Calmodulin-mediated cadmium inhibition of phosphodiesterase activity, in vitro

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Abstract. Ion-stripped bovine brain calmodulin (CaM) binds 4 moles Cd$^{2+}$ as well as 4 moles Ca$^{2+}$ per mole protein, with similar affinity; in the presence of 1 mM Mg$^{2+}$ the molar binding ratio of CaM for Ca$^{2+}$ decreased to 3, the apparent $K_{0.5}$ for Ca$^{2+}$ nearly doubled, but the binding characteristics of CaM for Cd$^{2+}$ were not changed. Saturating concentrations of Ca$^{2+}$ did not affect the molar binding ratio of CaM for Cd$^{2+}$, but increased the apparent $K_{0.5}$ for Cd$^{2+}$; vice versa, saturating concentrations Cd$^{2+}$ decreased the molar binding ratio for Ca$^{2+}$ to 2 without affecting the apparent $K_{0.5}$ for Ca$^{2+}$.

CaM-independent phosphodiesterase (PDE) activity was inhibited at $[Cd^{2+}] > 10^{-5}$ M. Cd$^{2+}$-CaM as well as Ca$^{2+}$-CaM activated PDE. However, the Cd$^{2+}$-CaM complex is less effective than the Ca$^{2+}$-CaM complex in stimulating CaM-dependent enzyme activities. Cd$^{2+}$ inhibits Ca$^{2+}$- and CaM-dependent PDE in a competitive way. Introduction of Cd$^{2+}$ in a medium containing Ca$^{2+}$ and CaM may, therefore, result in a reduction of CaM-dependent enzyme stimulation.

By its interference with Ca$^{2+}$- and CaM-dependent PDE activity, Cd$^{2+}$ could upset the catabolic pathway of cellular cyclic nucleotide metabolism.

Key words: Cadmium — Phosphodiesterase — Calmodulin — Alkaline phosphatase — Competitive inhibition

Introduction

Eukaryotic cells are characterized by low cytosolic Ca$^{2+}$ concentrations (generally below 0.1 $\mu$M Ca$^{2+}$). Fluctuations in cytosolic Ca$^{2+}$ concentrations play an essential information role in the physiology of the cell. Only minor changes in cytosolic Ca$^{2+}$ activities are required to give a Ca$^{2+}$-specific signal, as a result of the specific and high affinity for Ca$^{2+}$ of the cytosolic Ca$^{2+}$ receptors (Kretsinger 1977). Perhaps the now best-known intracellular Ca$^{2+}$ receptor is calmodulin (CaM). CaM was first reported (Cheung 1970) as an activator of cyclic nucleotide phosphodiesterase (PDE). Ever since, at least ten enzymic activities were shown to be Ca$^{2+}$- and CaM-dependent (Cheung 1984). CaM has four binding sites for Ca$^{2+}$ and on binding at least 3 Ca$^{2+}$-ions (Cox 1984) the protein changes conformation to become activator (Cheung 1981; Haiech et al. 1981).

Chao et al. (1984) have shown that heavy metal ions with ionic radii (0.1 $\pm$ 0.02 nm) close to the one of Ca$^{2+}$ (0.099 nm) may substitute — at least to some extent — for Ca$^{2+}$ in:

1. increasing tyrosine fluorescence of CaM,
2. altering the electrophoretic mobility of this protein, and
3. activation of PDE by CaM.

Both Tb$^{3+}$ (ionic radius 0.092 nm) and Cd$^{2+}$ (ionic radius 0.097 nm) have been reported to effectively occupy the four "Ca$^{2+}$-specific" binding sites in CaM and the use of such ions in CaM binding studies has proven a powerful tool (Forsen et al. 1980; Wallace et al. 1982). Moreover, Chao et al. (1984) and Suzuki et al. (1985) speculated that competition of such heavy metal ions with Ca$^{2+}$ for CaM may be a key to their toxicity at the molecular level. Characteristic symptoms of Cd$^{2+}$ poisoning, such as osteoporosis, hypercalciuria and altered protein synthesis (Yoshiki et al. 1975; Dudley et al. 1984) indeed strongly suggest that Cd$^{2+}$ interferes with Ca$^{2+}$-dependent processes.

