Arch. Environ. Contam. Toxicol. 24, 21–27 (1993)

ARCHIVES OF
Environmental
Contamination
and Toxicology

In Vitro Effects of Cadmium, Zinc and Lead on Calmodulin-Dependent Actions in *Oncorhynchus mykiss, Mytilus* sp., and *Chlamydomonas reinhardtii*

Renata Behra

EAWAG (Swiss Federal Institute for Water Resources and Water Pollution Control), CH-8600 Duebendorf, Switzerland

Abstract. The potential of cadmium, zinc, and lead to interact with calmodulin (CaM) was investigated by examining *in vitro* CaM-dependent protein phosphorylation in tissues from rainbow trout (*Oncorhynchus mykiss*) and sea mussel (*Mytilus* sp.) and CaM-dependent phosphodiesterase (PDE) activation by algal (*Chlamydomonas reinhardtii*) extracts. Cadmium, zinc, and lead proved effective in sustaining CaM-dependent protein phosphorylation in systems containing calcium, whereas only lead was capable of CaM activation in systems depleted of calcium. Cadmium lead to a small activation of CaM-dependent PDE activity by algal extracts, corresponding to ~25% of that induced by calcium. Cadmium-induced PDE-activation could be attributed to the residual calcium present in the extract. The results indicate that metal-induced CaM activation is primarily mediated in the case of cadmium and zinc by resulting calcium/CaM complexes and in the case of lead by lead/CaM complexes.

Heavy metals are widely distributed environmental pollutants, shown in laboratory and field experiments to be toxic to most forms of life (Nordberg *et al.* 1978; Förstner and Wittman 1981). Their effects on health are very diverse including neurological, cardiovascular, bronchial and renal as well as other disorders (Dreisbach 1983). Intensive toxicological and biochemical research has led to the identification of a large number of cellular targets for essential and toxic metals (Viarengo 1985). The relationships between the binding of various metals to proteins and other targets and the actual biochemical mechanisms leading to toxicity are still unknown. However, in many biological systems some deleterious effects have been linked to an alteration of cellular processes mediated by calcium and to an upsetting of calcium metabolism (Jones and Fowler 1980; Simons 1986).

A hypothesis concerning the basis for metal-induced toxicological effects involves calmodulin (CaM) as a primary intracellular target (Cheung 1984). CaM, a ubiquitous calcium-binding protein, regulates many cellular processes and stimulates the activity of several target proteins (Cheung 1980; Klee and Vanaman 1982). Its action is dependent on the presence of calcium and is mediated through the binding of the Ca^{2+}-CaM complex to the respective proteins. The potential role of CaM in metal toxicity rests upon the finding that, besides calcium, other metals are capable of binding to CaM (Habermann *et al.* 1983; Chao *et al.* 1984) and to activate CaM-dependent enzymes (see for example Mazzei *et al.* 1984; Suzuki *et al.* 1985; Flik *et al.* 1987).

So far, only a few studies comment on the potential of metals to interact with CaM from aquatic organisms (Verbost *et al.* 1988; Lewis *et al.* 1990; Behra and Gall 1991). Previously we showed that cadmium was effective in stimulating soluble CaM-dependent phosphorylations in cultured fish cells. This paper deals with the interaction of cadmium, lead and zinc with CaM from various tissues of rainbow trout and mussels and from algae. *In vitro* effects of the metals on CaM-dependent protein phosphorylation and phosphodiesterase activity are examined. A comparative approach, using species widely used for studies in aquatic toxicology, was applied with the goal to identify general principles that govern the interactions of metals with aquatic organisms.

Materials and Methods

Chemicals

Leupeptin, pepstatin A and phenylmethanesulfonylfluoride (PMSF) were obtained from Serva (Heidelberg, FRG); trifluoperazine and tosyl-L-phenylalanin-chlormethylketon (TPCK) were obtained from Fluka Chemie (Buchs, Switzerland). Calmidazolium was a gift of J. Krebs, Institut für Biochemie (ETH, Zürich); calmodulin and calmodulin-deficient 3′,5′-cyclic nucleotide phosphodiesterase were from Sigma, division of Fluka Chemie (Buchs, Switzerland); adenosine deaminase and alkaline phosphatase (both calmodulin-deficient) were from Boehringer (Rotkreuz, Switzerland). Amersham (Zürich, Switzerland) provided [γ-^{32}P]-ATP (5000 Ci/mmol). Molecular weight markers were from Bio-Rad Laboratories (Glattbrugg, Switzerland).

