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AFFINITY OF HEAVY METAL IONS TO INTRACELLULAR Ca^{2+}-BINDING PROTEINS

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Abstract—Parvalbumin, troponin C and vitamin D dependent Ca^{2+}-binding proteins (CaBP type I and II) share the property of calmodulin to interact with some heavy metal ions.

In flow dialysis and in spot tests the affinities of Cd^{2+} and Pb^{2+} to these proteins were comparable to those of Ca^{2+}. The relative affinities were for calmodulin: $Pb^{2+} > Ca^{2+} > Cd^{2+}$, for troponin C: $Ca^{2+} > Cd^{2+} > Pb^{2+}$, for CaBP I: $Ca^{2+} \sim Pb^{2+}$, for CaBP II; $Ca^{2+} > Pb^{2+} > Cd^{2+}$, and for parvalbumin: $Cd^{2+} \sim Ca^{2+} > Pb^{2+}$.

Upon gel filtration of the supernatant of a pig mucosal homogenate, binding for both Pb^{2+} and Ca^{2+} appeared in the MW range of 10,000, together with CaBP II.

We conclude that the investigated proteins bind heavy metal ions, in particular Pb^{2+} and Cd^{2+}, similar to calmodulin. Their role in transport, storage and toxicity remains to be assessed.

Intracellular Ca^{2+} can react with a series of cytoplasmatic constituents. Calmodulin is the best known member of this family of low molecular weight acidic proteins with high Ca^{2+}-affinity. It is present in all eukaryotic cells and serves as a Ca^{2+} sensor [1, 2]. Other Ca^{2+}-binding proteins are restricted to certain tissues and may be involved in muscle contraction (troponin C, parvalbumin) and in the renal and the intestinal Ca^{2+}-transport (CaBP I, CaBP II)§ [3, 4]. Each of these proteins possesses two or four Ca^{2+}-binding domains. In the search for a possible intracellular target of toxic heavy metal ions, we and others have shown that several of them, in particular Pb^{2+} and Cd^{2+}, can substitute for Ca^{2+} in the Ca^{2+}–calmodulin-complex and thus activate calmodulin-dependent intracellular processes [5–7]. Since the other Ca^{2+}-binding proteins resemble calmodulin with respect to their Ca^{2+}-binding sites which are arranged as EF-hand structures [1, 3, 8], interaction with heavy metals might not be a property restricted to calmodulin.

We studied the binding of some metal ions to Ca^{2+}-binding proteins, as a possible basis for their toxicological relevance.

MATERIALS AND METHODS

Materials

Calmodulin, parvalbumin and troponin C were purchased from Sigma (St. Louis) MO). CaBP I (MW = 28,000) was purified from rat kidney using heat treatment and gel filtration on a Sephadex G-100 column, but without final preparative gel electrophoresis [4]. CaBP II (MW 10,000) was prepared from pig duodenal mucosa [9]. Gel electrophoresis showed a single protein band for calmodulin, CaBP I and parvalbumin, four bands for troponin, and two bands for CaBP II. However, all protein preparations displayed only one band that bound $^{45}Ca^{2+}$ following electroblotting (see Methods).

Chelex 100 was obtained from Bio Rad (Richmond, CA), Tris from Calbiochem-Behring (La Jolla, CA). All metal salts were of analytical quality. $^{45}Ca^{2+}$ (specific activity 1.2 Ci/mmole) was a product of Amersham Buchler (Braunschweig, F.R.G.). $^{212}Pb^{2+}$ was prepared as a decay product of ^{228}Thorium ($^{228}Th \rightarrow {}^{224}Ra \rightarrow {}^{220}Rn \rightarrow {}^{216}Po \rightarrow {}^{212}Pb$) according to Jonas [10]. Aqueous solutions of 0.1 μCi/ml carrier-free $^{212}Pb^{2+}$ ($t/2 = 10$ hr, initial specific radioactivity about 3×10^8 Ci/mole) were regularly obtained, which corresponded to a lead content of 0.3 pM.

