MOLECULAR PHARMACOLOGY, 26:75–82

Activation of Calmodulin by Various Metal Cations as a Function of Ionic Radius

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Received December 29, 1983; Accepted March 27, 1984

SUMMARY

The active form of calmodulin is a Ca^{2+}·calmodulin complex. The purpose of this investigation was to determine whether other metal cations substitute for Ca^{2+} to activate calmodulin. Binding of Ca^{2+} resulted in an altered conformation of calmodulin with an increased quantum yield in its tyrosine fluorescence. Qualitatively similar results were obtained with Zn^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+}, Sr^{2+}, Pb^{2+}, Tb^{3+}, Sm^{3+}, and La^{3+}. The relative extents of fluorescence enhancement by these cations were related to their ionic radii: all cations with ionic radii close to Ca^{2+} (0.99 Å) increased tyrosine fluorescence, whereas those with different ionic radii were not effective, or much less so. The change in calmodulin conformation by the cations was confirmed by its altered electrophoretic mobility on polyacrylamide gels. Cations that change the conformation of calmodulin allow it to stimulate phosphodiesterase. The relative extents of stimulation of phosphodiesterase by cations were also related to their ionic radii. Finally, the ability of metal cations to inhibit Ca^{2+} binding was similarly related to their ionic radii. In general, the closer the radius of a metal cation was to that of Ca^{2+}, the more effective was the cation to substitute for Ca^{2+}. The range of effective ionic radii was approximately 1 ± 0.2 Å. Calmodulin-stimulated phosphodiesterase activity by the cations was reversed by trifluoperazine, an antagonist of calmodulin.

INTRODUCTION

Metal cations participate in many diversified cellular functions. Iron is a component of hemoglobin and the cytochrome system. Zinc, cobalt, and copper are components of certain coenzymes or metalloenzymes, and magnesium is required in many enzymatic reactions involving phosphorylated substrates. Calcium, however, is perhaps unique. It not only serves as vital structural components for bones and teeth, but also participates—as Ca^{2+} ions—in a wide spectrum of cellular processes such as intermediary metabolism, cyclic nucleotide metabolism, contractility and motility, endo- and exocytosis, chromosome movement, and cell division (1–5). In fact, Ca^{2+} plays a role in almost every aspect, directly or indirectly, of the multiplicity of cellular events. Many, but by no means all, of the Ca^{2+} effects are mediated by a class of Ca^{2+}-binding proteins of which calmodulin appears to be the most versatile and ubiquitous (6–11).

We have shown previously that Tb^{3+}, which has an ionic radius (0.92 Å) comparable to that of Ca^{2+} (0.99 Å), effectively substitutes for Ca^{2+} in calmodulin (12). The question arises whether other cations having comparable ionic radii also replace Ca^{2+}.

In this communication, we have examined many metal cations and found that all of those having ionic radii in the range of Ca^{2+} indeed substitute for Ca^{2+} in calmodulin. These ions include Cd^{2+}, Hg^{2+}, and Pb^{2+}, which are serious environmental pollutants. This raises the possibility that some of their toxicity in biological systems may be mediated through their activation of calmodulin. A preliminary account of this work has been presented (13).

MATERIALS AND METHODS

Reagents. [^{3}H]cyclic AMP (27 Ci/mmole) was purchased from ICN Pharmaceuticals (Irvine, Calif.) and purified before use (14). $^{45}CaCl_2$ (22 mCi/mg) and $^{109}CdCl_2$ (carrier-free, radionuclidic purity greater than 99%) were obtained from New England Nuclear Corporation (Boston, Mass.) Magnesium sulfate, calcium chloride, cadmium chloride, lanthanium chloride ($LaCl_3 \cdot 2H_2O$), lead chloride, mercury chloride, samarium chloride ($SmCl_3 \cdot 5H_2O$), strontium chloride ($SrCl_2 \cdot 6H_2O$), and terbium chloride ($TbCl_3 \cdot 6H_2O$) were of highest purity (99.999%) from Aldrich Chemical Company (Milwaukee, Wisc). Acryl-

This work was supported by Cancer Center Support Grant CA 21765, by Project Grants NS 08059 and GM 28178 from the National Institutes of Health, and by American Lebanese Syrian Associated Charities. A preliminary account of this work was presented at the 67th annual meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 10–15, 1983.

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[2] Recipient of National Research Service Award CA 09346.

0026-895X/84/040075-08$02.00/0

amide, *N,N*-methylenebisacrylamide, Coomassie brilliant blue, anionic exchange resin AG1-X2 and Chelex-100, and DEAE-cellulose were obtained from Bio-Rad Laboratories (Richmond, Calif.). Cyclic AMP, snake venom (*Crotalus atrox*), PIPES,[3] phenylmethylsulfonyl fluoride, and pepstatin A were from Sigma Chemical Company (St. Louis, Mo). All other reagents were of highest grade. Calmodulin was prepared from bovine brain (15). Doubly glass-distilled water was used throughout.

Removal of contaminating metals. PIPES (pH 7.0), Tris-HCl (pH 7.5 or 8.0), and KCl were passed through a Chelex-100 column (25 × 3 cm) separately to remove contaminating divalent cations. The Chelex column reduced Ca^{2+} in the reagent solutions to 0.10–0.30 μM. Cyclic AMP solutions were treated batchwise with Chelex-100. Calmodulin (2 mg/ml) was first dialyzed against 100 volumes of 20 mM Tris-HCl (pH 7.5) containing 1 mM EGTA and then against 20 mM Tris-HCl (pH 7.5) containing 10 μM EGTA. The sample was next dialyzed for 5 days against 200 volumes of 20 mM Tris-HCl with 10 changes, and further dialyzed for 1 week against doubly distilled, deionized water with 14 changes. Extensive dialysis precipitated calmodulin, which was dissolved by adjusting the pH to neutrality with 20 mM Tris-HCl (pH 7.5). Atomic absorption analysis indicated that calmodulin prepared by this procedure contained 0.01–0.05 mole of Ca^{2+} per mole of calmodulin.

