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Susceptibility of insulinoma cells to cadmium and modulation by L-type calcium channels

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Abstract

Cadmium (Cd), a toxic metal that induces apoptosis and necrosis in a variety of cells, accumulates in pancreas and may be a cause of diabetes in humans. In the insulinoma cells line HIT-T15 (HIT), we measured internal calcium (Ca) and Cd levels by the fluorescent dye Fura-2 and confirm that L-type voltage-dependent calcium channels (VDCC) play a major role in glucose response and represent a pathway of Cd influx in these cells. Therefore we examined the role of VDCC in acute Cd poisoning by comparing its accumulation and cytotoxic effect in HIT cells and in epithelial-like VDCC-free HeLa cells. Cultures were incubated with 10–300 μM Cd for 15 min–6 h. While negligible at the end of the treatment, HIT cell death was evident after 18–24 h, and it was time-, dose- and serum-dependent. Short (≤60 min) Cd treatments with lower doses (≤100 μM in serum-free medium) induced delayed apoptotic cell death, as demonstrated by DNA fragmentation on agarose gels and segmentation of DAPI-stained nuclei. Longer incubations and/or higher concentrations caused mainly necrosis. The same treatments were largely harmless in HeLa cells, in which neither death nor DNA fragmentation was observed. The Ca antagonist nimodipine was capable to prevent HIT cell death at lower doses of Cd and to restore the apoptotic condition at higher doses, indicating that reduction of Cd flux through VDCC modulates Cd toxicity. These data demonstrate a specific sensitivity to Cd of insulinoma cells that can be significant for pancreatic β-cell pathology.

Introduction

Cadmium (Cd), a widespread environmental pollutant, affects many cellular functions and is involved in a variety of pathological conditions. Its main target organs are the liver, the kidneys and the bones, but Cd accumulates also in pancreas and it has recently been proposed that it may be a cause of diabetes in humans (Schwartz *et al.* 2003). A recent work has indicated specific islet β-cells injury and diabetes clinical signs in monkeys as a consequence of Cd treatment (Kurata *et al.* 2003).

Due to a close structural similarity to calcium (Ca), Cd binds Ca-binding proteins, such as calmodulin (Sutoo 1994; Ouyang & Vogel, 1998), with high affinity and blocks voltage-dependent calcium channels (VDCC). The same VDCC represent a major pathway of Cd influx in excitable cells (Hinkle *et al.* 1987, 1992; Shibuya & Douglas, 1992; Hinkle & Osborne 1994), including mammalian neurons (Usai *et al.* 1999).

Cell death caused by cytotoxical chemicals may display features of apoptotic or necrotic condition (Robertson & Orrenius 2000). Apoptosis is an active process, characterized by several structural changes in the cell, including the exposure of phosphatidylserine on the extracellular side of the plasma membrane, the activation of specific proteases (caspases), chromatin condensation and specific DNA fragmentation (Nagata 2000;

Robertson *et al*. 2000). Conversely necrotic cells swell, lyse and disperse cytoplasmic material and chromatin in the surrounding medium (Collins *et al*. 1997). In the presence of cytotoxical chemicals, apoptosis may be a mean of elimination of critically damaged cells and precedes necrosis (Habeebu *et al*. 1998). Different from several other heavy metal ions, which did not induce apoptotic DNA fragmentation (Watjen *et al*. 2002), Cd has been shown to induce apoptosis in many tissues and cells both in vivo (Hamada *et al*. 1991; Habeebu *et al*. 1998) and in vitro (Li *et al*. 2000; Shen *et al*. 2001; Watjen *et al*. 2002; Lopez *et al*. 2003), even when applied in short pulse treatments (Chrestensen *et al*. 2000; Galan *et al*. 2000).

It is well-known that part of the cellular uptake of Cd involves VDCC in excitable cells and it has been shown that Ca antagonist can protect against Cd induced cell death (Hinkle *et al*. 1987; Hinkle & Osborne 1994; Li *et al*. 2000). However, in those studies, the presence of VDCC per se did not seem to enhance sensitivity to Cd; for example VDCC expressing (PC12) and non-expressing (PC18) cells showed comparable LD_{50} for Cd (Hinkle *et al*. 1987; Hinkle & Osborne 1994; Li *et al*. 2000) and the role of this pathway in the induction of cell death appears questionable. On the other hand, it is generally believed that, due to the presence of VDCC, excitable cells accumulate Cd faster and are more vulnerable to acute Cd poisoning.

In the present study, we tested the effect of Cd on an insulinoma cell model, HIT-T15 (HIT) cells, a simian virus transformed hamster β-cell line that secretes insulin in response to glucose and possess different types of VDCC (Marchetti *et al*. 1994; Satin *et al*. 1995). In these cells, glucose-dependent insulin secretion is largely inhibited by the dihydropyridine (DHP) antagonists (Satin *et al*. 1995), because of the involvement of L-type VDCC of the neuroendocrine subtype ($Ca_V1.3$ or α_{1D}, (Scholze *et al*. 2001). Preliminary data indicated that in these cells Cd permeation occurs mainly through L-type VDCC, because it was entirely blocked by nimodipine, an antagonist DHP (Usai *et al*. 1997). Here we address the question to which extent these channels are responsible of Cd-induced cell damage by comparing the consequences of relatively short pulses of Cd in HIT cells and in non-excitable and VDCC-free epithelial-like HeLa cells. We first studied the toxic effect of Cd and then try to protect cells from this injure by VDCC blockers. We found that insulinoma cells not only accumulate significantly more Cd, but delayed cell death was strikingly more prominent in these cells than in HeLa cells and suggest a specific susceptibility of pancreatic β-cells to Cd.

