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In vitro enzyme activation with calbindin-D_{28k}, the vitamin D-dependent 28 kDa calcium binding protein

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Purified porcine erythrocyte membrane Ca^{2+}-ATPase and 3′:5′-cyclic nucleotide phosphodiesterase were stimulated in a dose-dependent, saturable manner with the vitamin D-dependent calcium binding protein from rat kidney, calbindin-D_{28k} (CaBP-D_{28k}). The concentration of CaBP-D_{28k} required for half-maximal activation ($K_{0.5}$ act.) of the Ca^{2+}-ATPase was 28 nM compared to 2.2 nM for calmodulin (CaM), with maximal activation equivalent upon addition of either excess CaM or CaBP-D_{28k}. 3′:5′-Cyclic nucleotide phosphodiesterase (PDE) also showed equivalent maximum saturable activation by calbindin ($K_{0.5}$ act. = 90 nM) or calmodulin ($K_{0.5}$ act. = 1.2 nM). CaBP-D_{28k} was shown to effectively compete with CaM-Sepharose for PDE binding. Immunoprecipitation with CaBP-D_{28k} antiserum completely inhibited calbindin-mediated activation of PDE but had no effect on calmodulin's ability to activate PDE. While the physiological significance of these results remains to be established, they do suggest that CaBP-D_{28k} can activate enzymes and may be a regulator of yet to be identified target enzymes in certain tissues.

Calbindin; Ca^{2+}-ATPase; Phosphodiesterase; Calmodulin; Enzyme activation

1. INTRODUCTION

Calcium plays a central role in mediating a variety of biological processes, for instance secretion, contraction, maintenance of bones and teeth, cell division, growth and the functioning of various receptors and enzymes. An important link in the molecular mechanism by which calcium mediates these processes is a class of calcium binding proteins with dissociation constants for Ca^{2+} in the micromolar range that are postulated to be major targets of biological signals [1,2]. Members of this class of calcium binding proteins include calmodulin, troponin C, S100 protein, parvalbumin and the vitamin D-dependent calcium binding proteins, the 9 and 28 kDa calbindins. Calbindin-D_{9k} (CaBP-D_{9k}) is present in mammalian intestine and CaBP-D_{28k}, a phylogenetically conserved protein, is found in avian intestine and in reptilian, avian and mammalian kidney and brain as well as in squid brain [3,4]. Although much is known biochemically and functionally concerning other members of the family of calcium binding proteins, the exact physiological significance of the calbindins is still unknown. Studies indicate that both CaBP-D_{9k} and CaBP-D_{28k} can serve as facilitators of diffusion and intracellular buffers for calcium [5–7]. What remains to be determined is whether the vitamin D-dependent calcium binding proteins modulate specific enzyme activities similar to (calmodulin) CaM or function simply as intracellular calcium buffers. Several reports suggest that calcium may mediate conformational changes in the calbindin molecule which facilitate the interaction of calbindin with other cellular constituents [8,9]. Recently, studies by Walters have indicated that CaBP-D_{9k} can stimulate intestinal basolateral membrane calcium pumping activity [10,11]. In addition, James et al. identified the primary structure of a CaBP-D_{9k} binding domain in the plasma membrane Ca^{2+} pump [12]. In earlier studies, we reported that rat CaBP-D_{28k} specifically stimulates $Ca^{2+}Mg^{2+}$-ATPase activity in human erythrocyte plasma membrane preparations in a dose-dependent manner [13]. The work described here tested whether rat CaBP-D_{28k} could stimulate and bind to purified Ca^{2+}-ATPase and whether 3′:5′-cyclic nucleotide PDE, another CaM-regulated enzyme, could also be activated by the 28 kDa calbindin.