Methods

Removal of Ca^{2+}. All buffers and protein solutions were passed through Chelex 100 columns. By this means the Ca^{2+}-concentration of buffers was lowered to below 0.5 μM, whereas protein solutions still retained between 0.25 and 1 mole Ca^{2+} per mole of protein. Similar amounts of residual Ca^{2+} have also been found by others [11, 12] in Ca^{2+}-binding proteins purified over Chelex.

Detection of Ca^{2+}. Ca^{2+}-concentration was measured using an atomic absorption photometer (Hewlett Packard 360) with a detection limit of 0.1 μM.

$^{45}Ca^{2+}$*-autoradiography*. To test the functional homogeneity of the Ca^{2+}-binding proteins, $^{45}Ca^{2+}$-autoradiography was applied on nitrocellulose filters after SDS-polyacrylamide gel electrophoresis and electroblotting according to Maruyama *et al.* [13].

Radioactivity was counted in a Packard Tri-Carb 2660 after mixing the samples with Instagel II (Packard Instruments).

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§ Abbreviations used: CABP I and II: Ca^{2+}-binding proteins type I and II.

Mini-Chelex-assay. Ca^{2+}- and Pb^{2+}-binding of protein fractions was routinely followed in column eluates by a modified version of the Chelex-assay of Wasserman *et al.* [14]. This method is based on the competition between the insoluble chelator and the soluble Ca^{2+}-binding proteins. The sample (500 μl), 200 μl of Chelex suspension (regenerated and diluted 1:3 with the corresponding buffer) and 50 μl of a $^{45}Ca^{2+}$ or $^{212}Pb^{2+}$-solution (0.1 μCi/ml, which corresponds to 0.3 μM Ca^{2+} or 0.3 pM Pb^{2+}) were mixed (Vortex® shaker) for 5 sec and centrifuged for 1 min. The radioactivity in an aliquot of supernatant was measured.

Flow dialysis. Displacement by metal ions of $^{45}Ca^{2+}$ bound to proteins was determined at 25° in an apparatus originally described by Colowick and Womack [15] and improved by Feldmann [16]. Protein solution and $^{45}Ca^{2+}$ (for concentrations see the legends to the figures) were preincubated at room temperature for 30 min. An aliquot (500 μl) was filled into the upper chamber. The flow (0.2 M Tris acetate pH 6.8) through the lower chamber was maintained at 0.3 ml/min by a Desaga peristaltic pump. Six fractions of 0.3 ml each were collected before metal salts were added stepwise (5 μl) to the upper chamber. After every addition, six fractions were collected directly into scintillation vials. The K_D for Ca^{2+} given in the Results section were calculated from flow dialysis data according to Colowick and Womack [15]. The binding curves represent data from single experiments. To check reproducibility, the binding of Ca^{2+} to parvalbumin was examined in four consecutive flow dialysis experiments. The standard error of the results (percent $^{45}Ca^{2+}$ bound) was less than 4%.

Spot test. The test was developed to compare metal-binding patterns of the proteins. Like flow dialysis, it is based on competition between metal ions and $^{45}Ca^{2+}$ for Ca^{2+}-binding proteins. The protein solutions (1 or 2 mg/ml, 2 or 3 μl) were pipetted on discs of nitrocellulose (5 mm diameter), dried for 10 min and incubated in 500 μl M Tris acetate pH 6.8 containing $^{45}Ca^{2+}$ (1 μCi/ml) for a further 10 min. Competing metal salts in different concentrations had been added to the latter incubation buffer. Finally the nitrocellulose filters were soaked twice in 500 μl of distilled water to remove loosely bound radioactivity. The bound $^{45}Ca^{2+}$ was measured. Drying of the proteins should be for 5–10 min in order to fix them to the support. The pH must be controlled because $^{45}Ca^{2+}$-binding increases strongly between pH 6 and pH 8. $^{45}Ca^{2+}$-binding was independent of incubation time (5–20 min). Apparently due to absorption to nitrocellulose only approx. 10% of the calculated sites were available for Ca^{2+}-binding. The determinations were run in triplicate. The standard error of the results (bound dpm) was about 5%. Lead-binding experiments showed errors up to 10%.

