British Journal of Pharmacology (2007) 151, 1176–1186
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

IP_3 receptor antagonist, 2-APB, attenuates cisplatin induced Ca^{2+}-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis

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Background and purpose: Cisplatin drives specific types of tumour cells to apoptosis. In this study we investigate the involvement of intracellular calcium ($[Ca^{2+}]_i$) in triggering apoptosis in two different cell lines. As cisplatin is used for the treatment of several forms of cancer we choose HeLa-S3 and U2-OS as two examples of tumour cell lines.
Experimental approach: Cisplatin (1nM–10μM) was applied to HeLa-S3 and U2-OS cells and $[Ca^{2+}]_i$ measured with fluo-4, using laser scanning microscopy. Inositol-1,4,5-trisphosphate (IP_3) receptors were visualized with immunostaining. Membrane conductances were measured with patch-clamp techniques. Levels of calpain and caspases were assessed by western blots and apoptotic cells were stained with Hoechst 33342 and counted.
Key results: Cisplatin increases $[Ca^{2+}]_i$ concentration-dependently in HeLa-S3 but not in U2-OS cells. This elevation of $[Ca^{2+}]_i$ depended on extracellular Ca^{2+} but was reduced by the IP_3 receptor blocker, 2-APB. This effect was not due to a Ca^{2+} release triggered by Ca^{2+} entry. Immunostaining showed IP_3-receptors (type1-3) at the cellular membrane of HeLa-S3 cells, but not in U2-OS cells. Electrophysiological experiments showed an increased membrane conductance with cisplatin only when Ca^{2+} was present extracellularly. Increase of $[Ca^{2+}]_i$ was related to the activation of calpain but not caspase-8 and triggered apoptosis in HeLa-S3 but not in U2-OS cells.
Conclusions and implications: Our observations on the activation of IP_3-receptors, calcium entry and apoptotic rate by cisplatin in specific carcinogenic cells might open new possibilities in the treatment of some forms of cancer.
British Journal of Pharmacology (2007) **151,** 1176–1186; doi:10.1038/sj.bjp.0707335; published online 25 June 2007

Keywords: cisplatin; carboplatin; anticancer drugs; calcium signalling; HeLa-S3; U2-OS; calpain; apoptosis; IP_3-receptor; calcium stores; $[Ca^{2+}]_i$

Abbreviations: ATCC, American-type culture collection; 2-APB, 2-aminoethoxydiphenyl borate; $[Ca^{2+}]_i$, intracellular calcium; CTX, charybdotoxin; ER, endoplasmic reticulum; IP_3 receptor, inositol-1,4,5-trisphosphate receptor; K_{Ca} channels, calcium-activated potassium channels; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; TBS-T, Tris-buffered saline Tween-20; TRAIL, TNF-related apoptosis-inducing ligand

Introduction

Cisplatin (cis-diammindichloroplatin) is widely used as a potent anti-tumour agent for treating different forms of cancer, like colon, lung or cervix carcinoma. However, treatment with cisplatin is often accompanied by cytotoxic, ototoxic and nephrotoxic effects (Dehne *et al*., 2002; Schloffer *et al*., 2003; Goren, 2003; Noda *et al*., 2006). Owing to the impairments of kidney and hearing functions, many patients discontinue the treatment with cis-diammindichloroplatin (CDDP). Moreover, cisplatin is known to be carcinogenic and genotoxic in mammalian cells. This is a major concern because of the risk of inducing secondary malignancies. Additionally, the occurrence of cellular resistance limits the therapeutic efficiency of cisplatin (Marzano *et al*., 2004).

The aim of chemotherapy of human malignancies is the specific inhibition of tumour cell proliferation and/or induction of apoptosis. It has been demonstrated that intracellular calcium ($[Ca^{2+}]_i$) signals are involved in a number of events, including apoptotic pathways (Mandic *et al*., 2002; Arany *et al*., 2004; Jaffe, 2005). While Ca^{2+} could be released from the $[Ca^{2+}]_i$ stores, as it has been demonstrated for other metal compounds, such as trimethyltin or As_2O_3 (Florea and Büsselberg, 2005, 2006; Florea *et al*., 2005a, b, 2007), Ca^{2+} also could enter from the extracellular space through membrane channels. Recently it has been shown that the activation of inositol-1,4,5-trisphosphate (IP_3) receptors, found at the cellular membrane, results in an elevation of $[Ca^{2+}]_i$ (Dellis *et al*., 2006).

