Bull. Environ. Contam. Toxicol. (1989) 43:541–547

Environmental Contamination and Toxicology

Cadmium Inhibits Brain Calmodulin: *In vitro* and *in vivo* Studies

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The toxic effects of divalent cations especially Cd^{2+}, are related to their chemical and physical characteristics viz. ion polarizability, electronic structure and the hard and soft characteristics (Williams et al 1982). Cadmium ion, a hazardous environmental pollutant (Friberg et al 1974) in areas in which cadmium nickel batteries are manufactured, accumulates in tissues such as kidney, liver, brain or bone with a very slow turnover and can cause pathophysiological disorders (Samarawickarama 1979; Giles et al 1983; Nath 1986).

Calmodulin (CaM), a Ca^{2+}binding protein is found in many if not most eukaryotic cells. It is a low molecular weight, heat and acid stable protein whose amino acid sequence has been conserved, almost perfectly throughout evolution (Cheung 1984). The protein mediates the control of a large number of enzymes by Ca^{2+}fluxes and alterations in these fluxes have been postulated to be involved in steps leading to irreversible cell damage (Mergner et al 1981; Cox and Harrison 1983). The present study demonstrates an interaction of Cd^{2+} with CaM resulting in an impairment of CaM-dependent phosphodiesterase (PDE) stimulation in vitro. Biological activity of CaM is also affected in brain (cerebral cortex) of Cd-exposed rats in vivo.

MATERIALS AND METHODS

The activator deficient PDE, bovine brain CaM, PDE free snake venom, Dowex 1x8 (200-400 mesh) anion exchanger, used in these experiments were purchased from Sigma Chemical, St. Louis, MO. (2,8-^{3}H) adenosine 3'-5' cyclic phosphate (specific activity 41.7 Ci/mmole) was obtained from Amersham International Plc, England. All other chemicals used were highest grade commercial products. All glassware were acid washed in concentrated nitric acid (AR grade) and rinsed with glass-distilled deionized water.

Bovine brain CaM (2.8 μM) was incubated with varying concentrations of Cd^{2+} (0.044, 0.22, 0.44, 0.88 and 1.77 mM) for 24 hr at 25°C. After incubation, samples were dialysed against 20 mM Tris-HCl, pH 7.5 till no Cd^{2+} was detected in dialysates. Cadmium was estimated

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on Atomic Absorption Spectrophotometer, Perkin Elmer, Model 4000. Calmodulin activity was subsequently assayed by measuring stimulation of activator deficient PDE (Cox and Harrison 1983). The reaction mixture contained 40 mM Tris HCl pH 8.0, $CaCl_2$ (50 µM), PDE (60 µg) and CaM (400 ng) in a final volume of 0.1 ml. The reaction was started by the addition of (^3H) cAMP (2 mM, 0.01 µCi). After 10 minutes incubation at 30°C, the reaction was terminated by placing the tubes in water bath (for 2 min) maintained at 95°C. After an additional 10 min incubation with snake venom (1 mg/ml) at 30°C, (^3H) adenosine was separated from untreated (^3H) cAMP using Dowex 1x8 (active chloride form, anion exchanger) resin (33% slurry, pH 5) and radioactivity was counted by Liquid Scintillation Counter (Packard, Model 2425). Quench correction was done by external standard. Basal PDE activity was assayed by incubating with varying concentrations of Cd^{2+} in absence of CaM. Protein concentrations were determined by Lowry method (Lowry et al 1951).

Male adult Wistar rats (100-140 g) were divided into five groups of eight each. The animals were fed on a stock diet (Hindustan Lever Ltd., Bombay, India) and water ad-libitum. Group I animals served as control and received normal saline through gastric intubation. Group II, III, IV and V animals received 3, 6, 9 and 12 mg Cd/kg body weight daily (as $CdCl_2$ in normal saline) through gastric intubation for 4 weeks respectively. The animals were sacrificed and the brains were removed and immediately stored at -20°C.

Five percent rat brain (cerebral cortex) homogenates were prepared in 20 mM Tris-HCl, pH 7.5 containing 1mM EGTA (Wallace et al 1983). The homogenates were heat treated in a hot water bath at 95°C for 2 min. The heat treated supernatants were again centrifuged at 12,000 g for 20 min. Calmodulin was assayed by the method of Cox and Harrison (1983). Rat brain (cerebral cortex) homogenates before and after heat-treatment were electrophoresed on polyacrylamide gels (Davies 1964). Bovine brain calmodulin was used as standard. Five hundred milligrams of brain tissue was digested by the method of Ravi et al (1984). Cadmium was estimated on Atomic Absorption Spectrophotometer, Perkin Elmer, Model 4000.

RESULTS AND DISCUSSION

Calmodulin when incubated with varying concentrations of Cd^{2+}, demonstrated decreased ability to stimulate CaM-dependent PDE (Fig. 1). Figure 2 shows the activation curve of PDE by CaM and a heat-treated extract of rat brain. Approximately 12% of the protein in the heat-treated brain extract is CaM. Figure 3 shows the electrophoretic pattern of rat cerebral cortex homogenate before and after heat treatment and bovine brain calmodulin as standard. Heat treatment of the homogenate removed most of the contaminating proteins that might have interfered in calmodulin assay. The body weight of rats exposed to varying concentrations of Cd decreased by 34.70%, 33.68%, 40.82 % and 70.41% respectively (Table 1).

