported phosphodiesterase activity under specified conditions (Wallace et al., 1979).

Preparation of Subunits of CaM-BP_{80}. The 60 000- and 18 500-dalton subunits of CaM-BP_{80} were isolated by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Wallace & Dieckert, 1976). CaM-BP_{80} (2.1 mL, 0.57 mg/mL) was heated for 2 min in boiling water in the presence of 100 μL of $NaDodSO_4$ (10% w/v), 100 μL of bromphenol blue (0.05% w/v), and 25 μL of dithiothreitol (200 mM). The denatured protein was electrophoresed in a 10% polyacrylamide gel slab (14 × 10 × 0.3 cm) which contained 2.7% Bis. The phosphate buffer system of Weber & Osborn (1969) was used. Electrophoresis was discontinued when the bromphenol blue dye front reached the bottom of the gel. The gel was incubated at 4 °C overnight; chilling rendered the two subunits of CaM-BP_{80} to appear as white opaque bands in the gel (Wallace et al., 1974). The protein bands from two gels were cut out and macerated with a glass rod. Thirty milliliters of NaCl–P_i containing 0.02% NaN_3 was added, and the slurry was sonicated extensively; the suspension was allowed to stir at 22 °C overnight. Acrylamide was removed by centrifugation (12000*g* for 10 min). The supernatant fluid was dialyzed against NaCl–P_i containing 0.02% NaN_3, and the dialyzed solution was concentrated in an Amicon cell fitted with a UM-2 membrane. The concentrated sample contained only the 60 000- or the 18 500-dalton subunit as determined by analytical $NaDodSO_4$ gel electrophoresis.

Preparation of Antibodies against CaM-BP_{80}. Two male New Zealand white rabbits were immunized with CaM-BP_{80}. On day 1, 0.5 mg of CaM-BP_{80} dissolved in 0.5 mL of buffer A was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously at four sites on the backs of the rabbits just above the scapula. On days 21 and 135 injections with the same emulsion were repeated, and on day 142 the rabbits were bled from the marginal ear vein. The blood was allowed to clot, and the serum was stored in small aliquots at −90 °C. Whole serum was used for the radioimmunoassay.

Iodination of CaM-BP_{80}. CaM-BP_{80} was iodinated according to a modification of the chloramine-T procedure (Hunter & Greenwood, 1962). The reaction mixtures (75 μL) contained 0.1 M sodium phosphate (pH 7.2), 2 mCi of ^{125}I, 6 μg of CaM-BP_{80}, and 10 μg of chloramine-T, added sequentially at 22 °C. Immediately, 30 μg of sodium metabisulfite and then 275 μL of 0.1 M potassium iodide were added to terminate the reaction. Triton X-100 was added to make a final concentration of 0.05%. The iodinated CaM-BP_{80} was separated from the unreacted ^{125}I on a Sephadex G-25 column (14 × 0.7 cm) which had been equilibrated with NaCl–P_i containing 0.05% Triton X-100. The iodinated protein contained 1.6 mol of ^{125}I per mol of CaM-BP_{80}, equivalent to a specific activity of 43 μCi/μg. The sample was stored in small aliquots in 12 × 75 mm polypropylene tubes (Falcon) at −90 °C; no loss of immunological activity was apparent over a period of 3 months.

Radioimmunoassay of CaM-BP_{80}. Radioimmunoassay was performed in 12 × 75 mm BDS polymer tubes (Evergreen Scientific). The reaction mixture (final volume 300 μL) contained 20 mM sodium phosphate (pH 7.2), 0.15 M NaCl, 0.02% sodium azide, 0.05% Triton X-100, 3 mM EGTA, 30 μL of a 50-fold dilution of antiserum, and 9 μL of a 10-fold dilution of normal rabbit serum; each tube received serum protein equivalent to that in 1.5 μL of an undiluted rabbit serum. The serum was diluted in NaCl–P_i. Graded levels of noniodinated antigen were added, and the reaction mixture was incubated for 30 min at 30 °C. ^{125}I-Labeled antigen (13 000 cpm) was added, and the mixture was incubated with

