Study of the interactions of cadmium and zinc ions with cellular calcium homoeostasis using ^{19}F -NMR spectroscopy

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The effects of the heavy-metal ions Cd^{2+} and Zn^{2+} on the homoeostasis of intracellular free Ca^{2+} in E367 neuroblastoma cells were examined using ^{19}F -NMR spectroscopy with the fluorinated chelator probe 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (5F-BAPTA). First, the technique was used to quantify the uptake and intracellular free concentrations of the heavy metals after treatment of the cells with $20\ \mu\text{M}$ CdCl_2 or $100\ \mu\text{M}$ ZnCl_2 . Secondly, metal-induced transients in intracellular free Ca^{2+} were recorded. Addition of $20\ \mu\text{M}$ CdCl_2 , but not $100\ \mu\text{M}$ ZnCl_2 , evoked a transient increase

in Ca^{2+} from a resting level of 84 nM to approx. 190 nM within 15 min after addition of the metal. Zn^{2+} at $20\ \mu\text{M}$ completely prevented the induction of a Ca^{2+} transient by Cd^{2+} . Ca^{2+} was mobilized by Cd^{2+} from intracellular organelles, since depletion of these stores by thapsigargin abolished the effect of the toxic metal. Furthermore, $20\ \mu\text{M}$ Cd^{2+} evoked a transient rise in cellular $\text{Ins}(1,4,5)\text{P}_3$, reaching a maximum level within 5 min after addition of the metal. These results demonstrate that perturbation of the $\text{Ins}(1,4,5)\text{P}_3/\text{Ca}^{2+}$ messenger system is an early and discrete cellular effect of Cd^{2+} .

INTRODUCTION

The maintenance of cellular calcium homoeostasis is a prerequisite for the role of Ca^{2+} ions in mediating signals from the cell surface to the nucleus. Since Ca^{2+} serves as an almost universal messenger in cell activation, the intracellular free Ca^{2+} concentration has to be regulated tightly [1–3]. This is ensured by various Ca^{2+} transport systems, such as channels, ATP-driven pumps, exchanger proteins and Ca^{2+} -binding proteins [4,5]. Perturbations of Ca^{2+} -mediated signal transduction have been considered as underlying mechanisms for the action of various chemical toxicants [6,7]. Transient increases in intracellular free Ca^{2+} and subsequent activation of protein kinases modulate cell proliferation and/or differentiation [8], whereas persistent elevation of the Ca^{2+} concentration might be associated with necrotic or, in certain systems, apoptotic processes [9,10].

Cellular Ca^{2+} homoeostasis and Ca^{2+} -mediated functions are being increasingly recognized as sensitive and critical targets for the action of toxic metal compounds [11–13]. Heavy-metal ions can interact with cell surface receptors, Ca^{2+} influx channels and intracellular Ca^{2+} transport systems. The carcinogen cadmium has been shown to interfere at various levels of cellular Ca^{2+} signalling. First, with regard to cell surface effects, Cd^{2+} concentrations in the low micromolar range provoke the formation of inositol phosphates in human skin fibroblasts, causing a large, but short-lasting, mobilization of intracellular free Ca^{2+} [14]. On the other hand, hormone-evoked Ca^{2+} transients in PC12 cells are inhibited by Cd^{2+} at slightly higher concentrations [15]. Secondly, with regard to plasma membrane Ca^{2+} channels, Cd^{2+} is a well known blocker of Ca^{2+} influx into cells. Cd^{2+} inhibits receptor-operated Ca^{2+} channels in hepatocytes as well as voltage-operated Ca^{2+} channels in neuronal cells [16,17]. However, the latter type of channel is the predominant means by which Cd^{2+} ions enter mammalian cells [18,19]. Thirdly, once the heavy-metal ion has reached the cytosol, it interferes with all known active Ca^{2+} transport systems. The interaction of Cd^{2+} with

plasma membrane Ca^{2+} -ATPase reduces the Ca^{2+} extrusion capacity of the cell [20,21]. Similarly, Cd^{2+} inhibits the intracellular sequestration of Ca^{2+} into microsomal stores [22,23], and even Ca^{2+} uptake into nuclear compartments [24].

In contrast with the exclusively toxic nature of Cd^{2+} , Zn^{2+} at low concentrations is essential for cellular growth and differentiation [25–27]. There are various indications for a regulatory function for intracellular Zn^{2+} ions. Zn^{2+} is an essential constituent of enzymes involved in nucleic acid synthesis and of many transcription factors controlling the initiation of gene expression [28,29]. On the other hand, Zn^{2+} has been shown to interfere with intracellular Ca^{2+} sequestration by inhibiting Ca^{2+} transport systems of microsomal vesicles and nuclei [22,30], and to block voltage-activated Ca^{2+} channels in neuronal cells [31]. Furthermore, Zn^{2+} decreases the cellular uptake of Cd^{2+} , thereby protecting cells from the toxic effects of this metal. Zn^{2+} reduces the accumulation of Cd^{2+} in rat hepatocytes, as well as its binding to rabbit kidney cell membranes [32,33]. Furthermore, Zn^{2+} competitively inhibits the mobilization of Ca^{2+} caused by Cd^{2+} in human skin fibroblasts [14].