Preparation of Fish Tissues

Rainbow trout, *Oncorhynchus mykiss*, weighing ~250 g each, were obtained from a local commercial hatchery. Fish were killed with a blow on the head. Liver, kidney and heart were removed, washed several times in ice-cold isolation medium (150 mM KCl, 1 mM EGTA, 50 mM Tris nitrate, pH 7.6), blotted, frozen with liquid nitro-

gen and stored at −80°C. For preparation of cytosol fractions, the tissues were thawed on ice, resuspended in homogenization medium (150 mM KCl, 1 mM PMSF, 10 μg/mL leupeptin, 3 μg/mL pepstatin A, 100 μg/mL TPCK, 50 mM Tris nitrate, pH 7.6) and finely chopped with scissors. Homogenization was performed by 25 strokes with a Teflon pestle in a Potter Elvehjem homogenizer at 2,000 rpm and 4°C. Homogenates were centrifuged in a Kontron A 8.24 rotor at 12,000 rpm (12,400 × g_{max}) for 10 min at 4°C. The supernatants were further centrifuged in a Kontron TFT 65.13 rotor at 45,000 rpm (185,000 × g_{max}) for 1 h at 4°C to yield the cytosolic fractions. This material was stored at −80°C in small aliquots and was thawed only once prior to assay.

Preparation of Mussel Tissues

Cytosol fractions from the gills and the digestive gland of the mussel Mytilus sp. were prepared in the laboratory of A. Viarengo, Istituto di Fisiologia Generale, Genova. The homogenization medium contained 150 mM KCl, 1mM PMSF, 10 μg/mL leupeptin, 1 mM imidazole, 25 mM Tris chloride (pH 7.2). Homogenization and centrifugation followed the procedure described for the preparation of fish tissues.

Preparation of Algal Extracts

Samples of *Chlamydomonas reinhardtii* (EAWAG 149b), growing in continuous culture in Z-medium (Staub 1961), were obtained from H. Bachmann, EAWAG, Dübendorf.

For the preparation of extracts, cells were pelleted at 5,000 rpm (3000 × g), washed twice with homogenization buffer (50 mM Tris nitrate, pH 7.6, containing 1 mM PMSF and 10 μg/mL leupeptin) and resuspended in two volumes of the same buffer. Homogenization was performed as described above for processing of the fish tissues. After homogenization the extract was frozen in liquid nitrogen and thawed on ice. This procedure was repeated before a second homogenization. The resulting homogenate was centrifuged twice at 8,000 rpm (7700 × g) to yield the extract.

Protein Phosphorylation

Endogenous phosphorylation was assayed in a final volume of 50 μL containing 50 mM Tris nitrate (pH 7.6), 2 mM $MgCl_2$, 40–80 μg of cytosolic proteins, 220 μM [γ-^{32}P]-ATP (containing 1-2 μCi), 1 μg CaM where indicated and various concentrations of EGTA, Ca(II), and metals. Time and temperature were optimized for maximal ^{32}P-incorporation. Assays were preincubated for 30 sec at 30°C, and the reaction was initiated by the addition of [γ-^{32}P]-ATP. After incubation for 1 min, the reaction was terminated by adding 30 μL of sample buffer (Lämmli, 1970) for sodium dodecyl sulfate-polyacrylmide gel electrophoresis (SDS-PAGE) and heating in a boiling water bath for 5 min. Samples were then analyzed by SDS-PAGE on 12.5% slab gels and autoradiography. Molecular weights on SDS gels were calibrated using phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400) as standards. Proteins were dyed with Coomassie Brillant Blue R250. Autoradiography was performed on Kodak Xomat AR films, with or without Cronex Lightning Plus intensifying screen (Dupont Instruments) for 1–3 days at −80°C. Phosphorylation experiments were repeated at least three times.

Measurement of Phosphodiesterase Activity

The ability of the *Chlamydomonas* extract to activate CaM-deficient cyclic nucleotide PDE from bovine heart was monitored spectrophotometrically by the coupled enzyme assay described by Schiefer (1986) for CaM determination, with the following modifications. The assay contained in 500 μL final volume 50 mM Tris nitrate pH 7.6, 1 mU phosphodiesterase, 1 U adenosine deaminase, 6 U alkaline phosphatase, various concentrations of Ca(II) or Cd(II) in presence of 10 μM EGTA and 75 μg of algal protein. All reagent and enzyme solutions were depleted from calcium as described below. In this way, the final total Ca(II) concentration in the assay was ~2 μM. Data were statistically analyzed by the Mann-Whitney U-test. Statistical significance was accepted for $p < 0.05$.

Miscellaneous

Protein concentration was determined by the method of Bradford (1976), using the Bio-Rad Protein Assay Reagent and bovine serum albumin as a standard. The CaM content in the various tissues was determined as described earlier (Behra and Gall 1991).

The concentrations of the free metal ion species (mentioned in the Results and in Discussion sections) were estimated by speciation calculations taking into consideration complexes with defined ligands, as present at the various conditions. Calculations were computed with the Mac-MicroQL-program (Westall 1979). Stability constants of the ligands EGTA, 2-mercaptoethanol and chloride for the different metals were obtained from Smith and Martell (1975) and those of ATP from Sillen and Martell (1971) and Verbost *et al.* (1988). Stock solutions of metals were prepared from the chloride salts of Ca(II), Cd(II) and Zn(II) and from the nitrate salts of Pb(II).