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Received 11 January 2007; revised 18 April 2007; accepted 9 May 2007; published online 25 June 2007

The modulation of $[Ca^{2+}]_i$ influences the activation of calpain (Sharma and Rohrer, 2004), which controls cell-cycle regulation, differentiation and apoptosis (Alznauer *et al*., 2004). Activation of calpain from pro-calpain leads to apoptosome formation by cleavage of Bid, which in turn regulates Bax and activates caspase-3 (Mandic *et al*., 2002; Alznauer *et al*., 2004).

Cisplatin has been shown to trigger apoptotic pathways in tumour and nontumour cell models, *in vitro* or *in vivo* (Schloffer *et al*., 2003; Fan *et al*., 2005). However, details of early events leading to apoptosis after exposure to cisplatin are not yet understood. Here we have investigated the hypothesis that Ca^{2+} as a second messenger plays an important role in the cisplatin-induced apoptosis in HeLa-S3 cells.

Materials and methods

Cell culture

HeLa-S3 (human cervix adenocarcinoma) cells (ATCC, American-type culture collection) were cultured in Ham's F-12 medium (Sigma, Taufkirchen, Germany) supplemented with 10% foetal calf serum (Cambrex Biowhitaker, East Rutherford, NJ, USA), 100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

U2-OS (human osteosarcoma) cells (ATCC) were incubated in RPMI 1640 medium (GIBCO) containing 10% foetal calf serum, 100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin under the same conditions as HeLa-S3.

Confocal laser scanning microscopy

Twenty-four hours before experiments, HeLa-S3 and U2-OS cells were harvested and seeded to 7.5 cm^2 'easy grip' petri dishes (Falcon, Franklin Lakes, NJ, USA). For experiments the culture medium was changed to Tyrode buffer (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, pH 7.4 by 1 M NaOH, 1.8 mM $CaCl_2$ and 1.2 mM $MgCl_2$). In the experiments with nominal 'no calcium' in the extracellular solution, no calcium salts were added to the solution. For measurements under 'no calcium' conditions, the normal solution was replaced immediately before starting the experiment. Changes of $[Ca^{2+}]_i$ as well as the changes of the fluorescence in the calcium stores were measured as described previously (Florea *et al*., 2005a, b, 2007). Briefly, to study $[Ca^{2+}]_i$ modulation by cisplatin, two Ca^{2+} sensitive dyes were used: fluo-4/AM (Molecular Probes, Karlsruhe, Germany) to observe changes of $[Ca^{2+}]_i$ and rhod-2/AM (Sigma) to determine the changes of Ca^{2+} within $[Ca^{2+}]_i$ stores. Fluorescence images were collected at room temperature every 30 s. Full-screen images were taken which allows the analysis of the $[Ca^{2+}]_i$ rise in single cells. The cells were marked as 'regions of interest' and the calcium concentration was calculated offline.

Cisplatin (Bristol-Meyers Squibb, Munich, Germany) or carboplatin (Sigma) in the concentration range of 1 nM–10 μM was added to the cell culture using a constant flow application system (1 ml min^{-1}). 2-Aminoethoxydiphenyl borate (2-APB; Tocris, Bristol, UK) was dissolved in dimethylsulphoxide and used at its IC_{50} of 50 μM (Mignen *et al*., 2005). All experiments were performed at room temperature (24–26°C).

For determination of calcium concentrations, the META-Software (ZEISS) was used, with the equation: $[Ca^{2+}]_i = K_d{}^*(F-F_{min}/F_{max}-F)$, where K_d is the dissociation constant (taken from the data sheet for fluo-4 as provided by Molecular Probes) and F the fluo-4 intensity. The basal $[Ca^{2+}]_i$-concentration was considered 100 nM, as documented previously (Grynkiewicz *et al*., 1985; for review see Orrenius *et al*., 2003). All images were background substracted. $[Ca^{2+}]_i$ concentrations obtained under control conditions were set to 100% and all other values were calculated as percentage of increase in control.

