A major calmodulin-binding protein common to various vertebrate tissues
(avian erythrocytes/immunoprecipitation)

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ABSTRACT A major calmodulin-binding protein (CaM-BP) of membranes isolated from various rat tissues by using a 125I-labeled CaM gel overlay technique. This protein (designated p240) was detected in the particulate fraction and to a lesser extent in the cytosol of all tissues studied. Binding of CaM to p240 was completely dependent on Ca2+. A second, exclusively soluble, CaM-BP was found in both fractions and also in other tissues. Antibodies prepared against turkey erythrocyte CaM-BP recognized p240 in plasma membranes from avian erythrocytes but was absent from mammalian erythrocyte membranes. Antibodies prepared against turkey erythrocyte CaM-BP (anti-Tp240) and CaM were by demonstrating that 125I-labeled CaM could bind to p240 specifically immunoprecipitated from either rat brain or turkey erythrocytes by anti-Tp240. The p240 may be related to a previously described actin-binding protein and may represent a major site of action of CaM on the cytoskeleton.

Calmodulin (CaM), the ubiquitous Ca2+-binding protein, is responsible for mediating several physiological responses associated with increases in intracellular Ca2+ levels. This protein has been shown to bind to and activate a number of enzymes of defined function, such as cyclic nucleotide phosphodiesterases (1, 2), Ca2+, Mg2+ ATPases (3–5), a specific class of protein kinases (6–8), and a protein phosphatase that appears to be identical with calcineurin (9, 10). Several reports also suggest that CaM interacts with certain cytoskeletal elements—for example, microtubules (11, 12), brush border core proteins (13), and spectrin (14). These observations are of considerable interest because Ca2+ is widely believed to control various aspects of cell shape, motility, and translocation of intracellular organelles, and CaM may be intimately involved in these phenomena (15).

During the course of studies on the purification of CaM-dependent protein kinase from two sources, avian erythrocyte and Torpedo electroplaque, we noted the presence of a high molecular weight species that appeared to adsorb specifically to CaM affinity matrices. The presence of this protein in two such divergent tissues led us to investigate the possibility that the protein might have a more widespread distribution. For this purpose we used the recently developed 125I-labeled CaM (125I-CaM) gel overlay technique (13, 16) and have demonstrated the existence of a M, 240,000 CaM-binding protein (CaM-BP), designated p240, common to avian erythrocytes and a number of mammalian tissues.

METHODS

Preparation of 125I-CaM and 125I-CaM Gel Overlay. CaM was isolated from bovine brain by the method of Grand et al. (17) and iodinated with the Bolton–Hunter reagent (New England Nuclear) as described by Chafouleas et al. (18) to a specific activity of 110 Ci/nmol (1 Ci = 3.7 × 1010 becquerels). Those authors have demonstrated that CaM iodinated by this technique retains full biological activity. The gel overlay technique was similar to that of Carlin et al. (16). Briefly, NaDodSO4/polyacrylamide (6% or 5–10% gradient) gels (19) were fixed, equilibrated in buffer A (150 mM NaCl/25 mM Tris-HCl, pH 7.3/0.1% NaNO3/0.5% gelatin) with either 1 mM CaCl2 or 1 mM EGTA, and then incubated in sealed plastic bags with 125I-CaM (20 nM; 2.2 μCi/ml, diluted in buffer A) for 3 hr. The gels were then washed (1–2 hr each) with five changes of buffer A, stained, destained, and autoradiographed. For quantitation of relative amounts of 125I-CaM bound, pieces were excised from the dried gel and assayed by γ-emission spectrometry. The amount of 125I-CaM bound to the p240 region of gels containing turkey erythrocyte membrane proteins was found to be linearly proportional to the amount of protein applied (2–40 μg). All 125I-CaM binding experiments were conducted within this range. Molecular weights of CaM-BPs were estimated by reference to molecular weight markers (19) run on the same gel.

Preparation of Tissues. Adult rat tissues were dissected out, washed in physiological saline, and homogenized in 10 vol of 25 mM Tris-HCl, pH 7.3/1 mM EDTA/5 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride in a Teflon/glass homogenizer. After a low-speed spin (1,000 × g for 5 min) to eliminate debris, the supernatant was centrifuged at 150,000 × g for 45 min to obtain crude particulate and cytosol fractions. These were analyzed for protein concentration (20) and solubilized in a NaDodSO4 stop buffer (19) prior to electrophoresis.

Membranes from avian and mammalian erythrocytes were prepared as described (21). Extraction of turkey erythrocyte membranes with 0.5% Nonidet P-40 (NP-40), 15 mM lithium diodiosalicylate, or low-ionic strength dialysis was by the procedure of Beam et al. (21). Nonextractable material was pelleted by centrifugation at 50,000 × g for 20 min.