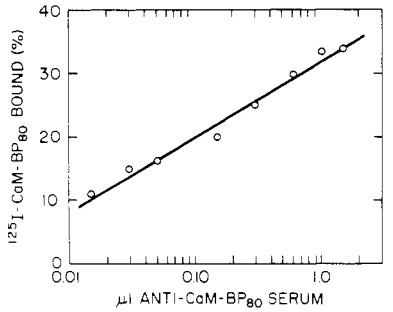

FIGURE 1: Binding of [^{125}I]CaM-BP$_{80}$ to rabbit anti-CaM-BP$_{80}$ serum. [^{125}I]CaM-BP$_{80}$ (13 000 cpm) was incubated 24 h with various amounts of anti-CaM-BP$_{80}$ serum in a reaction mixture (300 μL) containing NaCl–P$_i$, 0.05% Triton X-100, and 3 mM EGTA. All tubes contained a constant amount of serum protein (equivalent to 1.5 μL of undiluted rabbit serum) by the addition of an appropriate amount of normal rabbit serum. After overnight incubation, the bound [^{125}I]CaM-BP$_{80}$ was precipitated by the addition of 15 μL of undiluted goat antirabbit IgG serum. The precipitate was counted for [^{125}I]-CaM-BP$_{80}$. Each point on the curve represents the mean of three determinations.

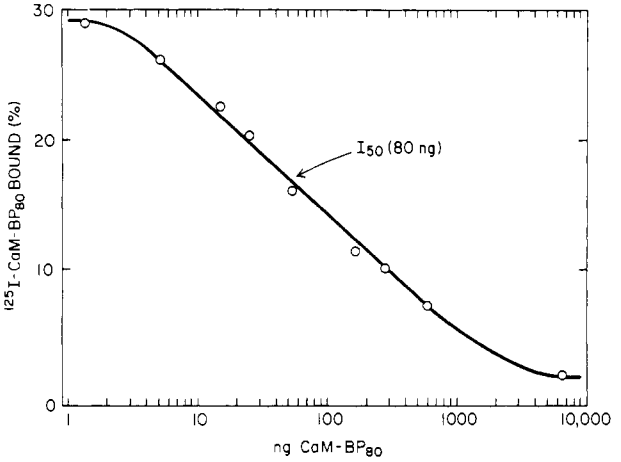

FIGURE 2: Competition of CaM-BP$_{80}$ with [^{125}I]CaM-BP$_{80}$ for binding to antiserum. The amount of CaM-BP$_{80}$ present in the assay mixture is indicated along the abscissa. The radioimmunoassay was conducted as described under Experimental Procedure.

shaking for 30 min at 30 °C, which was followed by 24 h at 4 °C. Fifteen microliters of undiluted goat antirabbit IgG serum was added, and the mixture was further incubated for 16 h at 4 °C. The precipitate was collected by centrifugation at 20000*g* for 20 min. The supernatant fluid was carefully removed, and the sediment in the tube was counted in a Nuclear Chicago γ spectrophotometer. The data were corrected for nonspecific binding to the tube and to goat antirabbit IgG and were expressed as the mean of three determinations.

Preparation of Tissue Extracts for Radioimmunoassay. Tissues were obtained fresh from a local abattoir, dissected, kept at −90 °C, and used over a 2-month period. The tissue (~1 g) was thawed, combined with 3 volumes of buffer A containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM EGTA, and homogenized for 30–60 s with a Polytron tissue homogenizer set at 30% maximum speed. The homogenate was centrifuged at 20000*g* for 20 min, and the supernatant fluid was recentrifuged at 100000*g* for 1 h. The 100000*g* supernatant fluid was dialyzed overnight against NaCl–P$_i$ containing 0.1 mM PMSF; the sample was stored at −90 °C .

Protein Determination. Protein was determined according to Lowry et al. (1951) with a slight modification. The protein was first precipitated with 10 volumes of 10% perchloric acid and 1% phosphotungstic acid. Bovine serum albumin was used as a standard.

Results

Production of Antibodies against CaM-BP$_{80}$ and Development of a Radioimmunoassay. CaM-BP$_{80}$ was used to immunize two rabbits; the serum from both animals contained high titers of antibodies after two booster injections with the antigen. The anti-CaM-BP$_{80}$ serum was used to develop a radioimmunoassay. As is shown in Figure 1, the binding of ^{125}I-labeled antigen to the antiserum was linear on a log scale between 0.01 and 1.0 μL of antiserum. A 0.6-μL amount of antiserum, which bound 30% of the iodinated antigen, was used to construct a standard curve for the radioimmunoassay. Figure 2 shows that CaM-BP$_{80}$ competed with the iodinated antigen for antibody binding over a 1000-fold concentration range. Approximately 80 ng or 1 pmol of nonlabeled CaM-BP$_{80}$ displaced 50% of the binding of [^{125}I]CaM-BP$_{80}$. The sensitivity of the radioimmunoassay is ~5 ng or 63 fmol of CaM-BP$_{80}$. Variations in the sensitivity of the assay were about twofold when different preparations of [^{125}I]CaM-BP$_{80}$ were used. The apparent discrepancy probably results from a different extent of chemical iodination of CaM-BP$_{80}$. For the purpose of comparison, the same preparation of [^{125}I]-CaM-BP$_{80}$ was always used within a series of experiments.