Ca^{2+}-free solutions were stored in polyethylene containers that had been treated with dilute HCl, maintained at about 95° for 5 min, and then rinsed several times in doubly-distilled, deionized water. In experiments where the concentration of Ca^{2+} was critical, plastic ware was used throughout. Doubly glass-distilled, deionized water contained less than 0.1 μM Ca^{2+}.

Tyrosine fluorescence spectroscopy. The tyrosine fluorescence change of calmodulin induced by metal cations was measured with a Perkin-Elmer MPT-44B fluorescence spectrophotometer equipped with a DCSU-I differential corrected spectrum unit at a spectral band width of 5 nm for excitation and 10 nm for emission. Calmodulin was dissolved in 20 mM PIPES (pH 7.0) containing 100 mM KCl in a final volume of 1 ml. The cuvette had a 1-cm pathlength for excitation and 0.4 cm for emission. Excitation was at 280 nm, and the change in tyrosine fluorescence intensity was monitored at 307 nm. Titrations with metal cations were conducted by sequential addition of 1 to 5 μl of a stock solution (1.0 or 10 mM). To preclude interference by fluorescence inner filter effects, optical densities of all titrated solutions were held below 0.05 at wavelengths of excitation and emission.

Preparation and assay of calmodulin-dependent phosphodiesterase. Calmodulin-dependent phosphodiesterase was purified from bovine brain to a specific activity of about 80 μmoles/mg/min under the standard assay conditions (16). The enzyme was extensively dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 mM Mg^{2+} and 10% glycerol and stored at −70°. Phosphodiesterase activity was assayed according to a two-stage procedure (17, 18). The reaction mixture (0.1 ml) was prepared sequentially as follows: 40 mM Tris-HCl (pH 8.0), 3 mM $MgSO_4$, an appropriate amount of $CaCl_2$ or other metal cations, 0.15 μg of calmodulin, 0.12 μg of enzyme, and 2 mM [^{3}H]cyclic AMP. The reaction was started by the addition of cyclic AMP and, after 10 min, was stopped by boiling for 2 min. Ca^{2+} contaminating the reaction mixture ranged from 1 to 1.5 μM as determined by atomic absorption analysis. The reaction product, 5′-AMP, was converted to adenosine and inorganic phosphate by 5′-nucleotidase of snake venom (*Crotalus atrox*) in a second stage of incubation. Heavy metal ions such as Zn^{2+}, Pb^{2+}, and Hg^{2+} inhibit the nucleotidase activity. Therefore, an appropriate concentration of EDTA was added to the second stage of incubation to relieve the inhibition of these cations on 5′-nucleotidase (19).

Polyacrylamide gel electrophoresis. Alkaline glycerol-gel electrophoresis was carried out according to the method of Perrie and Perry (20). The gel (14 × 10.5 × 0.12 cm) was made up with 10% (w/v) polyacrylamide in 25 mM Tris-80 mM glycine (pH 8.6) containing 40% (v/v) glycerol. The Tris-glycine was also used in the electrode solution. The gel was prerun without samples for 1 hr at 10 mamp at 4°. Calmodulin (1.8 μg) in 25 mM Tris-80 mM glycine (pH 8.6) containing 20% glycerol and 0.01% bromophenol blue (marker dye) was incubated with 300 μM EGTA and then with 600 μM various cations except Tb^{3+}, Sm^{3+}, and La^{3+}. These cations at concentrations higher than about 25 μM precipitated calmodulin, and they were used at 20 μM each without EGTA. For calmodulin without cations, 600 μM EGTA was added. All samples were electrophoresed at 5 mamp for 30 min, then at 10 mamp for 2 hr at 4°.

SDS-gel consisted of 10% polyacrylamide gel in 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS; a 3% stacking gel was used (21). Calmodulin, in 63 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, and 0.001% bromophenol blue, was incubated with cations under the same conditions described for alkaline glycerol-gel. Addition of β-mercaptoethanol to the sample was omitted, as was brief boiling. Samples were electrophoresed at 40 mamp for 2 hr at 18°.

After electrophoresis, the position of the marker dye was indicated; the gel was stained with Coomassie brilliant blue R, and the mobility of calmodulin and marker dye in each lane was noted.

Other measurements. Ca^{2+} was determined with a Perkin-Elmer Model 403 atomic absorption spectrophotometer using a graphite furnace and high-density graphite rod. The instrument response was tested before and after each determination to ensure that there was no drift in absorbance arising from rod aging. The sensitivity of this instrument was about 0.01 ppm for Ca^{2+}.

Protein concentration was determined according to the method of Lowry *et al.* (22), using bovine serum albumin as a standard.

RESULTS

Metal cation-induced enhancement of tyrosine fluorescence. Bovine brain calmodulin possesses two tyrosines—residues 99 and 138—and no tryptophan. Binding of Ca^{2+} to calmodulin results in a conformational change which increases the quantum yield of tyrosine fluorescence (12, 23, 24). The emission spectra of calmodulin with excitation set at 280 nm is shown in Fig. 1A; the peak at 307 nm is characteristic of the tyrosine fluorescence (12, 23, 24). The addition of Ca^{2+} increased the fluorescence sharply, whereas the addition of EGTA brought the fluorescence back to the original basal level, indicating that the Ca^{2+} contaminating the reaction mixture was minimal.