In order to keep calcium contamination low, glassware and plasticware were rinsed with 5 mM EDTA and then exhaustively with double-distilled water. Dyalisis tubing (Visking, Chemie Brunschwig AG, Basel, Switzerland) was successively boiled in (1) deionized water, (2) 0.5% $NaHCO_3$, (3) five changes of double-distilled water treated with Chelex-100, and (4) two changes in 5 mM EDTA. The tubing was stored in 5 mM EDTA, pH 7.6, 0.2% sodium azide at 4°C. Before use, the tubing was rinsed several times with bidistilled water.

For calcium depletion, buffers, reagents and enzymes were passed through small Chelex-100 columns, whereas the various cytosol fractions and the algal extract were incubated overnight with Chelex-100 suspended in 50 mM Tris nitrate, pH 7.6. Calcium concentrations were measured with an atomic absorption spectrometer (Perkin Elmer 5000).

Results

Calcium and Calmodulin-Dependent Phosphorylations and Effects of Cadmium

The patterns of phosphorylation of endogenous proteins in soluble fractions of various rainbow trout tissues are shown in Figure 1. In a typical experiment Ca^{2+}/CaM-dependent phosphorylations were identified by comparison of phosphorylation patterns obtained in presence of Ca(II) and with no further additions, EGTA or a CaM antagonist. In that way, in the heart cytosol, at least seven protein bands with molecular weights of ~28, 43, 44, 56, 58, 60, and 64 kDa could be attributed to phosphorylation by Ca^{2+}/CaM-dependent protein kinases. Phosphorylation of these substrates was not detectable in presence of 1 mM EGTA (Figure 1A, lane 1) but was visible in presence of 0.5 mM Ca(II) (Figure 1A, lane 2) and slightly enhanced by addition of exogenous CaM (Figure 1A, lane 3). The pattern of phosphorylation in presence of the CaM antagonist trifluoperazine (TFP; Figure 1A, lane 4) corresponded to that with EGTA, indicating the mediation of CaM in supporting