Immunostaining

HeLa-S3 and U2-OS cells were washed with Tyrode buffer and fixed with 1% paraformaldehyde. Cells were stained 1:200 with specific anti-IP_3 receptor antibodies ('anti-IP_3RI–III PAb polyclonal', Calbiochem, San Diego, CA, USA; and 'anti-IP_3R-I PAb polyclonal' Alexis, Lörrach, Germany) and washed after 20 min with Tyrode buffer. Secondary antibody immunoglobulin G (IgG)-anti-rabbit fluorescein isothiocyanate used diluted 1:1000. Cells were loaded for 30 min. Coverslips were mounted on slides and pictures were collected with ZEISS 510 imaging system. Single pictures and Z-stacks were taken with ZEISS lens (63 × /1.4).

Electrophysiology

Membrane currents in HeLa-S3 cells were recorded as described by Splettstoesser *et al*. (2005), but electrodes were backfilled with the following solution (140 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 11 mM EGTA and pH 7.4 by 0.1 M NaOH). Culture medium was replaced by Tyrodes buffer.

In the experiments where the influence of the current through calcium-activated potassium channels (K_{Ca}) on cisplatin-induced changes of the membrane conductance was analysed, charybdotoxin (3.3 nM, Alomone Labs, Jerusalem, Israel) was added to the extracellular Tyrodes buffer.

Membrane potential was clamped at −80 mV. Voltage ramps from −110 to + 20 mV over 1.3 s were performed and membrane currents were recorded. Currents were elicited every minute for at least 1 h. Cisplatin was applied by a bath application system, for total exchange a volume of 10 ml was flushed through the bath (volume 1 ml) with a continuous flow of 1 ml per min.

Changes of the membrane conductance at the resting membrane potential (−80 mV and at + 20 mV) were analysed over the time of the experiments. Control values were set to 100% and all other values were calculated in relation to control.

Preparation of cell extracts and western blots

Cells were grown in six-well dishes, 300 000 cells ml^{-1} were seeded and incubated overnight. Cultures were exposed to 1 μM cisplatin and/or 50 μM 2-APB as well as to TNF-related apoptosis-inducing ligand (TRAIL; 160 ng ml^{-1}; Calbiochem) for 15 min and 1 h. For preparation of cell lysates, the

monolayer was washed with ice-cold phosphate-buffered saline (PBS), drained, lysed on the plates with 50 μl lysis buffer (300 mM NaCl, 10 mM Tris (pH 7.9), 1 mM EDTA, 0.1% NP40, 1 × protease-inhibitor-cocktail; (Roche, Mannheim, Germany)), and then scraped and left for 20 min on ice. The extract was centrifuged (2300 *g*, 4°C, 5 min), and the protein content was quantified using a spectrophotometer and a protein assay reagent (bovine serum albumin; Bio-Rad Laboratories, Hercules, CA, USA). Lysates were stored at −80°C until western blots were performed.

SDS-PAGE and western blotting

Sample buffer (50 mM Tris (pH 6.8), 2% SDS (sodium dodecyl sulphate), 5% β-mercaptoethanol, 0.0125% bromphenol blue, 1% glycine) was added 1:4 to the whole-cell lysate and boiled for 5 min at 95°C. A 50–75 μg weight of protein was used per well of a 7.5% SDS-PAGE (polyacrylamide gel electrophoresis). Gels were run for ~90 min at 120 V at room temperature (running buffer: 25 mM Tris base, 190 mM glycine, 3.5 mM SDS; transfer buffer: 25 mM Tris base, 190 mM glycine, 20% methanol) and transferred for 80 min at 120 V to nitrocellulose membrane (0.2 μm pore size, Schleicher and Schuell, Dassel, Germany). A protein marker (SDS 7B, Sigma) was used as a protein size control. The efficiency of protein transfer and equal loading was confirmed by staining the nitrocellulose membrane with Ponceau S (Sigma). Membranes were blocked overnight at 4°C with 5% non-fat dry milk in TBS-T (Tris-buffered saline Tween-20) (10 mM Tris (pH 7.5), 100 mM NaCl, 0.05% Tween 20), then washed in TBS-T and incubated with the primary antibody at room temperature for 2 h with gentle shaking. Primary antibodies used were rabbit polyclonal antibody against calpain-1 (58 Kda) domain I (1:5000, Calbiochem) and goat polyclonal antibody against active caspase-8 (2 kDa large and 18 kDa small subunit) (R&D Systems, Minneapolis, MN, USA). After washing with TBS-T, the blot was incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG antibody (Sigma). Immunoreactive proteins were visualised using ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Uppsala, Sweden), followed by exposure to X-ray films (Agfa, Mortsel, Belgium).