Materials and methods

Cell culture

HIT-T15 clone (HIT) of Syrian hamster β-cells was purchased from American Type Culture Collection (Rockville, Maryland), at passage 59. These cells were maintained in Ham's F12K Kaighn's modification medium, supplemented with 2.5% FCS and 10% dialyzed horse serum, in a 5% CO_2 humidified atmosphere at 37 °C. HeLa cell line was obtained from European Collection of Cell Culture and maintained in MEM supplemented with 10% FBS.

Photometry

Measurements of intracellular Ca and Cd were performed as described previously (Marchetti *et al*. 1995; Usai *et al*. 1999). Monolayer HIT cultures were plated on glass coverslips and incubated with 5 μM fura-2AM for 30 min at 37 °C in a physiological solution containing (in mM): NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 5, pH 7.4. The coverslip was then washed and mounted on the stage of an inverted microscope (IM35 Zeiss, Germany), where it was continually superfused with the same solution, at room temperature. Excitation and emission were controlled and measured as in our previous works (Marchetti *et al*. 1995; Usai *et al*. 1999; Mazzolini *et al*. 2001). The final frequency of recording was 1 point every 0.5 s. The fluorescence ratio $R=E_{340}/E_{380}$ was calculated after subtraction of the background fluorescence. Cells were depolarized by superfusing a high potassium solution, which was prepared by equimolar substitution of sodium with potassium. In every experiment, cells were first challenged by this solution to verify the Ca response and assess their viability and health and then different test solutions were applied. The internal Ca concentration was calculated following a calibration procedure, as previously described (Marchetti *et al*. 1995). Cd treatment was performed in the absence of Ca and terminated by the addition of the membrane-permeant heavy metal

chelator (N,N,N′,N′-tetrakis-(2-pyridyl methyl) ethylenediamin (TPEN, 100 μm).

Cd treatment

Cells were incubated with 10–300 μM $CdCl_2$ for 15 min–3 h in medium with or without added serum or in phosphate buffered saline (PBS). In most experiments, the potassium concentration was elevated to 20 mM (HIT cells) and 50 mM KCl (HeLa cells) to depolarize the cell membrane and open VDCC. High KCl alone (no Cd added) had no effect on cell viability. Stock solutions of nimodipine and BayK (Sigma) were made up at 10 mM in 100% ethanol and diluted in medium to the final concentration. Neither nimodipine nor BayK had any effect on cell viability when applied without Cd. After incubation, cells were washed three times with PBS containing 1 mM EDTA and fresh medium containing serum was replaced. Cultures were kept in the incubator for another 18–24 h; then floating cells and adherent cells were harvested separately for further applications. Cell viability in each sample was assessed by trypan-blue exclusion assay.

Cd determination

Cells were exposed to Cd for 30 min–6 h in culture medium with or without added serum. After treatment, cultures were washed three times with PBS containing 1 mM EDTA and harvested immediately. Viable cells were counted in triplicate in each sample. Cells were then washed again three times with the above buffer, re-suspended in distilled water and disrupted by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 2 min in an ice bath. The total Cd content of each sample was measured by Flameless Atomic Absorption Spectroscopy (FAAS) using a Perkin Elmer Spectrophotometer (Model 1100 B) equipped with a graphite furnace (Model HGA 700). The Cd content was normalized to the total cell volume (see results).

DNA extraction and gel electrophoresis

Total genomic DNA isolation was performed using the classical phenol/chloroform extraction procedure. Attached and floating cells were collected as separated samples of about 10^7 cells/ml, incubated first with 20 μg/ml pancreatic ribonuclease-A for 3 h and then with 100 μg/ml proteinase K overnight at 37 °C in the presence of EDTA and SDS. The aqueous phase containing DNA was extracted once with phenol/chloroform/isoamylic alcohol and once with chloroform/isoamylic alcohol, then precipitated with Na chloride and cold ethanol. After centrifugation, the pellet was washed and dissolved in TE buffer. DNA was quantified by UV spectrophotometry. DNA oligonucleosomal ladder was assessed on 1.5% agarose gel containing ethidum bromide, loading about 3 μg of DNA sample per lane and running the gel at 40–50 V. A DNA marker (gene Ruler DNA ladder mix from MBI-Fermentas) was used as reference.

DAPI staining

Monolayer cultures on glass coverslips were incubated with 4,6-diamidino-2-phenylindole (DAPI, Sigma) 1 μg/mL in PBS for 5–10 min; floating cells were mixed in the same solution and left to settle down in glass chamber before performing the fluorescence analysis. Cells were observed under fluorescence microscopy on a Zeiss Axiovert 100 equipped with filters for UV excitation. Photographs were collected by a digital camera (Nikon Coolpix 995) for further examination; this procedure was suitable to overcome fluorescence loss due to photobleaching. Cell counts were performed on fields containing a comparable number of cells (20–30 stained nuclei); therefore samples containing too few cells were not included in the DAPI analysis. Nuclei were judged apoptotic when showing segmentation and masses mostly associated with the nuclear envelope or apoptotic bodies (Collins *et al.* 1997; Barrett *et al.* 2001).

Statistical analysis

Experiments were run in triplicate. Data are presented as mean ± standard error of the mean in at least two experiments. Statistical significance was estimated by unpaired two-tailed Student's *t* test.