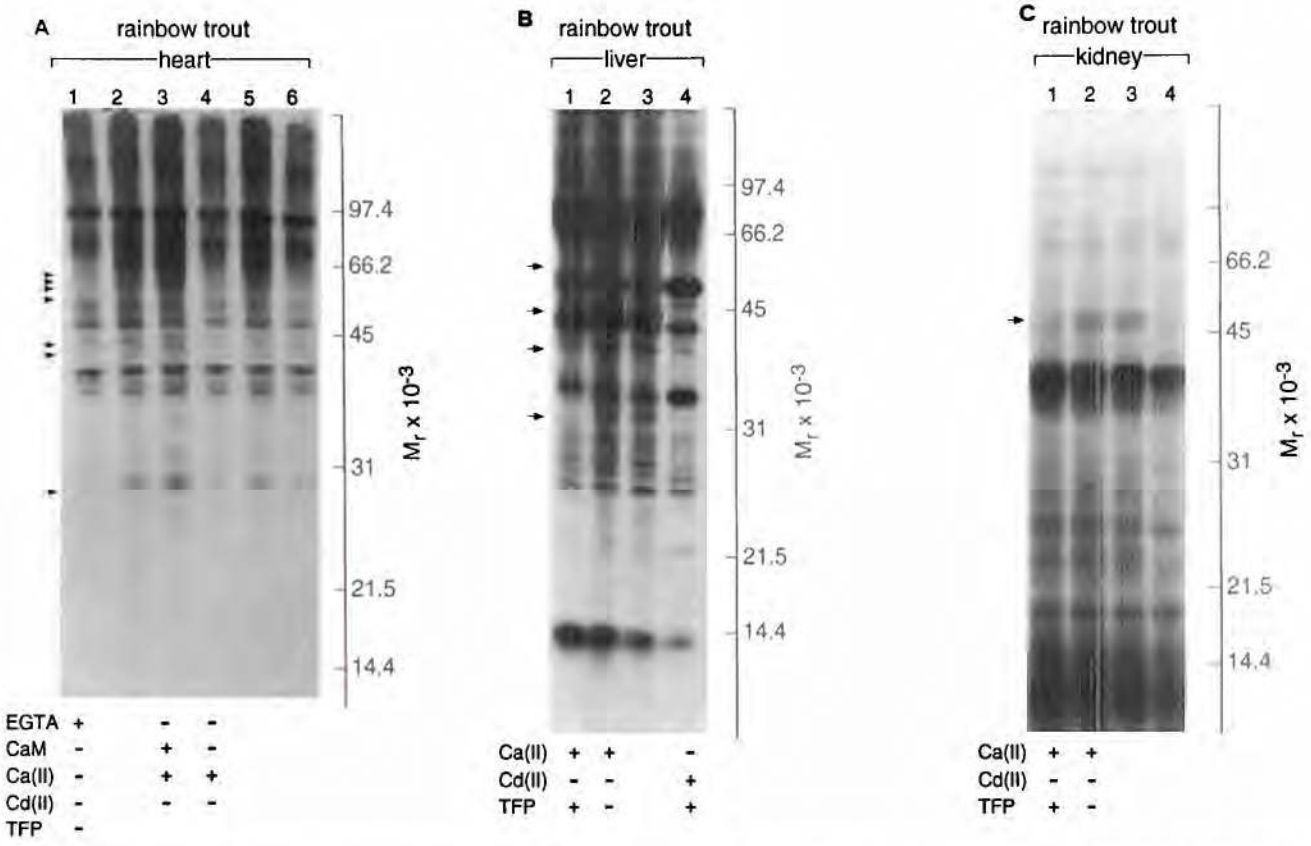

Fig. 1. Autoradiographs showing soluble phosphorylation in rainbow trout using: (A) 60 μg heart; (B) 50 μg liver; (C) 43 μg kidney proteins. Samples were assayed with the following additions: 1 mM EGTA (A, lane 1); 0.5 mM Ca(II) (A, B, C, lane 2); 0.5 mM Ca(II) and 1 μg CaM (A, lane 3); 0.5 mM Ca(II) and 20 μM TFP (A, lane 4; B, C, lane 1); 10 μM EGTA and 50 μM Cd(II), (~6 μM Cd^{2+}; A, lane 5; B, C, lane 3); 10 μM EGTA, 50 μM Cd(II) and 20 μM TFP (~6 μM Cd^{2+}; A, lane 6; B, C, lane 4). Total Ca(II) concentration in assays without addition of exogenous Ca(II) was: heart 11; liver, 9; kidney 6 μM. Endogenous CaM content in assays without addition of exogenous CaM was: heart ~40; liver, ~100; kidney ~150 ng. M_r of major substrates for Ca^{2+}/CaM-dependent protein kinases are indicated by arrows. Their corresponding M_r are: heart, ~28, 43, 44, 56, 58, 60, and 64 kDa, liver ~23, 34, 38, 45, and 62 kDa, kidney, ~45 kDa

the observed Ca^{2+}-dependent phosphorylations. In kidney fractions (Figure 1C), a protein of M_r ~45 kDa was apparently the sole substrate phosphorylated in a Ca^{2+}/CaM-dependent manner, whereas in liver cytosol (Figure 1B) at least five proteins with M_r ~23, 34, 38, 45, and 62 kDa were found to be dependent on Ca^{2+} and CaM for phosphorylation. All these substrates were found to be phosphorylated in presence of 0.5 mM Ca(II) (Figures 1B and C, lane 2) but not upon addition of both, Ca(II) and TFP (Figures 1B and C, lane 1).

In all examined tissues CaM-dependent protein phosphorylation was triggered also upon addition of 50 μM Cd(II) and 10 μM EGTA, the resulting calculated Cd^{2+} concentration being ~6 μM (Figure 1A, lane 5; Figures 1B, C, lane 3). Phosphorylation the same substrates was absent by concomitant addition of Cd(II) and TFP (Figure 1A, lane 6; Figures 1B, C, lane 4). Phosphorylation patterns obtained in presence of Cd(II) were indistinguishable from those observed in presence of Ca(II) alone.

Phosphorylation of soluble proteins from tissues of the sea mussel (Figure 2) revealed only one protein from the digestive gland as substrate of Ca^{2+}/CaM-dependent kinases. As such, this protein of M_r ~47 kDa was phosphorylated in presence of 0.5 mM Ca(II) but not after addition of 1 mM EGTA or TFP (Figure 2B, lanes 2, 1 and 3, respectively). This protein was found to be phosphorylated at calculated ~6 μM Cd^{2+} upon addition of 50 μM Cd(II) and 10 μM EGTA to the phosphorylation mixture (Figure 2, lane 4); phosphorylation of the same protein was blocked in presence of Cd(II) and TFP (Figure 2, lane 5).

Examination of the phosphorylation patterns of soluble sea mussel proteins further showed one protein of M_r ~33 kDa from the gills and two proteins of M_r ~35 and 36 kDa from the digestive gland. Their phosphorylation was prevented by TFP but not by EGTA (Figure 2A and B, lanes 3 and 1, respectively). The involvement of CaM was confirmed by the observation that phosphorylation of the three proteins was also blocked by 1 μM calmidazolium (not shown), a potent CaM antagonist (Van Belle 1981).

Phosphorylation experiments with the algal extract were not indicative of Ca^{2+}/CaM-dependent protein kinase activities.