We have previously shown that Tb^{3+} enhances tyrosine fluorescence of calmodulin and substitutes for Ca^{2+} effectively in calmodulin (12), apparently because Tb^{3+} has an ionic radius comparable to Ca^{2+}. The question is raised whether other metal cations that have comparable radii also increase intrinsic fluorescence in calmodulin. The low level of Ca^{2+} contaminant in our reaction mixture allowed us to examine the effect of these cations individually, relatively free from the interference of Ca^{2+}. We chose Be^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+} from the alkaline earth metals; Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} from the fourth, fifth, and sixth periods; and La^{3+}, Sm^{3+}, and Tb^{3+} from the lanthanide series. The ionic radii of these cations range from 0.35 Å for Be^{2+} to 1.34 Å for Ba^{2+}. Figure 1B, C, and D illustrates the fluorescence change caused by Cd^{2+}, Hg^{2+}, and Pb^{2+}. These changes are qualitatively representative of those by other cations such as Mn^{2+}, Zn^{2+}, Sr^{2+}, La^{3+}, Sm^{3+}, and Tb^{3+} (data not shown).

The extent of tyrosine fluorescence was measured as a function of the concentration of each of these cations.

[3] The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate.

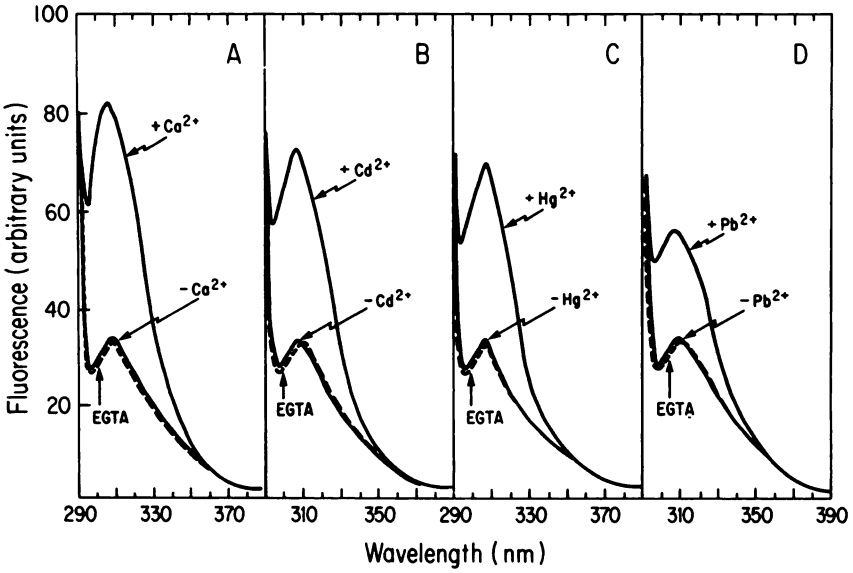

FIG. 1. *Enhancement of calmodulin tyrosine fluorescence upon binding metal cations*

The fluorescence emission spectra, determined as described under Materials and Methods, of calmodulin (10 μM) with excitation wavelength set at 280 nm are recorded.

A. Calmodulin (*lower solid curve*), in the presence of Ca^{2+} (50 μM, *solid curve*), and in the presence of EGTA (100 μM, *lower dashed curve*).

B. Calmodulin (*lower solid curve*), Cd^{2+} (50 μM, *upper solid curve*), and EGTA (100 μM, *lower dashed curve*).

C. Calmodulin (*lower solid curve*), Hg^{2+} (50 μM, *upper solid curve*), and EGTA (300 μM, *lower dashed curve*).

D. Calmodulin (*lower solid curve*), Pb^{2+} (20 μM, *upper solid curve*), and EGTA (100 μM, *lower dashed curve*).

The fluorescence change for each concentration was determined by taking the difference between the peak height obtained in the presence and absence of the cation. Figure 2A shows that, for Ca^{2+}, tyrosine fluorescence increased linearly until a ratio of approximately 7 Ca^{2+} per mole of calmodulin was reached, and the fluoresence remained at the plateau level thereafter. The increase of fluorescence induced by Sr^{2+} was also linear, but more gradual, and the maximal level (about 70% that by Ca^{2+}) was not reached until a molar ratio of 40:1. Be^{2+}, Mg^{2+}, and Ba^{2+} did not induce any significant change in fluorescence. Co^{2+} (Fig. 2B) induced a slight increase in fluorescence only at high concentrations; Ni^{2+} was virtually ineffective. Mn^{2+}, Zn^{2+}, and Cd^{2+} induced fluorescence changes to various extents, and the level remained elevated. The slope of fluorescence increase by Mn^{2+} was less steep than that by Ca^{2+}, and the extent of increase was about 40% less. The slope by Zn^{2+} was steeper than that by Mn^{2+}, but their maximal levels were comparable. The slope by Cd^{2+} was slightly steeper than that by Ca^{2+}, but the level of fluorescence was some 20% less. Hg^{2+} and Pb^{2+} exhibited fluorescence profiles that were biphasic. The fluorescence peak by Hg^{2+} was obtained at a molar ratio of approximately 10:1, whereas that by Pb^{2+} at approximately 2:1. Tb^{3+}, La^{3+}, and Sm^{3+} (Fig. 2C) also induced a biphasic fluorescence profile. Maximal fluorescence, approximately 75% that of Ca^{2+}, was obtained at a molar ratio of approximately 3:1.

It should be noted that these measurements do not represent the stoichiometry of metal binding, nor their relative binding affinity. They do, however, indicate their abilities to substitute qualitatively for Ca^{2+}.

When the ionic radii of these metals are compared with their ability to enhance fluorescence, the results clearly indicate that all of the cations with ionic radii comparable to Ca^{2+} increase fluorescence of calmodulin (see Table 3), whereas those with significantly different radii are ineffective, or much less so.