Previous studies of the effects of Cd^{2+} and Zn^{2+} on intracellular Ca^{2+} homoeostasis using fluorescent probes such as fura-2 have been hampered by the fact that bivalent metal ions other than Ca^{2+} also bind to the probes and cause fluorescence often more intense than that of the Ca^{2+} complex. Hence neither the changes in intracellular free Ca^{2+} induced by the toxic metal ions nor the intracellular concentrations of the intruding toxic ions could be assessed unambiguously. In order to quantify the intracellular concentrations of various ions, we have used ^{19}F -NMR spectroscopy in combination with the intracellular bivalent cation chelator 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (5F-BAPTA). This technique, originally developed by Smith et al. [34], permits the simultaneous detection of both intracellular free Ca^{2+} and other bivalent metal ions [35]. It overcomes the problems of specificity and interference which are the major drawbacks for the utilization of fluorescent and

Abbreviations used: 5F-BAPTA, 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; 5F-BAPTA/AM, acetoxymethyl ester of 5F-BAPTA; DMEM, Dulbecco's modified Eagle's medium.

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bioluminescent indicators in the presence of heavy-metal ions. In the present study we have employed ^{19}F -NMR spectroscopy in 5F-BAPTA-loaded E367 neuroblastoma cells to (i) quantify the uptake and intracellular free ion concentrations of Cd^{2+} and Zn^{2+} , and (ii) investigate the modulation of the intracellular free Ca^{2+} concentration by these heavy-metal ions.

EXPERIMENTAL

Materials

All tissue culture reagents were obtained from Gibco (Eggenstein, Germany). The bivalent cation chelator 5F-BAPTA and its acetoxymethyl ester (5F-BAPTA/AM) were purchased from Calbiochem (Bad Soden, Germany). Stock solutions of CdCl_2 and ZnCl_2 were obtained from Merck (Darmstadt, Germany). Collagen-coated microcarrier beads (Cytodex 3) and the assay kit for the determination of $\text{Ins}(1,4,5)\text{P}_3$ were obtained from Amersham (Braunschweig, Germany). All other chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany).

Cell culture procedures

Rat E367 neuroblastoma cells [36] were cultivated and grown to confluence on 10 cm plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and buffered with 2.4 g/l NaHCO_3 . The cells were incubated in a humidified atmosphere of 10 % CO_2 at 37 °C. The medium was changed twice per week. At 5 days prior to NMR experiments, collagen-coated microcarriers (Cytodex 3) were prepared for binding of cells. The dry microcarriers were swollen and hydrated in Ca^{2+} - and Mg^{2+} -free PBS for at least 3 h at room temperature. After sterilization by autoclaving, the beads were equilibrated in DMEM. Microcarrier beads (500 mg) were added in 50 ml of culture medium to a siliconized glass spinner vessel (Wheaton Scientific). The suspension culture system was inoculated with 5×10^7 cells, mixed gently (20 rev./min) and incubated at 37 °C in a humidified atmosphere of 10 % CO_2 . After 24 h the culture volume was increased to 150 ml with a stirring speed of 50 rev./min, and cells were grown for a further 3 days with daily changes of medium. After achievement of confluence, a thick slurry of carriers with a final cell concentration of $(1-5) \times 10^8$ cells per ml was reached, and this was transferred into a 10 mm diameter NMR tube, which contained the cell perfusion medium (DMEM supplemented with 10 % fetal calf serum). The total time needed for transferring the cells into the NMR tube was 5 min, and perfusion with well oxygenated medium at a rate of 1 ml/min was started immediately after loading. The perfusion technique was as described before [35,37].

NMR measurements

All NMR spectra were recorded on a Bruker AM 360 FT NMR spectrometer equipped with an 8.4 T/52 mm superconducting magnet and a 10 mm $^1\text{H}/^{19}\text{F}$ or $^1\text{H}/^{31}\text{P}$ dual probe. $^2\text{H}_2\text{O}$ in a sealed capillary was placed in the NMR tube for magnetic field lock. Shimming was carried out on the free induction decay of the water signal, and a line width at half height of 15 Hz could be obtained routinely. The temperature was maintained at 30 °C by a thermostatically controlled air jacket around the NMR tube.

The energy state in the presence of 5F-BAPTA was assessed by ^{31}P -NMR spectra, in comparison with spectra recorded in the absence of the metal indicator. ^{31}P -NMR spectra were collected at a transmitter frequency of 145.8 MHz using a 66 ° flip angle

with a spectral width of 5300 Hz, a repetition time of 2.5 s, a composite pulse decoupling with WALTZ-16 [38], a data size of 4K and zero filling to 8K. Each spectrum represents the sum of 360 scans.