Abbreviations: CaBP-D_{28k}, calbindin-D_{28k}; CaBP-D_{9k}, calbindin-D_{9k}; CaM, calmodulin; PDE, phosphodiesterase; EGTA, ethylene glycol bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Materials*

Calmodulin was isolated from bovine testes [14] and subsequently linked to Sepharose 4B [15]. CaBP-D_{28k} was isolated from rat kidney utilizing a final step of preparative gel electrophoresis followed by storage at −20°C in 0.01 M Tris-HCl [16]. Activator-deficient 3′:5′-cyclic nucleotide phosphodiesterase (PDE) from bovine brain was purchased from Sigma and reconstituted in a 50% glycerol solution for all assays. Rabbit anti-CaBP-D_{28k} antiserum was raised against rat kidney CaBP-D_{28k} and shown to be specific for CaBP-D_{28k} as previ-

ously described [16]. Human erythrocyte membrane Ca^{2+}-ATPase from outdated bank blood or porcine erythrocyte membrane Ca^{2+}-ATPase from fresh porcine blood was purified by CaM-Sepharose affinity column chromatography [17].

Phosphodiesterase I, (5'-nucleotidase) and Protein A-Sepharose were obtained from Sigma as were all other chemicals used unless otherwise noted.

2.2. *Ca^{2+}-ATPase activity assays*

Detergent solubilized Ca^{2+}-ATPase was assayed as originally described [17] in a 200 μl reaction mixture containing 1 μg of Ca^{2+}-ATPase. Reactions were carried out at 37°C for 10 min in the presence of CaM concentrations ranging from 0–30 nM, or CaBP-D_{28k} concentrations ranging from 0–250 nM. ATP hydrolysis was measured colorimetrically by the detection of released inorganic phosphate [18]. Ca^{2+}-ATPase stimulation was calculated by subtracting basal ATPase activity in the absence of CaM or CaBP-D_{28k} from maximal activity obtained in the presence of excess CaM. This difference was given the designation of 100% stimulation. Appropriate controls were included to discount the possibility of background phosphate contamination or any effect that CaM or CaBP-D_{28k} preparations had on ATP hydrolysis in the absence of added Ca^{2+}-ATPase. Each point is the average of 2 independent determinations (±S.D.).

2.3. *3':5'-Cyclic nucleotide phosphodiesterase activity assays*

Cyclic nucleotide PDE activity was measured using a two-stage assay procedure described by Wallace et al. [19] in a 200 μl reaction mixture containing 2×10^{-3} U PDE; however, released inorganic phosphate was measured colorimetrically [18]. PDE stimulation was achieved by inclusion of CaM concentrations ranging from 0–30 nM and CaBP-D_{28k} concentrations ranging from 0–250 nM. Additional PDE assays were performed in the presence of 150 nM CaM or CaBP-D_{28k} (Fig. 1B) either in a reaction mixture containing 0.1 mM $CaCl_2$ or with the addition of EGTA to a final concentration of 10 mM. PDE stimulation was calculated by subtracting basal activity in the absence of CaM from maximal activity in the presence of excess CaM and designating this as 100% stimulation. Appropriate controls were included to discount the possibility of background phosphate contamination or any effect of CaM or CaBP-D_{28k} on cAMP hydrolysis in the absence of PDE. Each point represents the average of 3 independent determinations (±S.D.).

2.4. *Immunoprecipitation of phosphodiesterase stimulating activity*

20 ng of CaM or 500 ng of CaBP-D_{28k} in 20 μl Tris-buffered saline were exposed to 5 μl of rabbit anti-CaBP-D_{28k} antiserum or 5 μl of preimmune rabbit serum for 1 h at 4°C. Afterwards, 10 μl of a Protein A-Sepharose slurry was added and the mixture allowed to incubate with gentle agitation at 4°C for 1 h. Following centrifugation at 10,000 $\times$ g for 30 s, 25 μl of supernatant was removed and assayed for its ability to activate PDE as described above. Stimulation values were corrected for dilution of activator during the immunoprecipitation. Values obtained from blanks containing only serum and Protein A-Sepharose were subtracted to obtain the indicated stimulation values. Percentage of maximum stimulation is represented as described for the normal PDE assay above.

