Calmodulin-mediated cadmium inhibition of phosphodiesterase activity, in vitro

Gert Flik, Jan G. J. van de Winkel*, Peter Pärt**, Sjoerd E. Wendelaar Bonga, and Robert A. C. Lock

Department of Zoology II, Catholic University, Toernooiveld 25, 6525 ED Nijmegen, The Netherlands

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 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 μM Ca^{2+}). Fluctuations in cytosolic Ca^{2+} concentrations play an essential informatory 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 ac-

tivities 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 ± 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 activation is described by a sigmoidal curve, the pCd dependence follows a biphasic, more or less parabolic 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

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* *Present address:* Department of Nephrology, Catholic University, Geert Grooteplein Zuid 16, 6225 GA Nijmegen, The Netherlands

** *Present address:* Institute of Zoophysiology, University of Uppsala, S-751 22 Uppsala, Sweden

Offprint requests to: Gert Flik

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 highest purity from Merck Co. Chelex-100 was purchased from Bio-Rad Laboratories. Cadmium was used as $Cd(NO_3)_2.4H_2O$ (RCB, Brussels) or as $CdCl_2$.

N-(propionyl-^{3}H)-CaM (925 GBq/mol), $^{45}CaCl_2$ (30.5 GBq/mol) and $^{115m}CdCl_2$ (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 $g \times 2$ 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 $-20\,^\circ$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 $^{45}Ca^{2+}$ 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.

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 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 $22 \pm 1\,^\circ$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 $^{45}Ca/^3$H or $^{115m}Cd/^3$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.

Assay of cAMP-PDE. Stimulation of PDE was measured by a modification of the one-step procedure of Teo and Wang (1973). Phosphate 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 $(CH_3CO_2)_2$, 20 µM $CaCl_2$, 2 mU/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_{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.

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.
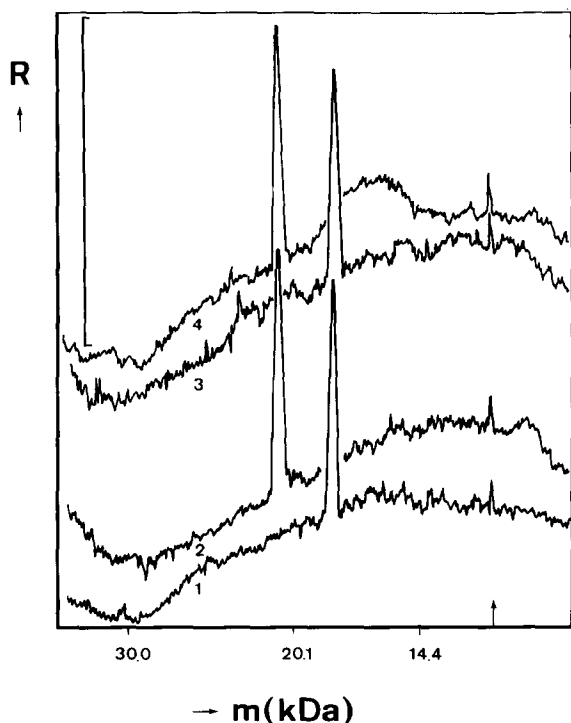
R ↑

30.0   20.1   14.4

→ m(kDa)

Fig. 1. Densitometric scan of bovine brain CaM after SDS-PAGE and silver staining of the gel. Y-axis: reflectance; *bar* indicates 100% reflectance. X-axis: position of the marker proteins for molecular mass, *m*, in kDa. *Arrow* indicates bromphenol blue front. *(1, 3)* Samples run in the presence of 1 mM $CdCl_2$ or $CaCl_2$, respectively; *(2, 4)* samples run in the presence of 2 mM EGTA and 1 mM $CdCl_2$ or $CaCl_2$, respectively

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 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 Ca^{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 μM, the concentration used in the CaM-PDE assay) essentially all