Effects of Cadmium, Zinc and Lead on Ca^{2+}/CaM-Dependent Phosphorylations in Calcium-Depleted Systems

The observed Cd^{2+}-induced CaM-dependent phosphorylation activities may be due to a direct interaction of the metal with

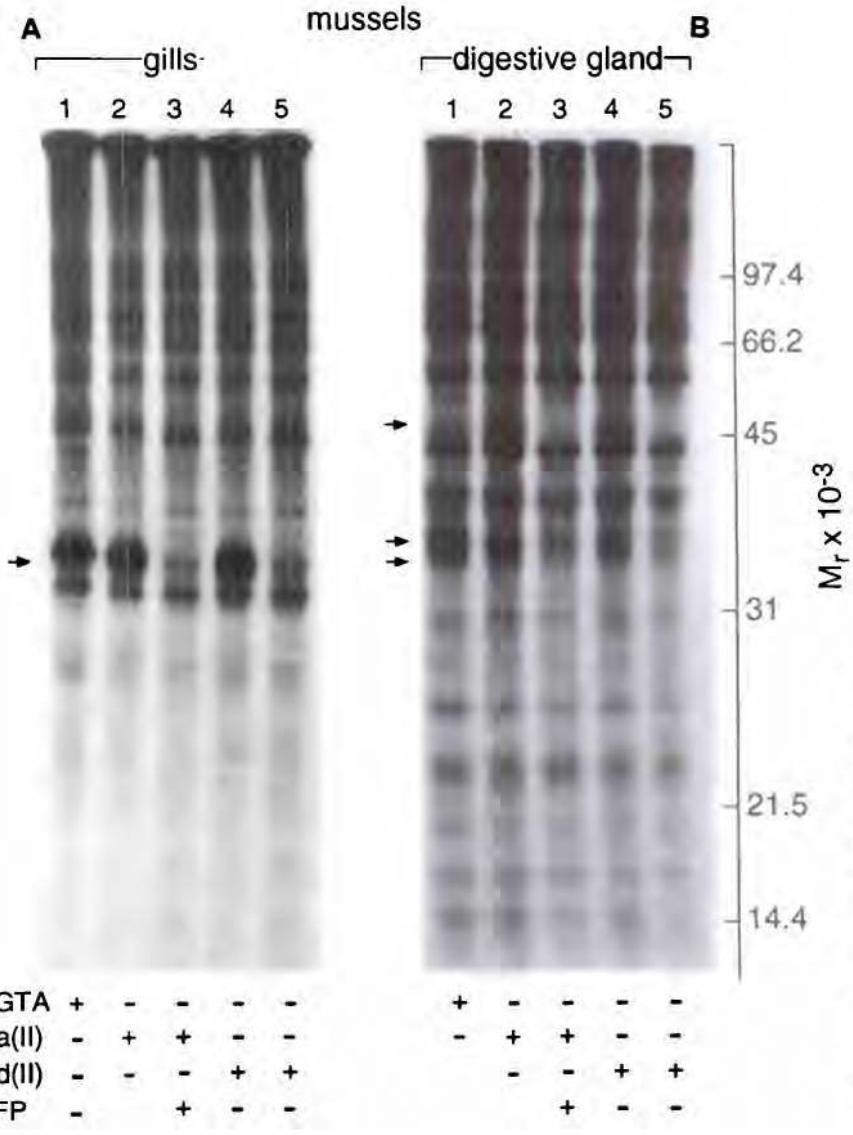

Fig. 2. Autoradiographs illustrating phosphorylation of cytosolic mussel proteins (80 μg each) from: (A) gills; (B) digestive gland. Samples were assayed in presence of 1 mM EGTA (lane 1); 0.5 mM Ca(II) (lane 2); 0.5 mM Ca(II) and 20 μM TFP (lane 3); 10 μM EGTA and 50 μM Cd(II), (~6 μM Cd^{2+}; lane 4); 10 μM EGTA, 50 μM Cd(II) and 20 μM TFP (~6 μM Cd^{2+}; lane 5). Total Ca(II) concentration in assays without addition of exogenous Ca(II) was: gills, 20 and digestive gland, 15 μM. Endogenous CaM content in assays without addition of exogenous CaM was: gills and digestive gland, ~300 ng each. Arrows indicate proteins discussed. The corresponding M_r are: gills, ~33 kDa, digestive glands, ~47, ~35, and 36 kDa

CaM or to an indirect activation through displacement of Ca^{2+} from other cytosolic Ca^{2+}-binding sites to CaM. In order to distinguish between these mechanisms, phosphorylations were further examined in systems previously depleted of endogenous Ca(II). Treatment of the various cytosols with Chelex-100 lowered the endogenous Ca^{2+} content so that activation of CaM-dependent phosphorylation became dependent on the addition of Ca(II). As shown in Figure 3 for the liver extract, CaM-dependent phosphorylation was minimal at the residual total Ca(II) ~1 μM, increased upon addition of Ca(II) and was maximal at a Ca^{2+} concentration of ~10 μM (Figure 3, lanes 1–4). Compared to the phosphorylation pattern obtained at the residual Ca^{2+} concentration, (Figure 3, lane 1), additions of Cd(II) or Zn(II) did not enhance ^{32}P-incorporation into substrates of Ca^{2+}/CaM-dependent protein kinases. In presence of 10 μM EGTA and at concentrations as high as 100 μM Cd(II) and 30 or 50 μM Zn(II) (corresponding to calculated free ion concentrations of ~14 μMCd^{2+} and ~18 μM or ~35 μM Zn^{2+}), both metals inhibited the CaM-dependent as well as other cytosolic phosphorylation activities (Figure 3, lanes 5, 10, and 11). Both metals showed to be ineffective in stimulating CaM-dependent phosphorylations also in all other previously mentioned Ca^{2+}-limited cytosols (not shown).