A note of technical interest concerning the radioimmunoassay should be added. The iodinated CaM-BP$_{80}$ binds avidly to the surface of glass or plastic tubes. The addition of bovine serum albumin (1%), NaSCN (0.2 M), or urea (0.5 M) to the reaction mixture did not alleviate the nonspecific binding. The addition of nonionic detergents such as Triton X-100, Lubrol-PX, or Triton N-101 effectively eliminated the nonspecific binding. In the radioimmunoassay, 0.05% Triton X-100 was included in the incubation mixture, although a concentration of 0.01% was equally effective. The addition of the detergent diminished the background counts from 15% to ~2% of the radioactivity in each assay mixture.

Previous measurement of CaM-BP$_{80}$ in this and other laboratories made use of the ability of CaM-BP$_{80}$ to inhibit calmodulin-supported phosphodiesterase activity; an example of this is included to contrast with the radioimmunoassay. As Figure 3 shows, 50% inhibition of calmodulin-supported phosphodiesterase activity was obtained with ~500 ng of CaM-BP$_{80}$, the sensitivity of the assay being ~400 ng. The steepness of the inhibition curve in the phosphodiesterase assay limits the useful range to between 300 and 600 ng of CaM-BP$_{80}$. Clearly, the radioimmunoassay affords a greater sensitivity as well as a wider sample range.

Cross-Reactivity of the Individual Subunits of CaM-BP$_{80}$ with the Antibodies. Since the rabbits were immunized with intact CaM-BP$_{80}$, it was of interest to determine whether the antibodies recognize the individual subunits of the antigen. The two subunits were isolated, and their reactivities with the immune serum were examined. Figure 4 shows that both subunits inhibited the binding of [^{125}I]CaM-BP$_{80}$ to the antiserum. The large subunit proved to be almost as effective as CaM-BP$_{80}$, whereas the small subunit was only ~0.02 as active. The inhibition curves generated by the two subunits appear parallel to the curve by CaM-BP$_{80}$, suggesting that the procedure used for subunit isolation did not affect the antigenic determinants. These data suggest that the large subunit contains most of the antigenic determinants of CaM-BP$_{80}$ for the antibodies.

Specificity of Radioimmunoassay for CaM-BP$_{80}$. Before the immunoassay could be used to determine the level of CaM-BP$_{80}$ in various tissues, it was necessary to establish the

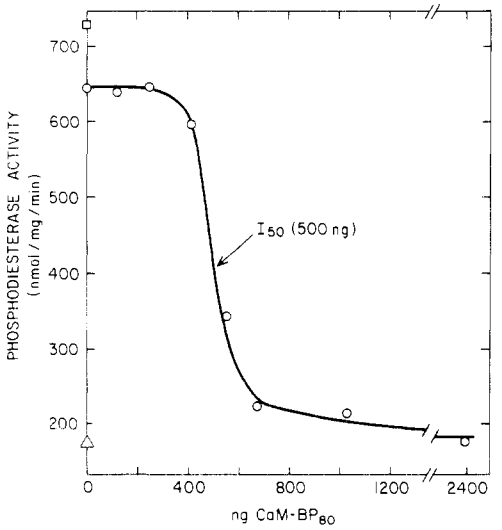

FIGURE 3: Standard curve for the assay of CaM-BP_{80} using the phosphodiesterase system. The assay is based on the ability of CaM-BP_{80} to inhibit calmodulin-supported phosphodiesterase activity. The reaction mixture contained 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 μM $CaCl_2$, 8 μg of calmodulin-deficient phosphodiesterase, 90 ng of calmodulin, and different amounts of CaM-BP_{80} as indicated in the figure. (△) Basal phosphodiesterase activity observed in the presence of 1 mM EGTA; (□) phosphodiesterase activity observed in the presence of excess calmodulin to give maximum stimulation.