Metal cation-induced change in electrophoretic mobility of calmodulin. Calmodulin migrates more slowly on polyacrylamide gels under nondenaturing conditions in the presence of Ca^{2+} than in the presence of EGTA, presumably because of a decrease in net charge upon binding Ca^{2+} (25). In the presence of Mn^{2+}, Tb^{3+}, Cd^{2+}, Sm^{3+}, La^{3+}, Sr^{2+}, or Pb^{2+}, calmodulin also migrated more slowly (Fig. 3A), whereas in the presence of Be^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, or Ba^{2+}, no significant change in mobility was seen. The effect of these cations on calmodulin mobility was further examined by SDS-polyacrylamide gel electrophoresis (Fig. 3B). Under this condition, calmodulin migrated more quickly in the presence of Mn^{2+}, Tb^{3+}, Cd^{2+}, Ca^{2+}, Sm^{3+}, La^{3+}, or Pb^{2+} than in EGTA. The larger mobility probably results from a change toward a more compact conformation of calmodulin. No change in mobility was seen with the other metal cations. A borderline case appears to be Sr^{2+}, which induced only a slight change. In the presence of Ca^{2+}, calmodulin is known to exhibit more than one band in SDS gels (12, 26). In the presence of Cd^{2+}, and to some extent Sm^{3+}, calmodulin behaved similarly (Fig. 3B).

According to the data showing fluorescence change in Fig. 2, Zn^{2+} and Hg^{2+} also should induce a mobility change during electrophoresis in the acrylamide gel. The apparent lack of change may be attributable to a difference in the buffer systems and experimental conditions. During electrophoresis, calmodulin migrates toward the

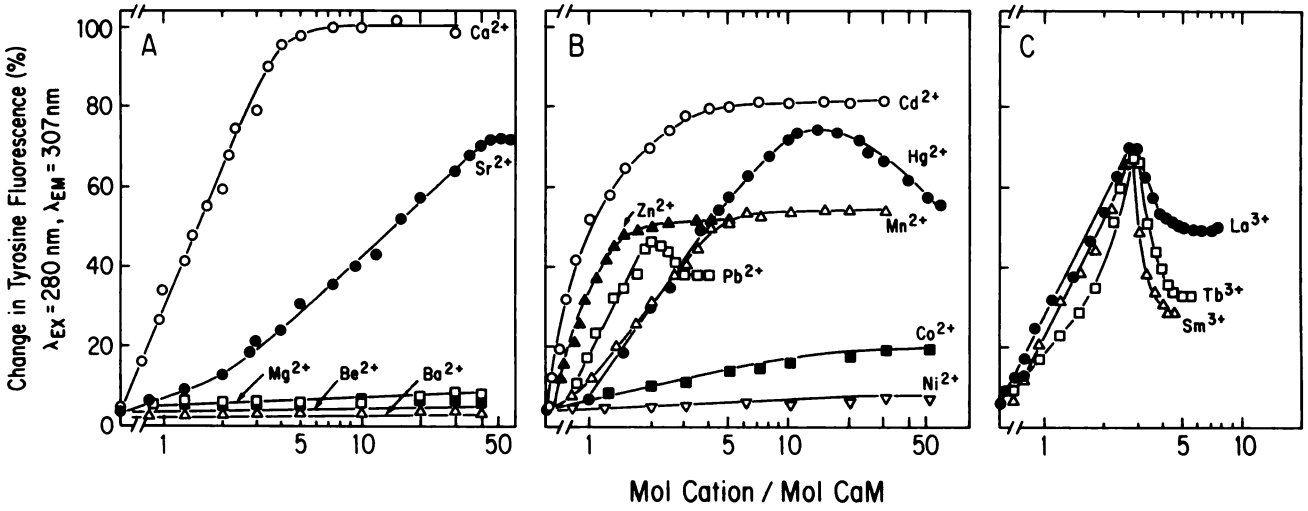

FIG. 2. *Changes in calmodulin tyrosine fluorescence upon addition of metal cations*

Bovine brain calmodulin (10 μM) was titrated with different concentrations of metal cations; the increase in fluorescence at 307 nm was measured as described under Materials and Methods. The difference between the fluorescence peaks with and without the addition of cation at each concentration was determined. A. Be^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, and Ca^{2+}. B. Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Pb^{2+}, Hg^{2+}, and Cd^{2+}. C. La^{3+}, Tb^{3+}, and Sm^{3+}. The Ca^{2+}-induced fluorescence is expressed as 100; the fluorescence induced by each cation is thus directly comparable.

anode, whereas the free cations migrate toward the cathode. Whether the cation-induced conformation in calmodulin can be maintained to cause the differential mobility depends on the affinity of the cation for calmodulin. A cation with a low affinity may well be released from calmodulin during the course of electrophoresis. Zn^{2+} may be such an example. Hg^{2+}, which has strong affinity for the amino group of glycine in the running buffer during electrophoresis, most probably is not available to form a complex with calmodulin.

Efficacy of metal cations to activate calmodulin. In the next series of experiments, we determined whether the change in fluorescence and in mobility caused by these metal cations correlated with their ability to activate calmodulin, using phosphodiesterase as an assay. As shown in Fig. 4A, Be^{2+}, Mg^{2+}, and Ba^{2+} were essentially inactive. Sr^{2+} activated phosphodiesterase to 85% of the maximal activity by Ca^{2+}; as in Ca^{2+}, the activation curve by Sr^{2+} was monophasic, and no inhibition was observed up to 1 mM. None of these alkaline earth metals altered the basal activity of phosphodiesterase. The effect of Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} on both calmodulin-stimulated and basal phosphodiesterase activities is shown in Fig. 4B. Ni^{2+} and Co^{2+} were essentially inactive. Zn^{2+} and Mn^{2+} supported calmodulin-stimulated activity with a biphasic response. Cd^{2+} and Hg^{2+} gave qualitatively similar responses. Zn^{2+}, Cd^{2+}, and Hg^{2+} inhibited basal phosphodiesterase activity at high cation concentrations, and this could explain their biphasic profiles. Pb^{2+}, which inhibits many enzymes, as does Hg^{2+}, activated phosphodiesterase to 90% of the maximal activity by Ca^{2+}. Hg^{2+} was less effective. Figure 4C shows the effect of La^{3+}, Sm^{3+}, and Tb^{3+}. All stimulated phosphodiesterase at low concentrations (3–5 μM) and inhibited phosphodiesterase at high concentrations (5 μM for Tb^{3+}, 20 μM for Sm^{3+}, and 30 μM for La^{3+}). None of the lanthanides affected basal enzyme activity.

