Calmodulin-mediated cadmium inhibition of phosphodiesterase activity, in vitro

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Abstract. Ion-stripped bovine brain calmodulin (CaM) binds 4 moles Cd²⁺ as well as 4 moles Ca²⁺ per mole protein, with similar affinity; in the presence of 1 mM Mg²⁺ the molar binding ratio of CaM for Ca²⁺ decreased to 3, the apparent Kₐ₅ for Ca²⁺ nearly doubled, but the binding characteristics of CaM for Cd²⁺ were not changed. Saturating concentrations of Ca²⁺ did not affect the molar binding ratio of CaM for Cd²⁺, but increased the apparent Kₐ₅ for Cd²⁺; vice versa, saturating concentrations Cd²⁺ decreased the molar binding ratio for Ca²⁺ to 2 without affecting the apparent Kₐ₅ for Ca²⁺.

CaM-independent phosphodiesterase (PDE) activity was inhibited at [Cd²⁺] > 10⁻⁵ M. Cd²⁺-CaM as well as Ca²⁺-CaM activated PDE. However, the Ca²⁺-CaM complex is less effective than the Ca²⁺-CaM complex in stimulating CaM-dependent enzyme activities. Cd²⁺ inhibits Ca²⁺- and CaM-dependent PDE in a competitive way. Introduction of Cd²⁺ in a medium containing Ca²⁺ and CaM may, therefore, result in a reduction of CaM-dependent enzyme stimulation.

By its interference with Ca²⁺- and CaM-dependent PDE activity, Cd²⁺ 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 Ca²⁺ concentrations (generally below 0.1 μM Ca²⁺). Fluctuations in cytosolic Ca²⁺ concentrations play an essential informative role in the physiology of the cell. Only minor changes in cytosolic Ca²⁺ activities are required to give a Ca²⁺-specific signal, as a result of the specific and high affinity for Ca²⁺ of the cytosolic Ca²⁺ receptors (Kretsinger 1977). Perhaps the now best-known intracellular Ca²⁺ 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 Ca²⁺- and CaM-dependent (Cheung 1984). CaM has four binding sites for Ca²⁺ and on binding at least 3 Ca²⁺-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±0.02 nm) close to the one of Ca²⁺ (0.099 nm) may substitute — at least to some extent — for Ca²⁺ 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 Cd²⁺ (ionic radius 0.097 nm) have been reported to effectively occupy the four “Ca²⁺-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 Ca²⁺ for CaM may be a key to their toxicity at the molecular level. Characteristic symptoms of Cd²⁺ poisoning, such as osteoporosis, hypercalciumia and altered protein synthesis (Yoshiki et al. 1975; Dudley et al. 1984) indeed strongly suggest that Cd²⁺ interferes with Ca²⁺-dependent processes.

To our knowledge there are no reports in the literature available at this moment that evaluate the effects of Cd²⁺ on cyclic nucleotide levels in, e.g. isolated cells. Obviously, by interference with Ca²⁺- and CaM-dependent PDE activity, Cd²⁺ could disturb the catabolic pathway of cyclic nucleotide metabolism in the cell: Cd²⁺ mimicks Ca²⁺ 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 activation is described by a sigmoidal curve, the pCd dependence follows a biphasic, more or less parabolic pattern (Suzuki et al. 1985). Apparently, Cd²⁺ is able to disturb the kinetics of CaM-dependent PDE activity. Thus, although Cd²⁺ may mimick Ca²⁺ to some extent in activating CaM, the biological activity of the Cd²⁺-CaM complex may be such that Cd²⁺ eventually upsets a key mechanism in cellular cyclic nucleotide metabolism.

We report here on the effects of Cd²⁺ on the kinetics of CaM-dependent PDE activity. The study of Cd²⁺ interference with CaM-stimulated PDE activity is complicated by the fact that Cd²⁺ interference with the CaM-PDE enzyme system is not necessarily restricted to an interaction with CaM only. A priori, one cannot exclude Cd²⁺ 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 of Cd\(^{2+}\) with CaM in the presence of Ca\(^{2+}\) or Mg\(^{2+}\). Next, the interaction of Cd\(^{2+}\) with PDE activity was evaluated by testing its effects on basal (CaM-independent) PDE activity and on the kinetics of CaM stimulation of PDE activity.