RESULTS

Competition between metal ions and $^{45}Ca^{2+}$ for Ca^{2+}-binding proteins

With calmodulin, the K_D calculated from flow dialysis was 4 μM for Ca^{2+}. The slope of the displacement curve was steeper with Pb^{2+} than with Ca^{2+} (Fig. 1). Cd^{2+}, Mn^{2+} and Al^{3+} were less potent with respect to displacement, while Hg^{2+} and other cations like Mg^{2+}, Ba^{2+}, Ni^{2+} or Na^{+} (not shown) had no distinct effect in the concentration range chosen.

With parvalbumin, Ca^{2+} and Cd^{2+} yielded nearly the same displacement curves in flow dialysis, whereas the latter ion was slightly more potent in the spot test. Pb^{2+} and Mn^{2+} were less potent competing agents in both tests. Other cations were without effect up to 1 mM (Fig. 2). The K_D calculated for Ca^{2+} was 7 μM.

Ca^{2+} was also the most potent displacing agent with troponin C, closely followed by Cd^{2+} and Pb^{2+}. Mn^{2+} and Al^{3+} were less potent. Other cations, e.g. Hg^{2+}, below 1 mM did not compete with $^{45}Ca^{2+}$ binding (Fig. 3).

For CaBP II, Ca^{2+} had a higher affinity than Pb^{2+}, Cd^{2+} and Mn^{2+} (Fig. 4); its K_D was about 7 μM.

With CaBP I, only Ca^{2+} and Pb^{2+} were compared in flow dialysis. The K_D for Ca^{2+} was 5 μM and the affinity of Pb^{2+} to CaBP I was not significantly different (Fig. 5).

The IC_{50} values are compiled in Table 1.

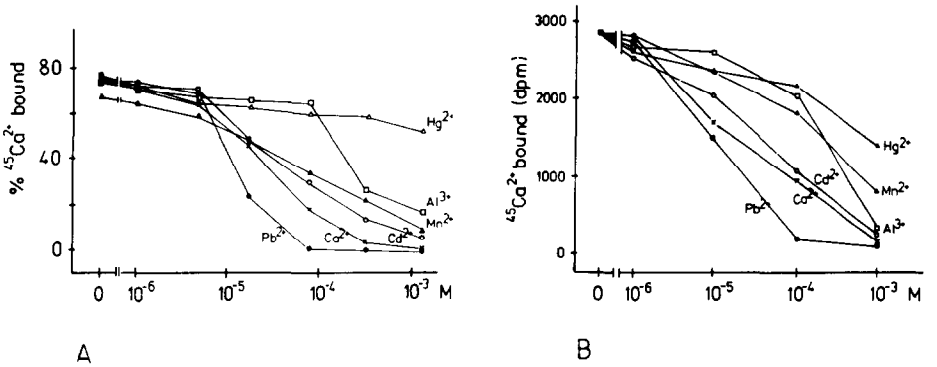

Fig. 1. Binding of $^{45}Ca^{2+}$ to calmodulin in presence of various cations. (A) Flow dialysis. Calmodulin (6 μM) had been preincubated with 190,000 dpm corresponding to 0.7 μM Ca^{2+}: ordinate, % $^{45}Ca^{2+}$ bound; abscissa, final concentrations of the respective cations added. (B) Spot test. 60 pmole calmodulin/spot, 1 100,000 dpm $^{45}Ca^{2+}$, corresponding to 3 μM Ca^{2+} in the incubation solution: ordinate, $^{45}Ca^{2+}$ bound (dpm); abscissa, final concentrations of the respective cations added.