Results

Voltage-dependent calcium channels in HIT cells

HIT cells express different types of VDCC (Marchetti *et al.* 1994; Satin *et al.* 1995). In Fura2-loaded

cells, depolarization induced by 15–30 mM KCl caused a rise in internal Ca that was robustly, but not completely antagonized by 1 μM nimodipine, an antagonist DHP that blocks L-type VDCC ($Ca_V1.3$ type). Application of elevated (20 mM) external glucose caused a rise in the internal Ca concentration, which was approximately of the same extent of that caused by 15 mM KCl in basal conditions. The increase was completely reversed by nimodipine, indicating that, in agreement with previous studies (Satin *et al.* 1995), L-type VDCC play a major role in glucose response in these cells.

When HIT cells were exposed to 100 μM Cd in a Ca-free solution, membrane depolarization elicited an increase in the fluorescence ratio R (E_{340}/E_{380}). The increase was completely reversed by addition of TPEN (100 μM), a non-fluorescent cell membrane-permeant heavy metal chelator, whose affinity for Cd is 12 orders of magnitude higher than that for Ca (Arslan *et al.* 1985; Usai *et al.* 1999). Thus the increase of R is due to influx and accumulation of Cd into the cells (Figure 1c). In addition, we had previously shown that, different from neuronal cells, the ratio elevation due to Cd influx was completely prevented in the presence of nimodipine (Usai *et al.* 1997).

Conversely, control experiments in epithelial-like HeLa cells confirmed that these cells do not possess VDCC. When Fura2-loaded cells were challenged by the high KCl solution, we never observed any Ca rise and the fluorescence ratio was similarly unaffected by Cd application (data not shown).

Cadmium cytotoxicity

We investigated how VDCC channels are involved in Cd cytotoxicity by treating the cultures with similar pulses of Cd and examining the effect on cell viability. Experiments were run in parallel in the two cell types.

When HIT cultures were incubated with Cd for 30–180 min, cell mortality was negligible immediately and up to 8 h after the treatment, but it became evident after 16–24 h, without notable differences within this lapse of time. Thus it appears that Cd accumulates into the cell during incubation, but the toxic mechanism leading to cell death becomes effective at a later time. In contrast, Cd did not apparently damage non-excitable epithelial-like HeLa cells after similar exposures. Optical microphotographs in Figure 2A were obtained 24 h after an incubation of 30 or 60 min with 100 μM Cd in serum-free high potassium medium for both cell types. HIT cells exposed to Cd lost adhesion and showed a reduction in the diameter. Because dead HIT cells tend to detach from the substrate, as previously reported (Loweth *et al.* 1997), the number of viable cells was estimated from the number of adherent, trypan-blue excluding cells and this number was compared to that of adherent viable cells in control conditions. As Cd influx is favored by depolarization, we examined the effect of raising the KCl concentration. With a 30 min treatment, the number of

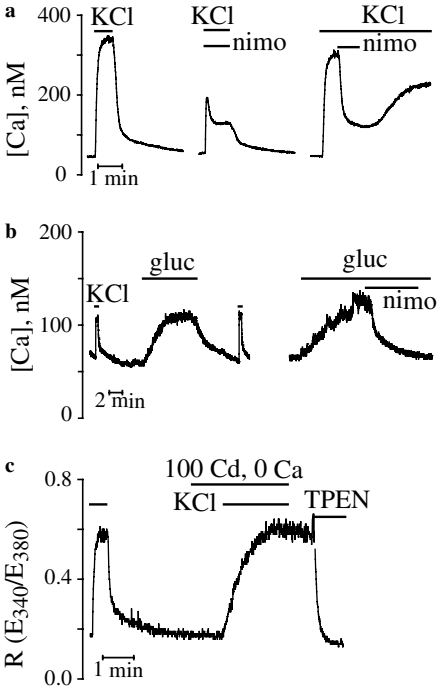

Figure 1. Role of VDCC in the physiological and toxicological response of HIT cells. (a) Depolarization driven Ca influx. Fura-2 loaded cells were stimulated with 25 mM external KCl, which caused a substantial internal Ca elevation. This elevation was only partly reversed by 1 μM nimodipine. (b) Internal Ca elevation during application of 20 mM glucose. Fura-2 loaded cells were first stimulated with 15 mM external KCl and then with 20 mM glucose, with roughly equivalent Ca rise (left panel). The glucose effect was reversed by 1 μM nimodipine (right panel), indicating the involvement of DHP-sensitive, L-type VDCC. (c) Depolarization driven Cd influx. Cells were first depolarized by exposure to 25 mM KCl in the presence of 1.8 mM Ca and subsequently exposed to 100 μM Cd, in a Ca-free solution. Cd permeation was negligible in basal condition and was triggered by 25 mM KCl depolarization, indicating a role of VDCC also in this response.