**Materials and methods**

**Reagents.** Adenosine 3':5'-cyclic monophosphate (cAMP), adenosine 5'-monophosphate (AMP), PDE, bovine alkaline phosphatase (APiase), CaM, ethylene glycol-bis N'-tetraacetic acid (EGTA) and trishydroxymethylaminomethane (Tris) were purchased from Sigma Chemical Co. Imidazol, calcium chloride and magnesium acetate were of the 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\(_2\).

**N-(propionyl-^3H)-CaM (925 GBq/mol), ^4CaCl\(_2\) (30.5 GBq/mol) and ^115mCdCl\(_2\) (3 GBq/mol) were obtained from Amersham International plc, UK.**

**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 × 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 sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a mobility identical to CaM run in the presence of 2 mM EGTA. The Ca\(^{2+}\)-free CaM solution was rapidly frozen in liquid N\(_2\), stored at -20 °C and used within 1 week. Chelex-100 was also used to remove Ca\(^{2+}\) from the Tris buffer solutions we used in the binding studies; these solutions were kept in stock above 2 cm of chelex-100. Atomic absorption spectrophotometry showed that the [Ca\(^{2+}\)] of these buffers was below 10\(^{-8}\) M.

**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 ^4Ca\(^{2+}\) and ^115mCd\(^{2+}\) 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 ^4Ca\(^{2+}\) or ^115mCd\(^{2+}\) 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.

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 ^3H-CaM in 1 ml Tris (40 mM, pH 7.4) and the ^3H-CaM specific activity established. To 900 μl of this mixture 2.1 ml of the same buffer containing ^4Ca\(^{2+}\) or ^115mCd\(^{2+}\) 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 (Aqualamina) to the 25 μl samples, the radioactivity of ^4Ca/ ^3H or ^115mCd/ ^2H 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 APiase. The assay medium (0.8 ml per assay) contained 40 mM Tris/HCl (pH 7.4), 40 mM imidazol, 1 mM Mg\((CH_2COO)_2\), 20 μM CaCl\(_2\), 2 μU/assay PDE and 1.5 U/assay APiase. 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\(_{232}\) 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.

The effect of Cd\(^{2+}\) on the CaM-stimulated PDE activity was studied at concentrations ranging from 10\(^{-7}\) to 10\(^{-2}\) M Cd (NO\(_3\))\(_2\). Total Cd and Ca concentrations were measured by atomic absorption spectrophotometry. Since phosphate production in this PDE assay is strictly dependent on the APiase activity, we first studied the effect of Cd\(^{2+}\) on APiase separately. APiase activity was determined under conditions comparable to the PDE assay; in this case AMP (1 mM) was used as substrate and the incubation time was 10 min. The effect of Cd on the kinetic parameters (K\(_{0.5}\) and V\(_{max}\)) of CaM-stimulated PDE activity was evaluated after linearization of the results by Eadie-Hofstee plots (Flik et al. 1984). Experiments were carried out independently at least three times.
Cd\(^{+}\) was added before or after SDS treatment of Ca\(^{2+}\). The shift in SDS-PAGE mobility identical to the Ca\(^{2+}\) concentration used in the CaM-PDE assay essentially all the molar binding ratio of CaM is 3 for Ca\(^{+}\) and 4 for Cd\(^{+}\). As shown in Fig. 2a, in the presence of 1 mM Mg\(^{+}\) or Ca\(^{+}\), respectively; (2, 4) samples run in the presence of 2 mM EGTA and 1 mM CdCl\(_2\), or CaCl\(_2\), respectively.