Effect of various cations on binding of Ca^{2+} to calmodulin. The ability of cation metals to substitute for Ca^{2+} in the activation of calmodulin implies that these cations bind to the same Ca^{2+} sites on calmodulin. To explore this possibility, we examined the effect of these metals on the binding of $^{45}Ca^{2+}$ to calmodulin. Table 1 shows that Be^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+}, and Ni^{2+} did not significantly affect the binding of $^{45}Ca^{2+}$, whereas Sr^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and the lanthanides La^{3+}, Sm^{3+}, and Tb^{3+} decreased the binding of $^{45}Ca^{2+}$ to various extents. In general, cations that stimulate calmodulin inhibit $^{45}Ca^{2+}$ binding. An exception is Hg^{2+}, which was quite effective in activating calmodulin, but virtually inactive in affecting $^{45}Ca^{2+}$ binding, possibly because of its potential interaction with the Tris buffer and the dialysis membrane used in equilibrium dialysis. These results strongly suggest, but do not prove, that the effective metals bind to calmodulin at the Ca^{2+} sites.

Reversal of calmodulin-stimulated phosphodiesterase activity by trifluoperazine. In the presence of Ca^{2+}, calmodulin exhibits a high affinity for trifluoperazine. Binding of the drug prevents calmodulin from interacting with and thereby activating phosphodiestease (27, 28). Table 2 shows the effect of trifluoperazine on calmodulin-stimulated phosphodiesterase activity in the presence of the various cations. The concentrations of these cations were chosen not to alter basal phosphodiesterase activity. Trifluoperazine at a concentration that did not affect basal phosphodiesterase activity suppressed the calmodulin-dependent phosphodiesterase activities to their basal levels in a manner qualitatively similar to the effect of Ca^{2+}. These results are in accord with the notion that these cations substitute for Ca^{2+} in calmodulin.

The higher basal level in the presence of Hg^{2+} resulted from its stimulation on phosphodiesterase per se[4]; under our assay conditions, stimulation depended on the length of time in which the enzyme was preincubated with Hg^{2+}

[4] J. R. Zysk, S. H. Chao, and W. Y. Cheung, Mercuric ion activates calmodulin-dependent phosphodiesterase, submitted for publication.

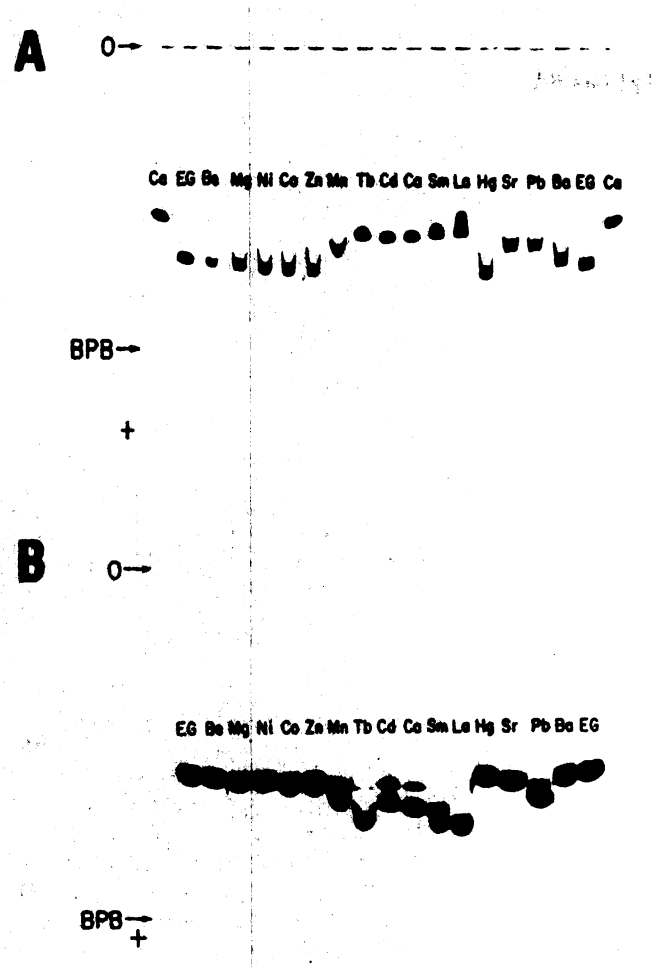

FIG. 3. *Mobility of calmodulin in polyacrylamide gel in the presence of various cations*

Calmodulin (1.8 μg) was incubated in 300 μM EGTA to chelate the contaminating Ca^{2+} in the sample and then in 600 μM of various cations indicated. In the case of Tb^{3+}, Sm^{3+}, and La^{3+}, calmodulin was incubated with the cations at a final concentration of 20 μM; no EGTA was added. A. Alkaline glycerol gel. B. SDS gel. *O*, origin; *BPB*, bromophenol blue (marker dye); +, anode; *EG*, EGTA.

