Characterization of a calmodulin-binding protein that is deficient in trifluoperazine-resistant variants of the macrophage-like cell line J774

(phenothiazines/calcium/phagocytosis)

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ABSTRACT A calmodulin-binding protein is present in extracts of the macrophage-like mouse cell line J774 and in extracts of thioglycollate-stimulated mouse peritoneal macrophages; it is deficient in variants of J774 resistant to trifluoperazine and in resident peritoneal macrophages. The calmodulin-binding protein [CaMBP (J7)^{0.5}] was purified from J774 and resolved from endogenous cyclic nucleotide phosphodiesterase and protein kinase activities. The protein has an apparent native M_r of 125,000–150,000 and binds calmodulin in a calcium-dependent manner with a K_d of 20 nM. It inhibits the ability of calmodulin to activate phosphodiesterase. Its sedimentation constant in glycerol gradients containing calmodulin was dependent upon the relative concentrations of calmodulin and the calmodulin-binding protein.

Macrophages express a number of functions—such as phagocytosis, migration, and enzyme secretion—in which calcium ions may play a role (1–3). Many of the intracellular actions of calcium ions are mediated by calmodulin (CaM) (4–8): myosin ATPase activity in smooth muscle and nonmuscle cells (9–11), polymerization of actin in brain and smooth muscle (12–14), and calcium transport across membranes (15–17). We have studied the role of CaM in the macrophage-like mouse cell line J774 (18, 19). Trifluoperazine (Tfp), an inhibitor of CaM function *in vitro*, reversibly inhibited the growth of J774 as well as its ability to engage in phagocytosis. Levels of cAMP in intact cells were not affected by Tfp and the activities of cyclic nucleotide phosphodiesterase (PDEase) and adenylate cyclase *in vitro* were neither Ca^{2+} -CaM dependent nor Tfp sensitive.

Variants capable of growing in Tfp were selected after mutagenesis with ethyl methanesulfonate (18). The CaM content of C2, the variant studied in greatest detail, was unaltered, as was its ability to stimulate PDEase and to be inhibited by Tfp. However, extracts of all of the Tfp-resistant variants were found to be uniquely deficient in their content of a major ^{125}I -labeled CaM (^{125}I -CaM) complex found in parental line J774 and designated CaM-binding protein (J7)^{0.5} [CaMBP (J7)^{0.5}]. This deficiency was not dependent upon the continuous presence of Tfp and was not found in unrelated variants of J774 (18). Mixing experiments suggested it was not due to a transferable inhibitor or proteolysis. The ability of CaMBP (J7)^{0.5} to bind CaM was Ca^{2+} dependent and was destroyed by boiling or by treatment with urea or trypsin (18). We now report the characterization and partial purification of this CaMBP and suggest that it may be macrophage specific.

MATERIALS AND METHODS

Unless otherwise described, materials and methods are cited in ref. 18. Calcineurin was a gift from C. Klee (National Insti-

tutes of Health). Mouse peritoneal macrophages were kindly provided by John Loike and Samuel Silverstein (Rockefeller University). Dimethyl suberimidate was from Pierce. J774.2 and J774.16 are both wild-type clones derived from the same mouse tumor, J774. J774.2 was used in earlier studies, and J774.16 in later studies. C2 is a clonally selected variant of J774.2 capable of growing in 35 μM Tfp. Clone CBI was obtained without mutagenesis by selecting for cells that survived exposure to gradually increasing concentrations of Tfp (15 μM to 40 μM).

CaMBPs were assayed as in ref. 18 by a method based on that of LaPorte and Storm (20). CaM binding activity was expressed either as cpm bound or as the percent of total cpm bound in a particular band per 100 μg of protein applied to the gel. The assay was linear up to the point at which 10% of the ^{125}I -CaM was bound.

Gradient Sedimentation. Gradients (4.2 ml) were 10–40% (vol/vol) glycerol in 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM CaCl_2 , 2 mM MgSO_4 , and 0.2 mM dithiothreitol. The 10% and 40% glycerol solutions were made 0.3 nM in ^{125}I -CaM and additional CaM was added to achieve the desired final concentration. Samples were applied in 0.2 ml of gradient buffer containing 10% glycerol and contained the same concentration and specific activity CaM as the gradient solutions. Centrifugation was performed in a Beckman SW 60 Ti rotor at 55,000 rpm for 16 hr at 4°C.