Preparation of Antibodies to Turkey Erythrocyte p240 and Antibody Overlay. Turkey erythrocyte membranes were prepared as above and their proteins were separated on NaDodSO4/4% polyacrylamide slab gels (20 cm long, 1.2 mm thick; 33 mg of protein per 10 gels). Proteins were localized by immersing the gels in 4 M Na acetate for 30 min (22), and the band corresponding to p240 was excised and stacked onto a 5-mm-thick NaDodSO4/4% polyacrylamide gel. After reelectrophoresis the region containing p240 (approximately 100 μg) was visualized

Abbreviations: SAC, Staphylococcus aureus; p240, protein of M, 240,000; anti-Tp240, antibody against turkey erythrocyte p240; CaM, calmodulin; CaM-BP, calmodulin-binding protein; NP-40, Nonidet P-40.

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as above and excised, and the gel strip was washed in physiological saline to decrease its NaDdSO₄ content. It was then homogenized with a Teflon/glass homogenizer in 3 ml of the same buffer, mixed with 3 ml of complete Freund's adjuvant, and emulsified by repeated passage through a 24-gauge syringe needle. Rabbits were injected intradermally with this material per injection site. Two booster injections of material without Freund's adjuvant were given after 1 and 2 weeks, and blood was collected 2 weeks later. Preimmune serum was obtained from the same rabbits prior to antigen injection.

The antibody overlay technique was similar to that described above for ¹²⁵I-CaM binding and followed the original method of Burridge (23). Gels were first incubated in antibody to turkey erythrocyte p240 (anti-Tp240; 1:10 in buffer A) for 3 hr, washed four times (1–2 hr each) in buffer A, and then incubated in ¹²⁵I-labeled protein A (¹²⁵I–protein A; Amersham; 0.5–1 μCi/ml in buffer A) for a further 2 hr. After a second series of washes in buffer A, gels were either dried directly or were stained and destained before autoradiography. All experiments were conducted within the range of protein concentrations in which anti-Tp240–¹²⁵I-protein A binding was found to be linear.

Immunoprecipitation. The immunoprecipitation technique was modified slightly from Ivarie and Jones (24) and Goetz et al. (25). Briefly, dialyzed extract (30–40 μg of protein) of turkey erythrocyte membranes (21), or synaptosomal lysate (80–150 μg of protein) prepared as described (26), was incubated at 4°C in NET buffer (25) in the presence of 0.1% NaDdSO₄ for 5 min (final volume, 120 μl). Then 40 μl of NET containing 25 mg of bovine serum albumin per ml and 2% NP-40 was added, followed by 10 μl of a 10% solution of freshly prepared Staphylococcus aureus (SAC; Pansorbin, Calbiochem) in NET/0.25% NF-40. Incubation was continued for 5 min, followed by sedimentation at 12,000 X g. The “precleared” supernatant was transferred to a second tube and incubated with 5 μl of anti-Tp240 (or preimmune serum) for 20 min. An additional 10 μl of SAC was added and 5 min later the SAC–IgG precipitate was collected as above. This pellet was washed twice with NET/0.25% NF-40 and then solubilized in 100 μl of NaDdSO₄stop solution. Gel electrophoresis was carried out in NaDdSO₄/7.5% polyacrylamide gels and the products were analyzed by staining or by ¹²⁵I-CaM gel overlay as described above.

RESULTS

¹²⁵I-CaM Binding to Erythrocyte Membranes and to Various Rat Tissues. Membranes prepared from erythrocytes of five species were compared for their ability to bind ¹²⁵I-CaM, by using the gel overlay technique. The results shown in Fig. 1 illustrate the high levels of ¹²⁵I-CaM binding to a protein of Mr 240,000 present in avian erythrocyte membranes (turkey, goose, and duck). In contrast, mammalian (human and rat) erythrocyte membranes were devoid of such binding. The interaction of ¹²⁵I-CaM with p240 was totally dependent on Ca²⁺ being eliminated in the presence of EGTA. In addition, Ca²⁺-dependent binding was reversed by subsequent washing of the gels in EGTA-containing solutions or by including triluoperazine (0.1 mM) in the binding buffer (data not shown).