^{19}F -NMR measurements for the determination of intracellular free ion concentrations were performed as previously described [35,39,40], with minor modifications. The cells were loaded with 5F-BAPTA by perfusion with membrane-permeant 5F-BAPTA/AM. Prior to loading the culture with 5F-BAPTA/AM, the cell perfusion medium (DMEM plus 10 % fetal calf serum) was replaced by an incubation medium, containing 10 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4 , 1 mM MgCl_2 , 1.3 mM CaCl_2 , 0.3 % BSA and 5 mM glucose. After 15 min of perfusion with incubation medium, 50 μM 5F-BAPTA/AM was added to the perfusate from a 30 mM stock solution in DMSO. The medium was removed after adequate loading had been achieved (60 min). The perfusate was then switched back to the cell perfusion medium, and the cells were washed with 15 ml of fresh medium to remove any extracellular chelator and allowed to equilibrate for 1 h. For acquisition of ^{19}F -NMR spectra, a repetition time of 0.5 s was used. The data were recorded at an operating frequency of 338.8 MHz using a 45 ° flip angle with a spectral width of 33000 Hz, composite pulse decoupling with WALTZ-16, a data size of 8K, zero filling to 16K and an apodization of 40 Hz; 600, 1800 and 7200 transients were routinely collected, resulting in temporal resolutions of 5 min, 15 min and 1 h respectively. The intracellular free Ca^{2+} , Cd^{2+} and Zn^{2+} concentrations were calculated from the integrated areas under the metal-bound 5F-BAPTA (M^{2+} -5F-BAPTA) and free chelator (5F-BAPTA) signals using the equation:

$$[\text{M}^{2+}] = K_d \frac{[\text{M}^{2+}\text{-5F-BAPTA}]}{[\text{5F-BAPTA}]}$$

where M^{2+} is the metal of concern and K_d is the dissociation constant for the respective M^{2+} -5F-BAPTA complex [34,35,41,42]. The apparent dissociation constants were 5.0×10^{-7} M for Ca^{2+} -5F-BAPTA, 5.7×10^{-11} M for Cd^{2+} -5F-BAPTA and 7.9×10^{-9} M for Zn^{2+} -5F-BAPTA at 30 °C. For studies of intracellular free calcium concentration during extracellular cation addition, ^{19}F -NMR spectra were first obtained under control perfusion conditions until a stable intracellular free Ca^{2+} concentration was reached. To examine the reversibility of the effects, the perfusion constituents were brought back to the starting point, and spectra were run until a stable reading for the intracellular free Ca^{2+} concentration was again obtained.

Determination of $\text{Ins}(1,4,5)\text{P}_3$

$\text{Ins}(1,4,5)\text{P}_3$ was determined by the isotope dilution assay (Amersham), as described by the manufacturer with the following modifications. One aliquot of the cell suspension was mixed with 0.5 vol. of ice-cold 20 % (w/v) perchloric acid and incubated at 0 °C for 20 min. The resulting protein precipitate was sedimented at 4000 g for 5 min at 4 °C. The supernatant was titrated to pH 7.5 with 1.5 M KOH containing 60 mM Hepes buffer. Protein was determined by the bicinchoninic acid assay [43].

Statistics

In each experiment, the mean of three measurements in approx. 10^8 cells was usually obtained. Data points shown in the figures are expressed as means $\pm$ S.D.

RESULTS

In order to quantify intracellular ion concentrations, we performed a ^{19}F -NMR study in E367 neuroblastoma cells loaded with the fluorinated chelator 5F-BAPTA. This method allows the measurement of intracellular free Ca^{2+} and other bivalent metal ions simultaneously. To assess the metabolic integrity of the cells, the energy state was first monitored by recording ^{31}P -NMR spectra of the same cell preparation used subsequently for ^{19}F -NMR spectroscopy. The levels of phosphocreatine, phosphomonoesters (phosphoethanolamine and phosphocholine) and the α -, β - and γ -phosphates of nucleoside phosphates (mainly ATP) are sensitive criteria for the energy state and the viability of the cells. As shown in Figure 1, ^{31}P -NMR spectra before (Figure 1A) and after (Figure 1B) loading of the 5F-BAPTA indicator showed no significant differences in the normal energy state, which remained constant for more than 8 h. Thus these conditions permit the measurement of intracellular free ion concentrations without significant perturbation of the energy state of E367 neuroblastoma cells.