CaM-Dependent PDEase Inhibition Assay for CaMBPs. The assay was performed with bovine brain PDEase (18) in a volume of 80 μl with 1 mM cAMP and 10 nM CaM. The sample to be tested was added and the mixture was incubated on ice for 10 min. The [^3H]cAMP was then added and the reaction was allowed to proceed for 20 min at 37°C. Blanks without added brain PDEase were assayed in parallel. The concentration of CaM required for 50% activation of the PDEase was 7.5 nM, as previously reported (5).

RESULTS

Characteristics of CaMBP (J7)^{0.5} in Cell Extracts. We previously reported that the major ^{125}I -CaM complex seen at R_f 0.5 in J774.2 was not found in other mouse tissues (18). A complex with the same mobility in nondenaturing gel electrophoresis in the presence of calcium is, however, detectable in mouse peritoneal macrophages (Fig. 1). The CaMBP^{0.5} activity in resident macrophages was approximately 10% that observed in activated macrophages and in J774. Variants of J774.2 resistant to Tfp, like resident peritoneal macrophages, have a dramatically reduced content of CaMBP (J7)^{0.5} (Fig. 2). This reduction

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Abbreviations: CaM, calmodulin; CaMBP, CaM-binding protein; Tfp, trifluoperazine; PDEase, cyclic nucleotide phosphodiesterase.

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FIG. 1. CaMBP (J7)^{0.5} in extracts of resident and activated murine peritoneal macrophages. Extracts (3 μ g of protein) of J774.16 (lane 1), "resident" peritoneal macrophages from Nelson Collins strain mice from the Rockefeller University colony (lane 2), and "activated" peritoneal macrophages from the same strain obtained 4 days after thioglycollate stimulation (lane 3) were subjected to nondenaturing gel electrophoresis in the presence of calcium and ¹²⁵I-CaM. Shown is an autoradiogram of the gel. The brackets indicate CaMBP^{0.5}. The amount of CaM in the cell extracts was insufficient to significantly alter the specific activity of the added ¹²⁵I-CaM.

is a consistent feature of the Tfp-resistant variants selected either by cloning directly in 30 μ M Tfp (18) or by batch culture selection in incremental Tfp.

To estimate the M_r of the CaMBP (J7)^{0.5} complex, ¹²⁵I-CaM was crosslinked to proteins in extracts of J774.2 and C2. A major complex that migrates as a broad band of M_r 77,000 is formed in extracts of J774.2 and is not present in C2 (Fig. 3). A number of less prominent radioactive bands noted in C2 may be CaMBPs visualized only in the absence of CaMBP (J7)^{0.5}. Complexed CaM was not detected in either extract in the absence of calcium.

Approximately 65–80% of the CaMBP (J7)^{0.5} in both parent and variant CBI was found in the soluble (50,000 $\times$ g supernatant) fraction. The total amount of CaMBP (J7)^{0.5} activity found in CBI was 20% that of the parent; however, CaMBP (J7)^{0.5} was undetectable in most of the Tfp-resistant variants (18).

Purification of CaMBP (J7)^{0.5}. Wild-type J774.16 cells (10¹⁰) were suspended in 100 ml of iced 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA, 2 mM MgSO₄, 0.2 mM dithiothreitol, 0.34 M sucrose, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 2 μ g/ml, and pepstatin at 10 μ g/ml (buffer A). The cell suspension was divided into 20-ml aliquots, frozen at -70°C, thawed, and sonicated such that the cells were broken but nuclei remained intact. Breakage was monitored with trypan blue. Three successive centrifugations were performed (550

FIG. 2. CaMBP (J7)^{0.5} activity in J774.2 and Tfp-resistant variant CBI. Extracts (10 μ g of protein) of J774.2 (■) and the Tfp-resistant variant CBI (▲) were prepared and subjected to nondenaturing gel electrophoresis in the presence of Ca²⁺ and ¹²⁵I-CaM. The gels were sliced into 80 $\times$ 1 mm slices and ¹²⁵I-CaM was determined in a gamma counter. Only the eight slices covering the region containing CaMBP (J7)^{0.5} (R_f 0.45–0.55) are shown.

FIG. 3. Crosslinking of ¹²⁵I-CaM to protein extracts of J774.2 and Tfp-resistant variant C2. Extracts (165 μ g of protein) of J774.2 (lanes 1–4) and C2 (lanes 5–8) were incubated with ¹²⁵I-CaM (10⁶ cpm, 20 ng) for 30 min at 4°C in the presence of either 1.7 mM Ca²⁺ (lanes 1, 2, 5, and 6) or 3.4 mM EGTA (lanes 3, 4, 7, and 8). Crosslinking was performed in 50 mM sodium borate, pH 9.0. Extracts containing ¹²⁵I-CaM were incubated for 30 min at room temperature with dimethylsuberimide at either 200 μ g/ml (lanes 1, 3, 5, and 7) or 1 mg/ml (lanes 2, 4, 6, and 8) and then subjected to electrophoresis on a 0.1% sodium dodecyl sulfate/8.5% polyacrylamide gel.