Contrary to Cd^{2+} and Zn^{2+}, Pb^{2+} proved very efficient in triggering CaM-dependent phosphorylations in the Ca^{2+}-limited liver cytosol; the Pb^{2+}-induced phosphorylation activity was blocked by TFP (Figure 3, lanes 6–9). At the lowest tested Pb(II) concentration of 50 μM and in presence of 10 μM EGTA (corresponding to a calculated Pb^{2+} concentration of ~11 μM), ^{32}P-incorporation into substrates of CaM-dependent protein kinases was higher than that at the corresponding Ca^{2+} concentration (Figure 3, lanes 6 and 3). The minimal requirement of Pb^{2+} for activation of CaM was not established. Pb^{2+} was found to mimic Ca^{2+} in activating CaM also in Ca(II) depleted cytosols from the heart and kidney of rainbow trout and from the digestive gland of mussels (not shown).

Effects of Cadmium on Activation of Phosphodiesterase by Calmodulin from Chlamydomonas

In order to examine the interaction of heavy metals with *Chlamydomonas* CaM, the activity of CaM-deficient cyclic

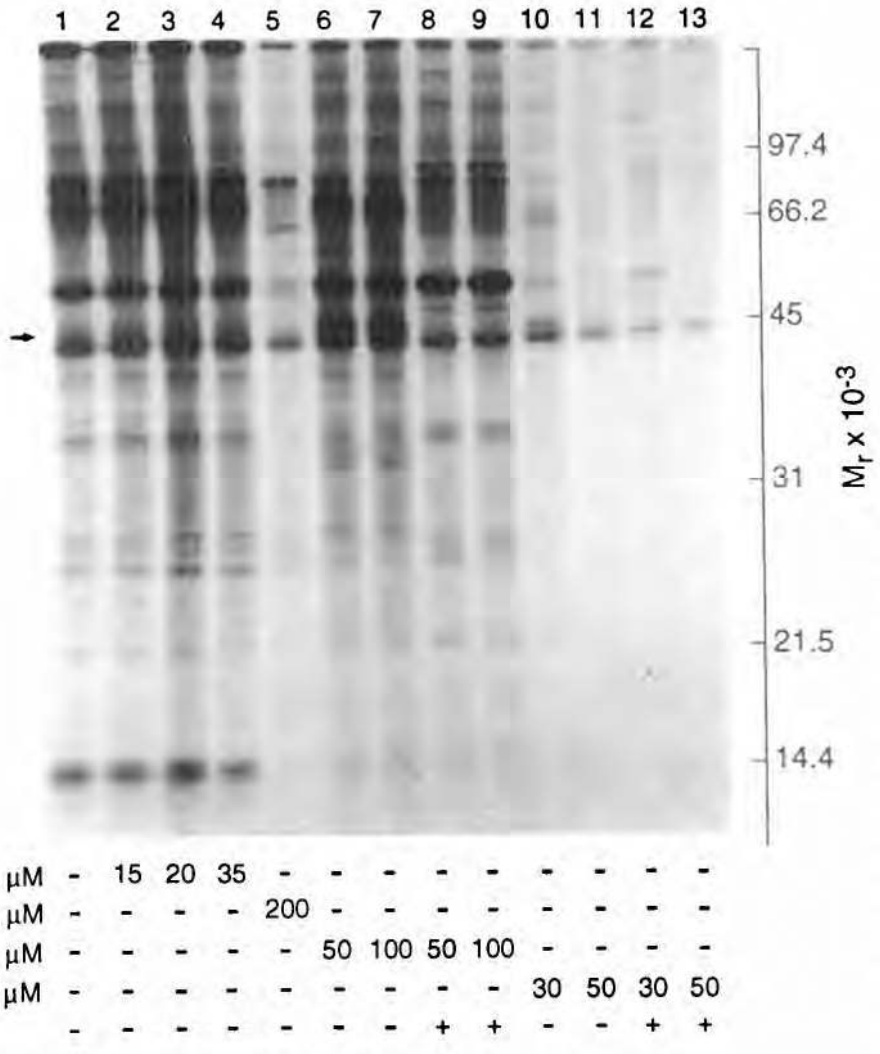

Fig. 3. Autoradiograph showing effects of cadmium, lead and zinc on phosphorylation of cytosolic liver proteins from rainbow trout after depletion of calcium. Samples were assayed directly (lane 1) or in presence of 10 µM EGTA (lanes 2–13) and the following additions: 15, 20, and 35 µM Ca(II) (5, 10, and 25 µM Ca^{2+}; lanes 2–4, respectively); 100 µM Cd(II) (~14 µM Cd^{2+}, lane 5); 50 and 100 µM Pb(II) (~11 and ~26 µM Pb^{2+}, respectively) in absence (lanes 6, 7) or in presence of 20 µM TFP (lanes 8, 9); 30 and 50 µM Zn(II) (~18 and ~35 µM Zn^{2+}, respectively) in absence (lanes 10, 11) or in presence of 20 µM TFP (lanes 12, 13). Residual total Ca(II) concentration in assays without addition of exogenous Ca(II) was ~1 µM. The arrow indicates one major substrate for Ca^{2+}/CaM-dependent protein kinases with M_r of ~45 kDa