In order to identify unambiguously their resonance positions in whole cells, we first measured the ^{19}F -NMR signals of various bivalent metal ions in aqueous solution. Figure 2 illustrates the relative ^{19}F chemical shifts of the free chelator (5F-BAPTA) and of various metal complexes in aqueous solution. Distinct resonance peaks for the metal-5F-BAPTA chelates were observed for Zn^{2+} (3.7 p.p.m. downfield of that of free 5F-BAPTA), Pb^{2+} (4.6 p.p.m.), Cd^{2+} (4.8 p.p.m.), Hg^{2+} (5.3 p.p.m.) and Ca^{2+} (5.8 p.p.m.). Furthermore, the resonance frequencies of Fe^{2+} , Co^{2+} and Ni^{2+} appeared at 28.1, 32.4 and 31.1 p.p.m. respectively.

Figure 1 ^{31}P -NMR spectra of perfused E367 neuroblastoma cells, before (A) and after (B) incubation with 5F-BAPTA/AM

During the incubation, cells were perfused with Hank's balanced salt solution enriched with 0.3% BSA and 5 mM glucose. The resonances of PCr (phosphocreatine), PME (phosphomonoesters), P_i and the α -, β - and γ -phosphate groups of intracellular NTPs (mainly ATP) are as indicated. The difference in the heights of the resonance peaks of P_i (between A and B) is due to the change in the buffer composition during the loading procedure. Chemical shifts were referenced to PCr at -2.33 p.p.m.

Figure 2 Chemical shifts of 5F-BAPTA in the ^{19}F spectrum for various metal ions in aqueous solution

Shifts are given in p.p.m. downfield from the signal of free 5F-BAPTA.

Mg^{2+} has a very low affinity for 5F-BAPTA and gave no distinct resonance signal under the conditions employed, as also reported by Smith et al. [34].

Next, we monitored the intracellular free ion concentrations of Ca^{2+} and Cd^{2+} before and during incubation of E367 neuroblastoma cells with $20\ \mu\text{M}$ CdCl_2 (Figure 3A). Under control conditions (untreated cells), the free 5F-BAPTA (peak a) and Ca^{2+} -5F-BAPTA (peak b) resonances were detected at 0 p.p.m. and 5.8 p.p.m. respectively. A stable steady-state level of intracellular free Ca^{2+} with a value of 84 ± 10 nM was obtained. This result is in accordance with the intracellular free Ca^{2+} concentration measured in the same cell line using the fura-2 fluorescent probe (results not shown).

A crucial step in cadmium toxicity is cellular uptake and the yield of a significant intracellular free concentration of the toxic metal. ^{19}F -NMR spectra obtained after the addition of $20\ \mu\text{M}$ CdCl_2 to the perfusion medium exhibited a consistently increasing resonance peak at 4.8 p.p.m. (Figure 3A, peak c). The intracellular free Cd^{2+} concentration was calculated from the integrated intensities using a dissociation constant of 5.7×10^{-11} M for the Cd^{2+} -5F-BAPTA complex. The time course of the signal showed that the intracellular free Cd^{2+} concentration increased without any observable saturation (Figure 3B). Treatment with $20\ \mu\text{M}$ CdCl_2 over a period of 8 h yielded an intracellular free Cd^{2+} concentration of 32.5 pM.

It is of interest to compare the uptake and intracellular free ion concentration of Cd^{2+} with those of Zn^{2+} , which has closely related chemical, but fundamentally different biological, properties. Figure 3(A) (peak d) and Figure 4(A) show that, in untreated cells, a ^{19}F resonance at 3.7 p.p.m., which is attributed to Zn^{2+} -5F-BAPTA, is slightly above the noise level, but does not permit accurate quantification of the free Zn^{2+} concentration. However, the addition of $100\ \mu\text{M}$ ZnCl_2 to the perfusion medium increased the intracellular free Zn^{2+} concentration in the cells. It was then possible to quantify the metal ion concentration from the integrated ratio of Zn^{2+} -5F-BAPTA and free 5F-BAPTA, using a dissociation constant of 7.9×10^{-9} M for the Zn^{2+} -5F-BAPTA complex. Figure 4(B) represents the time course of Zn^{2+} uptake. The intracellular free concentration increased with time over several hours without any detectable saturation. After 2.5 h a concentration of approx. 1 nM free Zn^{2+} was reached.

To examine the effects of heavy metals on intracellular calcium homeostasis in intact, metabolically stable cells, we investigated alterations in the intracellular free Ca^{2+} concentration after the addition of Cd^{2+} or Zn^{2+} to the perfusion medium. The time intervals in these experiments were as short as possible, i.e. about 5 min. At first, four consecutive spectra were acquired to establish the control level of intracellular free Ca^{2+} in untreated cells, prior to the addition of Cd^{2+} or Zn^{2+} . After addition of $20\ \mu\text{M}$ CdCl_2

Figure 3 Detection of intracellular free Cd^{2+} in perfused E367 neuroblastoma cells

(A) Alterations in ^{19}F -NMR spectra (accumulation time 60 min) after incubation with $20\ \mu\text{M}$ CdCl_2 . The resonance peaks are free 5F-BAPTA (a), Ca^{2+} -5F-BAPTA (b), Cd^{2+} -5F-BAPTA (c) and Zn^{2+} -5F-BAPTA (d). (B) Time course of the calculated intracellular free Cd^{2+} concentration over a period of 8 h.