$\times$ g, 10 min; 12,000 $\times$ g, 20 min; 50,000 $\times$ g, 30 min). The final supernatant was applied to DEAE-cellulose (10 mg of protein per ml of DEAE-cellulose) that had been equilibrated with buff-

FIG. 4. DEAE-cellulose chromatography of an extract of J774.6. The 50,000 $\times$ g supernatant fluid was chromatographed on DEAE-cellulose. Fractions were collected and assayed for: (A) CaMBP (J7)^{0.5} by nondenaturing gel electrophoresis in the presence of calcium and ¹²⁵I-CaM (●) and by inhibition of PDEase activation (○); (B) histone kinase activity measured in the presence of 3 μ M cAMP minus activity measured in its absence (○) and endogenous PDEase activity (●); (C) endogenous substrate phosphorylation in the presence of calcium (●) or EGTA (○); and (D) myosin light chain kinase (MLCK) activity in the presence of calcium (●) or EGTA (○).

Table 1. Purification of CaMBP (J7)^{0.5}

Step	CaM-binding activity, cpm $\times 10^{-6}$	Protein, mg	Specific activity, cpm $\times 10^{-6}$ CaM bound/mg protein	PDE inhibition,* units $\times 10^{-3}$ /mg protein	Purification, [†] fold	Recovery, [†] %
50,000 $\times g$ supernatant fluid	1,488	890	1.67	—	—	100
DEAE-cellulose eluate	1,042	100	10.4	15.5	6.2	70
CaM-Sepharose eluate	610	8	76.3	100	46	41

* One unit of PDEase inhibitory activity was defined as the amount that decreased conversion of [³H]cAMP by 1%.

[†] Purification and recovery were calculated from CaM binding after nondenaturing gel electrophoresis in the presence of calcium.

er A lacking sucrose and protease inhibitors. The column was washed with 4 vol of buffer A and then eluted with 10 vol of a 0–500 mM NaCl gradient in buffer A.

CaMBP (J7)^{0.5} eluted from DEAE-cellulose in a broad peak at 50–175 mM NaCl (Fig. 4A). When tested for inhibition of PDEase, the peak of inhibitory activity coincided with the peak of CaMBP (J7)^{0.5} activity (Fig. 4A). Calcium–CaM-dependent myosin light chain kinase eluted at 75 mM NaCl (Fig. 4D) and cAMP-dependent histone kinase eluted at 40 mM NaCl just before myosin light chain kinase. Treatment of fractions containing the cAMP-dependent protein kinase with 8-azido-[³²P]cAMP followed by 0.1% sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed a radioactive band, *M*_r 48,000, consistent with the type I regulatory subunit previously described in these cells (21). A calcium–CaM-dependent protein kinase and an endogenous substrate eluted in the same fractions as the calcium–CaM-dependent myosin light chain kinase (Fig. 4C). A calcium–CaM-independent PDEase eluted after CaMBP (J7)^{0.5} at 175 mM NaCl (Fig. 4B). Peak fractions containing CaMBP (J7)^{0.5} resolved from the other activities described were pooled and adjusted to 200 mM NaCl. To remove any endogenous CaM still present, DEAE-cellulose equilibrated in buffer A containing 200 mM NaCl was added at 1 ml of DEAE-cellulose per 5 ml of sample. After 30 min, the DEAE-cellulose was removed by centrifugation. The supernatant fluid was made 1.5 mM in CaCl₂ and applied to a 25-ml column of CaM-Sepharose equilibrated with buffer A containing 1.5 mM CaCl₂. The sample was recirculated through the column overnight and washed with 8 vol of buffer A containing 200 mM NaCl and 1.5 mM CaCl₂. The CaMBP (J7)^{0.5} was eluted with 3 vol of buffer A (which contains EGTA) containing 300 mM NaCl and 10% (vol/vol) glycerol and concentrated 40-fold by using an Amicon PM-10 membrane. This material could be stored at –70°C for 6 months without loss of activity. Unless

otherwise indicated, the 46-fold purified material was used for further characterization (Table 1).

Heat Sensitivity of CaMBP (J7)^{0.5}. The pooled DEAE-cellulose fractions (2 mg of protein per ml) were heated and CaMBP (J7)^{0.5} activity was then determined. After 10 min at 60°C, 15% of the activity was lost; 70% of the activity was lost after heating at 75°C for 1 min.