To our knowledge there are no reports in the literature available at this moment that evaluate the effects of Cd$^{2+}$ on cyclic nucleotide levels in, e.g. isolated cells. Obviously, by interference with Ca$^{2+}$ and CaM-dependent PDE activity, Cd$^{2+}$ could disturb the catabolic pathway of cyclic nucleotide metabolism in the cell: Cd$^{2+}$ mimicks Ca$^{2+}$ in "activating" CaM, but the pattern of activation of CaM-dependent PDE by either ion (Chao et al. 1984; Suzuki et al. 1985) differs remarkably. The pCa dependence of CaM-PDE activity is described by a sigmoidal curve, the pCd dependence follows a biphasic, more or less parallel pattern (Suzuki et al. 1985). Apparently, Cd$^{2+}$ is able to disturb the kinetics of CaM-dependent PDE activity. Thus, although Cd$^{2+}$ may mimick Ca$^{2+}$ to some extent in activating CaM, the biological activity of the Cd$^{2+}$-CaM complex may be such that Cd$^{2+}$ eventually upsets a key mechanism in cellular cyclic nucleotide metabolism.

We report here on the effects of Cd$^{2+}$ on the kinetics of CaM-dependent PDE activity. The study of Cd$^{2+}$ interference with CaM-stimulated PDE activity is complicated by the fact that Cd$^{2+}$ interference with the CaM-PDE enzyme system is not necessarily restricted to an interaction with CaM only. A priori, one cannot exclude Cd$^{2+}$ interference...
with, e.g. Mg$^{2+}$ binding to PDE. Mg$^{2+}$ binding is essential for the activity of PDE to catalyze cAMP hydrolysis (Teo and Wang 1973). Therefore, we first studied the interaction for the activity of PDE to catalyze cAMP hydrolysis (Teo et al. 1973). Therefore, we first studied the interaction for the activity of PDE to catalyze cAMP hydrolysis (Teo et al. 1973). Therefore, we first studied the interaction for the activity of PDE to catalyze cAMP hydrolysis (Teo et al. 1973). Therefore, we first studied the interaction for the activity of PDE to catalyze cAMP hydrolysis (Teo et al. 1973).

Materials and methods

Reagents. Adenosine 3'5'-cyclic monophosphate (cAMP), adenosine 5'-monophosphate (AMP), PDE, bovine alkaline phosphatase (APase), CaM, ethylene glycol-bis N' -tetraacetic acid (EGTA) and trishydroxymethylamino- methane (Tris) were purchased from Sigma Chemical Co. Imidazol, calcium chloride and magnesium acetate were of highest purity from Merck Co. Chelex-100 was purchased from Bio-Rad Laboratories. Cadmium was used as Cd(NO$_3$)$_2$·4H$_2$O (RCB, Brussels) or as CdCl$_2$·2H$_2$O (Aqualuma) to the 25 µl samples, the radioactivity of $^{45}$Ca/ $^{115m}$Cd$^{2+}$ or $^{115m}$Cd$^{2+}$/ was determined in a liquid scintillation counter (Rackbeta 1216, LKB Wallac, Sweden), and the total concentrations of CaM and Ca or Cd were calculated. The Me$^{2+}$ bound to CaM was calculated as the difference between the total and the free Me$^{2+}$ concentration, the latter being measured in the filtrate. Experiments were carried out independently at least three times. Assay of cAMP-PDE. Stimulation of PDE was measured by a modification of the one-step procedure of Teo and Wang (1973). Phosphatase release was measured following the conversion of cAMP to adenosine by PDE and bovine APase. The assay medium (0.8 ml per assay) contained 40 mM Tris/HCl (pH 7.4), 40 mM imidazol, 1 mM MgCl$_2$, 20 µM CaCl$_2$, 2 mU/assay PDE and 1.5 U/assay APase. Stimulation of PDE by CaM was studied by the addition of up to 250 ng CaM per assay. After preincubation at 30 °C for 1 min, the reaction was started by the addition of 1 mM (final concentration) cAMP. Incubation lasted for 30 min at 30 °C. The inorganic phosphate release was quantified spectrophotometrically as ΔA$_{623}$ by the use of a colorimetric method with malachite green (Flik et al. 1984). The PDE concentration was reduced 8-fold in comparison with the method of Teo and Wang (1973). Blanks were prepared by replacing the PDE reaction mixture with distilled water. To determine non-specific substrate hydrolysis, the reaction mixture was incubated without enzymes. Enzyme reactions were stopped by the addition of the malachite green mixture.