Ca^{2+} is eliminated from CaM at 20 μM $^{115m}Cd^{2+}$ (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 μM Ca^{2+}) and the occurrence of $^{115m}Cd^{2+}$ complexes upon addition of $^{115m}Cd^{2+}$. Figure 2c shows that CaM binds 4 moles Cd^{2+} per mole protein in the absence of other divalent ions (line A). In the presence of saturating concentrations of Cd^{2+} (20 μM) maximally 2 moles Ca^{2+} were bound to CaM at 20 μM Ca^{2+}. Values for apparent $K_{0.5}$ and maximum binding (B_{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 Ca^{2+}-saturated CaM, 50% of the Ca^{2+} binding sites of CaM were occupied by Cd^{2+} at 4.50±0.68 μM Cd^{2+}. The results suggest that differences in association and dissociation velocities of CaM and Ca^{2+} and of CaM and Cd^{2+} exist. When Ca^{2+} was added to Cd^{2+}-saturated CaM, a binding ratio of 2.50±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 *c*AMP 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^{-5} 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 Cd^{2+} or Ca^{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 μM) was stimulated 5-to 6-fold compared to basal PDE activity. As shown in Fig. 4, with 10 μM Cd^{2+} and a fixed 20 μM 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.
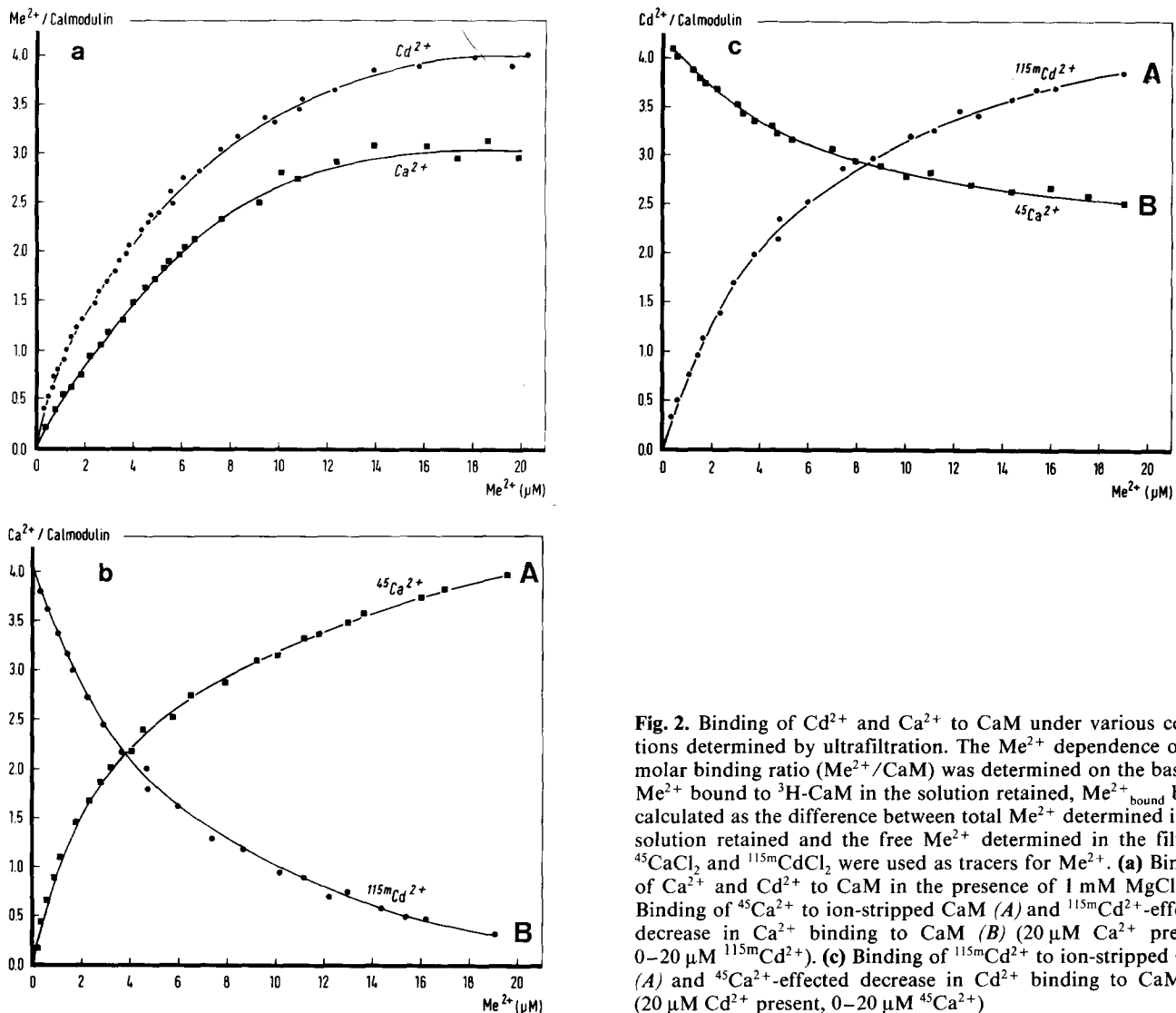
Fig. 2. Binding of Cd^{2+} and Ca^{2+} to CaM under various conditions determined by ultrafiltration. The Me^{2+} dependence of the molar binding ratio (Me^{2+}/CaM) was determined on the basis of Me^{2+} bound to ^3H-CaM in the solution retained, Me^{2+}_{bound} being calculated as the difference between total Me^{2+} determined in the solution retained and the free Me^{2+} determined in the filtrate. $^{45}CaCl_2$ and $^{115m}CdCl_2$ were used as tracers for Me^{2+}. (a) Binding of Ca^{2+} and Cd^{2+} to CaM in the presence of 1 mM $MgCl_2$. (b) Binding of $^{45}Ca^{2+}$ to ion-stripped CaM *(A)* and $^{115m}Cd^{2+}$-effected decrease in Ca^{2+} binding to CaM *(B)* (20 μM Ca^{2+} present, 0–20 μM $^{115m}Cd^{2+}$). (c) Binding of $^{115m}Cd^{2+}$ to ion-stripped CaM *(A)* and $^{45}Ca^{2+}$-effected decrease in Cd^{2+} binding to CaM *(B)* (20 μM Cd^{2+} present, 0–20 μM $^{45}Ca^{2+}$)