Interaction of CaMBP (J7)^{0.5} with CaM. The ability of the purified CaMBP (J7)^{0.5} to inhibit the activation of brain PDEase by CaM was tested. Inhibition was concentration dependent and could be reversed by addition of excess CaM (Fig. 5). The CaMBP (J7)^{0.5} activities assessed by nondenaturing gel electrophoresis and PDEase inhibition copurified (Table 1).

The binding affinity of CaMBPs for CaM was estimated by velocity sedimentation of CaMBP through glycerol gradients containing calcium and ¹²⁵I-CaM. In the absence of CaMBPs, a uniform distribution of CaM through the bottom two-thirds of the tube was obtained, while the concentration of CaM decreased toward the top of the tube. The midpoint of inflection corresponds to the mobility of CaM determined by conventional velocity sedimentation. As increasing amounts of CaMBP were sedimented through gradients containing 10 nM ¹²⁵I-CaM, free CaM was displaced from the top of the tube into a peak corresponding to the complex formed by the binding protein and CaM. For purified calcineurin, the amount of CaM bound was a linear function of the amount of calcineurin loaded on gradients of identical CaM concentrations (data not shown). When a constant amount of calcineurin was sedimented through gradients of different CaM concentrations, the peak of bound ¹²⁵I-CaM decreased as the specific activity of the CaM decreased (Fig. 6). The concentrations of bound and free CaM can be determined from the binding curves and the data can be expressed in a Scatchard plot.[§] The linearity suggests one class of non-cooperative sites for binding of CaM to calcineurin. The dissociation constant derived is 8 nM, in agreement with the value derived from PDEase inhibition (22, 23).

When CaMBP (J7)^{0.5} was sedimented in gradients containing 10 nM ¹²⁵I-CaM a peak of bound CaM was evident (Fig. 7A). The amount of CaM bound was a linear function of the amount of CaMBP (J7)^{0.5} added. When CaMBP (J7)^{0.5} was sedimented through gradients containing different CaM concentrations the binding curve shown in Fig. 7B was obtained. Like calcineurin, CaMBP (J7)^{0.5} exhibited binding of ¹²⁵I-CaM that could be displaced by unlabeled CaM, with a dissociation constant of 20 nM (Fig. 7B *Inset*). In contrast to calcineurin the mobility of the CaM complex was markedly dependent upon the concentration of CaM. The direction of the sedimentation shift when the con-

FIG. 5. CaM reversal of the inhibition of PDEase by CaMBP (J7)^{0.5}. PDEase was assayed in the absence (○) or presence (●) of 3 μ g of CaMBP (J7)^{0.5}.

[§] *B/F* can be calculated as follows: *F* = free CaM in a fraction = cpm present in blank (just CaM, no CaMBP); *B* + *F* = free + bound CaM in a fraction = cpm present in fractions with CaM + CaMBP; *B* = bound CaM = (*B* + *F*) – *F*.

FIG. 6. Velocity sedimentation of calcineurin. Samples of calcineurin ($1 \mu\text{g}$) were applied to 10–40% glycerol gradients. All gradients contained $0.3 \text{ nM } ^{125}\text{I-CaM}$. The indicated concentrations of CaM are in nM. (Inset) Scatchard plot of the binding data.

centration of CaMBP was held constant (Fig. 7B) is consistent with the direction of the shift when the CaM concentration was held constant and the amount of binding protein was varied (Fig. 7A).

To establish that the complexes with different S values contained CaMBP ($J7$) $^{0.5}$, aliquots from peak fractions of different gradients were subjected to nondenaturing gel electrophoresis in the presence of calcium (Fig. 7C). A characteristic complex with R_f 0.5 was observed whose distribution in the gradient corresponded to the distribution of bound $^{125}\text{I-CaM}$.

When purified CaMBP ($J7$) $^{0.5}$ was sedimented through gradients containing 1 mM EGTA instead of calcium, then assayed for CaM binding activity in the presence of calcium and $^{125}\text{I-CaM}$, CaMBP ($J7$) $^{0.5}$ was found to have a sedimentation value of $6 S$. The mobility in EGTA did not vary as a function of CaM concentration in the gradients. The value of $6 S$ is consistent with the M_r of $150,000$ estimated from gel filtration of the pooled DEAE-cellulose fractions on Ultrogel Aca 34 (data not shown).

The unusual behavior of the CaMBP suggests that the protein may aggregate or associate with other molecules in the preparation and that these interactions are influenced by calcium-CaM.