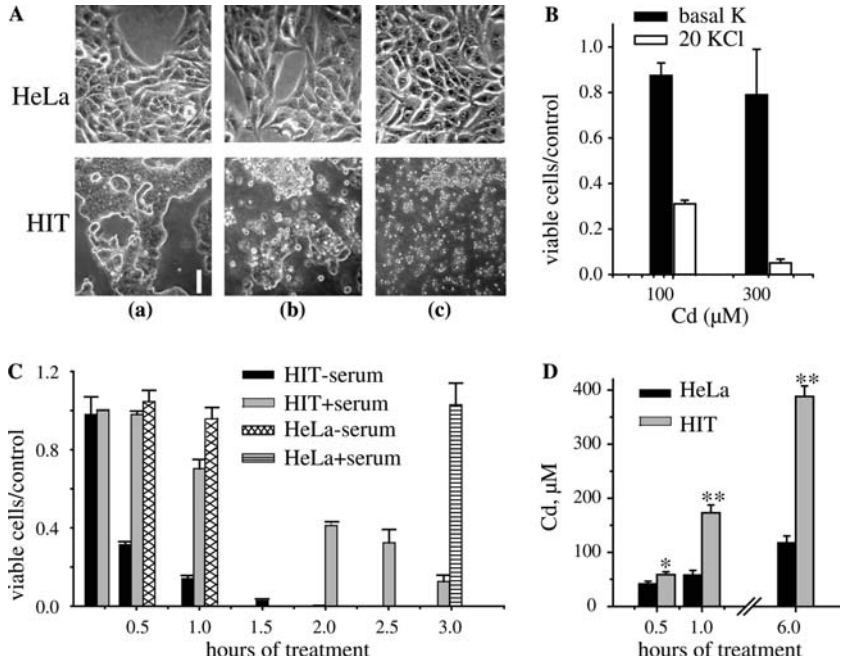

Figure 2. Cytotoxic effect of pulse treatment with Cd in HIT and lack of toxicity in HeLa cells (A) Microphotographs of cell cultures in control and after treatment with 100μM Cd in serum-free MEM supplemented with 50 mM KCl (HeLa cells, upper row) and in serum-free F12K medium containing 20 mM KCl (HIT, lower row). Pictures were taken 24 h after wash of the metal. (a) control cells; (b) and (c) cells treated for 30 and 60 min respectively. Bar=35μM (B) Effect of KCl-induced depolarization on HIT cell viability, with 30 min pulse treatment at the indicated doses of Cd. Viable cells were estimated from counts of trypan-blue excluding adherent cells 24 h after wash of the metal. The incubation medium was serum-free F12K with either 5 (basal KCl, filled bars) or 20 mM KCl (empty bars). (C) Effect of a pulse treatment with 100 μM Cd on cell viability, as a function of the duration of treatment. Viable cells were estimated from counts of trypan-blue excluding adherent cells 24 h after wash of the metal. Incubation media always contained 50 mM KCl (HeLa cells) or 20 mM KCl (HIT). In the presence of serum, the percentage of viable HIT cells was significantly higher ($P < 0.0001$ by Student's t test) than in serum-free medium with treatments from 0.5 to 2 h. HeLa cells were very resistant to Cd cytotoxicity with this kind of protocol. (D) Total Cd accumulation measured by FAAS. In both cell types, treatments of 0.5 and 1 h were performed in serum-free medium, while the 6 h treatment was in the presence of serum. Incubation media always contained 50 mM KCl (HeLa cells) or 20 mM KCl (HIT). In all cases, the difference between the concentration of Cd accumulated by HIT and HeLa was significant with (*) $P < 0.0005$ and (**) $P < 0.005$.

viable cells was significantly lower with 20 mM KCl both in 100 and 300 μM Cd ($P < 0.0001$, Figure 2B) than in basal (5 mM) KCl. Therefore, most of the experiments were performed in elevated KCl (Figure 2C).

As previously reported in neurons (Lopez *et al.* 2003), serum had a protective effect: a 30 min exposure to 100 μM Cd in a serum-free medium was already sufficient to induce 70% cell death, whereas with serum cells were largely spared after the same treatment (Figure 2C). In the relatively short time of our incubations, serum withdrawal by itself never affected cell viability. Also, the percentage of dead cells was not significantly different if the incubation was performed in serum-free medium or PBS (not shown); therefore results in the two latter conditions were pooled.

HeLa cells were very resistant to Cd with this kind of protocol. After 24 h from 30 min exposure to 100 μM Cd in serum-free medium, viable cells were 1.04 ± 0.06 of control and when treatment was continued up to 3 h in the presence of serum, viability was still 1.03 ± 0.11 of control. (Figure 2C). In addition, the culture was not significantly affected even with a 6 h treatment (not shown).

HIT cells mortality was dose-dependent. In serum-free medium with 20 mM KCl, the Cd concentration that reduces cell viability by 50% with respect to control cultures was 84 μM with 30 min and 35 μM with 60 min incubation. In the

same conditions, but in the presence of serum, the same Cd concentrations required longer applications to produce the same toxicity and the Cd concentration that reduces cell viability by 50% was 35 μM when Cd was applied for 2.5 h. In agreement with previous observations (Hinkle *et al.* 1987; Lopez *et al.* 2003), in the presence of serum the effective concentration of free Cd is unknown and for this reason most of the subsequent experiments were performed in serum-free medium.

The amount of total Cd accumulated during the treatments was evaluated by FAAS and normalized to the cell volume. The mean cell volume was estimated from the single cell capacitance. From electrophysiological measurements, HIT cells have an average electrical capacitance of 12.8 $\pm$ 4.5 pF (mean $\pm$ sd, n=75), while the capacitance of HeLa cells is 30 $\pm$ 7 pF (n=17). Assuming that both cell types are approximately spherical and have a specific membrane capacitance of 1 μF/cm^2, 10^6 cells comprise a volume of 4.4 μl (HIT) and 15.4 μl (HeLa). This calculation allows an approximate determination of cellular Cd content and the comparison of Cd accumulation in the two different cell types and, with this method, results were considerably more reproducible than using a standard normalization to the total protein content. In 30 min, HIT cells accumulate a significant larger concentration of Cd than HeLa cells and the difference increases considerably with longer treatments (Figure 2D).