A survey of CaM-BPs in crude particulate and cytosol fractions of several rat tissues was made with the same technique (Fig. 2). It is apparent that ¹²⁵I-CaM binding to a protein of Mr 240,000 is widespread in the tissues examined, with much of the activity concentrated in the particulate fraction. Although this technique cannot yield an absolute quantitative measure, the relative amount of binding to the p240 region in different tissues was remarkably similar when normalized for protein concentration. However, skeletal muscle clearly contained sig-
Anti-Tp240 Binding to Erythrocyte Membranes and to Various Rat Tissues. In addition to the major CaM-BP in various tissues appearing to have the same molecular weight on NaDodSO₄/polyacrylamide gels, peptide fragments, generated by thermolysin, of the protein from different sources showed extensive homology (data not shown). Therefore, it became of interest to evaluate the possibility that this protein would display immunological similarity in all the tissues under study. To this end, an antibody to the prominent p240 of turkey erythrocytes was developed and the abilities of various tissues to recognize anti-Tp240 antiserum were compared.

In erythrocyte membrane samples, anti-Tp240 binding occurred about equally in the p240 region of the three avian species tested but was absent from mammalian erythrocyte ghosts (data not shown). Positive immunological reactions were also obtained in both particulate (Fig. 3) and cytosol (data not shown) fractions from those rat tissues that had been analyzed for ¹²⁵I-CaM binding. Moreover, the relative amounts of anti-Tp240 bound to both erythrocyte and rat tissue samples corresponded well qualitatively with ¹²⁵I-CaM binding, supporting the idea that the proteins responsible for the two activities were identical. The anti-Tp240 antibodies also interacted with a series of proteins of \( M_r < 240,000 \) (Fig. 3). These were more prevalent in the particulate fraction and appeared to be similar in the several tissues studied; they may represent proteolytic breakdown products of p240.

Immunoprecipitation of \( M_r 240,000 \) CaM-BP. In order to examine the possible identity of the immunoreactive p240 with the CaM-BP, the ability of anti-Tp240 immunoprecipitates to bind ¹²⁵I-CaM was tested. Low ionic strength extracts of turkey erythrocyte membranes that were found to contain substantial amounts of p240 CaM-BP (see below) were preincubated in 0.1% NaDodSO₄ and then were incubated with antibody preparation or preimmune serum and precipitated with SAC. Under these conditions, a p240 was immunoprecipitated from the extract.
tract by anti-Tp240 but not by preimmune serum (Fig. 4A). After gel electrophoresis, the immunoprecipitate was found to bind 125I-CaM (Fig. 4B), confirming that a protein recognized by anti-Tp240 also bound CaM. Preincubation in 0.1% NaDodSO4 was found to be essential to obtaining maximal specificity in the assay, but this specificity was obtained at the expense of a decrease in the quantity of antigen precipitated by the antibody (cf. ref. 25).

Similar experiments carried out with synaptosomal lysates also led to the immunoprecipitation of a M, 240,000 CaM-BP (Fig. 4 C and D). In this case, preincubation in 0.1% NaDodSO4 was also essential to solubilize the sample. Traces of a protein of M, 235,000 were precipitated along with the p240 CaM-BP (Fig. 4C), a phenomenon that could be abolished by preincubation of the lysate in 0.5% NaDodSO4. This is probably indicative of a relatively tight interaction between p240 and the M, 235,000 protein (see Discussion), but the possibility of a direct but weaker immunological interaction between anti-Tp240 and p235 cannot be excluded.

Extraction of 125I-CaM-BP and Anti-Tp240 Binding Activities from Turkey Erythrocyte Membranes. The above results clearly demonstrated that a component of the anti-Tp240 antiserum recognized the p240 CaM-BP. Further evidence that these two activities resided on the same molecule came from experiments in which the extractability of 125I-CaM-BP and anti-Tp240 binding protein from turkey erythrocyte membranes was compared. The three extraction procedures used were chosen for their ability to solubilize certain proteins differentially from the membrane (cf. ref. 21). Good agreement between the amounts of the two M, 240,000 binding components released from the membrane under these three conditions was obtained (Fig. 5). These results suggest that a single protein is responsible for the majority of both binding activities.

**DISCUSSION**

CaM is now considered to be a ubiquitous intracellular receptor for Ca2+, mediating several of the second-messenger activities of this ion. The various CaM-BPs thus represent a next level of response in the sequence from Ca2+ increase in the cytoplasm to physiological effect. It is evident from the present work that the p240 represents a major binding site for CaM in most tissues. Our results suggest that this protein is homologous, and may be highly conserved, in different tissues and species, based on its ability to be recognized by an antibody directed against the protein from turkey erythrocytes (anti-Tp240). Immunoprecipitation experiments confirmed that the p240 band recognized by the antibody was the same protein that bound CaM (Fig. 4).