Ultrafiltration was carried out in 3 ml ultrafiltration cells (Amicon Inc., USA) fitted with Diaflo YM-5 membranes. CaM (100 µg/ml) was mixed with $^3$H-CaM in 1 ml Tris (40 mM, pH 7.4) and the $^3$H-CaM specific activity was established. To 900 µl of this mixture 2.1 ml of the same buffer containing $^{45}$Ca$^{2+}$ or $^{115m}$Cd$^{2+}$ of known specific activity was added. Ultrafiltration was carried out at 22±1 °C in stirred cells operated at 25 psig N$_2$ gas. The filtration rate was 100 µl/min. Every 4 min, 25 µl samples were collected from the filtrate and the solution retained. It was ascertained in control experiments (without CaM) that equilibrium was reached for the Me$^{2+}$-free, as indicated by identical Me$^{2+}$ concentrations in the filtrate and the solution retained. After sampling, the volume was readjusted to 3 ml with the appropriate buffer solutions and filtration continued. Sampling was repeated up to 40 times which decreased the free Me$^{2+}$ concentration from an initial 20 µM to 0.2 µM at the completion of the experiment. Due to sampling, the CaM concentration decreased by 35% maximally. After addition of 4 ml scintillation fluid (Aqualuma) to the 25 µl samples, the radioactivity of $^{45}$Ca/$^{115m}$Cd/H was determined in a liquid scintillation counter (Rackbeta 1216, LKB Wallac, Sweden), and the total concentrations of CaM and Ca or Cd were calculated. The Me$^{2+}$ bound to CaM was calculated as the difference between the total and the free Me$^{2+}$ concentration, the latter being measured in the filtrate. Experiments were carried out independently at least three times.

Removal of Ca$^{2+}$ from CaM. Chelex-100 was used to remove Ca$^{2+}$ from CaM. The resin was prepared according to the manufacturer's instructions. The pH of the resin was adjusted to pH 7.4 by incubation overnight in a 1 M Tris solution, followed by several rinses with double-distilled, deionized water ([Ca$^{2+}$] < 10$^{-7}$ M). The pH of the chelex-100 remained stable after this treatment. CaM was freed of divalent ions through shaking in 1 ml double-distilled water containing 300 µl chelex-100 suspension. After pelleting the resin by centrifugation (3000 g x 2 min), the supernatant containing the CaM was collected; this procedure was repeated twice. Atomic absorption spectrophotometry showed that the residual Ca$^{2+}$ content of CaM prepared by this procedure was less than 0.02 mol Ca/mol CaM. Furthermore, CaM thus prepared showed a single band on SDSPAGE with a mobility identical to CaM run in the presence of 1 mM Me$^{2+}$ or 1 mM Me$^{2+}$ + 2 mM EGTA, a modification of the procedure described by Gitelman and Witman (1980). Carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as markers for molecular radius.

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SDS-PAGE. SDS-PAGE was performed in 15% polyacrylamide slab gels that were silver stained. CaM samples were run in the presence of 1 mM Me$^{2+}$ or 1 mM Me$^{2+}$ + 2 mM EGTA, a modification of the procedure described by Gitelman and Witman (1980). Carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as markers for molecular radius.