Figures 2a, b and c show the concentration dependency of Ca\(^{+}\) or Cd\(^{+}\) binding to CaM under various conditions. As shown in Fig. 2a, in the presence of 1 mM Mg\(^{2+}\), the molar binding ratio of CaM is 3 for Ca\(^{2+}\) and 4 for Cd\(^{2+}\). Figure 2b shows that in the absence of Mg\(^{2+}\), the molar binding ratio of CaM for Ca\(^{2+}\) is 4 (line A). In the presence of saturating concentrations Ca\(^{2+}\) (20 \(\mu\)M) maximally 2 moles Ca\(^{2+}\) were bound to CaM at 20 \(\mu\)M Ca\(^{2+}\). Values for apparent K\(_d\) and maximum binding (B\(_{\text{max}}\), derived from plots as shown in Fig. 2, are summarized in Table 1. Apparently, Cd\(^{2+}\) eliminates Ca\(^{2+}\) from CaM at saturating Ca\(^{2+}\) levels, whereas even a surplus of Ca\(^{2+}\) eliminated Cd\(^{2+}\) from CaM at saturating Cd\(^{2+}\) concentrations only to a limited extent. When Cd\(^{2+}\) was added to Cd\(^{2+}\)-saturated CaM, 50% of the Ca\(^{2+}\) binding sites of CaM were occupied by Cd\(^{2+}\) at 4.50 \pm 0.68 \(\mu\)M Cd\(^{2+}\). The results suggest that differences in association and dissociation velocities of CaM and Cd\(^{2+}\) and of CaM and Cd\(^{2+}\) exist. When Ca\(^{2+}\) was added to Cd\(^{2+}\)-saturated CaM, a binding ratio of 2.50 \pm 0.36 Cd/CaM was found at equimolar concentrations of Ca\(^{2+}\) and Cd\(^{2+}\).

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

We then tested the effects of either Ca\(^{2+}\) or Cd\(^{2+}\) on CaM-dependent PDE activity. Table 2 shows that maximum stimulation of PDE at \(10^{-5}\) M Cd\(^{2+}\) or Ca\(^{2+}\) 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 Ca\(^{2+}\)-stimulated CaM-dependent activity was not significantly lower than at 250 ng/assay) Cd\(^{2+}\) was significantly less stimulatory than Ca\(^{2+}\).

Subsequently, the effects of Cd\(^{2+}\) concentrations of \(10^{-5}\) M and lower were tested on Ca\(^{2+}\) - and CaM-dependent PDE activity, at high and low CaM concentrations. In the absence of Cd\(^{2+}\), the PDE activity at 20 ng CaM/assay (twice the K\(_{0.5}\) for CaM) and a saturating concentration of Ca\(^{2+}\) (20 \(\mu\)M) was stimulated 5-to 6-fold compared to basal PDE activity. As shown in Fig. 4, with 10 \(\mu\)M Cd\(^{2+}\) and a fixed 20 \(\mu\)M Ca\(^{2+}\), 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 Cd\(^{2+}\), 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 Cd\(^{2+}\) and Ca\(^{2+}\) was of the same order of magnitude. However, the apparent affinity constant of the enzyme for the CaM-Me\(^{2+}\) complex increased upon addition of Cd\(^{2+}\). Figure 5 shows that in the presence of Cd\(^{2+}\) the half maximum activation concentration of CaM-Me\(^{2+}\) for PDE more than doubled from 10 to 22 ng CaM/assay. These observations led us to conclude that Cd\(^{2+}\)-containing CaM inhibits Ca\(^{2+}\)- and CaM-dependent PDE activity competitively.

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 electrophoretograms of CaM run in the presence of Ca\(^{2+}\) (1 mM), Cd\(^{2+}\) (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 (M\(_r\)) of 21 kDa (2 and 4). A decrease in M\(_r\) of CaM of approximately 3 kDa was observed both in the presence of Cd\(^{2+}\) and of Ca\(^{2+}\) (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 Cd\(^{2+}\) provoked a shift in SDS-PAGE mobility identical to the Ca\(^{2+}\)-induced shift. No difference was observed in M\(_r\) whether Ca\(^{2+}\) or Cd\(^{2+}\) was added before or after SDS treatment of CaM (results not shown).