2.5. *CaM-Sepharose competition assay*

A 200 μl PDE reaction mix in 50 mM Tris, pH 8.0, 25 mM ammonium acetate, 5 mM dithiothreitol, 0.10 mM $CaCl_2$ and 3 mM $MgCl_2$ containing 2×10^{-3} U of PDE activity was aliquotted into 1.5-ml mifrofuge tubes. The minimum amount of a CaM-Sepharose slurry required to completely remove all PDE activity batchwise from the reaction mixture was determined by titration to be 20 μl from a representative CaM-Sepharose preparation. Increasing quantities of CaM (0–1 μg) or CaBP-D_{28k} (0–8 μg) diluted in 20 μl of reaction buffer were added to the 20 μl of CaM-Sepharose slurry. This mixture was then added to individual 200 μl PDE reaction mixes allowing simultaneous exposure of the PDE to CaM-Sepharose and free CaM or CaBP-D_{28k}. Fig. 4 represents the final concentration of CaM or CaBP-D_{28k} after addition to the PDE reaction mix. The mixture was incubated for 10 min at room temperature followed by centrifugation at 10,000 $\times$ g for 1 min to remove CaM-Sepharose-bound PDE. The supernatant was transferred to a fresh tube and cAMP and CaM were added to 0.25 mM and 150 nM, respectively, followed by incubation at 37°C for 10 min to determine PDE activity remaining in the supernatants. The excess CaM was included in assays to insure maximal activation of all remaining PDE in the supernatant. Activity was measured as described above. No CaM-Sepharose added corresponds to 100% activity remaining in supernatant and 20 μl CaM Sepharose added corresponds to 0% activity remaining in supernatant. A control experiment involved substitution for CaBP-D_{28k} in the pre-incubation with either 2 μg concanavalin A or 2 μg bovine serum albumin. No PDE activity was detected in the supernatants of these controls.

2.6. *Other methods*

Calmodulin concentration for use in all assays was determined by amino acid analysis [20]. CaBP-D_{28k} concentration for assays was determined by the method of Lowry [21] or using the BCA assay protocol (Pierce). SDS-PAGE was carried out according to Laemmli [22]. Silver staining of SDS-polyacrylamide gels was carried out as described by Morrissey [23].

3. RESULTS

3.1. *Activation of bovine brain 3':5'-cyclic nucleotide phosphodiesterase with rat kidney CaBP-D_{28k}*

The effect of CaBP-D_{28k} on activator-deficient PDE activity was measured and compared to the known stimulatory effect of CaM on PDE as shown in Fig. 1 [24]. In both instances, maximum PDE stimulation above basal activity was achieved and the stimulatory activity based on activator concentrations was saturable. Although maximum stimulation by CaM and CaBP-D_{28k} was comparable, there was a significant shift of the dose–response curves. The concentration for half-maximal stimulation by CaM was 1.2 nM and by CaBP-D_{28k} was 90 nM. Maximal stimulation was achieved at a CaM concentration between 8 and 10 nM but only at CaBP-D_{28k} concentrations above 200 nM. From the results in Fig. 1A it was not clear if the activation of PDE by CaBP-D_{28k} was a Ca^{2+}-dependent phenomenon. To address this question, PDE activation assays were performed (Fig. 1B) in the presence of either $CaCl_2$ alone, or $CaCl_2$ plus excess EGTA. All other conditions were exactly as used for Fig. 1A. The sequestration of Ca^{2+} by EGTA effectively abolished the ability of CaM to activate PDE but had no effect on the stimulatory activity of CaBP-D_{28k}. Controls for the experiments represented in Fig. 1A and B confirmed that CaBP-D_{28k} had no effect on the hydrolysis of cAMP in the absence of PDE (data not shown). Similar results were obtained whether cAMP hydrolysis was measured by phosphate release as described in Materials and Methods or using the [^{3}H]cAMP assay of Wallace [19].