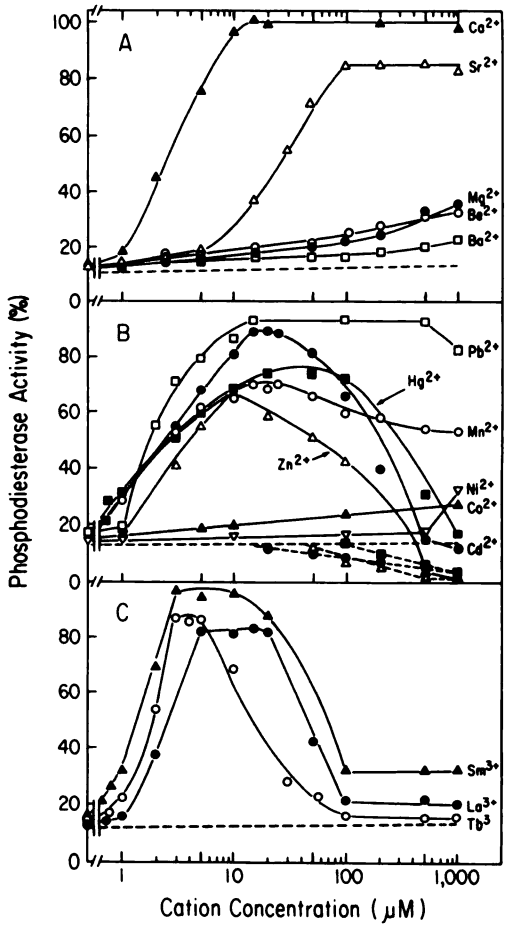

FIG. 4. *Effect of various metal cations on the activity of phosphodiesterase as a function of cation concentration*

The concentration of each cation is indicated in the abscissa. Phosphodiesterase was assayed in the presence or absence of calmodulin as described under Materials and Methods. Basal phosphodiesterase activity (in the absence of calmodulin) is indicated by a *dashed line* or as identified by the same *symbols*; calmodulin-dependent phosphodiesterase activity is indicated by a *solid line*. Metal cations not affecting basal enzyme activity are also depicted by a *dashed line*. The calmodulin-stimulated phosphodiesterase activity in the presence of Ca^{2+} was 81 μmoles/mg/min.

before the reaction was initiated with cyclic AMP. Stimulation of basal phosphodiesterase by Hg^{2+} was not observed if preincubation time exceeded 1 hr at 30°.

Correlation between ionic radius of metal cations and their efficacy to activate calmodulin. Table 3 lists the ionic radii of the various cations in an ascending order and some of the data extracted from Figs. 2, 3, and 4 and Table 1 in order to compare the efficacy of these cations to induce change in tyrosine fluorescence, electrophoretic mobility, calmodulin activity, and $^{45}Ca^{2+}$ binding.

The data indicate that all cations having ionic radii between Zn^{2+} (0.74 Å) and Pb^{2+} (ionic radius 1.2 Å) enhance intrinsic fluorescence. The effectiveness of these cations to induce mobility change of calmodulin in acrylamide gel is approximately comparable, nearly as expected from their effects on fluorescence enhancement. The possible exceptions were Zn^{2+} and Hg^{2+}, and, as stated above, their apparent lack of effect is probably due to the difficulty of calmodulin to retain them during electrophoresis. The ability of cations to stimulate calmodulin-supported phosphodiesterase activity appears to correlate quite well with that to induce tyrosine fluorescence change. In general, however, the increase of phosphodiesterase activity by a cation seems slightly higher than the corresponding increase in tyrosine fluorescence. On the basis of their ability to inhibit Ca^{2+} binding to calmodulin, the metal cations also show a close relationship to their other three attributes. Collectively, these data demonstrate a rather good correlation between the ionic radii of the cations and their ability to substitute for Ca^{2+} in calmodulin. Metal cations with ionic radii in the range of 1 ± 0.2 Å induce the right conformation in calmodulin and allow it to activate phosphodiesterase. It should be added that Na^{+}, whose ionic radius is 0.95 Å, did not cause any change in tyrosine fluorescence, nor did it support calmodulin-dependent phosphodiesterase activity (data not shown). Thus, to activate calmodulin, the metal cation has to possess at least two charges.

TABLE 1

Effect of various cations on $^{45}Ca^{2+}$ binding to calmodulin

Equilibrium dialysis was performed in an acrylic plastic dialysis cell (1 ml/side, Fisher Scientific, Pittsburgh, Pa.). The volume of solution in the inner and outer chamber was 0.5 ml. Dialysis membrane sheets were boiled for 5 min in 50 mM EDTA and 5% $NaCO_3$, and washed extensively for 1 week in 10 changes of doubly distilled water. Calmodulin (3.6 μM in the inner solution) was dialyzed at 22° for 20 hr against 20 mM Tris-HCl (pH 7.4) containing 25 μM $^{45}CaCl_2$ (0.5 μCi). The concentration of the competing cation was 100 μM. Aliquots of inner and outer solutions were removed and the radioactivity was counted. The amount of Ca^{2+} bound to calmodulin was calculated from the difference in radioactivity between the two solutions. $^{45}Ca^{2+}$ bound to calmodulin in the absence of other cations was 1.9 moles/mole of calmodulin, a value taken as 100%. Each value represents the mean ± standard deviation of three to four determinations.

Addition	$^{45}Ca^{2+}$ binding
None	100
Be^{2+}	97 ± 2
Mg^{2+}	97 ± 7
Sr^{2+}	73 ± 6
Ba^{2+}	96 ± 3
Mn^{2+}	67 ± 6
Co^{2+}	98 ± 1
Ni^{2+}	100 ± 3
Zn^{2+}	79 ± 6
Cd^{2+}	31 ± 5
Hg^{2+}	103 ± 3
Pb^{2+}	37 ± 5
La^{3+}	5 ± 3
Sm^{3+}	1 ± 1
Tb^{3+}	4 ± 2

TABLE 2

Reversal of calmodulin-stimulated phosphodiesterase activity by trifluoperazine

Phosphodiesterase was determined as described under Materials and Methods in the presence or absence of 0.5 μg of calmodulin. Trifluoperazine, 100 μM; phosphodiesterase, 29 μg. The enzyme was partially purified to the stage of DEAE-cellulose column chromatography (29). At 100 μM, trifluoperazine did not affect basal phosphodiesterase activity. The calmodulin-stimulated phosphodiesterase activity in the presence of Ca^{2+} was 280 nmoles/mg/min.