Figures 2a, b and c show the concentration dependency of Cu\(^{2+}\) or Cd\(^{2+}\) binding to CaM under various conditions. As shown in Fig. 2a, in the presence of 1 mM Mg\(^{2+}\), the molar binding ratio of CaM is 3 for Ca\(^{2+}\) and 4 for Cd\(^{2+}\). Figure 2b shows that in the absence of Mg\(^{2+}\), the molar binding ratio of CaM for Ca\(^{2+}\) is 4 (line A). In the presence of saturating concentrations Ca\(^{2+}\) (20 \(\mu\)M, the concentration used in the CaM-PDE assay) essentially all Ca\(^{2+}\) is eliminated from CaM at 20 \(\mu\)M Cd\(^{2+}\) (line B). The decrease in Ca\(^{2+}\) bound to CaM, represented by line B, was calculated as the difference between maximally saturated CaM (Ca\(_4\) CaM at 20 \(\mu\)M Ca\(^{2+}\)) and the occurrence of Cd\(^{2+}\) complexes upon addition of Cd\(^{2+}\). The molar binding ratio of CaM is 3 for Ca\(^{2+}\) and 4 for Cd\(^{2+}\). Values for apparent K\(_d\) and maximum binding (B\(_{\text{max}}\), derived from plots as shown in Fig. 2, are summarized in Table 1.
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>
<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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<tbody>
<tr>
<td>K$_{0.5}$ (Ca)</td>
<td>3.65 ± 0.28</td>
<td>3.00 ± 0.20</td>
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<tr>
<td>B$_{max}$ (Ca)</td>
<td>3.78 ± 1.48</td>
<td>6.15 ± 1.40</td>
<td>3.77 ± 0.55</td>
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<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>K$_{0.5}$ (Cd)</td>
<td>3.56 ± 0.70</td>
<td>4.68 ± 0.97</td>
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<tr>
<td>B$_{max}$ (Cd)</td>
<td>5.03 ± 0.87$^b$</td>
<td>3.90 ± 0.38$^{ab}$</td>
<td>3.95 ± 0.65$^{bc}$</td>
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<td></td>
<td>(n = 4)</td>
<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$^{2+}$; $P < 0.005$
activator for PDE than Ca\(^{2+}\)-CaM. This Cd\(^{2+}\) 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\(^{2+}\) further indicates that Cd\(^{2+}\)-containing CaM complexes do interact at the same site of the enzyme as Ca\(^{2+}\)-containing CaM.

**Binding of Ca\(^{2+}\) and Cd\(^{2+}\) to calmodulin**

The contention that Cd\(^{2+}\) binds to CaM at its four Ca\(^{2+}\)-binding sites is substantiated not only by our SDS-PAGE results but also by the very similar binding constants of CaM for Cd\(^{2+}\) and Ca\(^{2+}\) in the absence of other divalent metal ions. In their studies on Ca\(^{2+}\) 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\(^{2+}\) is as effective as Ca\(^{2+}\) 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\(^{2+}\) in activating CaM and this has been ascribed to a similarity in their ionic radius with that of Ca\(^{2+}\) (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\(^{2+}\) among other divalent and trivalent metal ions was found to only partly mimic Ca\(^{2+}\) in its interaction with CaM (Chao et al. 1984). The question arose then, whether differences in interaction of CaM with Ca\(^{2+}\) and Cd\(^{2+}\) or in the interaction of the Cd\(^{2+}\)-containing CaM with, e.g. PDE underlie the aberrant Cd\(^{2+}\)-activation pattern of CaM-dependent PDE activity.