Figure 4 Detection of intracellular free Zn^{2+} in perfused E367 neuroblastoma cells

(A) Alterations in ^{19}F -NMR spectra (accumulation time 15 min) after incubation with $100\ \mu\text{M}$ ZnCl_2 (time intervals 1–3 h). The resonance peaks are free 5F-BAPTA (a), Ca^{2+} -5F-BAPTA (b) and Zn^{2+} -5F-BAPTA (c). (B) Time course of the calculated intracellular free Zn^{2+} concentration over a period of 3 h.

the Ca^{2+} level rose within 15 min to reach a maximum value of approx. 190 nM (Figure 5). This transient Ca^{2+} peak was followed by a gradual decline towards the control level. The complete time

course of the Ca^{2+} transient induced by Cd^{2+} is shown in Figure 6(A).

In contrast, treatment with $20\ \mu\text{M}$ ZnCl_2 caused no increase in

Figure 5 Sequential ^{19}F -NMR spectra of perfused E367 neuroblastoma cells treated with $20\ \mu\text{M}$ CdCl_2

Spectra (accumulation time 5 min) were obtained immediately before treatment, 15 min after treatment and 60 min after treatment, indicating a transient rise in the ^{19}F signal for the Ca^{2+} -5F-BAPTA complex. The resonance peaks are free 5F-BAPTA (a) and Ca^{2+} -5F-BAPTA (b).

Figure 6 Effects of Cd^{2+} and Zn^{2+} on the intracellular free Ca^{2+} concentration in perfused E367 neuroblastoma cells

(A) Addition of $20\ \mu\text{M}$ CdCl_2 transiently increases the intracellular free Ca^{2+} level. (B) Addition of $20\ \mu\text{M}$ ZnCl_2 does not alter the intracellular free Ca^{2+} level. (C) Preincubation with $20\ \mu\text{M}$ ZnCl_2 prevents the Cd^{2+} -induced increase in the Ca^{2+} concentration.

intracellular free Ca^{2+} in E367 neuroblastoma cells (Figure 6B). After the addition of $20\ \mu\text{M}$ ZnCl_2 we obtained a constant baseline for intracellular free Ca^{2+} , without any significant alteration. Pretreatment with $20\ \mu\text{M}$ Zn^{2+} prevented the Cd^{2+} -inducible increase in Ca^{2+} concentration (Figure 6C). This observation demonstrates that Zn^{2+} abolishes the Cd^{2+} -induced Ca^{2+} transient completely.

In order to elucidate the origin of the Ca^{2+} release caused by Cd^{2+} , we employed thapsigargin, an inhibitor of intracellular Ca^{2+} -ATPases that is known to deplete intracellular Ca^{2+} stores [44]. As expected, this drug elevated the intracellular Ca^{2+} level (Figure 7). However, the subsequent addition of Cd^{2+} evoked no further increase in Ca^{2+} , demonstrating that the intracellular pools are the main source of Cd^{2+} -induced Ca^{2+} transients.

Figure 7 Effect of Cd^{2+} on the intracellular free Ca^{2+} concentration in perfused E367 neuroblastoma cells after depletion of intracellular Ca^{2+} stores by thapsigargin

Incubation with $100\ \text{nM}$ thapsigargin was followed by addition of $20\ \mu\text{M}$ CdCl_2 to the perfusion medium, as indicated.

Finally, we wished to find out whether Cd^{2+} triggers the mobilization of Ca^{2+} via the $\text{Ins}(1,4,5)\text{P}_3$ signalling pathway. Figure 8 shows that the concentration of intracellular $\text{Ins}(1,4,5)\text{P}_3$ underwent a transient 2-fold increase within 5 min after the administration of $20\ \mu\text{M}$ CdCl_2 under the same conditions that evoked the Ca^{2+} transient in E367 neuroblastoma cells. We conclude that the stimulation of the $\text{Ins}(1,4,5)\text{P}_3$ /calcium signalling system is an early and discrete cellular effect of Cd^{2+} in E367 neuroblastoma cells.

Figure 8 Effect of Cd^{2+} on the intracellular $\text{Ins}(1,4,5)\text{P}_3$ concentration in E367 neuroblastoma cells

After the addition of $20\ \mu\text{M}$ CdCl_2 , the $\text{Ins}(1,4,5)\text{P}_3$ (IP_3) concentration was determined by the isotope dilution assay. The metal evoked a transient rise in cellular $\text{Ins}(1,4,5)\text{P}_3$, reaching a maximum level within 5 min after addition.