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

Additional Me^{2+}		Mg^{2+} (1 mM)	Ca^{2+} (20 μM)	Cd^{2+} (20 μM)
$K_{0.5}$ (Ca)	3.78 ± 1.48	6.15 ± 1.40^a	–	3.77 ± 0.55
B_{max} (Ca)	3.65 ± 0.28	3.00 ± 0.20^a	–	1.66 ± 0.14^a
	$(n = 6)$	$(n = 3)$		$(n = 3)$
$K_{0.5}$ (Cd)	3.56 ± 0.70	4.68 ± 0.97	$7.25 \pm 1.06^{a,c}$	–
B_{max} (Cd)	5.03 ± 0.87^b	$3.90 \pm 0.38^{a,b}$	$3.95 \pm 0.65^{a,c}$	–
	$(n = 4)$	$(n = 4)$	$(n = 3)$	

[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; $P < 0.005$

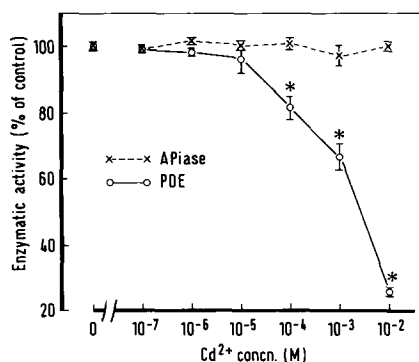[c] Significantly different from corresponding values in the presence of Cd^{2+}; $P < 0.005$