Although our data show that CaM exhibits similar affinity and binding capacity for Cd\(^{2+}\) as for Ca\(^{2+}\) in the absence of other divalent metal ions, we further demonstrate that CaM does indeed discriminate between Cd\(^{2+}\) and Ca\(^{2+}\) in the presence of physiological concentrations of Mg\(^{2+}\); in this case the molar binding ratio was 4 for Cd\(^{2+}\) and 3 for Ca\(^{2+}\). We suggest that Cd\(^{2+}\) binds with higher affinity to one of the four Ca\(^{2+}\)-binding sites of CaM for which Mg\(^{2+}\) successfully competes with Ca\(^{2+}\). This result is in line with the results of Andersson et al. (1982), who showed that Mg\(^{2+}\) decreases the \(^{45}\)Ca-CaM NMR signal.

**Table 2. Effects of Cd\(^{2+}\) or Ca\(^{2+}\) on calmodulin-dependent phosphodiesterase activity. Values are expressed as percentages of maximum CaM-dependent PDE activity observed at 10\(^{-4}\) M Ca\(^{2+}\) and 60 or 250 ng CaM/assay (ΔA\(_{620}\)/30 min = 1212 ± 21 = 100 ± 2 %). Values in parentheses represent basal, CaM-independent PDE activities.**

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<thead>
<tr>
<th>Me(^{2+}) (10 μM)</th>
<th>PDE activity (%)</th>
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<tr>
<td></td>
<td>CaM (ng/assay)</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>62 ± 2(^{a}) (15 ± 1)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>94 ± 4 (16 ± 2)</td>
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\(^{a}\) Significantly different (\(P < 0.001\)) from Ca\(^{2+}\)-stimulated, CaM-dependent PDE activity; \(n = 5\)

**Fig. 3. Effects of Cd\(^{2+}\) 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 (\(P < 0.01\))**

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

**Fig. 5. Effect of Cd\(^{2+}\) on the apparent \(K_{s,5}\) values for CaM stimulation of PDE activity in the presence of 20 μM Ca\(^{2+}\). CaM concentrations ranged from 0–100 ng/assay. \(K_{s,5}\) values were derived from Eadie-Hofstee plots. At all Cd\(^{2+}\) concentrations tested \(K_{s,5}\) values were found to increase significantly (\(P < 0.01\)) in a Cd\(^{2+}\)-concentration-dependent way**
(and therefore the binding of \( \text{Ca}^{2+} \) to CaM) but not the \(^{111}\text{Cd}\)-CaM NMR signal. A higher affinity of CaM for \( \text{Cd}^{2+} \) was further indicated by the fact that \( \text{Cd}^{2+} \) eliminates \( \text{Ca}^{2+} \) from \( \text{Ca}^{2+} \)-saturated CaM almost completely at equimolar concentrations of either metal ion. This result corroborates data of Chao et al. (1984), who reported that 100 \( \mu \text{M} \text{Cd}^{2+} \) gives a 69% inhibition of \(^{45}\text{Ca}^{2+} \) binding to CaM, albeit that in their studies \( \text{Mg}^{2+} \) appeared to have no effect on this binding and that the molar ratio for \(^{45}\text{Ca}^{2+} \) binding to CaM was only 1.9 in the absence of other divalent metal ions.