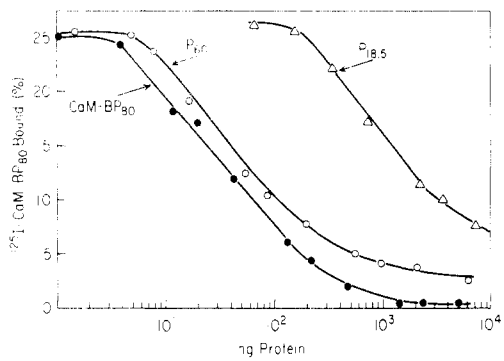

FIGURE 4: Competition by the individual subunits of CaM-BP_{80} with [^{125}I]CaM-BP_{80} for binding to the antiserum. P_{60} refers to the large subunit of CaM-BP_{80} and $P_{18.5}$ to the small subunit. The isolation of the two subunits is described under Experimental Procedure.

specificity and validity of the method. For examination of the specificity of the anti-CaM-BP_{80}, the reactivity with calmodulin (M_r 17 600), troponin (M_r 80 000), and its individual subunits Tn C (M_r 18 000), Tn I (M_r 24 000), and Tn T (M_r 39 000) was investigated (Figure 5). Troponin was chosen because its molecular weight is comparable to that of CaM-BP_{80}, and the molecular weight of one of its subunits, Tn C, is comparable to that of the smaller subunit of CaM-BP_{80}. In addition, both Tn I and Tn T interact with calmodulin. As Figure 5 shows, none of these proteins recognized the antibody. Nevertheless, the possibility exists that in the two troponin subunits the domains which interact with calmodulin may be structurally similar to those in CaM-BP_{80}.

The reaction mixture in the radioimmunoassay contains 3 mM EGTA, a condition that would prevent the binding of calmodulin to CaM-BP_{80}. With tissue extracts which contain calmodulin, binding of calmodulin to CaM-BP_{80} may mask certain antigenic sites and thus affect the radioimmunoassay for CaM-BP_{80}. To investigate this possibility, we conducted the radioimmunoassay in the presence of calmodulin with Ca^{2+} or EGTA. Figure 6 shows that calmodulin did not alter the standard curve in the presence of either Ca^{2+} or EGTA.

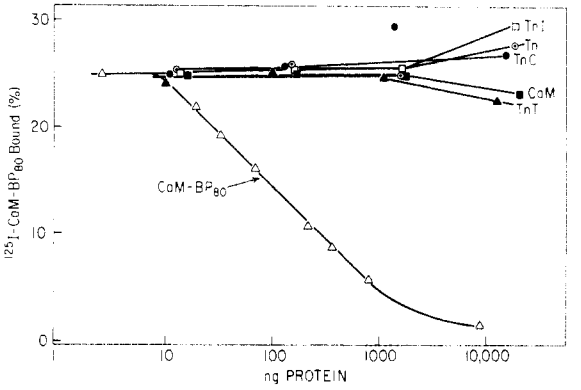

FIGURE 5: Lack of cross-reactivity of anti-CaM-BP_{80} serum with calmodulin (CaM), troponin (Tn), and its components troponin C (Tn C), troponin I (Tn I), and troponin T (Tn T). Stock solutions of Tn and Tn C were dissolved in NaCl-P_i and Tn I and Tn T in 1 mM HCl. Details of the radioimmunoassay are described under Experimental Procedure.

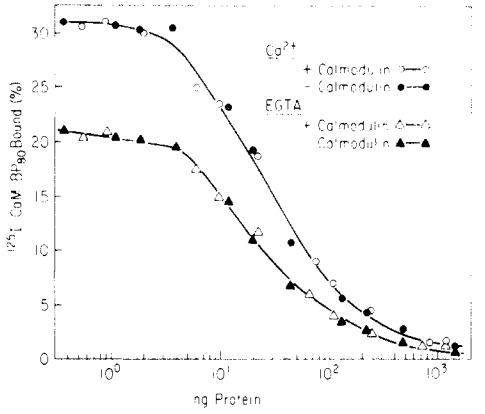

FIGURE 6: Effect of Ca^{2+} and calmodulin on the radioimmunoassay for CaM-BP_{80}. The assay was conducted in the presence of Ca^{2+} (100 μM) with (○) or without (●) calmodulin or in the presence of EGTA (3 mM) with (△) or without (▲) calmodulin. When present, calmodulin was used at a 15-fold molar excess over CaM-BP_{80}. Details of the radioimmunoassay are described under Experimental Procedure.