Hoechst staining and scoring of apoptotic cells

Chamber slides (LabTek, Wiesbaden, Germany) were seeded with 100 000 cells, each well containing 1 ml culture medium were incubated overnight. One micromolar of cisplatin was applied for 1 h. After the incubation, cells were washed twice with PBS, fixed and treated with cold methanol (−20°C) and left overnight at −20°C. After fixation, slides were air dried and stained with 10 μM Hoechst 33342 (Molecular Probes) for 30 s and mounted with cover slips. The cells were analysed using a × 63 magnification with an Axiovert fluorescent microscope.

Trypan blue cytotoxicity test

For determination of the cell viability, treated as well as untreated cells were taken for the experiments. Non-confluent cell monolayers were exposed to 1 μM cisplatin in cell culture flasks for 1, 2, 6, 24, 48 and 72 h. All experiments were repeated twice. After the treatment with cisplatin, cells were harvested using trypsin and resuspended in culture medium, washed, centrifuged (2 min, 100 *g*) and resuspended again in complete culture media. Then, a small aliquot of the cell suspension (50 μl) was mixed with the same volume of 0.4% trypan blue (Sigma) solution and the sample was counted after 3 min of staining using an improved Neugebauer Chamber. The number of bright (viable) cells and blue cells (nonviable) were evaluated using a light microscope with a 20-fold magnification. After counting, the cell viability (CV) was expressed as the percentage of surviving cells compared to the total number of cells: CV = (viable cells/total number of cells)100. Cisplatin was considered to be cytotoxic when it induced a decrease of cell viability of more than 50% compared to the negative control.

Data analysis

Results are presented as means ± s.d. from the number of assays shown. Statistical analysis was carried out using Student's *t*-test (two tailed, paired or unpaired as appropriate) and values of $P < 0.05$ was considered significant.

Results

Cisplatin induces a concentration-dependent increase in $[Ca^{2+}]_i$ in HeLa-S3 but not in U2-OS cells

Measurements of $[Ca^{2+}]_i$ concentration in untreated HeLa-S3 cells using Fluo-4 ranged between 120 and 220 nM (154.6 ± 48.8 nM). With the application of cisplatin (10 μM), $[Ca^{2+}]_i$ increased over a time interval of 15–35 min until it reached a plateau at 250–400 nM (Figure 1a). The increase in $[Ca^{2+}]_i$ caused by cisplatin in HeLa-S3 cells was concentration dependent with a threshold below 1 nM, the lowest concentration tested (Figure 1b). Between 1 nM and 10 μM, the increase in $[Ca^{2+}]_i$ ranged from 39.5 ± 19.1 to 101.3 ± 24.9%, respectively. Concentrations above 10 μM were not tested because they are clinically irrelevant. The elevation of $[Ca^{2+}]_i$ was only slightly (<20%) reversible at any of the concentrations used. The increase in $[Ca^{2+}]_i$ is not a general effect caused by cisplatin, since the application of 10 μM CDDP did not elevate $[Ca^{2+}]_i$ in U2-OS cells (Figure 1c). Furthermore, the effect in HeLa-S3 cells was restricted to cisplatin since the related compound, carboplatin (up to a concentration of 10 μM), did not substantially elevate $[Ca^{2+}]_i$ (Figure 1d).