Binding of $^{45}$Ca$^{2+}$ and $^{115m}$Cd$^{2+}$ by CaM. Binding of Ca$^{2+}$ or Cd$^{2+}$ to CaM was studied in 40 mM Tris buffer (pH 7.4) with an ultrafiltration technique. The binding of $^{45}$Ca$^{2+}$ or $^{115m}$Cd$^{2+}$ to CaM was studied under three different conditions: 1) in the presence of 1 mM Mg$^{2+}$, 2) with ion-stripped CaM, and 3) with Cd$^{2+}$- or Ca$^{2+}$-saturated CaM.
Data were statistically analyzed by Student's t-test or the Mann-Whitney U-test, where appropriate. Statistical significance was accepted at the 5% level. Linear regression analysis was based on the least squares method.

Results

Figure 1 shows SDS-gel electrophoretographs of CaM run in the presence of Ca2+ (1 mM), Cd2+ (1 mM) or in the presence of each of these ions (1 mM) with a surplus of EGTA (2 mM). CaM run in the presence of divalent metal ions and a surplus of EGTA has an apparent molecular radius (Mₐ) of 21 kDa (2 and 4). A decrease in Mₐ of CaM of approximately 3 kDa was observed both in the presence of Cd2+ and of Ca2+ (1 and 3). Of a variety of divalent cations tested (results for ions of Pb, Hg, Zn, Mg, Ba, Sr, Ni, Sn, Cu, Co and Mn not shown), only Cd2+ provoked a shift in SDS-PAGE mobility identical to the Ca2+-induced shift. No difference was observed in Mₐ, whether Ca2+ or Cd2+ was added before or after SDS treatment of CaM (results not shown).

Figures 2a, b and c show the concentration dependency of Cu2+ or Cd2+ binding to CaM under various conditions. As shown in Fig. 2a, in the presence of 1 mM Mg2+, the molar binding ratio of CaM is 3 for Ca2+ and 4 for Cd2+. Figure 2b shows that in the absence of Mg2+ the molar binding ratio of CaM for Ca2+ is 4 (line A). In the presence of saturating concentrations Ca2+ (20 μM, the concentration used in the CaM-PDE assay) essentially all Ca2+ is eliminated from CaM at 20 μM 115mCd2+ (line B). The decrease in Ca2+ bound to CaM, represented by line B, was calculated as the difference between maximally saturated CaM (Ca4 CaM at 20 μM Ca2+) and the occurrence of 115mCd2+ complexes upon addition of 115mCd2+. Figure 2c shows that CaM binds 4 moles Cd2+ per mole protein in the absence of other divalent ions (line A). In the presence of saturating concentrations of Cd2+ (20 μM) maximally 2 moles Ca2+ were bound to CaM at 20 μM Ca2+. Values for apparent Kₐ and maximum binding (Bₚₐₚ), derived from plots as shown in Fig. 2, are summarized in Table 1. Apparently, Cd2+ eliminates Ca2+ from CaM at saturating Ca2+ levels, whereas even a surplus of Cd2+ eliminated Cd2+ from CaM at saturating Cd2+ concentrations only to a limited extent. When Cd2+ was added to Cd2+-saturated CaM, 50% of the Ca2+ binding sites of CaM were occupied by Cd2+ at 4.50±0.68 μM Cd2+. The results suggest that differences in association and dissociation velocities of CaM and Ca2+ and of CaM and Cd2+ exist. When Ca2+ was added to Cd2+-saturated CaM, a binding ratio of 2.50±0.36 Cd/CaM was found at equimolar concentrations of Ca2+ and Cd2+.

As shown in Fig. 3, AMP hydrolysis by APase was not affected by Cd2+ concentrations up to 10⁻² M; basal, CaM-independent PDE activity, however, was significantly inhibited at [Cd2+] > 10⁻⁵ M. We conclude, therefore, that the cAMP catalytic activity of PDE is not affected at Cd2+ concentrations up to 10⁻³ M and that the decrease in the phosphate release in this one-step PDE assay at Cd2+ concentrations higher than 10⁻⁵ M results from an inhibition of basal PDE activity. We conclude, therefore, that the effects of Cd2+ on CaM-dependent PDE activity cannot be studied at concentrations above 10⁻⁵ M.