Fig. 3. Effects of Cd^{2+} on alkaline phosphatase (APiase) and CaM-independent PDE activities. Enzymic activities were determined as described in Materials and methods. $n = 4$; *asterisks* indicate significant inhibition ($P < 0.01$)
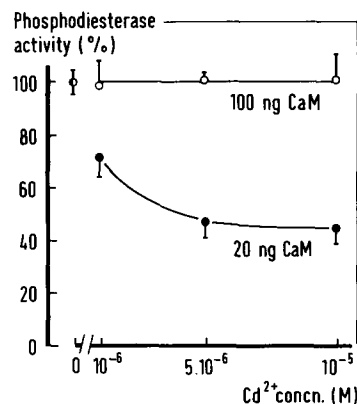

Fig. 4. Calmodulin concentration dependence of Cd^{2+} inhibition of PDE activity. At 100 ng CaM/assay, CaM-stimulated PDE activity was not affected by Cd^{2+} concentrations up to 10^{-5} M. At 20 ng CaM/assay, up to 60% inhibition of CaM-dependent PDE activity occurred at 10^{-5} M Cd^{2+}. The Ca^{2+}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

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 di- and trivalent metal ions was found to only partly mimick 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+} succesfully competes with Ca^{2+}. This result is in line with the results of Andersson et al. (1982), who showed that Mg^{2+} decreases the ^{43}Ca-CaM NMR signal
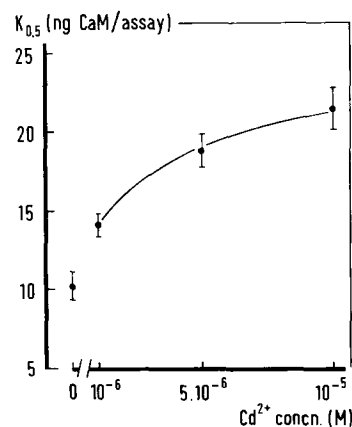
Table 2. Effects of Cd^{2+} or Ca^{2+} on calmodulin-dependent phosphodiesterase activity. Values are expressed as percentages of maximum CaM-dependent PDE activity observed at 10^{-5} M Ca^{2+} and 60 or 250 ng CaM/assay ($\Delta A_{623}/30$ min $= 1212 \pm 21 = 100 \pm 2$ %). Values in parentheses represent basal, CaM-independent PDE activities

Me^{2+} (10 μM)	PDE activity (%) CaM (ng/assay)	
	60	250
Cd^{2+}	62 ± 2^a (15 ± 1)	97 ± 1 (15 ± 1)
Ca^{2+}	94 ± 4 (16 ± 2)	100 ± 2 (17 ± 5)

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

Fig. 5. Effect of Cd^{2+} on the apparent $K_{0.5}$ values for CaM stimulation of PDE activity in the presence of 20 μM Ca^{2+}. CaM concentrations ranged from 0–100 ng/assay. $K_{0.5}$ values were derived from Eadie-Hofstee plots. At all Cd^{2+} concentrations tested $K_{0.5}$ values were found to increase significantly ($P < 0.01$) in a Cd^{2+} concentration-dependent way

(and therefore the binding of Ca^{2+} to CaM) but not the ^{113}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, per-

formed 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 $K_{0.5}$ for CaM activation of PDE activity. From this doubling of the $K_{0.5}$ 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 (Akerman 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.