However, the presence of Ca^{2+} increased significantly the maximum binding of [^{125}I]CaM-BP_{80} as well as the slope of the inhibition curve. One explanation for the apparent lack of calmodulin effect in the presence of Ca^{2+} is that the serum already contains a sufficient concentration of endogenous calmodulin. This explanation seems unlikely since calmodulin could not be detected in either the whole or the fractionated serum. Binding of calmodulin to CaM-BP_{80} apparently does not cover any of the antigenic determinants. The higher binding of [^{125}I]CaM-BP_{80} in the presence of Ca^{2+} may mean that Ca^{2+} exposes more antigenic sites. These results show that the antiserum was highly specific for CaM-BP_{80} and that calmodulin did not affect the radioimmunoassay. The interference of calmodulin with the enzyme assay for CaM-BP_{80} by the phosphodiesterase system limits its usefulness, particularly with respect to its application to tissue extracts.

Measurement of CaM-BP_{80} in Various Bovine Tissues. Having established the specificity of the radioimmunoassay, we proceeded to measure the level of CaM-BP_{80} in a variety of bovine tissues and in several regions of the cerebrum. The curves depicting the inhibition of binding of [^{125}I]CaM-BP_{80} to the antiserum by the various tissue extracts are shown in Figure 7. The titration curves given by the various brain tissues were parallel to the standard curve for the radioimmunoassay whereas those by the nonnervous tissues were not. Nonparallel titration curves could result from the tissue spe-

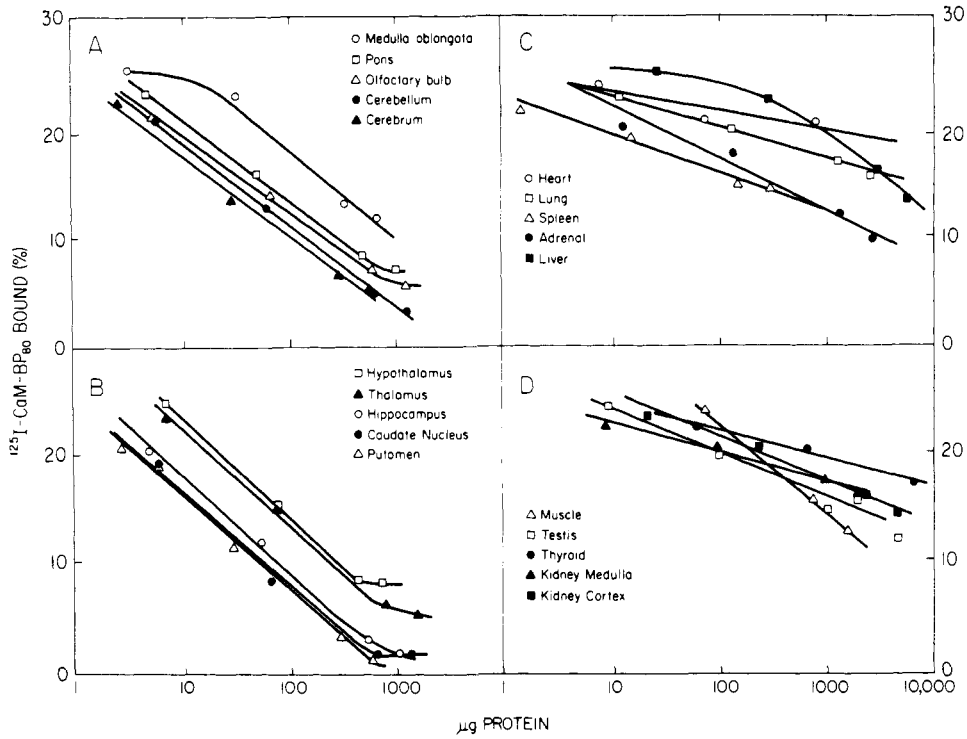

FIGURE 7: Radioimmunoassay of CaM-BP$_{80}$ in the extract of various bovine tissues. Preparation of the tissue extracts is described under Experimental Procedure. The same batch of [^{125}I]CaM-BP$_{80}$ was used in all assays.

Table I: Level of CaM-BP$_{80}$ in Various Bovine Tissues As Determined by Radioimmunoassay[a]

tissue	CaM-BP$_{80}$ (mg of protein per kg of tissue)
adrenal	3.3 ± 0.2
cerebellum	29.4 ± 12.0
cerebrum	
grey matter	36.0 ± 5.0
white matter	15.9 ± 2.5
caudate nucleus	62.9 ± 10.9
hippocampus	36.4 ± 7.3
hypothalamus	31.3 ± 19.7
putamen	84.8 ± 20.0
thalamus	19.8 ± 7.0
heart	0.7 ± 0.3
kidney	
cortex	2.3 ± 0.7
medulla	3.4 ± 1.8
liver	1.8 ± 0.5
lung	3.2 ± 1.9
medulla oblongata	2.5 ± 1.5
olfactory bulb	17.2 ± 11.8
pons	6.3 ± 3.9
skeletal muscle	1.9 ± 0.6
spleen	1.8 ± 0.6
testis	3.1 ± 0.9
tongue	2.8 ± 0.9
thyroid	2.1 ± 1.5

[a] An appropriate volume (0.1–200 μL) of a 100000*g* supernatant fluid from each tissue extract was assayed for CaM-BP$_{80}$ as described under Experimental Procedure. The data were taken from Figure 7, and they represent the mean ± SD of four determinations on tissues from two animals; each assay was done in duplicate.