3.2. *Activation of purified erythrocyte membrane Ca^{2+}-ATPase with rat kidney CaBP-D_{28k}*

An additional CaM-sensitive enzyme, erythrocyte membrane Ca^{2+}-ATPase was also activated in a dose-dependent manner by CaBP-D_{28k} as shown in Fig. 2. Detergent-solubilized porcine erythrocyte membrane

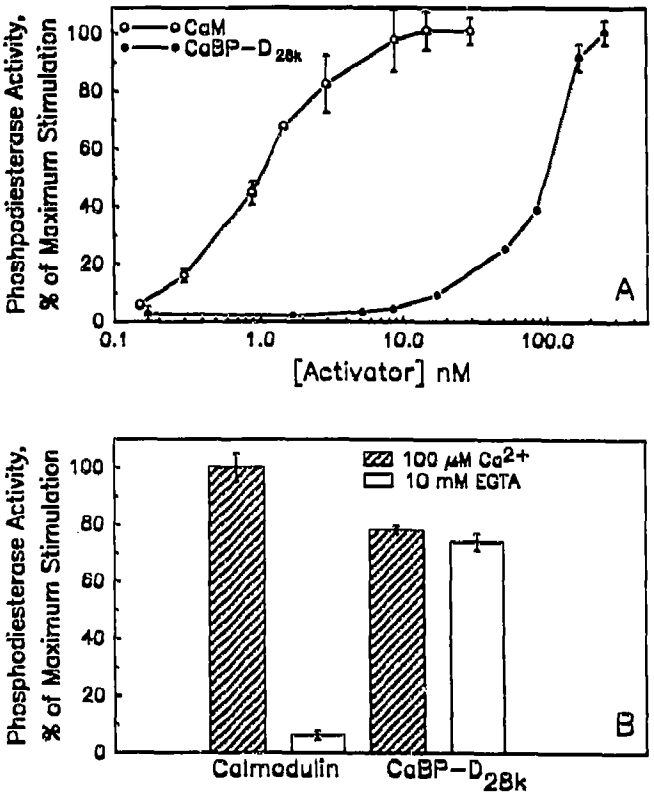

Fig. 1. Effect of CaBP-D_{28k} and CaM on bovine 3':5'-cyclic nucleotide phosphodiesterase activity. (A) Representation of PDE activity assays performed in the presence of increasing concentrations of CaM (○) or CaBP-D_{28k} (●). (B) The effect of EGTA on CaM and CaBP-D_{28k} stimulation of PDE activity. Assays were performed and results are expressed as described in Materials and Methods.

Ca^{2+}-ATPase activity was monitored as described in Materials and Methods in the presence of CaBP-D_{28k} or CaM. In both cases, Ca^{2+}-ATPase activity was fully stimulated in a saturable manner. The concentrations of CaM or CaBP-D_{28k} required for half-maximal activation were 2.2 nM and 28 nM, respectively. Full stimulation of Ca^{2+}-ATPase was observed at a CaM concentration of 15–20 nM and a CaBP-D_{28k} concentration of 150–200 nM. Subsequent studies showed that human erythrocyte Ca^{2+}-ATPase isolated from outdated bank blood, as described by Niggli et al. [17] and assayed as described in Materials and Methods, was also stimulated by nM concentrations of CaBP-D_{28k} (data not shown). In the absence of Ca^{2+}-ATPase, no ATP hydrolysis was observed with CaBP-D_{28k}.

3.3. *Evidence for the mechanism of CaBP-D_{28k} activation of PDE and Ca^{2+}-ATPase*

CaM-Sepharose batch adsorption (Fig. 3) was used to assess the ability of CaBP-D_{28k} to effectively compete with CaM for binding to PDE. Fig. 3 demonstrates that the CaM concentration required to effectively release 50% of PDE activity from CaM-Sepharose binding was 3 nM (Fig. 3, open circles), as compared to 600 nM for