Cisplatin-induced $[Ca^{2+}]_i$ increase depends on extracellular Ca^{2+}

To determine the source of the increase in $[Ca^{2+}]_i$ during the application of cisplatin, we tested whether Ca^{2+} is released from the internal stores or enters from the extracellular space. Ca^{2+} entry from the extracellular solution was examined by using an external buffer solution with no added Ca^{2+}. In this solution, cisplatin (10 μM) applied to HeLa-S3 cells did not significantly elevate $[Ca^{2+}]_i$ (Figure 2a).

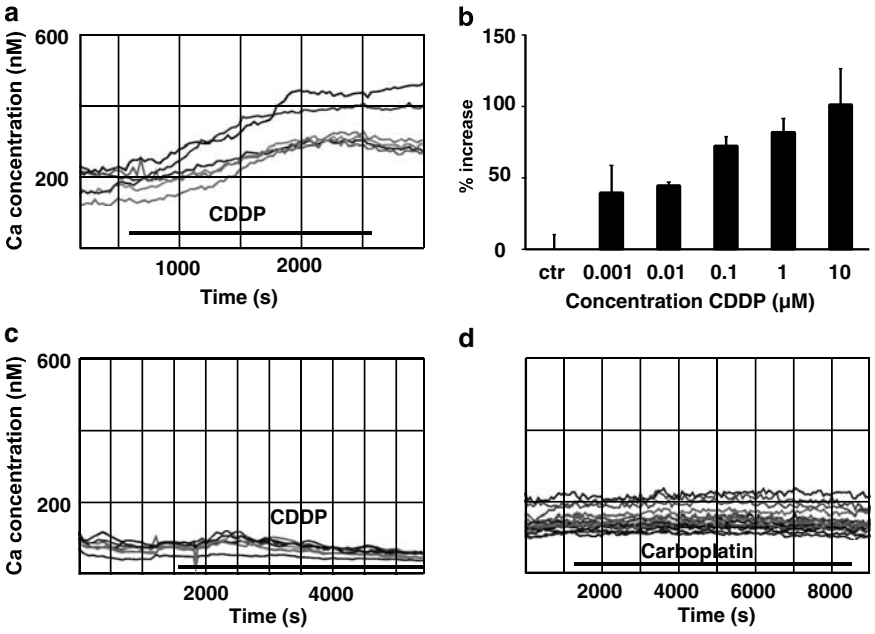

Figure 1 Modulation of $[Ca^{2+}]_i$ by platinum compounds under different conditions. (**a**) Cisplatin (CDDP; 10 μM) increases $[Ca^{2+}]_i$ in HeLa-S3 cells ($n=47$). Illustrated is the calcium rise in six cells. The basal $[Ca^{2+}]_i$ levels ranged from 120 to 220 nM. CDDP raised $[Ca^{2+}]_i$ over a time interval of 1000 s to a ~two-fold higher level. The effect was only slightly reversible (less than 20%; data not shown). (**b**) Concentration dependency of the $[Ca^{2+}]_i$ increase by cisplatin. Threshold concentration was below 1 nM and the $[Ca^{2+}]_i$ was doubled with 10 μM cisplatin (1 nM: $n=28$; 10 nM: $n=18$; 100 nM: $n=29$; 1 μM: $n=32$; 10 μM: means$\pm$s.d., $n=47$). (**c**) $[Ca^{2+}]_i$ did not increase in U2-OS cells during cisplatin (10 μM) application ($n=26$). (**d**) Carboplatin (10 μM) did not elevate $[Ca^{2+}]_i$ in HeLa-S3 cells ($n=39$). $[Ca^{2+}]_i$, intracellular calcium.

In four experiments (47 cells), in nominally Ca^{2+}-free buffer, the $[Ca^{2+}]_i$ rise was significantly diminished compared to experiments in a solution containing 1.8 mM Ca^{2+} (Figure 2a, right side; $P<0.001$). This is an indication that cisplatin-induced Ca^{2+} enter from the extracellular side. The increase in $[Ca^{2+}]_i$ observed when no Ca^{2+} was added to the extracellular solution is most likely due to impurities of the substances used (for example, NaCl has an impurity of 0.002% of calcium, which could result in a total extracellular Ca^{2+} concentration of up to 3 μM). We were unable to 'clamp' the extracellular calcium concentration, using 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) or EGTA, since the cell cultures did not survive under such conditions over the course of the experiments.