Table II: Recovery of Exogenous CaM-BP$_{80}$ from the Extract of Several Bovine Tissues As Determined by Radioimmunoassay[a]

addition	CaM-BP$_{80}$ obsd (ng)	% recovery
CaM-BP$_{80}$	11.6	
heart	12.6	
heart + CaM-BP$_{80}$	22.8 (24.2)	94
kidney medulla	13.9	
kidney medulla + CaM-BP$_{80}$	26.5 (25.5)	104
caudate nucleus	12.5	
caudate nucleus + CaM-BP$_{80}$	22.5 (24.1)	93

[a] An appropriate aliquot of a 100000*g* supernatant fluid from each tissue extract was assayed for CaM-BP$_{80}$ as described under Experimental Procedure with or without the addition of 11.6 ng of CaM-BP$_{80}$. The numbers in parentheses are the theoretical values. The volume of tissue extracts used in the assay: heart, 15 μL; kidney medulla, 25 μL; caudate nucleus, 0.5 μL.

cificity of CaM-BP$_{80}$ or the presence of interfering substances in the various nonnervous tissues. Because of the nonparallel curves, the concentration of radioimmunoassayable CaM-BP$_{80}$ in these tissues could only be estimated. The amount of protein needed to give 25% inhibition of the binding of iodinated antigen was used to estimate the tissue level of CaM-BP$_{80}$ from the standard curve. The data summarized in Table I show that CaM-BP$_{80}$ is found predominantly in the cerebrum, olfactory bulb, and cerebellum. Within the cerebrum, the level of CaM-BP$_{80}$ is especially high in the caudate nucleus and putamen. The trace level of CaM-BP$_{80}$ in nonnervous tissues needs a word of comment. Because of the apparently low level of CaM-BP$_{80}$ in these tissues, a large volume of the tissue extract was used in the radioimmunoassay. The addition of a bulk of extraneous proteins would accentuate any nonspecific displacement of the ^{125}I-labeled antigen from anti-CaM-BP$_{80}$ in the assay mixture. In other words, the levels of CaM-BP$_{80}$ in the nonnervous tissues are in the range of the sensitivity of the radioimmunoassay, raising the uncertainty that CaM-BP$_{80}$ is truly present in any significant level in these tissues.

To investigate whether the nonparallel titration curves arose from the presence of potential interfering substances in the tissue extracts, we performed recovery experiments in the extract of heart and kidney medulla. As a comparison, an extract of caudate nucleus was included in the assay (Table II). The amount of exogenous CaM-BP$_{80}$ recovered from the three tissue extracts was essentially quantiative, indicating the absence of interfering substances. These results suggest that the nonparallel curves could result from the tissue specificity

of CaM-BP_{80}, a notion that cannot be discounted unless pure CaM-BP_{80} can be isolated from these tissues.

Discussion

Previously, CaM-BP_{80} has only been estimated by an enzyme assay using the phosphodiesterase system. Tissue extracts contain calmodulin and phosphodiesterase, which interfere with the assay for CaM-BP_{80}. The availability of an antibody against CaM-BP_{80} allows the development of a radioimmunoassay to determine quantitatively its level in the various tissues, a study that cannot be attempted by the enzyme assay.

The radioimmunoassay appears superior to the enzyme assay in several respects. It is approximately 100-fold more sensitive, and it affords a much wider range of sample concentration, making it more versatile. Most important, however, is the finding that endogenous calmodulin in the tissue extract does not affect the accuracy of the assay.

The results of the tissue survey using the radioimmunoassay show that CaM-BP_{80} is found predominantly in the brain. The high levels found in the cerebrum, especially in the caudate nucleus and the putamen, suggest that the protein may have an important function in the neostriatum. Brain also contains a high level of calmodulin, and the level is exceeded only by that in the testis (Cheung et al., 1975b). The level of calmodulin in the testis is approximately twice that in the brain. Yet, the level of CaM-BP_{80} in the testis is barely detectable (Table I). This would strongly suggest that CaM-BP_{80} is not a true inhibitor of calmodulin and that its physiologic function is primarily associated with the nervous activity of the neostriatum, a part of the extrapyrimidal system concerned with the generation and regulation of motor command.