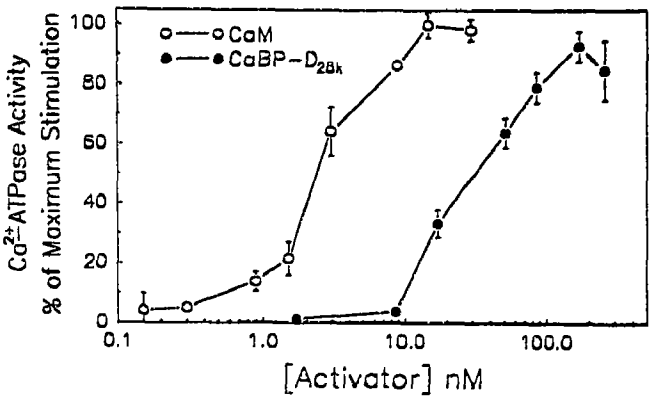

Fig. 2. Effect of CaBP-D_{28k} and CaM on purified porcine erythrocyte membrane Ca^{2+}-ATPase activity. Activity assays on detergent-solubilized porcine Ca^{2+}-ATPase purified and assayed as described in Materials and Methods were performed in the presence of increasing concentrations of either CaM (○) or CaBP-D_{28k} (●).

CaBP-D_{28k} (Fig. 3, closed circles). These numbers are not strictly quantitative due to the nature of batch adsorption. However, they do serve to illustrate that CaBP-D_{28k} interacts with PDE in a manner competitive with CaM, but with much lower affinity. Bovine serum albumin or concanavalin A had no effect on the interaction of PDE with CaM-Sepharose (data not shown). The results suggest that CaBP-D_{28k} activates PDE by binding to the enzyme at or near the CaM binding domain in a manner analogous to CaM. Additionally, calcium-independent binding of ^{125}I-labelled CaBP-D_{28k} to nitrocellulose-bound human erythrocyte Ca^{2+}-ATPase has been demonstrated (data not shown).

Several experiments were performed in order to demonstrate that PDE or Ca^{2+}-ATPase were not being activated by limited proteolysis in the presence of CaBP-D_{28k}. There were necessary since it has been previously shown that limited proteolysis activates these enzymes by making them independent of CaM [23,24].

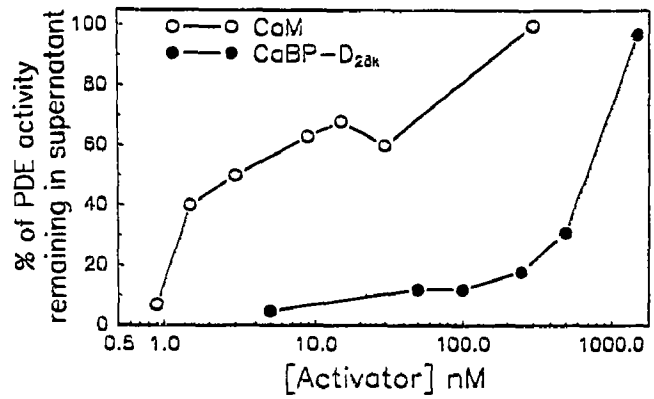

Fig. 3. Inhibition of 3':5'-cyclic nucleotide phosphodiesterase binding to CaM-Sepharose with free CaBP-D_{28k} or CaM. Increasing amounts of CaBP-D_{28k} (●) or CaM (○) were used to inhibit the interaction between CaM-Sepharose and PDE. Following centrifugation, supernatants were assayed for PDE activity as described in Materials and Methods.

PDE was incubated with 150 nM CaBP-D_{28k} in an assay reaction buffer (see Materials and Methods) for 10 min at 37°C followed by the addition of excess CaM-Sepharose. After centrifugation, all PDE activity was effectively removed from the supernatant indicating that the ability of PDE to interact with CaM-Sepharose was unimpaired by pre-exposure to CaBP-D_{28k}. Thus, CaBP-D_{28k} does not irreversibly alter the CaM binding domain (data not shown). Additionally, PDE and human erythrocyte Ca^{2+}-ATPase were pre-incubated individually with 150 nM CaBP-D_{28k} followed by SDS-PAGE and silver stain analysis. A control lane containing no CaBP-D_{28k} was included for comparison. No proteolytic breakdown of PDE was observed (data not shown).