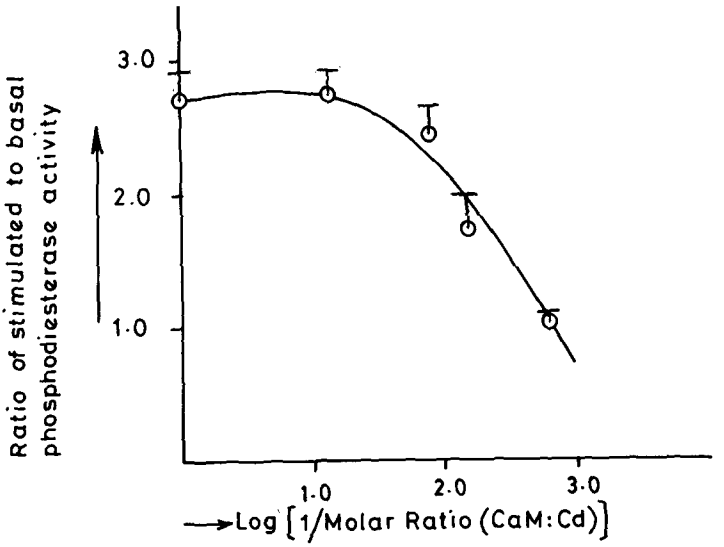

Figure 1. Effect of varying concentrations of Cd on bovine brain calmodulin in vitro reflected as reduced activation of calmodulin-dependent cyclic nucleotide phosphodiesterase. Each point is a mean ± S. D. of two independent experiments (n=6).

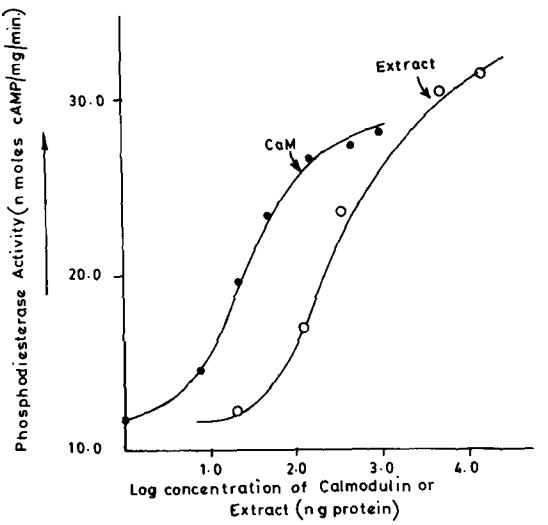

Figure 2. Activation curves of PDE by bovine brain CaM and heat-treated extract of rat brain. A heat-treated rat brain extract is prepared and assayed for CaM activity as described in the text. The abscissa refers to the amount of CaM or the amount of protein in the heat-treated extract. By comparing the points giving 50% activation of PDE, we estimated that CaM may account for 12% of the protein in the heat-treated extract. Each point is a mean of two independent experiments (n=6).

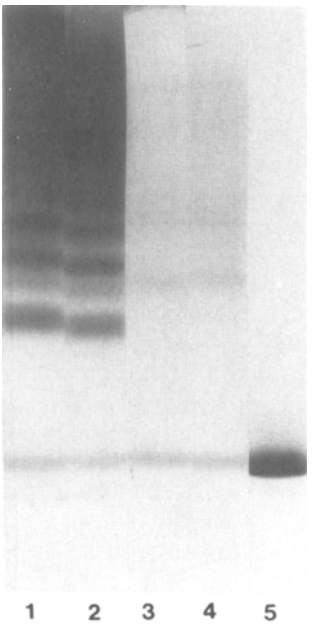

Figure 3. Electrophoretic pattern of rat brain calmodulin: Cerebral cortex homogenate (1 and 2); heat-treated cerebral cortex homogenate (3 and 4); bovine brain calmodulin as standard (5).

Table 1. Effect of varying doses of cadmium on body weight of rats

Group	Initial weight (g)	Final weight (g)	% gain in weight
I (Control)	113±6.13	211±25.02	86.7
II (3mg Cd/kg)	123±17.02	187±23.62	52.0
III (6mg Cd/kg)	110±12.70	175±10.28	59.0
IV (9mg Cd/kg)	109±16.89	167±14.44	53.2
V (12mg Cd/kg)	135±18.25	164±14.61	21.4

Each value is Mean ± S. D. of 6-8 animals. Animals were treated for 4 weeks.