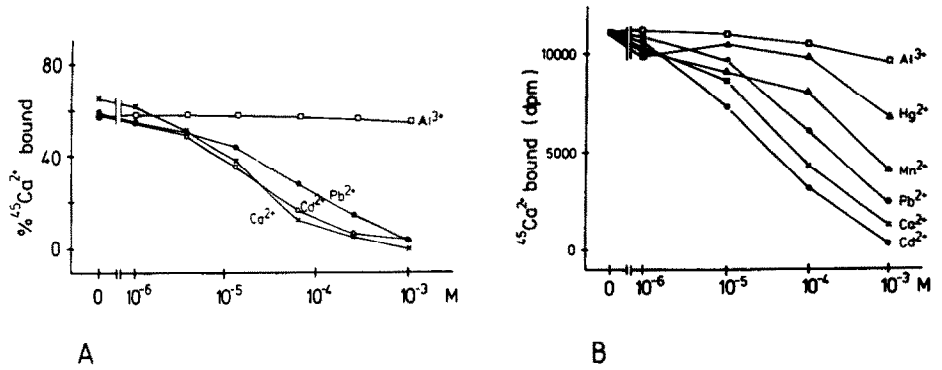

Fig. 2. Binding of $^{45}Ca^{2+}$ to parvalbumin in presence of various cations. (A) Flow dialysis. Parvalbumin (8 μM) had been preincubated with 190,000 dpm corresponding to 0.7 μM Ca^{2+}. (B) Spot test. 900 pmole protein/spot, 1 100,000 dpm $^{45}Ca^{2+}$, corresponding to 3 μM Ca^{2+} in the incubation solution. Ordinate and abscissa: see Fig. 1.

Binding of $^{45}Ca^{2+}$ and $^{212}Pb^{2+}$ to supernatant of mucosal homogenate

The 32,000 *g* supernatant of pig duodenal mucosa was fractionated by gel filtration on Sephadex G 100 and the eluates assayed by the chelex assay for Ca^{2+}- and Pb^{2+}-binding. The column had been calibrated with several molecular weight markers. Two peaks, both binding Ca^{2+} and Pb^{2+}, were detected (Fig. 6). The first had a low binding capacity per protein weight and eluted with the void volume. The second peak at MW 10,000 had a much higher binding capacity, as no maximum of protein was discernible. It was most likely due to CaBP II since this protein could be enriched from the fractions of the region.

DISCUSSION

The importance of Ca^{2+} in mediating numerous cell functions is well recognized. Potential interactions of heavy metals with cellular Ca^{2+} appear as a new perspective in the possible molecular mechanisms of heavy metal toxicity [7, 17].

The Ca^{2+}-signal is intracellularly transferred by calmodulin. This chelating protein also binds several metal ions, in particular Pb^{2+} and Cd^{2+} [5, 7]. Like Ca^{2+} these ligands induce functional changes after complex formation. For instance, they activate calmodulin dependent phosphodiesterase and calmodulin dependent phosphorylation of rat brain membranes, increase tyrosine fluorescence of calmodulin and alter the mobility of calmodulin in polyacrylamide gel electrophoresis [5–7, 18]. Numerous other Ca^{2+}-binding proteins share with calmodulin the high affinity for Ca^{2+} whereas the functional consequences of the complex formation are less well established [3, 19]. Their structural similarities with calmodulin are obvious [20, 21].