Cation (μM)	Phosphodiesterase activity		
	None	Calmodulin	Calmodulin + trifluoperazine
		%	
Zn^{2+} (50)	9	58	11
Mn^{2+} (20)	9	79	7
Tb^{3+} (25)	10	93	8
Cd^{2+} (30)	7	95	8
Ca^{2+} (20)	8	100	10
Sm^{3+} (20)	9	98	10
La^{3+} (20)	5	96	9
Hg^{2+} (100)	26	70	29
Sr^{2+} (500)	9	72	7
Pb^{2+} (500)	5	95	9

Effectiveness of Ca^{2+} and Cd^{2+} in stimulating calmodulin-dependent phosphodiesterase activity in the presence of each other. Figure 5 shows the activity of phosphodiesterase as a function of Ca^{2+} in the presence or absence of 5 μM Cd^{2+} (Fig. 5A), or as a function of Cd^{2+} in the presence or absence of 5 μM Ca^{2+} (Fig. 5B). In the presence of 5 μM Cd^{2+} (Fig. 5A), which raised phosphodiesterase activity to some 60% of the maximum, the titration curve by Ca^{2+} gradually increased the activity of the maximum, which remained unchanged thereafter, similar to the titration curve by Ca^{2+} alone. The peak phosphodiesterase activity was obtained at 10 μM Ca^{2+} in the presence of 5 μM Cd^{2+} and at 15 μM Ca^{2+} in the absence of Cd^{2+}. The activity of phosphodiesterase was generally higher in the presence of Cd^{2+} at each of the Ca^{2+} concentrations below 10 μM, but the final maximal activities were identical, suggesting that the effects of the two cations are not strictly additive. In the presence of 5 μM Ca^{2+} (Fig. 5B), which increased phosphodiesterase activity to some 70% of the maximum, the titration curve by Cd^{2+} also gradually increased to the maximum (comparable to that in Fig. 5A). Cd^{2+} at concentrations higher than 20 μM inhibited phosphodiesterase activity in a manner qualitatively similar to that by Cd^{2+} alone. The peak phosphodiesterase activity was obtained in the presence of 10 μM Cd^{2+} in the presence of 5 μM Ca^{2+}, and 15 μM Cd^{2+} in the absence of Ca^{2+}. Although Cd^{2+} alone could not activate phosphodiesterase to the maximal level, the addition of 5 μM Ca^{2+} did. Ca^{2+} at 5 μM could only stimulate phosphodiesterase submaximally. These data suggest that the presence of Cd^{2+} does not interfere with the effectiveness of Ca^{2+} to activate calmodulin. In fact, Cd^{2+} accentuates the effectiveness of Ca^{2+}. Similarly, Ca^{2+} accentuates the effectiveness of Cd^{2+}. In a separate experiment, the combined effect of Pb^{2+} and Ca^{2+} was studied, and the results were qualitatively similar to those obtained with Cd^{2+} and Ca^{2+}. These results are compatible with the notion that these cations occupy the same binding sites as Ca^{2+} on calmodulin, and that these sites, when occupied by Ca^{2+}, afford calmodulin optimal activity.

DISCUSSION

The work reported here represents a systematic, albeit preliminary, study of the efficacy of the various cations that substitute for Ca^{2+} in calmodulin. In this study, we have used several criteria to examine the efficacy of these cations: the ability to enhance intrinsic tyrosine fluorescence, to induce electrophoretic mobility change in polyacrylamide gel, to support calmodulin-stimulated phosphodiesterase activity, and to inhibit Ca^{2+} binding to calmodulin. Our findings show that Ca^{2+} is not specific for calmodulin, and that other cations with ionic radii comparable to that of calcium are effective to various extents. The efficacy appears to be largely a function of the ionic radius. For example, Sm^{3+}, whose ionic radius is nearly identical with that of Ca^{2+}, is nearly as effective in stimulating calmodulin-dependent phosphodiesterase activity, whereas Ba^{2+}, whose ionic radius is 30% larger than Ca^{2+}, is virtually inactive (Fig. 4). The ionic radius of a cation, however, is not the only deciding factor. Na^{+} has an ionic radius comparable to Ca^{2+}, but it did not activate calmodulin; presumably it has a lower affinity for the Ca^{2+}-binding sites on calmodulin.

The apparent effectiveness of some of these cations does not necessarily mean that they serve a physiological function. In fact, Cd^{2+}, Hg^{2+}, and Pb^{2+} are not normal

TABLE 3

Correlation between ionic radius of metal cations and their efficacy to induce change in tyrosine fluorescence, electrophoretic mobility, and calmodulin activity, and to inhibit Ca^{2+} binding

Ionic radius refers to the crystal structure of corresponding salts (30). Other data were taken from Figs. 2, 3, and 4. The tyrosine fluorescence and phosphodiesterase activity are expressed as percentage of Ca^{2+}, which is taken as 100. +, A mobility change; ±, slight change; −, no change. The data on the ability to inhibit Ca^{2+}-binding in the last column are recalculated from Table 1.