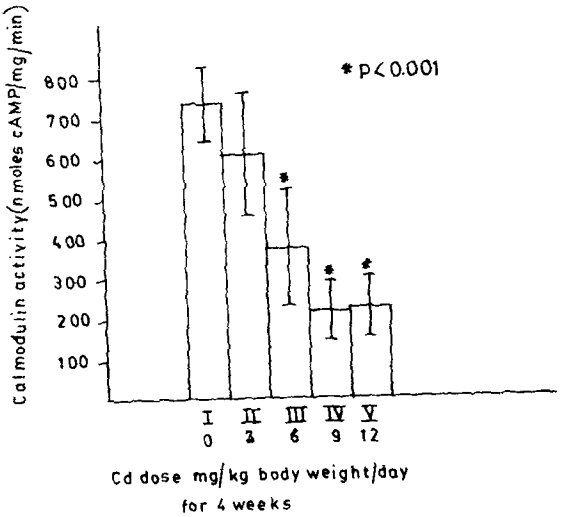

Figure 4. Calmodulin activity decreased *significantly* ($p < 0.001$) after 6, 9 and 12 mg Cd/kg body weight (in normal saline) treatment. Cd (as $CdCl_2$) was given through *gastric intubation* daily for 4 weeks. Calmodulin activity is expressed in terms of calmodulin dependent phosphodiesterase stimulation (nmoles cAMP/mg protein/min).

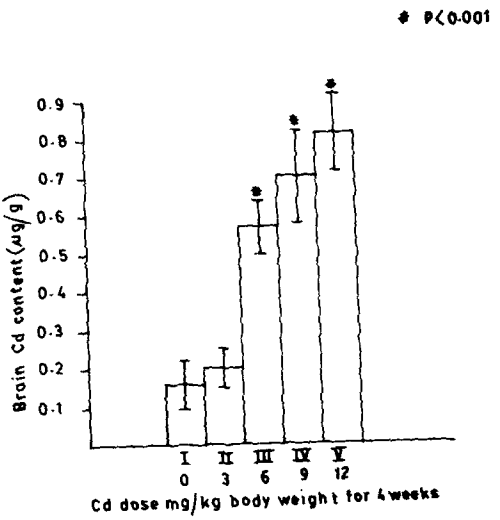

Figure 5. Cadmium content in rat brain after exposure to different concentrations of cadmium. Cadmium accumulated significantly ($p<0.001$) after 6, 9 and 12 mg Cd/kg body weight treatment.

A significant decrease in CaM activity was observed in brain (cerebral cortex) of rats treated with 6, 9 and 12 mg Cd/kg body weight respectively ($p < 0.001$). However, the rats treated with 3 mg Cd/kg body weight did not show any significant change (Fig. 4). A significant amount of Cd-accumulated in cerebral cortices of rats exposed to 6, 9 and 12 mg Cd/kg body weight whereas the group treated with 3 mg Cd/kg body weight did not show any significant change (Fig. 5).

In vitro interaction of Cd^{2+} with CaM, resulting in an impaired ability of CaM to stimulate CaM-dependent PDE can be attributed to the similar ionic radii of Ca^{2+} (0.99 A°) and Cd^{2+} (0.97 A°) (Cheung, 1984; Akerman et al 1985). *In vitro* inhibition of CaM by Cd^{2+} also corroborates data of Chao et al (1984) who reported that 100 µM Cd^{2+} gives a 69% inhibition of ^{45}Ca binding to CaM.

There was a considerable decrease in the body weight of rats chronically exposed to Cd and these results are in agreement to the findings of Weber and Singhal (1964) and Tandon and Tewari (1987). The decrease in body weight after Cd-exposure can be attributed to the enhanced synthesis of glucose from non-carbohydrate sources that is mobilization and utilization of fat deposits in liver and kidneys (Tandon and Tewari 1987). With chronic exposure, Cd^{2+} accumulates in many mammalian tissues, and liver and kidneys accumulate amounts significantly higher than other organs (Friberg et al 1974; Nath 1986). In these organs cadmium remains bound to cadmium induced metallothioneins (Friberg et al 1974; Samarawickarama 1979; Nath 1986) and thus is rendered nontoxic to a significant extent. Metallothionein like Zn^{2+} binding protein found in brain (Ebadi 1986) may not be the expected target of Cd. This brain metallothionein-like protein can be differentiated from the hepatic metallothionein in that the brain type binds neither copper nor cadmium; hence it may not participate in a metal detoxification mechanism (Ebadi and Swanson 1987). Therefore, there is a possibility that Cd^{2+} may interact with brain calmodulin. We found that there was a significant accumulation of cadmium in rat brain after chronic Cd-exposure and it increased with increasing doses of Cd. This correlated well with rat brain CaM inhibition. Mills and Johnson (1985) in their felodipine binding studies reported that Cd^{2+} is capable of not only occupying the Ca^{2+} binding sites of CaM but can bind to sites on CaM which are exposed or formed after Ca^{2+} has bound. However, the possible effect of Cd^{2+} on synthesis and degradation of CaM cannot be ruled out. Altered regulatory function of rat brain CaM after Cd-exposure may either be due to the direct effect on regulation of intracellular Ca^{2+} fluxes and/or by interaction of Cd^{2+} with some specific sites of CaM.

Acknowledgments. The suggestions and help given by Prof. D. Desaiah and Dr. Y. P. Rao in the preparation of this manuscript is gratefully acknowledged.

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Received January 11, 1989; accepted April 11, 1989.