Recently, antibodies against CaM-BP_{80} as well as calmodulin have been utilized for immunocytochemical localization of these proteins in mouse basal ganglia at the electron microscopic level. Antibodies for both proteins decorate the postsynaptic densities and the microtubules of the postsynaptic dendrites. Furthermore, the patterns of localization with both antisera appeared identical (Wood et al., 1980), suggesting that they may have a role in postsynaptic function.

Since the primary structure of calmodulin appears to have been highly conserved irrespective of its source (Grand et al., 1979), all calmodulin-regulated enzymes or proteins could share a common structural domain for interacting with calmodulin. If this domain represents one of the antigenic determinants of CaM-BP_{80}, it is conceivable that anti-CaM-BP_{80} may also recognize other calmodulin-regulated proteins, such as phosphodiesterase, adenylate cyclase, phosphorylase kinase, myosin light chain kinase, and Ca^{2+}-ATPase. The presence of these proteins in the tissue extracts could give rise to the nonparallel titration curves seen in Figure 7. Along the same line of argument, the recognition of anti-CaM-BP_{80} for these calmodulin-regulated proteins would permit the radioimmunoassay to detect all of these proteins indiscriminately. Thus, the tissue levels of CaM-BP_{80} presented in Table I should be taken with this reservation in mind.

The rapidly increasing interest in the role of calmodulin in cellular functions has added impetus to the identification of calmodulin-binding proteins. The availability of antibodies against and radioimmunoassays for each of these proteins will be highly useful in elucidating their biological functions. The high levels of CaM-BP_{80} in the cerebrum and its localization at the postsynaptic area as well as the dendritic microtubules (Wood et al., 1980) would suggest a role in the basal ganglion at the site of neurotransmitter action and at the level of microtubular function. Moreover, the finding that CaM-BP_{80} is concentrated in the neostriatum suggests that the protein may be involved in the generation and regulation of motor commands.

In summary, the results presented in this communication bear considerable significance from the biological viewpoint. The availability of a specific antibody for CaM-BP_{80} affords a highly useful tool to address questions concerning its biological function. More importantly, however, is the finding that CaM-BP_{80} is primarily associated with the cerebrum, especially with the neostriatum. This suggests that future efforts toward elucidating its identity may be directed most profitably from the point of neurophysiology, with emphasis on its function in the neostriatum.

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References

Amphlett, G. W., Vanaman, T. C., & Perry, S. V. (1976) *FEBS Lett. 72*, 163–168.

Anderson, J. M., & Cormier, M. J. (1978) *Biochem. Biophys. Res. Commun. 84*, 595–602.

Brostrom, C. O., & Wolff, D. J. (1974) *Arch. Biochem. Biophys. 165*, 715–727.

Brostrom, C. O., & Wolff, D. J. (1976) *Arch. Biochem. Biophys. 172*, 301–311.

Brostrom, C. O., Huang, Y. C., Breckenridge, B. McL., & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 64–68.

Brostrom, M. A., Brostrom, C. O., Breckenridge, B. M., & Wolff, D. J. (1976) *J. Biol. Chem. 251*, 4744–4750.

Cheung W. Y. (1980) *Science 207*, 19–27.

Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M., & Tallant, E. A. (1975a) *Biochem. Biophys. Res. Commun. 66*, 1055–1062.

Cheung, W. Y., Lin, Y. M., Liu, Y. P., & Smoake, J. A. (1975b) in *Cyclic Nucleotides in Disease* (Weiss, B., Ed.) pp 321–350, University Park Press, Baltimore, MD.

Cheung, W. Y., Lynch, T. J., & Wallace, R. W. (1978) *Adv. Cyclic Nucleotide Res. 9*, 233–251.

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., & Nairn, A. C. (1978) *FEBS Lett. 92*, 287–293.

Dabrowska, R., & Hartshorne, D. J. (1978) *Biochem. Biophys. Res. Commun. 85*, 1352–1359.

Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., & Hartshorne, D. J. (1978) *Biochemistry 17*, 253–258.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977) *J. Biol. Chem. 252*, 8415–8422.

Gopinath, R. M., & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun. 77*, 1203–1209.

Grand, R. J. A., Perry, S. V., & Weeks, R. A. (1979) *Biochem. J. 177*, 521–529.

Hanahan, D. J., Taverna, R. D., Flynn, D. D., & Ekholm, J. E. (1978) *Biochem. Biophys. Res. Commun. 84*, 1009–1015.