To investigate whether Ca^{2+} was also released from $[Ca^{2+}]_i$ stores, we blocked the activation of IP_3 receptors using a specific antagonist (2-APB; 50 μM) to reduce Ca^{2+} release from the intracellular stores (Mignen *et al*., 2005). In regard to the results described above, we expected that 1 μM cisplatin, which alone caused a substantial increase in $[Ca^{2+}]_i$ (Figure 1b), would have a similar effect when coapplied with 2-APB in the 1.8 mM Ca^{2+} containing external solution. Instead, 2-APB blocked the increase in $[Ca^{2+}]_i$ caused by cisplatin (Figure 2b). The difference between the $[Ca^{2+}]_i$ rise with and without 2-APB was highly significant (Figure 2b, right side; $P<0.001$).

Our observations are in agreement with two possible explanations of the increase in $[Ca^{2+}]_i$ caused by cisplatin: (1) an IP_3 receptor mediated Ca^{2+} entry from the extracellular space, or (2) Ca^{2+} release from internal stores triggered by entry of extracellular Ca^{2+} (Ca^{2+}-dependent Ca^{2+}-release). The second possibility was addressed by depleting the calcium stores with 10 mM caffeine (Thayer *et al*., 1988) before cisplatin (1 μM) was coapplied (Figure 2c). With caffeine, $[Ca^{2+}]_i$ increased significantly by 31.2$\pm$25.2% ($P<0.05$) and reached a steady state within 5 min. When cisplatin was added, there was a further, highly significant, increase in $[Ca^{2+}]_i$ to 102.2$\pm$50.2% (Figure 2c) compared to 81.9$\pm$9.6% as measured when the calcium stores were not depleted previously (Figure 2c, right side; $P<0.001$). Therefore, Ca^{2+} release from the stores induced by Ca^{2+} entry from the extracellular space is unlikely.

In addition, we have analysed the calcium dynamics in the calcium stores by labelling Ca^{2+} with the store-specific calcium sensitive dye, rhod-2. While the intracellular stores were hardly visible under control conditions (Figure 3A), the signal did not further decline after the application of cisplatin, but the calcium concentration in the stores increased about 60 min after the application of 1 μM cisplatin was started and reached a plateau about 30 min later (Figures 3Ab, c and 3B). Therefore we conclude that the cisplatin-induced increase in $[Ca^{2+}]_i$ results in an activation of Ca^{2+} transport mechanisms to extrude Ca^{2+} from the cytosol, which will transport Ca^{2+} to the extracellular space as well as to the Ca^{2+} stores, resulting in a loading of the stores with Ca^{2+}. Overall, the increase in the fluorescence in the stores was highly significant (Figure 3C; $P<0.001$).

Membrane conductance of HeLa-S3 cells is increased by cisplatin since the elevation of $[Ca^{2+}]_i$ activates K_{Ca} channels

Electrophysiological experiments were performed to evaluate the possibility of an increased membrane conductance due to Ca^{2+} uptake in the cells via open Ca^{2+}-selective ion