To investigate the role of L-type VDCC in Cd-induced cell death, HIT cells were treated with Cd in the presence of nimodipine or BayK-8644, a DHP Ca agonist. Nimodipine was able to prevent detachment from substrate and cell death, as appears in the microphotographs of Figure 3A. With one hour treatment, nimodipine (1 μM) prevented cell death caused by 100 and 300 μM and shifted the dose required to induce 50% of cell death from 35 to 62 μM (Figure 3Ba). As a function of incubation duration, nimodipine had a protective effects when applied during 100 μM Cd treatments from 30 to 90 min (Figure 3Bb). The role of VDCC is confirmed by measurements of total Cd accumulation. In 30 min exposure, cells accumulated significantly ($P < 0.005$) more Cd in the presence of 20 mM KCl than in basal condition (5 mM KCl) and nimodipine was able to prevent this increase and even to reduce accumulation

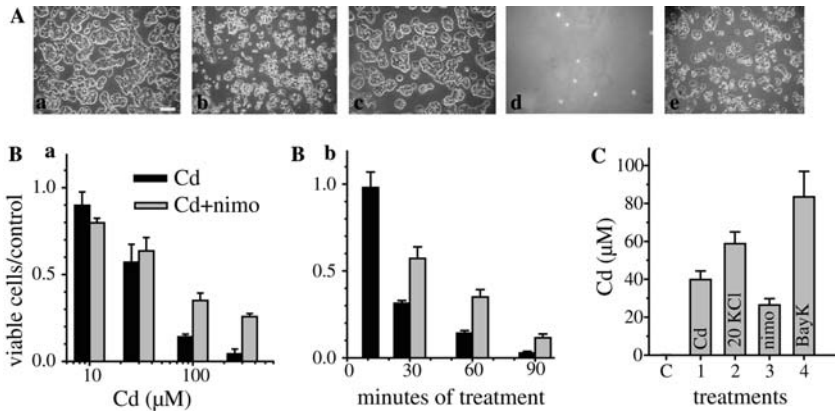

Figure 3. Protective effect of nimodipine on Cd cytotoxicity in HIT cells. Cells were subjected to the different treatments in serum-free F12K medium containing 20 mM KCl and examined 18–24 h after wash of the metal. (A) Microphotographs of cultures treated for 30 min in (a) control medium, (b) 100 μM Cd; (c) 100 μM Cd + 1 μM nimodipine; (d) 300 μM Cd; (e) 300 μM Cd + 1 μM nimodipine. Pictures were taken following wash out of the medium, which eliminated all floating cells. Bar=50 μM (Ba) Nimodipine protection as a function of Cd concentration. Treatments lasted 60 min. Viable cells were estimated from counts of trypan-blue excluding adherent cells 24 h after wash. Protection was significant with 100 ($P < 0.005$) and 300 μM Cd ($P < 0.05$). (Bb) Nimodipine protection as a function of the duration of treatment with 100 μM Cd. The number of viable cells was significantly higher in the presence of nimodipine than in Cd alone for 30 min ($P < 0.001$), 60 and 90 min ($P < 0.05$) incubation. (C) Total Cd accumulation cells in different conditions. Cells were incubated in serum-free F12K for 30 min; the concentration of Cd was 100 μM. In C (control), the Cd content was practically undetectable. In treatment (1) the cells were incubated in Cd in serum-free medium without other additions; in (2), the medium contained both Cd and 20 mM KCl, in (3) and (4), it was further supplemented with 1 μM nimodipine or BayK respectively. The total Cd content in (2) was significantly higher than in (1) with $P < 0.005$ and in (3) the Cd content was significantly lower than in (2) with $P < 0.00001$, but also lower than in (1) with $P < 0.05$. The difference between (4) and (2) was not significant.

below resting values. On the contrary, BayK determined a higher uptake.

Cd-induced apoptosis

In order to distinguish whether Cd induces necrotic or apoptotic cell death, living cells were stained with DAPI to visualize nuclear morphology. No significant modifications in nuclear morphology were observed up to 8 h after treatment. At later times (18–24 h after wash of Cd), treated cells were partly or totally detached from the substrate and were examined in separated samples of adherent and floating cells. Several nuclei were condensed and segmented, as typical of apoptotic cells (Figure 4Ab). The number of apoptotic nuclei were evaluated in each field and normalized to the number of total stained nuclei. The results of counts are summarized in Figure 4B. Condensed nuclei were more numerous in the floating cell sample than in the cells that were still attached to the substrate. Untreated cells were tightly attached to the substrate and were stained as monolayers; their nuclei appeared homogeneously bright (Figure 4Aa).

As a second marker to detect apoptosis, total genomic DNA was extracted and analyzed by agarose gel electrophoresis. The DNA of the untreated sample consisted of high molecular weight fragments, as seen in the lane marked with 0 in Figure 5a and the lane marked with C in Figure 5b. In contrast DNA extracted from samples treated with 10 or 30 μM Cd for 2.5 h in the presence of serum (lane 3–4 in Figure 5a) showed oligonucleosomal fragments, indicating induction of apoptosis in a dose-dependent manner. The oligonucleosomal ladder was never observed in DNA extracted from cells collected immediately and up to 8 h after Cd treatment, but only in samples collected after 18–24 h; moreover it was always more remarkable in the DNA extracted from floating than from adherent cells, indicating that at this stage of apoptosis cells have already lost adhesion to the substrate. When treatment was performed with higher Cd concentration (100 μM in Figure 5a), internucleosomal DNA fragmentation decreases, but random fragmentation, indicative of necrosis, appears as a 'smear' on the agarose gel, as previously described (Collins *et al*. 1997). It seems therefore that, when Cd level rises over a threshold, the mechanism that directs the cell to enter apoptosis is somehow overcome and necrosis takes over. Gel patterns also confirm