Cation	Radius	Fluorescence change	Mobility change		Phosphodiesterase activity	Ability to inhibit Ca^{2+} binding
			Native gel	SDS gel		
	Å	%			%	%
Be^{2+}	0.35	6	−	−	33	3 ± 2
Mg^{2+}	0.65	8	−	−	35	3 ± 7
Ni^{2+}	0.69	8	−	−	33	0 ± 3
Co^{2+}	0.72	20	−	−	27	2 ± 1
Zn^{2+}	0.74	52	−	−	67	21 ± 6
Mn^{2+}	0.80	54	+	+	70	33 ± 6
Tb^{3+}	0.92	67	+	+	89	96 ± 2
Cd^{2+}	0.97	81	+	+	90	69 ± 5
Ca^{2+}	0.99	100	+	+	100	—
Sm^{3+}	1.00	66	+	+	98	99 ± 1
La^{3+}	1.02	70	+	+	94	95 ± 3
Hg^{2+}	1.10	75	−	−	73	0 ± 3
Sr^{2+}	1.13	72	+	±	86	27 ± 6
Pb^{2+}	1.20	47	+	+	92	63 ± 5
Ba^{2+}	1.34	4	−	−	23	4 ± 3

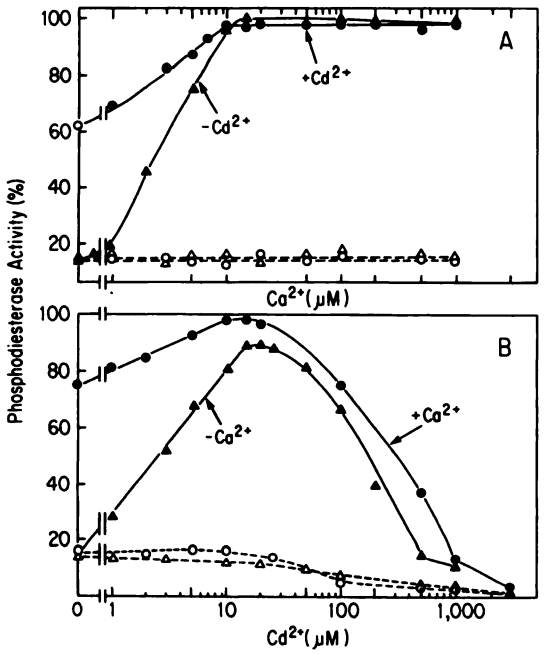

FIG. 5. *Effect of Ca^{2+}, Cd^{2+}, or both on the activity of calmodulin-dependent phosphodiesterase*

A, 5 μM Cd^{2+} and indicated concentration of Ca^{2+}; B, 5 μM Ca^{2+} and indicated concentration of Cd^{2+}. Other conditions were as described in Fig. 4.

constituents of tissues; they represent serious environmental pollutants in certain industrial regions, and accumulate in various human tissues, causing neurological, muscular, renal and bronchial disorders (31–33). The findings that these cations effectively substitute for Ca^{2+} in calmodulin, and that Cd^{2+} (and conceivably other cations) accentuates the effectiveness of Ca^{2+} raise the possibility that a combination of effective cations may fully activate calmodulin at a suboptimal concentration of Ca^{2+}. Such a condition would deprive the cell of its normal regulation through Ca^{2+}. Chronic activation of calmodulin could precipitate far-reaching disturbances to the many vital cellular functions, and this may constitute, at least in part, a molecular basis of their toxicity. This notion offers an explanation alternative to the conventional view that heavy metals affect cellular reactions by interacting with certain essential sulfhydryl groups of target enzymes.

It is not known whether an elevated tissue level of these cations does indeed activate calmodulin *in vivo*. Mammalian cells contain millimolar glutathione (34), which chelates Cd^{2+}, Pb^{2+}, and particularly Hg^{2+}. Moreover, metallothioneins, whose synthesis is induced by heavy metals (35), effectively bind heavy metals, acting as it were to protect the cell. However, if some of the heavy metals do find their way to calmodulin, it is conceivable that they may activate this versatile regulator, and upset the activity of calmodulin normally regulated by the cellular flux of Ca^{2+}.

In the context of our discussion for heavy metal toxicity, we are more concerned with their lower rather than higher range of concentration. Cd^{2+}, for example, could accumulate in tissues up to micromolar concentration (32), a level that effectively activates calmodulin.

A recent study (36) correlated metal toxicity with *in vitro* calmodulin inhibition; it describes the inhibition of calmodulin by various metals at high concentrations. Earlier, Brewer *et al.* (37) argued that Zn^{2+} toxicity may be attributable to its inhibitory effect on calmodulin function. As shown in Fig. 4, the effect of Zn^{2+} on calmodulin is biphasic: stimulatory at low concentrations and inhibitory at high concentrations. The apparent inhibition may be only a consequence of the assay system; high concentrations of Zn^{2+} inhibit basal phospho-

diesterase activity. The tissue concentration of Zn^{2+}, like that of other heavy cations, is relatively low, and appears more likely to stimulate rather than to inhibit calmodulin.

In the foregoing discussion, it has been implicitly assumed that the effective metal cations substitute Ca^{2+} by binding to the Ca^{2+} sites. Although this appears to be a reasonable assumption, on the basis that these metals mimic Ca^{2+} in inducing intrinsic tyrosine fluorescence, differential electrophoretic mobility, activation of phosphodiesterase, and inhibition of Ca^{2+} binding to calmodulin, direct binding of these metals to the Ca^{2+} sites has not been demonstrated, an undertaking technically not easily achievable. Fortunately, however, this does not distract from our general conclusion that these cations substitute for Ca^{2+} on calmodulin on the basis of their ionic size.

Although the idea that heavy metals may exert their toxicity through the activation of calmodulin appears attractive, it is speculative; the studies reported here are preliminary. It is desirable, and indeed necessary, to examine the efficacy of these metals to substitute for Ca^{2+} in other calmodulin-dependent enzymes. More importantly, these studies need to be substantiated with an *in vivo* system.

ACKNOWLEDGMENTS

We thank Dr. Gregory Schonbaum and Dr. Michael Dockter for many helpful discussions, Ms. Pat Nicholas for excellent manuscript preparation, and Fineberg Packing Company, Memphis, for fresh bovine brains.

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