References

Akerman KEO, Honkaniemi J, Scott IG, Andersson LC (1985) Interaction of Cd^{2+} with the calmodulin-activated (Ca^{2+} + Mg^{2+})-ATPase activity of human erythrocyte ghosts. Biochim Biophys Acta 845: 48–53

Andersson T, Drakenberg T, Forsen S, Thullin E (1982) Characterization of the Ca^{2+}-binding sites of calmodulin from bovine testis using ^{43}Ca and ^{113}Cd NMR. Eur J Biochem 126: 501–505

Brostrom CO, Wolff DJ (1981) Properties and functions of calmodulin. Biochem Pharmacol 30: 1395–1405

Burgess WH, Jemiolo DK, Kretsinger RH (1980) Interaction of calcium and calmodulin in the presence of sodium dodecyl sulphate. Biochim Biophys Acta 623: 257–270

Chao SH, Suzuki Y, Zysk JR, Cheung WY (1984) Activation of calmodulin by various metal cations as a function of ionic radius. Mol Pharmacol 26: 75–82

Cheung WY (1970) Cyclic 3'-5'-nucleotide phosphodiesterase. Demonstration of an activator. Biochem Biophys Res Commun 38: 533–537

Cheung WY (1981) Calmodulin and the adenylate cyclase-phosphodiesterase system. Cell Calcium 2: 263–280

Cheung WY (1984) Calmodulin: its potential role in cell proliferation and heavy metal toxicity. Fed Proc 43: 2995–2999

Cox JA (1984) Sequential events in calmodulin on binding with calcium and interaction with target enzymes. Fed Proc 43: 3000–3004

Dudley RE, Svoboda DJ, Klaassen CD (1984) Time course of cadmium induced ultrastructural changes in rat liver. Toxicol Appl Pharmacol 76: 150–160

Flik G, Van Rijs JH, Wendelaar Bonga SE (1984) Evidence for the presence of calmodulin in fish mucus. Eur J Biochem 138: 651–654

Forsen S, Thullin E, Drakenberg T, Krebs J, Seamon K (1980) A ^{113}Cd NMR study of calmodulin and its interaction with calcium, magnesium and trifluoperazin. FEBS Lett 117: 189–194

Gitelman SE, Witman GB (1980) Purification of calmodulin from Chlamydomonas: calmodulin occurs in cell bodies and flagella. J Cell Biol 98: 764–770

Habermann E, Crowell K, Janicki P (1983) Lead and other metals substitute for Ca^{2+} in calmodulin. Arch Toxicol 54: 61–60

Haiech J, Klee CB, Demaille JG (1981) Effects of cations on affinity of calmodulin for calcium: ordered binding of calcium ions allows the specific activation of calmodulin-stimulated enzymes. Biochemistry 20: 3890–3897

Kretsinger RH (1977) Evolution of the informational role of calcium in eukaryotes. In: Wasserman RH, Corrinadis RA, Carafoli E, Kretsinger RH, MacLennan DH, Siegel FL (eds) Calcium binding proteins and calcium function. Elsevier/North Holland Ppubl, Amsterdam and New York, pp 63–72

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 227: 680–685

Morrissey SE (1981) Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal Biochem 117: 307–310

Suzuki Y, Chao S-H, Zysk JR, Cheung WY (1985) Stimulation of calmodulin by cadmium ion. Arch Toxicol 57: 205–211

Teo TS, Wang JH (1973) Mechanism of activation of cyclic adenosine 3'-5'-monophosphate phosphodiesterase from bovine heart by calcium ions. J Biol Chem 248: 5950–5955

Wallace RW, Tallant EA, Dockter ME, Cheung WY (1982) Calcium binding domains of calmodulin. Sequence of fill as determined with terbium luminescence. J Biol Chem 257: 1845–1854

Wolff DJ, Brostrom CO (1979) Properties and functions of the calcium dependent regulator protein. In: Greengard P, Robison GA (eds) Advances in cyclic nucleotide research, Vol 11. Raven Press, New York, pp 27–88

Yoshiki SYT, Kimura MO, Suzuki SMT (1975) Bone and kidney lesions in experimental cadmium intoxication. Arch Environ Health 30: 559–564