We then tested the effects of either Cd2+ or Ca2+ on CaM-dependent PDE activity. Table 2 shows that maximum stimulation of PDE at 10⁻⁵ M Cd2+ or Ca2+ and 250 ng CaM/assay is of the same order. However, when the CaM concentration was reduced to 60 ng/assay (at which concentration the Ca2+-stimulated CaM-dependent activity was not significantly lower than at 250 ng/assay) Cd2+ was significantly less stimulatory than Ca2+.

Subsequently, the effects of Cd2+-concentrations of 10⁻⁵ M and lower were tested on Ca2+- and CaM-dependent PDE activity, at high and low CaM concentrations. In the absence of Cd2+, the PDE activity at 20 ng CaM/assay (twice the Kₐ for CaM) and a saturating concentration of Ca2+ (20 μM) was stimulated 5-to 6-fold compared to basal PDE activity. As shown in Fig. 4, with 10 μM Cd2+ and a fixed 20 μM Ca2+, the CaM-stimulated PDE activity was not affected at 100 ng CaM/assay but decreased by 50% at 20 ng CaM/assay.

Both in the absence and in the presence of low concentrations of Cd2+, the CaM dependency of the PDE activation could be described by Michaelis-Menten kinetics. Apparently, maximum stimulation of the enzyme by CaM in the presence of low concentrations of Cd2+ and Ca2+ was of the same order of magnitude. However, the apparent affinity constant of the enzyme for the CaM-Me2+ complex increased upon addition of Cd2+. Figure 5 shows that in the presence of Cd2+ the half maximum activation concentration of CaM-Me2+ for PDE more than doubled from 10 to 22 ng CaM/assay. These observations led us to conclude that Cd2+-containing CaM inhibits Ca2+- and CaM-dependent PDE activity competitively.
Discussion

We have concluded from our data that Cd$^{2+}$ firmly binds to CaM at its four Ca$^{2+}$-binding sites and that Cd$^{2+}$, even at submicromolar concentrations, displaces Ca$^{2+}$ from the protein. By inference, Cd$^{2+}$-CaM complexes will occur when Cd$^{2+}$ is introduced in a PDE assay system as used in this study. Cd$^{2+}$-CaM, similarly to Ca$^{2+}$-CaM, is able to stimulate CaM-dependent PDE activity. However, at an equimolar basis, the Cd$^{2+}$-CaM complex seems less effective than the Ca$^{2+}$-CaM complex, because the addition of submicromolar concentrations of Cd$^{2+}$ reduces Ca$^{2+}$-activated CaM-dependent PDE activity. Thus, the displacement of Ca$^{2+}$ from CaM by Cd$^{2+}$ makes it a less effective

Table 1. Binding constants of CaM for Ca$^{2+}$ and Cd$^{2+}$. $K_{0.5}$ (μM) and $B_{max}$ (mole Me$^{2+}$/mole CaM), determined in the absence of other Me$^{2+}$ or in the presence of Mg$^{2+}$, Ca$^{2+}$ or Cd$^{2+}$ were obtained from plots as shown in Fig. 2. Mean values ± SD are given; $n$ = number of observations.

<table>
<thead>
<tr>
<th>Additional Me$^{2+}$</th>
<th>Mg$^{2+}$ (1 mM)</th>
<th>Ca$^{2+}$ (20 μM)</th>
<th>Cd$^{2+}$ (20 μM)</th>
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<tr>
<td>$K_{0.5}$ (Ca)</td>
<td>3.78 ± 1.48</td>
<td>6.15 ± 1.40$^a$</td>
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<tr>
<td>$B_{max}$ (Ca)</td>
<td>3.65 ± 0.28</td>
<td>3.00 ± 0.20$^a$</td>
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<td>(n = 6)</td>
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<tr>
<td>$K_{0.5}$ (Cd)</td>
<td>3.56 ± 0.70$^b$</td>
<td>4.68 ± 0.97$^b$</td>
<td>7.25 ± 1.06$^{ac}$</td>
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<tr>
<td>$B_{max}$ (Cd)</td>
<td>5.03 ± 0.87$^{b}$</td>
<td>3.90 ± 0.38$^{bc}$</td>
<td>3.95 ± 0.65$^{bc}$</td>
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<td>(n = 4)</td>
<td>(n = 3)</td>
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$^a$ Significantly different from corresponding values obtained in the absence of other Me$^{2+}$; $P < 0.01$