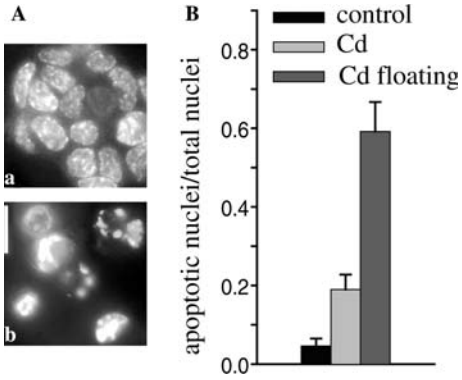

Figure 4. Evidence of Cd-induced apoptosis in HIT cells: nuclear staining. Cells were incubated with 100 μM Cd in serum-free 20 mM KCl F12K medium for 30 min. (A) DAPI imaging of cell nuclei after a treatment with 0 (a) or 100 μM Cd (b). Cells were stained with DAPI 24 h after wash of the metal. The cells in (a) are adherent cells, whereas in (b) are from the floating fraction, with several apoptotic nuclei visible in this field. Bar= 12 μM. (B) Counts of apoptotic nuclei in the different samples, 24 h after treatment. Control: vehicle only; Cd: adherent cells 24 h after wash of Cd; Cd floating: cells collected from the medium 24 h after wash of Cd. Floating control samples contained a negligible number of cells and were not counted.

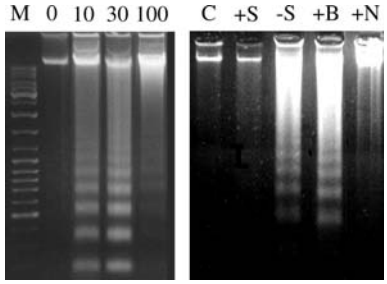

Figure 5. Evidence of Cd-induced apoptosis in HIT cells: DNA fragmentation. Gel electrophoresis of total genomic DNA extracted from the floating component collected 18 h after wash out of the metal. Cells were subjected to the different treatments in F12K medium containing 20 mM KCl with or without serum. (a) Cells were incubated with different doses of Cd for 150 min in the presence of serum; lane M is the marker and lane 0 is the control (complete medium, 0 Cd). DNA fragmentation appears at low doses of Cd and disappear at the highest dose (100 μM); (b) Cells were incubated with 100 μM Cd for 30 min in C=control (complete medium, 0 Cd), + S, complete medium + 100 μM Cd; -S, serum-free medium + 100 μM Cd. +B, serum-free medium + 100 μM Cd + 1 μM BayK 8644; +N, serum-free medium + 100 μM Cd + 1 μM nimodipine. DNA fragmentation is present only with Cd alone and Cd with BayK, but absent in the presence of serum and in Cd plus nimodipine.

the protective effect of serum, because a 30 min treatment with 100 μM Cd caused DNA fragmentation only in the absence of serum (Figure 5b). Note that serum withdrawal never induced DNA fragmentation by itself, as verified in routinely performed control experiments (not shown). In addition, DNA fragmentation was largely prevented by 1 μM nimodipine, but not by 1 μM BayK (Figure 5b).

The protection offered by nimodipine was further investigated as a function of concentration and duration of treatment. In Figure 6A, control nuclei of adherent cells (Aa) are shown and compared with nuclei of floating cells, 24 h after a 30 min treatment with 100 μM Cd or 300 μM without and with nimodipine. The apoptotic nuclear appearance was frequent in cells treated with 100 μM Cd for 30 min (Figure 6Ab), whereas with the higher concentration dead cell nuclei displayed a round and homogeneous staining, suggesting the occurrence of necrosis (Figure 6Ac). The number of apoptotic nuclei were evaluated in each field