According to our data all Ca^{2+}-binding proteins investigated have in common the ability to bind certain heavy metals. Whereas the interaction of these cations with calmodulin can be assessed by both competition experiments and enzymatic tests, affinity to the other proteins can be characterized by binding studies only. We have used the classical flow dialysis and a newly developed spot test on nitrocellulose. The former has the advantage that ligands and binding sites react in solution, but nonspecific binding to the membrane and other surfaces

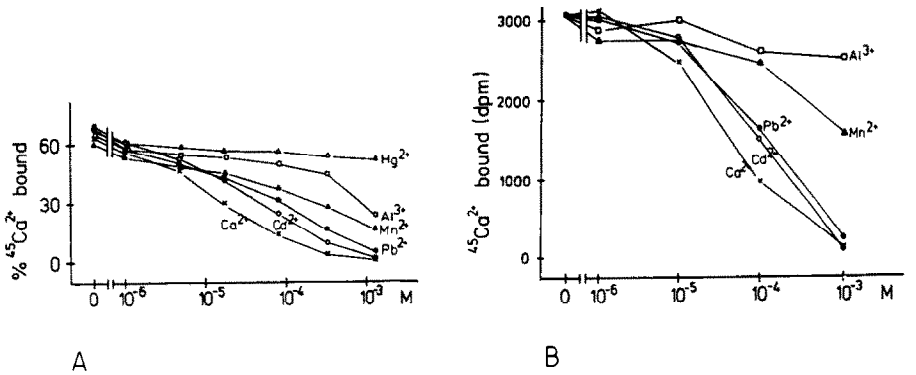

Fig. 3. Binding of $^{45}Ca^{2+}$ to troponin C in presence of various cations. (A) Flow dialysis. Troponin C (approx. 8 μM) had been preincubated with 190,000 dpm corresponding to 0.7 μM Ca^{2+}. (B) Spot test. Approximately 60 pmole protein/spot, 1 100,000 dpm $^{45}Ca^{2+}$ corresponding to 3 μM in the incubation solution. Ordinate and abscissa: see Fig. 1.

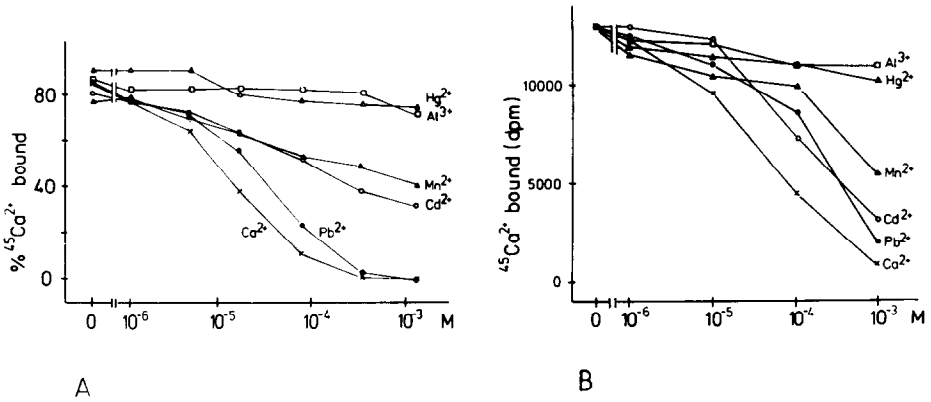

Fig. 4. Binding of $^{45}Ca^{2+}$ to CaBP II in presence of various cations. (A) Flow dialysis. CaBP II (approximately 6 μM) had been preincubated with 190,000 dpm corresponding to 0.7 μM Ca^{2+}. (B) Spot test. Approximately 400 pmole protein/spot, 1 100,000 dpm $^{45}Ca^{2+}$, corresponding to 3 μM in the incubation solution. Ordinate and abscissa: see Fig. 1.

may pose problems. In the spot test, previous drying of the binding proteins on nitrocellulose is unavoidable and may cause some denaturation. However, probably due to the stability of the Ca^{2+}-binding structures [13], flow dialysis and spot test gave essentially the same results with all proteins investigated.

Among the various cations Pb^{2+}, Cd^{2+} and Ca^{2+} were in the same affinity range but with different sequences between the binding proteins examined. The most affine cations were Pb^{2+} in calmodulin, Cd^{2+} and Ca^{2+} in parvalbumin, Ca^{2+} in troponin C and in CaBP II, and Ca^{2+} and Pb^{2+} in CaBP I.