nucleotide phosphodiesterase (PDE) from bovine heart was assayed with the algal extract as source of CaM and previously depleted of Ca(II) and in presence of various amounts of Ca^{2+} or Cd^{2+}. As shown in Figure 4, activation of PDE by the algal extract was dependent on the addition of exogenous Ca(II), half-maximal stimulation occuring at ~10 µM Ca^{2+}. Also depicted in Figure 4 is the effect of Cd^{2+} on both basal and CaM-dependent PDE activity. Addition of Cd(II) actually led to a weak but significant stimulation of CaM-dependent PDE activity. Maximal activity (14.76 nmol/mL/min; S.D. = 0.33; N = 6) corresponded to ~25% of that induced by Ca^{2+} and occurred at ~7.5 µM Cd^{2+} upon addition of 20 µM Cd(II) and 10 µM EGTA. CaM-dependent PDE activity decreased gradually and significantly ($p < 0.05$) compared to the basal activity, at concentrations higher than 7.5 µM Cd^{2+}. Inhibition of basal PDE activity was observable at Cd^{2+} concentrations higher than 100 µM Cd^{2+}. The effects of Pb^{2+} on algal CaM could not be examined because concentrations as low as 1 µM Pb^{2+} proved already inhibitory on basal PDE activity.

Discussion

The studies reported here were undertaken in order to examine possible interactions between some metals of toxicological interest and CaM from various aquatic organisms. For this purpose tissues of the rainbow trout and sea mussel and algal extracts were first characterized with respect to soluble CaM-dependent protein phosphorylations and activation of cyclic nucleotide PDE.

The results of this investigation indicate that CaM-dependent protein phosphorylation is a common feature of rainbow trout tissues. The highest CaM-dependent kinase activity was observed in the heart tissue, followed by that in the liver, whereas only one substrate could be identified in the kidney. In a previous study we have characterized soluble CaM-dependent phosphorylations in a rainbow trout gonadal cell line and found a very high phosphorylation activity (Behra and Gall 1991). Although the cell line was shown to have a high content of CaM, the CaM-dependent kinase activities described in the present study apparently do not correlate with the CaM content of the various tissues, the highest soluble CaM concentration having been found in the kidney. On the basis of a molecular weight comparison no common cytosolic substrate for CaM-dependent protein kinases could be found.

Examination of mussel tissues led to the identification of only one substrate of CaM-dependent protein kinases in the digestive gland. Although not directly relevant in the context of this study one additional observation is worth making. Two

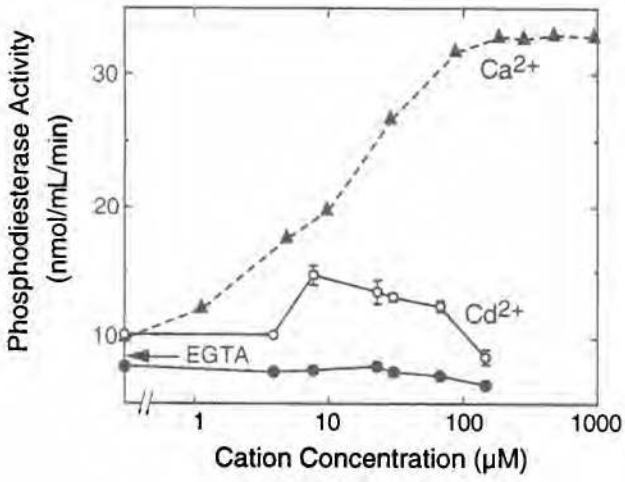

Fig. 4. Effect of Ca^{2+} and Cd^{2+} on PDE activation by algal extracts depleted of endogenous calcium. Metals were tested in presence of 10 μM EGTA. Added metal and calculated free metal concentrations were: 11, 20, 40, 100, 200, 300, 500 and 1000 μM Ca(II), corresponding to ~1.1, 9.6, 28.9, 86.7, 183, 279, 472 and 954 μM Ca^{2+} or 15, 20, 40, 50, 100 and 200 μM Cd(II), corresponding to ~3.8, 7.6, 22.8, 30, 68 and 145 μM Cd^{2+}. The calculated free ion concentration of each cation is indicated in the abscissa. Effect of cadmium on basal PDE activity in absence of algal extract is indicated by open circles. Bars represent ± 1.96 standard error (95% confidence interval; N = 6). The arrow shows the basal activity in presence of algal extract and 1 mM EGTA. Residual total Ca(II) concentration in samples without addition of exogenous Ca(II) was 2 ± 0.25 μM

additional proteins from the digestive gland, as well as one protein from the gills, were unexpectedly found to be phosphorylated in the presence of EGTA or Ca(II) but not in presence of the CaM antagonists TFP or calmidazolium. These results, together with only few other reports (Geiser 1991 and references cited therein), suggest a calcium-independent function for CaM.