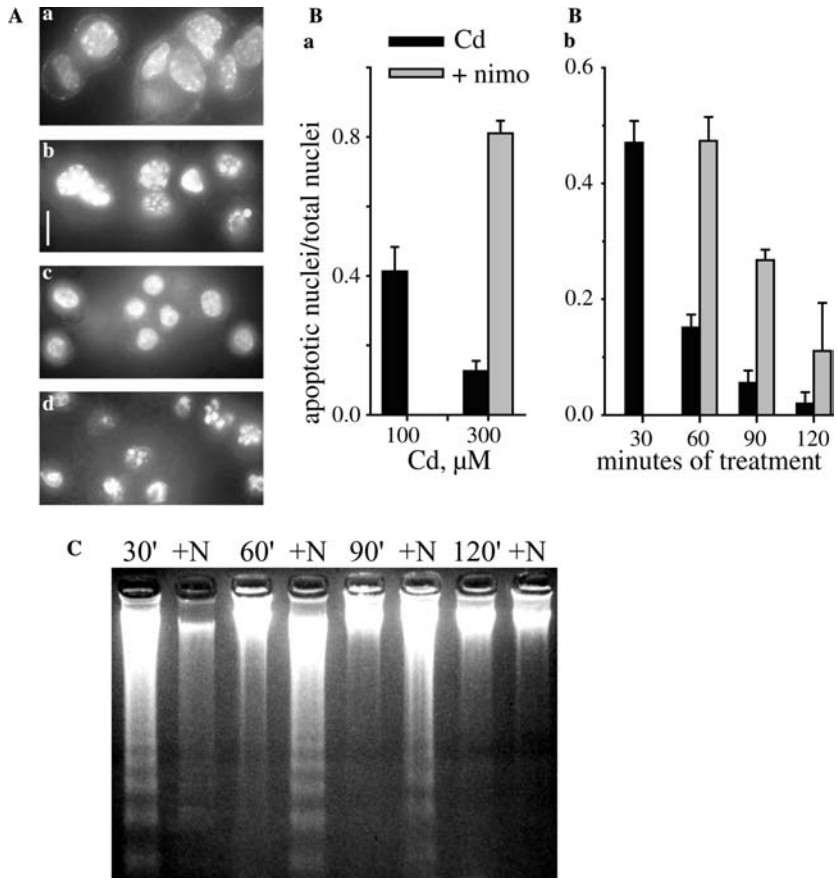

Figure 6. Modulation of Cd-induced apoptosis and necrosis by nimodipine in HIT cells. Cells were subjected to the different treatments in serum-free F12K medium containing 20 mM KCl. (A) DAPI imaging of nuclei. Cells were stained 24 h after treatment in (a) control medium, (b) 30 min in 100 μM Cd, (c) 30 min in 300 μM Cd, (d) 30 min in 300 μM Cd and 1 μM nimodipine. Bar=12 μM. (B) Counts of apoptotic nuclei in the different conditions. (a) Floating cells, after treatment for 30 min with 100 or 300 μM Cd, without (black bars) or with 1 μM nimodipine (gray bars). The sample treated with 100 μM Cd and nimodipine contained a negligible number of cells and counts are not included. Nimodipine significantly ($P < 0.0001$) increased the number of apoptotic nuclei in cells treated with 300 μM Cd, which were mostly necrotic without DHP. (b) Floating cells, after treatments of different durations with 100 μM Cd, with or without nimodipine. The sample treated for 30 min with Cd and nimodipine contained a negligible number of cells and counts are not included. Nimodipine significantly ($P < 0.0001$) increased the number of apoptotic nuclei in cells treated for 60 and 90 min, in agreement with results of gel electrophoresis in part C. (C) Gel electrophoresis of total genomic DNA from floating cells. Cells were incubated for the indicated time with 100 μM Cd or 100 μM Cd and 1 μM nimodipine (+N). DNA ladder is visible with incubation of 30 min with Cd alone and incubation of 60 and 90 min with Cd plus nimodipine.

and normalized to the number of total stained nuclei; the results of these counts are summarized in Figure 6B. Nimodipine rescued most of the cells in the presence of 100 μM Cd, making counts of the floating sample non-significant. On the contrary, a high percentage of cells treated with 300 μM Cd and nimodipine displayed nuclei with apoptotic features (Figure 6Ad), as seen with lower doses of Cd. A similar result was obtained when comparing treatment with 100 μM Cd of different durations in the absence and in the presence of nimodipine. Both DAPI staining of floating cell nuclei (Figure 6Bb) and agarose gel electrophoresis of total genomic DNA (Figure 6C) indicate that nimodipine protects cells from death in the short (30 min) treatment and is capable to redirect cells toward programmed cell death in longer treatment. Thus the protective action of nimodipine depends on both time of incubation and Cd concentration, suggesting that in all cases its effect is that of reducing Cd influx.

Discussion

Our data show that, in HIT cells, short pulses of Cd cause delayed cell injury and that blocking VDCC modulates the pattern of cell death. This clonal line represents an appropriate model because, as native β-pancreatic cells, HIT cells express multiple types of VDCC (Marchetti *et al.* 1994; Satin *et al.* 1995) that mediate both physiological function and toxicological effects. In particular, it was shown that glucose-induced insulin secretion is dependent on L-type VDCC in native β-pancreatic cells (Liu *et al.* 2003; Schulla *et al.* 2003), and in HIT cells (Satin *et al.* 1995). The same channels seem to be responsible for the depolarization-driven Cd influx, as shown by the observation that DHP antagonists inhibited Cd entry (Usai *et al.* 1997). The present study confirms that L-type VDCC are a major pathway mediating rapid Cd influx and toxicity, because the effect of Cd on cell viability was enhanced by an elevated KCl concentration (Figure 2B) and reduced by the VDCC antagonist nimodipine (Figure 3B). In resting condition, no sizeable Cd permeation was revealed by intracellularly entrapped Fura-2 (Figure 1C; see also Usai *et al.* 1999), but the total Cd content as measured by FAAS was increased with 30 min incubation (Figure 2D). These observations are not in contrast, because the two measurements are not exactly equivalent: FAAS measurements were performed on entire cells, while Fura-2 only binds Cd that enters the cytoplasm. It may be that the total Cd content comprises also an additional fraction, which is associated to membranes or to high-affinity binding proteins.

Previous studies demonstrated the possibility to protect VDCC containing cells against Cd toxicity by Ca antagonists (Hinkle *et al.* 1987; Hinkle & Osborne, 1994; Li *et al.* 2000), but the question whether the mere presence of VDCC can make cells more vulnerable to Cd injury was not clearly addressed. Our protocol, consisting of short pulses in serum-free high KCl medium and delayed death observation, is able to unmask completely the role of VDCC in triggering the Cd accumulation that leads to cell death. We chose to test the Cd effect in the absence of serum, even if this may represent a distance from *in vivo* conditions. However, Cd binds largely to serum proteins, such as albumin, and free rather than protein-bound Cd is transported into the cells (Hinkle *et al.* 1987), making it difficult to relate effects to precise doses. Moreover, serum proteins bind also DHPs, and the effect of these L-type VDCC ligands can be studied accurately only in the absence of serum. Our results are in fair quantitative agreement with those of Lopez et al. (Lopez *et al.* 2003), who reported a six time higher uptake of Cd in the absence than in the presence of serum in cortical neurons. In the present work, the effect of treatment with 100 μM Cd for 2.5 h in serum was comparable to that in serum-free medium for 0.5 h (Figure 2C).