We have also investigated Al^{3+} because of its possible role in dialysis encephalopathy. This metal has been claimed to interact with calmodulin [22].

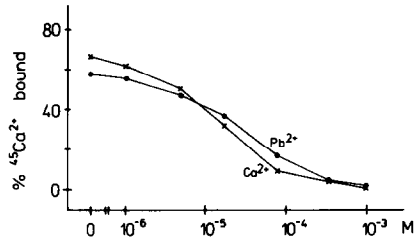

Fig. 5. Binding of $^{45}Ca^{2+}$ to CaBP I in presence of Ca^{2+} and Pb^{2+} in flow dialysis. Approximately 5 μM CaBP I, 190,000 dpm were added corresponding to 0.7 μM Ca^{2+}. Ordinate and abscissa: see Fig. 1.

However, in accordance with our own negative findings with calmodulin and Al^{3+} [18], the effects of this ion on the Ca^{2+}-binding proteins investigated was negligible. The same was true for Hg^{2+}, Mg^{2+}, Ni^{2+}, Ba^{2+}, or Na^{+}. Mn^{2+} took an intermediate position.

A role in intestinal Ca^{2+} absorption has been ascribed to CaBP II [19]. The vitamin D dependent intestinal Ca^{2+}-transport is inhibited in Pb^{2+} intoxi-

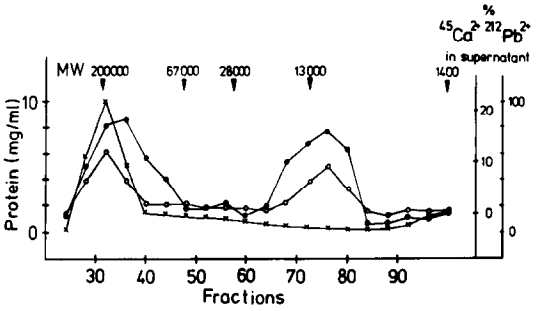

Fig. 6. Pb^{2+}-binding to 32,000 *g* supernatant of pig mucosal homogenate after Sephadex G 100 gel filtration. The volume of the column was 500 ml, that of the sample 20 ml, and the protein concentration of the sample was 50 mg/ml. Fractions of 5 ml were collected. Ca^{2+}- and Pb^{2+}-binding activity was determined by the mini-chelex assay (see Methods): Left ordinate, protein (mg/ml, calculated from extinction at 280 nm) (×—×); right ordinate, % in supernatant of $^{45}Ca^{2+}$ (○—○) or $^{212}Pb^{2+}$ (●—●).

Table 1. Compilation of the IC_{50} values (in μM) for metal ions, taken from the flow dialysis experiments

	Calmodulin	Parvalbumin	Troponin C	CaBP II	CaBP I
Ca^{2+}	26	18	19	16	18
Pb^{2+}	11	52	100	36	34
Cd^{2+}	42	22	46	310	
Mn^{2+}	80		360	1000	
Al^{3+}	210	>1000	1000	>1000	
Hg^{2+}	>1000		>1000	>1000	

cation [23]. Therefore we have assessed whether CaBP II might serve as a predominant binding site for both Ca^{2+} and Pb^{2+} in intestinal homogenates. This would add some functional relevance to the binding of both cations common to this protein. Indeed a fraction that bound Ca^{2+} and Pb^{2+} was eluted in the MW range expected for CaBP II.

We conclude that calmodulin has been only the first example of a Ca^{2+}-binding protein with comparable affinities to certain heavy metals, in particular Pb^{2+} and Cd^{2+}. Our recent data show that all investigated binding proteins share this property, apparently on the basis of their common chelating structure.

The role of these proteins in toxicity, storage and transport of heavy metals is still unsettled.

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