$^b$ Significantly different from corresponding values in the same column; $P < 0.005$

$^c$ Significantly different from corresponding values in the presence of Cd$^{2+}$; $P < 0.005$
Table 2. Effects of Cd\textsuperscript{2+} or Ca\textsuperscript{2+} on calmodulin-dependent phosphodiesterase activity. Values are expressed as percentages of maximum CaM-dependent PDE activity observed at 10\textsuperscript{-4} M Ca\textsuperscript{2+} and 60 or 250 ng CaM/assay (ΔA\textsubscript{620} 30 min = 1212±21 = 100±2 %). Values in parentheses represent basal, CaM-independent PDE activities

<table>
<thead>
<tr>
<th>Me\textsuperscript{2+} (10 μM)</th>
<th>PDE activity (%)</th>
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<td>CaM (ng/assay)</td>
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<td></td>
<td>60</td>
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<td>250</td>
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Cd\textsuperscript{2+}  
62±2\textsuperscript{a} (15±1)  97±1 (15±1)

Ca\textsuperscript{2+}  
94±4 (16±2)  100±2 (17±5)

\textsuperscript{a} Significantly different (P < 0.001) from Ca\textsuperscript{2+}-stimulated, CaM-dependent PDE activity; n = 5

activator for PDE than Ca\textsuperscript{2+}-CaM. This Cd\textsuperscript{2+} inhibition is of a purely competitive character, mediated via CaM and is obscured when CaM-dependent PDE is assayed in the presence of surplus CaM. The competitive inhibition brought about by Cd\textsuperscript{2+} further indicates that Cd\textsuperscript{2+}-containing CaM complexes do interact at the same site of the enzyme as Ca\textsuperscript{2+}-containing CaM.

**Binding of Ca\textsuperscript{2+} and Cd\textsuperscript{2+} to calmodulin**

The contention that Cd\textsuperscript{2+} binds to CaM at its four Ca\textsuperscript{2+}-binding sites is substantiated not only by our SDS-PAGE results but also by the very similar binding constants of CaM for Cd\textsuperscript{2+} and Ca\textsuperscript{2+} in the absence of other divalent metal ions. In their studies on Ca\textsuperscript{2+} binding to CaM, Burgess et al. (1980) concluded that the binding of four calcium ions to CaM causes a discrete conformational change. Because this also occurs in the presence of SDS, it can be visualized by SDS-PAGE as a decrease in the apparent molecular radius of the protein. As judged from our SDS-PAGE results, Cd\textsuperscript{2+} is as effective as Ca\textsuperscript{2+} in causing this change in conformation. This conformational change as visualized by SDS-PAGE has been interpreted to indicate the activation of CaM by its activating metal ion. A variety of divalent metal ions was shown by SDS-PAGE to substitute for Ca\textsuperscript{2+} in activating CaM and this has been ascribed to a similarity in their ionic radius with that of Ca\textsuperscript{2+} (0.1±0.02 nm; Chao et al. 1984). For two other parameters of CaM activation—tyrosine fluorescence and the activation of CaM dependent PDE—Cd\textsuperscript{2+} among other divalent metal ions was found to only partly mimic Ca\textsuperscript{2+} in its interaction with CaM (Chao et al. 1984). The question arose then, whether differences in interaction of CaM with Ca\textsuperscript{2+} and Cd\textsuperscript{2+} or in the interaction of the Cd\textsuperscript{2+}-containing CaM with, e.g. PDE underlie the aberrant Cd\textsuperscript{2+}-activation pattern of CaM-dependent PDE activity.