Hinds, T. R., Larsen, F. L., & Vincenzi, F. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 455–461.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Jarrett, H. W., & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun. 77*, 1210–1216.

Jarrett, H. W., & Penniston, J. T. (1978) *J. Biol. Chem. 253*, 4676–4682.
Kakiuchi, S., & Yamazaki, R. (1970) *Biochem. Biophys. Res. Commun. 41*, 1104–1110.
Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K., & Miyamoto, E. (1975) *Adv. Cyclic Nucleotide Res. 5*, 163–178.
Katz, S., & Remtulla, M. A. (1978) *Biochem. Biophys. Res. Commun. 83*, 1373–1379.
Klee, C. B. (1977) *Biochemistry 16*, 1017–1024.
Klee, C. B., & Krinks, M. H. (1978) *Biochemistry 17*, 120–126.
Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem.* (in press).
LaPorte, D. C., & Storm, D. R. (1978) *J. Biol. Chem. 253*, 3374–3377.
Larsen, F. L., & Vincenzi, F. F. (1979) *Science 204*, 306–309.
Liu, Y. P., & Cheung, W. Y. (1976) *J. Biol. Chem. 251*, 4193–4198.
Lowry, O. H., Rosebrough, M. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lynch, T. J., & Cheung, W. Y. (1979) *Arch. Biochem. Biophys. 194*, 165–170.
Lynch, T. J., Tallant, E. A., & Cheung, W. Y. (1977) *Arch. Biochem. Biophys. 182*, 124–133.
Marcum, J. M., Dedman, J. R., Brinkley, B. R., & Means, A. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3771–3775.
Schulman, H., & Greengard, P. (1978a) *Nature (London) 271*, 478–479.
Schulman, H., & Greengard, P. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5432–5436.
Sharma, R. K., Wirch, E., & Wang, J. H. (1978) *J. Biol. Chem. 253*, 3575–3580.
Sharma, R. K., Desai, R., Waisman, D. M., & Wang, J. H. (1979) *J. Biol. Chem. 254*, 4276–4282.
Sherry, J. M. F., Gőrecka, A., Aksoy, M. O., Dabrowska, R., & Hartshorne, D. J. (1978) *Biochemistry 17*, 4411–4418.
Teo, T. S., & Wang, J. H. (1973) *J. Biol. Chem. 248*, 5950–5955.
Waisman, D., Stevens, F. C., & Wang, J. H. (1975) *Biochem. Biophys. Res. Commun. 65*, 975–985.
Waisman, D. M., Singh, T. J., & Wang, J. H. (1978) *J. Biol. Chem. 253*, 3387–3390.
Wallace, R. W., & Dieckert, J. W. (1976) *Anal. Biochem. 75*, 498–508.
Wallace, R. W., Yu, P. H., Dieckert, J. P., & Dieckert, J. W. (1974) *Anal. Biochem. 61*, 86–92.
Wallace, R. W., Lynch, T. J., Tallant, E. A., & Cheung, W. Y. (1978a) *Arch. Biochem. Biophys. 187*, 328–334.
Wallace, R. W., Lynch, T. J., Tallant, E. A., Macleod, R. M., & Cheung, W. Y. (1978b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1302.
Wallace, R. W., Lynch, T. J., Tallant, E. A., & Cheung, W. Y. (1979) *J. Biol. Chem. 254*, 377–382.
Wang, J. H., & Desai, R. (1976) *Biochem. Biophys. Res. Commun. 72*, 926–932.
Wang, J. H., & Desai, R. (1977) *J. Biol. Chem. 252*, 4175–4184.
Wang, J. H., & Waisman, D. M. (1980) *Curr. Top. Cell. Regul. 15*, 47–108.
Wang, J. H., Teo, T. S., Ho, H. C., & Stevens, F. C. (1975) *Adv. Cyclic Nucleotide Res. 5*, 179–194.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wolff, D. J., & Brostrom, C. O. (1974) *Arch. Biochem. Biophys. 163*, 349–358.
Wolff, D. J., & Brostrom, C. O. (1979) *Adv. Cyclic Nucleotide Res. 11*, 28–88.
Wong, P. Y.-K., & Cheung, W. Y. (1979) *Biochem. Biophys. Res. Commun. 90*, 473–480.
Wood, J. G., Wallace, R., Whitaker, J., & Cheung, W. Y. (1980) *J. Cell Biol. 84*, 66–76.
Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., & Uenishi, K. (1978) *J. Biol. Chem. 253*, 1338–1340.