3.4. *The observed activation effects of CaBP-D_{28k} and CaM are protein-specific and independent of one another*

A variety of treatments have been shown to activate the Ca^{2+}-ATPase and PDE [2,26], not all of which are protein-mediated. Two experiments were performed to show that CaBP-D_{28k} specifically activates PDE and is virtually free from CaM contamination. As shown in Fig. 4, immunoprecipitation with anti-CaBP-D_{28k} (solid bars) or preimmune (hatched bars) sera had no effect on calmodulin's ability to activate PDE. However, immunoprecipitation with anti-CaBP-D_{28k} antisera (solid bars) completely inhibited CaBP-D_{28k} mediated activation of PDE. Preimmune serum had no effect on activation by CaBP-D_{28k} (open bars). This indicates that the effect of CaBP-D_{28k} on PDE is specific for CaBP-D_{28k} and is not the result of contamination with CaM or other undefined activating factors. SDS-PAGE with silver stain analysis of purified CaBP-D_{28k} also failed to detect any contaminating polypeptides including CaM (data not shown). Parvalbumin and troponin C, at concentrations greater than those required for activation by CaBP-D_{28k}, did not activate PDE or Ca^{2+}-ATPase (data not shown).

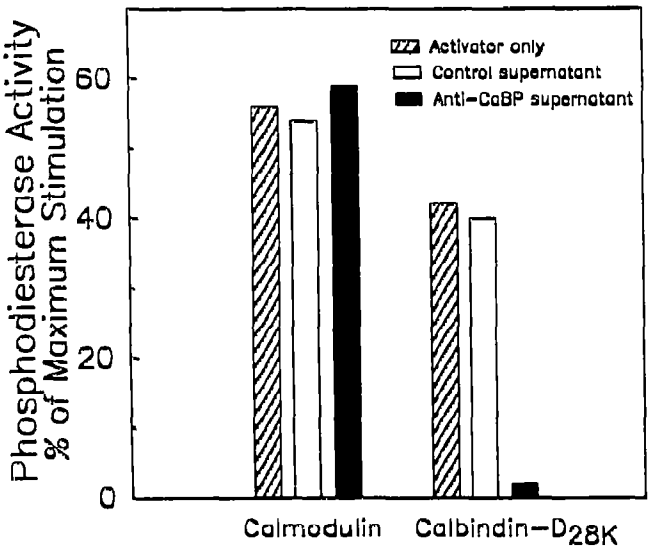

Fig. 4. Immunoprecipitation of PDE-stimulating activity from a purified CaBP-D_{28k} preparation using CaBP-D_{28k}-specific antiserum. CaM (20 ng) or CaBP-D_{28k} (500 ng) were immunoprecipitated with either no antisera (hatched bars), preimmune antiserum (solid bars) or CaBP-D_{28k}-specific antiserum (open bars) and supernatants were assayed for their ability to stimulate PDE activity as described in Materials and Methods. Each bar signifies a single representative determination.