Because of the many different protocols used in the different studies, it is difficult and somewhat misleading to try to compare sensitivity in term of LD_{50} estimation. As an example, in the original work by Hinkle et al. (1987), who first proposed a role of VDCC in Cd toxicity, LD_{50} is evaluated for 3 day treatment in serum. More recently, in anterior pituitary cells (Poliandri *et al.* 2003), an endocrine cell type that express several VDCC, the effect of Cd treatment on cell viability was less severe than in our cells, but cells were treated in the presence of serum and absence of depolarization. In most cases, the effect of Cd was found to be much different in different cell types. a Chrestensen *et al.* (2000) used a protocol similar to ours and reported significantly different effects

in H9 and Jurkat cells; however these authors do not have data on Cd influx and accumulation and it is not possible to correlate the difference in sensitivity on different modes of Cd influx. Despite these discrepancies in the experimental conditions, the striking sensitivity of the insulinoma for Cd-induced death is particularly relevant. Moreover, in insuline secreting cells, the effect of depolarization is pertinent because they are expected to be electrically active in the presence of a metabolic stimulus, such as glucose elevation.

This study also shows that HeLa cells are largely resistant to Cd and this observation poses the question if and to which extent the absence of functional VDCC by itself may be a protective factor for these kind of cells. HeLa cells have been reported to be significantly vulnerable to Cd poisoning (Szuster-Ciesielska *et al.* 2000) when exposed for 24 h; but in our short treatments, Cd did not induce either death or DNA fragmentation. Cd content measurements (Figure 2D) showed that HeLa cells do accumulate Cd with a time course similar to that reported for intestinal HT29 cells, where a contribution of VDCC was not relevant (Lecoeur *et al.* 2002). The accumulation of Cd in HeLa cells was quantitatively inferior to that in HIT cells and can be explained in several ways. First, as already observed, total Cd content measurements by FAAS cannot provide information on where Cd accumulation occurs and some of the metal can be associated to membranes. Secondly, alternative pathways of Cd influx, such as divalent metal transporters (Lecoeur *et al.* 2002; Okubo *et al.* 2003), undoubtely exist in all cell types. Indeed, VDCC are not the only pathway of Cd entry, even in excitable cells. However, Cd accumulation driven by these alternative mechanisms is apparently not sufficient to induce cell death in short incubations such as those we have used. It seems possible that cells are able to handle a certain amount of toxic metal without showing any acute consequence, and this amount would vary depending on the cell type; but above a certain threshold the processes of apoptosis and/or necrosis become irreversible. In view of the presence of this threshold, we propose that VDCC membrane density could be considered a discriminating factor for Cd sensitivity.

It can be argued that the HeLa cells resistance to Cd is not due only to limited influx, but also to other protective factors, such as an enhanced level of expression of detoxification proteins or metallothioneins (Kille *et al.* 1994). Induction of metallothioneius (MT) perturbed Cd-induced apoptotic cell death in several cellular models, including a HeLa cell-derived line (Shimoda *et al.* 2001). Cd has been shown to induce MT in the pancreas (Minami *et al.* 2002), but data on basal level and MT induction are not available for insulinoma cells. So it is not possible at present to reach a quantitative conclusion and this point needs further inspection. Inducible detoxification mechanisms may be involved in HeLa cell resistance to 6 h treatment, which determined a sizeable accumulation (Figure 2D), but no dramatic effects on cell viability. However, it is unlikely that even a substantial difference in MT content and induction can account completely for the difference in sensitivity to Cd of the two cell types.

Cd-induced apoptosis has been investigated intensively (Hamada *et al.* 1997; Pulido & Parrish, 2003). Here we show that Cd causes chromatin condensation and oligonucleosomal DNA fragmentation. Both of these marks appear late during apoptosis, when cells are largely detached from the substrate. On the other hand, these marks are not present in dead cells when the duration and intensity of treatment increased and necrosis takes over, as previously reported in different tissues and cell types (Habeebu *et al.* 1998; Lopez *et al.* 2003). Interestingly, death or toxicity signals in HIT cells appeared only after several hours from exposure and this delay was independent of the later occurrence of predominant apoptosis or necrosis. This suggests that necrosis may develop as a 'failure' of the apoptotic program, when energy levels in the cells are reduced (Robertson & Orrenius, 2000; Nicotera, 2003). With our protocol, the presence of a more noxious insult does not accelerate the death process, but modifies it. Moreover the occurrence of apoptosis or necrosis can be modulated by nimodipine, indicating that it is clearly dependent on Cd influx or internal Cd concentration. Also in this case, the effect of nimodopine can be interpreted as protective, because apoptosis is a cellular way of demise that causes minimal injury to neighbor cells and tissues, preventing the spreading of inflammation.

Our work suggests a specific susceptibility of insulinoma cells to Cd poisoning, with the possibility to control it by L-type VDCC antagonists. In view of the specific damage that Cd can exert on

pancreas, we propose that both the role of VDCC channels and the effect of Cd on β-cells are revaluated. However, further work is required to establish the relevance of these mechanisms in vivo.

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