DISCUSSION

NMR spectroscopy of ^{19}F -BAPTA-loaded E367 neuroblastoma cells was used to investigate the effects of Cd^{2+} and Zn^{2+} on intracellular Ca^{2+} homeostasis. Previously, this method has been used successfully to measure the intracellular free Ca^{2+} and Pb^{2+} concentrations after exposure of various cell lines to lead [40,45,46]. If the experiments are carried out in well-perfused cell cultures, the technique permits the study of processes extended over several hours. To exclude the possibility that under these conditions the metal chelator BAPTA might deplete essential metal ions and thus affect the metabolic integrity of the cells, we first monitored the cellular high-energy phosphates in the BAPTA-loaded cells by ^{31}P -NMR, but observed no significant decline.

Secondly, we measured the uptake of Cd^{2+} and Zn^{2+} by the ^{19}F -NMR technique, since knowledge of free intracellular Cd^{2+} and Zn^{2+} concentrations is crucial for the interpretation of the detrimental effects of these ions. Cd^{2+} was found to be taken up linearly with time, without saturation. After 8 h a concentration of free intracellular Cd^{2+} of 32.5 pM was achieved. The corresponding concentration of total Cd^{2+} is estimated to be several orders of magnitude greater than that of free Cd^{2+} . In a pituitary cell line, Cd^{2+} uptake has been measured using the ^{109}Cd radioisotope, and after 24 h a total amount of 2700 pmol of Cd/mg of protein was measured [18].

With respect to Zn^{2+} , the NMR technique used was not sensitive enough to quantify the basic level of free intracellular Zn^{2+} precisely, but it allowed us to estimate a concentration of approx. 0.5 nM. This value is similar to the free Zn^{2+} concentration measured by the NMR technique in synaptosomes, which are known to be rich in zinc [47], but it is much higher than the free Zn^{2+} level of only 0.024 nM in human red blood cells calculated from equilibrium studies [48]. After ZnCl_2 had been added to the medium, E367 neuroblastoma cells showed an increase in free Zn^{2+} , without saturation, over several hours. This finding is difficult to reconcile with the concept of stringent control of intracellular free Zn^{2+} , as expected for a regulatory metal ion.

The major objective of the present work was to examine the effects of Cd^{2+} and Zn^{2+} on cellular Ca^{2+} homeostasis. The most remarkable result was the transient increase in the intracellular free Ca^{2+} concentration evoked by Cd^{2+} in E367 neuroblastoma cells. This effect is comparable with the hormone-like stimulation of a Ca^{2+} transient by Cd^{2+} in human fibroblasts [14] and in cat adrenal cells [49]. In our system, the effect of Cd^{2+} was retarded and more extended in time compared with those cell types. On the other hand, Cd^{2+} did not cause sustained elevation of the basic intracellular Ca^{2+} level, as has been reported for the actions of Pb^{2+} in osteoblast cells and platelets [40,50,51], and for Hg^{2+} in PC12 cells [52].

At least three different mechanisms can be envisaged by which Cd^{2+} could evoke Ca^{2+} mobilization. First, Cd^{2+} may interact with plasma membrane Ca^{2+} channels and cause influx of Ca^{2+} into the cells. This would produce a sustained elevation of intracellular Ca^{2+} , which we did not observe in our system, but which has been found with Pb^{2+} and Hg^{2+} ions by others [40,50,51]. Secondly, after its uptake, Cd^{2+} could interact with intracellular proteins needed for maintenance of the low resting level of Ca^{2+} . The inhibition of intracellular Ca^{2+} -ATPases by Cd^{2+} [22,23] would impair the reloading of Ca^{2+} into storage organelles, similar to the action of the drug thapsigargin, and cause a sustained elevation of free Ca^{2+} . Thirdly, Cd^{2+} may interact with cell surface hormone or other receptors and trigger the $\text{Ins}(1,4,5)\text{P}_3$ signalling pathway, leading to Ca^{2+} release from

intracellular stores, as suggested for human fibroblasts [14]. Since we did not observe sustained elevation of the resting Ca^{2+} level by Cd^{2+} in our experiments, we can exclude the possibility that this toxic metal simply interferes with the control of Ca^{2+} fluxes from outside the cells or from intracellular organelles, as in the first and second hypotheses. To test the third mechanism, we employed thapsigargin to block intracellular Ca^{2+} -ATPases and thus empty the intracellular calcium stores in E367 cells. In this experiment we observed the expected sustained elevation of free Ca^{2+} , but the subsequent addition of Cd^{2+} was unable to evoke a Ca^{2+} transient. We conclude that the Cd^{2+} -induced Ca^{2+} transient in this system originated from intracellular stores. Furthermore, we conclude that Cd^{2+} causes a Ca^{2+} transient via the $\text{Ins}(1,4,5)\text{P}_3$ signalling pathway, since we observed that this metal also stimulated an increase in intracellular $\text{Ins}(1,4,5)\text{P}_3$ in E367 cells. However, there is still uncertainty regarding the type of receptor affected by Cd^{2+} and the mode of interaction. It has to be emphasized that the Cd^{2+} -evoked Ca^{2+} transient differs from the hormone-type signal with respect to the time course: the Cd^{2+} -stimulated Ca^{2+} transient is retarded compared with a hormone-like signal, and it lasts for about 10 min as compared with seconds in the case of hormones. The most probable hypothesis, still to be tested, is that Cd^{2+} indeed interacts with hormone receptors at the cell surface, but that the interaction leads to a conformation different from that of the hormone-bound receptor, which is slower in activating G-proteins.