4. DISCUSSION

The Ca^{2+}-pumping ATPase is a polypeptide of M_r of approximately 138,000 which is present in all mammalian cell plasma membranes [26]. The stimulation of Ca^{2+}-ATPase by CaM, which results in an increase in the affinity of the enzyme for calcium as well as an increase in the enzyme's maximal transport rate, has been well documented [27,28]. The distribution of calbindin along the villus-crypt axis in the intestine and in the distal tubule of the kidney correlates with the localization of epitopes of the plasma membrane calcium pump in these tissues [29,30], suggesting that the plasma membrane calcium pump and calbindin may be part of the same calcium transporting system, and that calbindin, similar to CaM, may also stimulate the Ca^{2+}-ATPase. However, previous reports have described either no effect or a stimulatory effect of the vitamin D-dependent calcium binding proteins, CaBP-D_{9k} and CaBP-D_{28k}, on $Ca^{2+}Mg^{2+}$-ATPase activity [13,31–35]. Similarly, inconsistent effects of vitamin D administration to vitamin D-deficient animals on intestinal Ca^{2+}-ATPase activity have been reported. Recently, James et al. [12] presented evidence that CaBP-D_{9k} interacts with the CaM binding domain of purified human erythrocyte Ca^{2+} pump in a calcium-dependent manner, although at a much lower affinity. However, activation of the purified Ca^{2+}-ATpase was not demonstrated. Our study indicates that CaBP-D_{28k} does activate purified porcine or human erythrocyte membrane Ca^{2+}-ATPase in a manner similar to CaM although with a 10-fold lower affinity. Similarly, activation of 3′:5′-cyclic nucleotide PDE with CaBP-D_{28k} was also demonstrated, but required 100-fold more CaBP-D_{28k} than CaM for equivalent activation. In addition, CaBP-D_{28k} effectively released PDE activity from CaM-Sepharose, suggesting that CaBP-D_{28k} does interact with PDE in a manner competitive with CaM but at a lower affinity under these conditions. Unlike James et al. [12] we did not observe calcium-dependent binding of CaBP-D_{28k} to the ATPase. Activation of PDE also was not Ca^{2+}-dependent. Similarly, using the gel overlay technique of Glenney et al. [36], binding of ^{125}I-labelled CaBP-D_{28k} to soluble rat brain and kidney proteins was consistently found to be calcium-independent (S. Christakos, unpublished results). In addition, Bikle et al. [37] using the same gel overlay technique, found that binding of rat kidney and chick gut calbindins to a

105-kDa CaM binding protein, found in intestinal brush border membrane vesicles, was actually increased in the presence of EGTA. Although CaM contains 4 EF hand helix-loop-helix structures that are required for the binding of calcium and CaBP-D_{9k} contains 2, it should be noted that CaBP-D_{28k} contains 6 EF hands. This could account for the observed differences between CaBP-D_{9k} and CaBP-D_{28k} in the Ca^{2+}-dependency of binding to the Ca^{2+}-ATPase. However, equilibrium dialysis studies have indicated that CaBP-D_{28k} binds only 3–4 mol of Ca^{2+}/mol protein [3] in agreement with the fact that 2 of the 6 potential Ca^{2+} loops lack some of the requisite ligand forming residues [39].

We previously reported that CaBP-D_{28k} did not activate PDE [13]. In the present studies care was taken not to freeze and thaw preparations of CaBP-D_{28k} (which we have observed to result in the loss of its stimulatory activity). Proteolytic enzyme inhibitors also were used throughout the purification procedure and all experiments were performed only with recent preparations of the protein. Since activation by CaBP-D_{28k} was critically dependent on the preparation of purified protein, we conclude that variability in the techniques used to prepare CaBP-D_{28k} may indeed have accounted for some of the conflicting data in the past.

The physiological relevance of the Ca^{2+}-independent activities of CaBP-D_{28k} shown here is unclear as has also been noted for CaBP-D_{9k} [12]. CaM is present at high concentrations in all animal tissues [2] and would clearly be the primary activator of these enzymes at elevated Ca^{2+} concentrations. It is intriguing, however, that specific neuronal sub-types which express relatively high levels of CaBP-D_{28k} show enhanced resistance to Ca^{2+} toxicity [7], suggesting an alteration in Ca^{2+} sequestering capability. It is possible that CaBP-D_{28k} might represent an alternative activation mechanism for the Ca^{2+} pump which would operate at free Ca^{2+} concentrations lower than required for CaM-mediated activation to ensure maintenance of resting state Ca^{2+} levels in specific cell types. The fact that CaBP-D_{28k} is a highly conserved member of the calcium binding protein family [4,38] which appears to possess target protein binding properties related to CaM also raises the possibility that it may function as a Ca^{2+}-dependent regulator of target proteins yet to be identified.

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