Although our data show that CaM exhibits similar affinity and binding capacity for Cd\textsuperscript{2+} as for Ca\textsuperscript{2+} in the absence of other divalent metal ions, we further demonstrate that CaM does indeed discriminate between Cd\textsuperscript{2+} and Ca\textsuperscript{2+} in the presence of physiological concentrations of Mg\textsuperscript{2+}: in this case the molar binding ratio was 4 for Cd\textsuperscript{2+} and 3 for Ca\textsuperscript{2+}. We suggest that Cd\textsuperscript{2+} binds with higher affinity to one of the four Ca\textsuperscript{2+}-binding sites of CaM for which Mg\textsuperscript{2+} successfully competes with Ca\textsuperscript{2+}. This result is in line with the results of Andersson et al. (1982), who showed that Mg\textsuperscript{2+} decreases the 35Ca-CaM NMR signal.

![Fig. 3. Effects of Cd\textsuperscript{2+} on alkaline phosphatase (APase) and CaM-independent PDE activities. Enzymic activities were determined as described in Materials and methods. n = 4; asterisks indicate significant inhibition (P < 0.01).](image)

![Fig. 4. Calmodulin concentration dependence of Cd\textsuperscript{2+} inhibition of PDE activity. At 100 ng CaM/assay, CaM-stimulated PDE activity was not affected by Cd\textsuperscript{2+} concentrations up to 10\textsuperscript{-5} M. At 20 ng CaM/assay, up to 60% inhibition of CaM-dependent PDE activity occurred at 10\textsuperscript{-5} M Cd\textsuperscript{2+}. The Ca\textsuperscript{2+} concentration was 20 μM.](image)

![Fig. 5. Effect of Cd\textsuperscript{2+} on the apparent K\textsubscript{50} values for CaM stimulation of PDE activity in the presence of 20 μM Ca\textsuperscript{2+}. CaM concentrations ranged from 0–100 ng/assay. K\textsubscript{50} values were derived from Eadie-Hofstee plots. At all Cd\textsuperscript{2+} concentrations tested K\textsubscript{50} values were found to increase significantly (P < 0.01) in a Cd\textsuperscript{2+} concentration-dependent way.](image)
(and therefore the binding of Ca$^{2+}$ to CaM) but not the $^{115}$Cd-CaM NMR signal. A higher affinity of CaM for Cd$^{2+}$ was further indicated by the fact that Cd$^{2+}$ eliminates Ca$^{2+}$ from Ca$^{2+}$-saturated CaM almost completely at equimolar concentrations of either metal ion. This result corroborates data of Chao et al. (1984), who reported that 100 µM Cd$^{2+}$ gives a 69% inhibition of $^{45}$Ca$^{2+}$ binding to CaM, albeit that in their studies Mg$^{2+}$ appeared to have no effect on this binding and that the molar ratio for $^{45}$Ca$^{2+}$ binding to CaM was only 1.9 in the absence of other divalent metal ions.

**Cd$^{2+}$ inhibition of phosphodiesterase**

Cd$^{2+}$ inhibits PDE activity in two ways, viz. directly via the enzyme and indirectly via interaction with its activator CaM. In our assay system, Cd$^{2+}$ concentrations higher than $10^{-5}$ M inhibited CaM-independent (basal) PDE activity; Cd$^{2+}$ concentrations below $10^{-5}$ M affected neither basal PDE nor the $V_{max}$ of CaM-stimulated PDE activity. Apparently, the lower concentrations did not influence the catalytic capacity of the enzyme. A plausible explanation for the direct inhibition of PDE would be that Cd$^{2+}$ competed with Mg$^{2+}$ at the catalytic unit of the PDE and by so doing decreased its catalytic activity. The fact that other plurivalent metal ions such as Mn$^{2+}$ and Co$^{2+}$ may substitute for Mg$^{2+}$ in activating PDE's catalytic activity (although to a smaller extent: Wolf and Brostrom 1979), supports our suggestion that Cd$^{2+}$ also interacts with this site. Assuming that Cd$^{2+}$ behaves similarly to Ca$^{2+}$ in this respect, inhibition of PDE catalytic activity may be predicted when Cd$^{2+}$ competes with Mg$^{2+}$ (Wolf and Brostrom 1979). However, such competition will be insignificant at Cd$^{2+}$ concentrations equal to or lower than $10^{-5}$ M, that will give Mg/Cd ratios higher than or equal to 100 in our assay system. Interactions other than with the catalytic site cannot be excluded and by affecting the tertiary structure such interactions could inhibit the PDE activity.