Phosphorylation experiments in the presence of Cd^{2+}, Pb^{2+}, or Zn^{2+} showed that in systems containing Ca^{2+} all metals supported CaM-dependent protein phosphorylations but that, in systems relatively free of Ca^{2+} contamination, only Pb^{2+} did. These results demonstrate the potential of Cd^{2+}, Pb^{2+}, and Zn^{2+} to trigger CaM actions, though through different activation mechanisms. Whilst Pb^{2+} stimulates CaM-dependent reactions through direct binding to CaM, indirect experimental evidence indicates that Cd^{2+} and Zn^{2+} act by a mechanism involving displacement of Ca^{2+} from intracellular binding sites and resulting in CaM activation through the increase in free calcium ion concentration. Indirect evidence for a rise in intracellular free Ca^{2+} concentration upon exposure of various cell types to Cd^{2+} has been reported in other studies (Verbost *et al.* 1989 and references cited therein).

Studies of PDE activation by CaM from algae further supported the mode of Cd^{2+}-induced CaM activation by a displacement mechanism. Introduction of Cd^{2+} in the assay induced a weak activation of CaM-dependent PDE. The residual Ca(II) concentration in the PDE assay system, estimated from Ca(II) determinations in the algal extract after Ca(II) depletion to be ~2 μM, may actually explain the observed PDE activation. Upon addition of Cd(II) displacement of Ca^{2+} from ligands present in the assay system would result in an increase of free Ca^{2+} concentration high enough, when compared to PDE activation by Ca^{2+}, to account for the observed PDE activation. According to a Ca^{2+}-displacement mechanism, maximal PDE activity would be dependent on the maximal Ca^{2+} concentration available for activation. This suggestion is also supported by the fact that concentrations of Cd^{2+} higher than those leading to maximal PDE activity only led to a progressive inhibitory action. Under these conditions, there is no further increase in free Ca^{2+} concentration and thus in PDE activity and the inhibitory effects of Cd^{2+} become visible. Similar mechanisms have been proposed by Verbost *et al.* (1988) as an explanation of the reported biphasic effect of Cd(II) on PDE (Chao *et al.* 1984; Suzuki *et al.* 1985; Flik *et al.* 1987) and other CaM-dependent enzymes (Mazzei *et al.* 1984).

In summary, the results from phosphorylation and PDE activation experiments indicate that in presence of cytosolic competing binding sites Cd^{2+} and Zn^{2+} have a poor, if any, accessibility to CaM, whereas Pb^{2+} is apparently available for binding to CaM. The results also indicate that observed CaM activations are mainly mediated in the case of Cd^{2+} and Zn^{2+} by resulting Ca^{2+}/CaM complexes and in the case of Pb^{2+} by Pb^{2+}/CaM complexes, thus confirming the suggested involvement of metals in activating CaM-dependent actions (Cheung 1984). This last suggestion was inferred from binding experiments that demonstrated that besides Ca^{2+} also other metals proved capable to bind to CaM (Chao *et al.* 1984; Suzuki *et al.* 1985; Habermann *et al.* 1983) and that the resulting metal-CaM complexes were efficient in activating CaM-dependent actions (Goldstein and Ar 1983; Mazzei *et al.* 1984).

The importance of CaM in mediating the effects of calcium to many different target proteins and as a regulator of many cellular processes (Cheung 1980; Klee and Vanaman 1982) ask for examination of the potential consequences of its activation with respect to metal-induced toxicity. At present, however, such a direct relationship has not been established. In this context it should be considered that Cd^{2+} and Pb^{2+} have been recently shown to activate skeletal muscle troponin C, a Ca^{2+}-binding protein highly homologous to CaM (Chao 1991) and also that other Ca^{2+} receptors have been shown to be highly sensitive to heavy metals. Nanomolar concentrations of Cd^{2+} inhibit branchial Ca^{2+} uptake (Verbost *et al.* 1988), and picomolar concentrations of Pb^{2+} activate protein kinase C (Markovac and Goldstein 1988).

Determination of the extent to which metals are available to these different targets *in vivo* awaits further experimental evaluation in order to delineate the biochemical determinants of metal toxicity.

Acknowledgments. I thank G. Genoni and H. Güttinger for their critical reading of the manuscript. I am indebted to A. Viarengo for providing the mussel tissues, to D. Kistler for assistance in measurement of calcium, to H. Bolliger for the artwork and to P. Schlup for the photography.

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Manuscript received April 6, 1992 and in revised form August 5, 1992