The 125I-CaM gel overlay technique used here appears to be a useful method for identifying CaM-BPs in crude tissue extracts. Thus far, only specialized preparations such as postsynaptic densities (16) and intestinal brush borders (13) have been

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**FIG. 4. Immunoprecipitation, by anti-Tp240, of proteins from turkey erythrocyte extracts (A and B) and synaptosomal lysates (C and D). Immunoprecipitated proteins were analyzed on NaDodSO4/7.5% polyacrylamide gels for protein (A and C) or by autoradiography after 125I-CaM overlay (B and D). Ext, total extract; Ab, anti-Tp240 immunoprecipitate; Pre, preimmune control; Ig, position of immunoglobulin.**

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**FIG. 5. Extraction, by three procedures, of protein (A), 125I-CaM-BP (B), and anti-Tp240-125I-protein A-reactive material (C) from turkey erythrocyte membranes. s, Supernatant; p, pellet; M, intact membranes. Numbers in B and C refer to the percentage radioactive material found in each lane. Only the relevant high molecular weight region of this NaDodSO4/6% polyacrylamide gel is shown. LIS, lithium diodosalicylate. In A, G indicates the position of the turkey erythrocyte protein goblin (21).**
investigated in detail with this method. It is not yet clear if artificial binding of CaM to certain proteins could take place in this procedure. This is unlikely to apply to p240 because specific interaction of the native protein with CaM affinity columns has been shown to take place with material derived from brain (ref. 28; see below), Torpedo electroplaque, and avian erythrocytes (unpublished results). However, we cannot be certain that binding of 125I-CaM to some of the other proteins shown in Fig. 2 is physiologically relevant. Conversely, certain CaM-BPs might be missed by using this method. These issues can best be addressed by application of CaM affinity chromatographic techniques to native tissue extracts.

It is likely that the protein described here corresponds to the higher molecular weight component of a doublet recently isolated from brain by two independent groups (29, 29). Davies and Klee (29) purified a doublet (Mr 235,000 and 230,000 in their gel system) from bovine brain on the basis of its ability to bind CaM. Shimo-Oka and Watanabe (29) purified a similar doublet from porcine brain on the basis of its ability to activate actomyosin ATPase activity. Both groups have shown that this protein doublet interacts directly with F-actin, although it is not known whether this activity resides in the p240 or the Mr 235,000 subunit. Interestingly, our immunoprecipitation experiments on synaptic proteins solubilized at low NaDodSO4 concentration did lead to sedimentation of a second protein of Mr 235,000 (Fig. 4) which could only be dissociated at higher NaDodSO4 concentrations. Kakiuchi et al. (30) have also purified a p240 from bovine brain by using CaM affinity chromatography in the presence of 6 M urea. Under these conditions, no evidence for the Mr 235,000 species was found. It is clear that p240 is a major constituent of brain [estimates of 2–4% (28) and 3% (30) of total brain protein have been given; see Fig. 4C where these proteins are conspicuously stained] and, as indicated here, this may be true for other tissues. The widespread distribution of p240 and the possibility that it interacts with F-actin suggest that it may play a major role in the modulation of cytoskeletal structure and function by intracellular Ca2+

The Mr 240,000 CaM-BP in turkey erythrocyte membranes comigrated with a gel band that stained heavily with Coomassie blue and that has previously been termed "turkey spectrin" (19, 21) on the basis of a mobility similar to that of human erythrocyte spectrin α on NaDodSO4 gels. However, it is apparent that these two proteins differ markedly in some of their properties. Human spectrin α does not bind either 125I-CaM or anti-Tp240 under the conditions used here. Nevertheless, the apparent association of p240 with stoichiometric amounts of a Mr 220,000 protein in extraction experiments (Fig. 5) resembles the behavior of human spectrin, and experiments with antibodies to purified human spectrin showed a weak crossreaction with avian erythrocyte p240 (unpublished data). Thus, some relationship between the two proteins cannot be excluded. It has been reported that human spectrin does bind CaM under certain conditions (14). We hypothesize that the reason for our negative results with human erythrocyte membranes in the 125I-CaM overlay technique (Fig. 1) may be partially due to the low affinity of spectrin for CaM [approximately 2.8 μM (14)]. It remains to be determined whether the human spectrin–CaM interaction is physiologically significant.

Further work is necessary to define the function and localization of p240 in various tissues and in different physiological states. The availability of an antibody to this protein should aid greatly in this undertaking.

Note Added in Proof. After this paper was communicated, an article by Glenney et al. (31) appeared, showing that a Mr 240,000 component purified from chicken intestinal brush borders and brain could bind CaM. The isolated protein from brain corresponded to a subunit of a previously identified brain doublet termed "fodrin" by Levine and Willard (32), who showed the presence of similar proteins in several tissues.

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