Because CaM-independent PDE activity appeared unaffected at Cd$^{2+}$ concentrations lower than $10^{-5}$ M, we further conducted our studies on Cd$^{2+}$ inhibition of CaM-dependent PDE activity only with Cd$^{2+}$ concentrations up to $10^{-5}$ M. At high CaM concentrations (250 ng/assay) both Cd$^{2+}$ and Ca$^{2+}$ (either ion separately tested) proved stimulatory to the same extent. Yet, at lower concentrations of CaM (60 ng/assay) and $10^{-5}$ M Cd$^{2+}$ or Ca$^{2+}$, the latter ion was preferred. This suggests that Cd$^{2+}$ is less effective as an activator of CaM-dependent PDE than Ca$^{2+}$. Brostrom and Wolff (1981) demonstrated that with decreasing concentrations of CaM, CaM-dependent PDE needs progressively higher Ca$^{2+}$ concentrations to become fully activated. Under those conditions, where due to low CaM levels the PDE becomes more Ca$^{2+}$ dependent, we found that Cd$^{2+}$ was less stimulating than Ca$^{2+}$. Of course, the possibility cannot be excluded that traces of Ca$^{2+}$ were present in the reagents and enzyme preparations composing our assay system. At higher concentrations of CaM then, traces of Ca$^{2+}$ could support the Cd$^{2+}$ activation progressively and lead to a full activation of PDE. Nevertheless, our data, which indicate that CaM-dependent PDE is not activated to the same extent by Cd$^{2+}$ as by Ca$^{2+}$, support the suggestion based on tyrosine fluorescence and binding studies (Habermann et al. 1983; Chao et al. 1984) that Ca$^{2+}$ and Cd$^{2+}$ activate CaM in a quantitatively different way. Moreover, in the PDE assay, performed under such conditions that the metal ion concentrations are crucial, Cd$^{2+}$ and every divalent metal ion other than Ca$^{2+}$ (except for Pb$^{2+}$) tested so far appeared less effective than Ca$^{2+}$ (Habermann et al. 1983). In our experiments we tested the effects of graded amounts of Cd$^{2+}$ on Ca$^{2+}$- and CaM-dependent PDE activity and found that at low Cd$^{2+}$ concentrations competitive inhibition occurred. At equimolar concentrations of Ca$^{2+}$ and Cd$^{2+}$, CaM-dependent PDE was inhibited by 50% as indicated by a doubling of the apparent $K_m$ for CaM activation of PDE activity. From this doubling of the $K_m$ value one may conclude to a 50% loss in the ability of CaM to activate PDE. Similarly, a competitive and CaM-mediated Cd$^{2+}$ inhibition has been reported for CaM-dependent Ca$^{2+}$, Mg$^{2+}$-ATPase activity in human erythrocyte membranes (Ackerman et al. 1985).

Apparently, Cd$^{2+}$ interaction with Ca$^{2+}$- and CaM-dependent enzyme systems is preferably exerted via CaM. This may be related to the relatively high affinity of CaM for Cd$^{2+}$. Our results suggest that Cd$^{2+}$, by interference with Ca$^{2+}$ and CaM-dependent PDE activity, could upset the catabolic pathway of cellular cyclic nucleotide metabolism. For evaluation of the physiological implications of this conclusion, studies on isolated cells are indicated.

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