**Cd\(^{2+}\) inhibition of phosphodiesterase**

\( \text{Cd}^{2+} \) inhibits PDE activity in two ways, viz. directly via the enzyme and indirectly via interaction with its activator CaM. In our assay system, \( \text{Cd}^{2+} \) concentrations higher than \( 10^{-5} \text{ M} \) inhibited CaM-independent (basal) PDE activity; \( \text{Cd}^{2+} \) concentrations below \( 10^{-5} \text{ M} \) affected neither basal PDE nor the \( V_{\text{max}} \) 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 \( \text{Cd}^{2+} \) competes with \( \text{Mg}^{2+} \) at the catalytic unit of the PDE and by so doing decreased its catalytic activity. The fact that other pluriivalent metal ions such as \( \text{Mn}^{2+} \) and \( \text{Co}^{2+} \) may substitute for \( \text{Mg}^{2+} \) in activating PDE’s catalytic activity (although to a smaller extent: Wolf and Brostrom 1979), supports our suggestion that \( \text{Cd}^{2+} \) also interacts with this site. Assuming that \( \text{Cd}^{2+} \) behaves similarly to \( \text{Ca}^{2+} \) in this respect, inhibition of PDE catalytic activity may be predicted when \( \text{Cd}^{2+} \) competes with \( \text{Mg}^{2+} \) (Wolf and Brostrom 1979). However, such competition will be insignificant at \( \text{Cd}^{2+} \) concentrations equal to or lower than \( 10^{-5} \text{ M} \), that will give \( \text{Mg}/\text{Cd} \) 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 \( \text{Cd}^{2+} \) concentrations lower than \( 10^{-5} \text{ M} \), we further conducted our studies on \( \text{Cd}^{2+} \) inhibition of CaM-dependent PDE activity only with \( \text{Cd}^{2+} \) concentrations up to \( 10^{-5} \text{ M} \). At high CaM concentrations (250 ng/assay) both \( \text{Cd}^{2+} \) and \( \text{Ca}^{2+} \) (either ion separately tested) proved stimulatory to the same extent. Yet, at lower concentrations of CaM (60 ng/assay) and \( 10^{-5} \text{ M} \text{Cd}^{2+} \) or \( \text{Ca}^{2+} \), the latter ion was preferred. This suggests that \( \text{Cd}^{2+} \) is less effective as an activator of CaM-dependent PDE than \( \text{Ca}^{2+} \). Brostrom and Wolff (1981) demonstrated that with decreasing concentrations of CaM, CaM-dependent PDE needs progressively higher \( \text{Ca}^{2+} \) concentrations to become fully activated. Under those conditions, where due to low CaM levels the PDE becomes more \( \text{Ca}^{2+} \)-dependent, we found that \( \text{Cd}^{2+} \) was less stimulating than \( \text{Ca}^{2+} \). Of course, the possibility cannot be excluded that traces of \( \text{Ca}^{2+} \) were present in the reagents and enzyme preparations composing our assay system. At higher concentrations of CaM then, traces of \( \text{Ca}^{2+} \) could support the \( \text{Cd}^{2+} \) 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 \( \text{Cd}^{2+} \) as by \( \text{Ca}^{2+} \), support the suggestion based on tyrosine fluorescence and binding studies (Habermann et al. 1983; Chao et al. 1984) that \( \text{Ca}^{2+} \) and \( \text{Cd}^{2+} \) activate CaM in a quantitatively different way. Moreover, in the PDE assay, performed under such conditions that the metal ion concentrations are crucial, \( \text{Cd}^{2+} \) and every divalent metal ion other than \( \text{Ca}^{2+} \) (except for \( \text{Pb}^{2+} \)) tested so far appeared less effective than \( \text{Ca}^{2+} \) (Habermann et al. 1983). In our experiments we tested the effects of graded amounts of \( \text{Cd}^{2+} \) on \( \text{Ca}^{2+} \)- and CaM-dependent PDE activity and found that at low \( \text{Cd}^{2+} \) concentrations competitive inhibition occurred. At equimolar concentrations of \( \text{Ca}^{2+} \) and \( \text{Cd}^{2+} \), 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 \( \text{Cd}^{2+} \) inhibition has been reported for CaM-dependent \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \)-ATPase activity in human erythrocyte membranes (Akerman et al. 1985).

Apparently, \( \text{Cd}^{2+} \) interaction with \( \text{Ca}^{2+} \)- and CaM-dependent enzyme systems is preferably exerted via CaM. This may be related to the relatively high affinity of CaM for \( \text{Cd}^{2+} \). Our results suggest that \( \text{Cd}^{2+} \), by interference with \( \text{Ca}^{2+} \)- 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.

**Acknowledgements.** It is our pleasure to acknowledge Dr. G. W. F. H. Borst Pauwels for his valuable comments during the preparation of the manuscript.

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Received April 29, 1986/Accepted September 16, 1986