Unlike Cd^{2+} , Zn^{2+} ions were unable to evoke an intracellular Ca^{2+} transient, and they even inhibited the effects of Cd^{2+} . Zn^{2+} is frequently protective against various biochemical and toxic effects of Cd^{2+} in a competitive manner. Indeed, Zn^{2+} ions seem to inhibit the binding of Cd^{2+} to a cell surface receptor in human fibroblasts [53]. In our system, too, we attribute the inhibition of Cd^{2+} -induced Ca^{2+} mobilization by Zn^{2+} to interference with the binding of Cd^{2+} to signalling protein(s). Inhibition by Zn^{2+} of cellular Cd^{2+} uptake has frequently been observed [33,54].

In summary, our experiments provide evidence for stimulation by Cd^{2+} of the $\text{Ins}(1,4,5)\text{P}_3$ /calcium signalling pathway in E367 neuroblastoma cells. Although there are differences in the detailed patterns of response to heavy metals between different cell types and different toxic metals, this signal transduction system seems to be an early and sensitive target of Cd^{2+} . The observed transient elevation of intracellular free Ca^{2+} may exert profound effects on the control of cellular proliferation and differentiation. It still remains to be elucidated which further elements in the cellular signal transduction pathway are involved. A possible role for protein kinase C and corresponding responsive elements at the genome level are under investigation in our laboratory.

REFERENCES

- Alkon, D. L. and Rasmussen, H. (1988) *Science* **239**, 998–1005
- Berridge, M. (1993) *Nature (London)* **361**, 315–325
- Clapham, D. E. (1995) *Cell* **80**, 259–268
- Carafoli, E. (1987) *Annu. Rev. Biochem.* **56**, 395–433
- Missaien, L., Wuytack, F., Raeymakers, L., De Smedt, H., Droogmanns, G., Declercq, I. and Casteels, R. (1991) *Pharmacol. Ther.* **50**, 191–232
- Pounds, J. (1990) *Environ. Health Perspect.* **84**, 7–15
- Nicotera, P., Bellomo, G. and Orrenius, S. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32**, 449–470
- Cerutti, P. A. and Trump, B. F. (1991) *Cancer Cells* **3**, 1–7
- Orrenius, S., McConkey, D. J., Bellomo, G. and Nicotera, P. (1989) *Trends Pharmacol. Sci.* **10**, 281–285
- Fawthrop, D. J., Boobis, A. R. and Davies, D. S. (1991) *Arch. Toxicol.* **65**, 437–444
- Pounds, J. (1984) *Neurotoxicology* **5**, 295–332
- Rossi, A. D., Manzo, L., Orrenius, S., Vahter, M. and Nicotera, P. (1991) *Pharmacol. Toxicol.* **68**, 424–429