DISCUSSION

Phenothiazines inhibit CaM *in vitro* by binding to it in a reversible, calcium-dependent fashion (24). Although the phenothiazines are highly lipid soluble drugs and possess detergent properties that may affect cellular membranes by mechanisms unrelated to CaM, they have been used to probe a variety of functions in intact cells (25, 26). In an earlier study, we showed that Tfp inhibited selected functions of the macrophage-like cell line J774 (18, 19). Growth and phagocytosis were maximally and reversibly inhibited by $25 \mu\text{M}$ Tfp, whereas secretion of lysozyme and plasminogen activator were not affected at this drug concentration.

A variant of J774 resistant to the growth inhibitory effects of Tfp was obtained. The resistance was attributable neither to decreased drug uptake nor to an alteration in the content, activity, or phenothiazine sensitivity of its CaM (18). The discovery of a decrease in a CaM binding activity in this variant as well

FIG. 7. Velocity sedimentation of CaMBP ($J7$) $^{0.5}$. (A) The indicated amounts (μg of protein) of CaM-Sepharose eluate were sedimented in gradients containing 10 nM CaM. (B) CaM-Sepharose eluate ($10 \mu\text{g}$ of protein) was sedimented in gradients containing the indicated concentrations of CaM in nM. In the absence of eluate the pattern was identical to that shown for 300 nM . (Inset) Scatchard plot derived from the data. (C) Peak fractions from a separate set of gradients were subjected without further manipulation to nondenaturing gel electrophoresis in the presence of calcium and then autoradiography. The concentrations of CaM in the gradient were 1 nM (a fractions); 30 nM (b fractions); and 100 nM (c fractions).

as in other independently selected Tfp-resistant variants suggested that the effect of Tfp on growth of J774 may be related to the action of this drug on CaM. A CaMBP with R_f 0.5 was found only in mouse peritoneal macrophages, not in a variety of other mouse tissues. Furthermore, the activity of this CaMBP was higher in "activated" than in "resident" peritoneal macrophages.

The enzymatic activity associated with CaMBP (J7)^{0.5}, if any, is unknown. The mobility of CaMBP (J7)^{0.5} and calcineurin in nondenaturing gel electrophoresis in the presence of calcium are different and CaMBP (J7)^{0.5} can be resolved from the PDEase and myosin light chain kinase activities of J774. Variant C2 has the same adenylate cyclase and CaM-dependent myosin light chain kinase activities as the parental cell.

A number of techniques were used to estimate the M_r of CaMBP (J7)^{0.5}. Electrophoresis of the CaM complex in nondenaturing gels of different porosities suggested a Stokes radius of 51 Å, corresponding to a M_r of about 125,000 for a globular protein (18). Crosslinking of ¹²⁵I-CaM to its binding proteins in extracts of J774.2 followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed a complex of M_r 77,000 that was absent from the Tfp-resistant variant lacking CaMBP (J7)^{0.5}. If one assumes that a single CaM is bound to each CaMBP monomer, the average M_r of CaMBP (J7)^{0.5} monomers would be about 60,000.

Partially purified CaMBP (J7)^{0.5} inhibited CaM activation of PDEase, and PDEase inhibitory activity paralleled CaM binding activity during purification. Because we were unable to calculate the protein mass of CaMBP (J7)^{0.5} in the affinity eluates, a CaM binding assay based on a glycerol gradient sedimentation method was devised. The assay was standardized with purified calcineurin. The Scatchard plot obtained from sedimentation analysis of CaMBP (J7)^{0.5} was linear and the derived K_d was 20 nM.

The pronounced dependency of the sedimentation rate of CaMBP (J7)^{0.5} on the concentration of CaM in gradients containing calcium may reflect the interaction of CaMBP (J7)^{0.5} with other proteins in the affinity column eluate or may be an intrinsic property of the CaMBP.

It is possible to estimate the purity of CaMBP (J7)^{0.5} in the CaM-Sepharose eluate from its affinity for CaM derived from sedimentation analysis coupled with the PDEase inhibition data. Assuming a monomeric M_r of 77,000 for the complex, the calculated purity is 12%. The mass of CaMBP (J7)^{0.5} in macrophages would then be similar to that of calcineurin in brain (22), and assuming 1 CaM per 77,000-dalton complex, CaMBP (J7)^{0.5} could bind 5% of the total CaM in the cell. If the amount of CaM bound to CaMBP (J7)^{0.5} represents a significant fraction of the CaM in a particular cellular compartment, absence of this CaMBP from Tfp-resistant variants might increase the CaM

available to processes involved in growth and confer resistance to Tfp. The discovery of an aberrancy in a CaMBP in cells resistant to the growth-inhibitory effect of Tfp may help to define the role of CaM in cell growth. Characterization of this CaMBP may also provide more information about the function of CaM in macrophages.

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