- 13 Beyersmann, D., Hechtenberg, S., Block, C. and Kirchherr, H. (1992) in *Metal Compounds in Environment and Life*, vol. 4, (Merian, E. and Haerdi, W., eds.), pp. 187–192, Science and Technology Letters, Northwood
- 14 Smith, J. B., Dwyer, S. D. and Smith, L. (1989) *J. Biol. Chem.* **264**, 7115–7118
- 15 Schäfer, T., Benders, T. and Beyersmann, D. (1994) in *Metal Ions in Biology and Medicine* (Collier, P., Poirier, L. A., Littlefield, N. A. and Etienne, J. C., eds.), pp. 149–154, John Libbey Eurotext, Paris
- 16 Hughes, B. P. and Barritt, G. J. (1989) *Biochim. Biophys. Acta* **1013**, 197–205
- 17 Théronod, F. and Jones, S. W. (1992) *Biophys. J.* **63**, 162–168
- 18 Hinkle, P. M., Kinsella, P. A. and Osterhoudt, K. C. (1987) *J. Biol. Chem.* **262**, 16333–16337
- 19 Blazka, M. E. and Shaikh, Z. A. (1991) *Toxicol. Appl. Pharmacol.* **110**, 355–363
- 20 Verboost, P. M., Flik, G., Lock, R. A. C. and Wendelaar Bonga, S. E. (1988) *J. Membr. Biol.* **102**, 97–104
- 21 Verboost, P. M., Flik, G., Pang, P. K. T., Lock, R. A. C. and Wendelaar Bonga, S. E. (1989) *J. Biol. Chem.* **264**, 5613–5615
- 22 Zhang, G. H., Yamaguchi, M., Kimura, S., Higham, S. and Kraus-Friedmann, N. (1990) *J. Biol. Chem.* **265**, 2184–2189
- 23 Hechtenberg, S. and Beyersmann, D. (1991) *Enzyme* **45**, 109–115
- 24 Hechtenberg, S. and Beyersmann, D. (1994) *Environ. Health Perspect.* **102**, 265–267
- 25 Grummt, F., Weinmann-Dorsch, C., Schneider-Schaulies, J. and Lux, A. (1986) *Exp. Cell Res.* **163**, 191–200
- 26 Chesters, J. K. and Boyne, R. (1991) *Exp. Cell Res.* **192**, 631–634
- 27 Petrie, L., Chesters, J. K. and Franklin, M. (1991) *Biochem. J.* **276**, 109–111
- 28 Vallee, B. L. and Auld, D. S. (1990) *Biochemistry* **29**, 5647–5659
- 29 Berg, J. M. (1990) *Annu. Rev. Biophys. Biophys. Chem.* **19**, 405–421
- 30 Yamaguchi, M. (1993) *Biochem. Pharmacol.* **45**, 943–948
- 31 Büsselberg, D., Michael, D., Evans, M. L., Carpenter, D. O. and Haas, H. L. (1992) *Brain Res.* **593**, 77–81
- 32 Foulkes, E. C. and Blanck, S. (1990) *Toxicol. Appl. Pharmacol.* **102**, 464–473
- 33 Blazka, M. E. and Shaikh, Z. A. (1992) *Toxicol. Appl. Pharmacol.* **113**, 118–125
- 34 Smith, G. A., Hesketh, R. T., Metcalfe, J. C., Feeney, J. and Morris, P. G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7178–7182
- 35 Schanne, F. A. X., Dowd, T. L., Gupta, R. K. and Rosen, J. F. (1990) *Environ. Health Perspect.* **84**, 99–106
- 36 Hossmann, K.-A., Mies, G., Paschen, W., Szabo, L., Dolan, E. and Wechsler, W. (1986) *Acta Neuropathol.* **69**, 139–147
- 37 Flögel, U., Wilker, W. and Leibfritz, D. (1994) *NMR Biomed.* **7**, 157–166
- 38 Shaka, A. J., Keeler, J., Frenkiel, T. and Freeman, R. (1983) *J. Magn. Reson.* **52**, 335–338
- 39 Bachelard, H. S., Badar-Goffer, R. S., Brooks, K. J., Dolin, S. J. and Morris, P. G. (1988) *J. Neurochem.* **51**, 1311–1313
- 40 Dowd, T. L. and Gupta, R. K. (1991) *Biochim. Biophys. Acta* **1092**, 341–346
- 41 Metcalfe, J. C., Hesketh, R. T. and Smith, G. A. (1985) *Cell Calcium* **6**, 183–195
- 42 Long, G. J., Rosen, J. F. and Schanne, F. A. X. (1994) *J. Biol. Chem.* **269**, 834–837
- 43 Smith, P. K., Krohn, J. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- 44 Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- 45 Schanne, F. A. X., Dowd, T. L., Gupta, R. K. and Rosen, J. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5133–5135
- 46 Schanne, F. A. X., Moskal, J. R. and Gupta, R. K. (1989) *Brain Res.* **503**, 308–311
- 47 Denny, M. F. and Atchinson, W. D. (1994) *J. Neurochem.* **63**, 383–386
- 48 Simons, T. J. B. (1991) *J. Membr. Biol.* **123**, 63–71
- 49 Yamagami, K., Nishimura, S. and Sorimachi, M. (1994) *Brain Res.* **646**, 295–298
- 50 Schanne, F. A. X., Dowd, T. L., Gupta, R. K. and Rosen, J. F. (1990) *Biochim. Biophys. Acta* **1054**, 250–255
- 51 Schanne, F. A. X., Gupta, R. K. and Rosen, J. F. (1992) *Biochim. Biophys. Acta* **1180**, 187–194
- 52 Rossi, A. D., Larsson, O., Manzo, L., Orrenius, S., Vahter, M., Berggren, P.-O. and Nicotera, P. (1993) *FASEB J.* **7**, 1507–1514
- 53 Smith, L., Pijuan, V., Zhuang, Y. and Smith, J. B. (1992) *Exp. Cell Res.* **202**, 174–182
- 54 Friedman, P. A. and Gesek, F. A. (1994) *Toxicol. Appl. Pharmacol.* **128**, 257–263