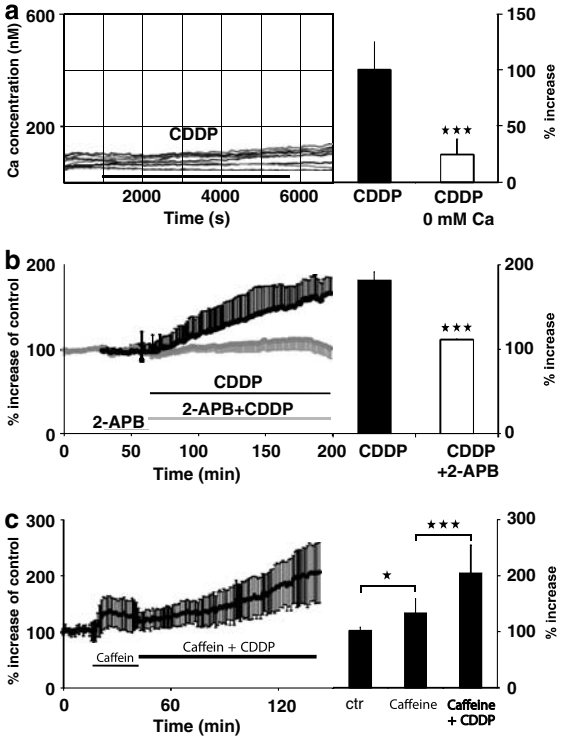

Figure 2 Cisplatin (CDDP)-induced $[Ca^{2+}]_i$ rise depends on extracellular calcium and IP_3 receptors but not calcium release from stores. (**a**) Left side: in HeLa-S3 cells, $[Ca^{2+}]_i$ did not increase when no calcium was added to the extracellular solution. Right side: averaged increase in $[Ca^{2+}]_i$ by cisplatin (10 μM) in HeLa-S3 is highly significantly (***$P<0.001$; means$\pm$s.d., $n=27$) reduced when no calcium is added to the extracellular solution. (**b**) Left side: averaged time course of the $[Ca^{2+}]_i$ concentration in HeLa-S3 during the application of cisplatin (1 μM) and with the coapplication of the IP_3-receptor antagonist 2-APB (50 μM). Right side: with the coapplication of 2-APB, the $[Ca^{2+}]_i$ elevation is highly significantly reduced (***$P<0.001$; means$\pm$s.d., $n=41$). (**c**) Left side: pre-application of caffeine (10 mM) did not abolish the CDDP-induced $[Ca^{2+}]_i$ elevation (time course of averaged data; left side). Right side: summary of caffeine-induced rise in $[Ca^{2+}]_i$ and the further CDDP induced increase (*$P<0.05$; ***$P<0.001$; means$\pm$s.d., $n=19$). $[Ca^{2+}]_i$, intracellular calcium; IP_3 receptors, inositol-1,4,5-trisphosphate receptors; 2-APB, 2-aminoethoxydiphenyl borate.

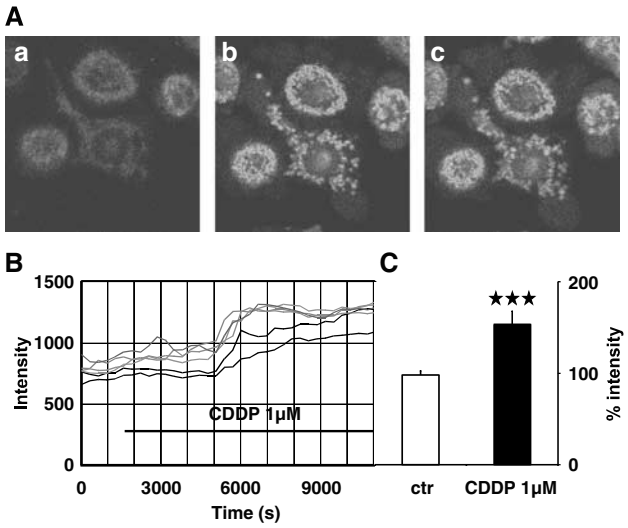

Figure 3 Using the store-located calcium-sensitive dye rhod-2, an increase in fluorescence was measured in the stores when cisplatin (CDDP; 1 μM) was applied. (**A**) Three images taken in an experiment (a) before, (b) after 80 min and (c) 140 min after cisplatin had been applied. The calcium concentration is indicated with false rainbow colours scale (blue = no calcium, green to yellow = low-to-medium calcium, red = high calcium). Clearly, during application of the anti-cancer drug, the calcium increases within the calcium stores. (**B**) Time course of the calcium increase in the stores measured as the total increase in the fluorescence in single cells. Note that the calcium increase in the stores occurs clearly later (the elevation begins about 60 min after the application) than the raise in the cytosolic calcium level (compare Figure 1). (**C**) The elevation of the fluorescence indicating the calcium rise in the stores is highly significant after cisplatin had been applied (***$P<0.001$; means$\pm$s.d., $n=20$) (for color figure see online version).

channels. Total membrane currents (Figures 4a, c and d) were elicited by a voltage ramp from -110 to $+20$ mV over 1.3 s (10 mV per 0.1 s): before and during the application of cisplatin (1 μM). During cisplatin application in normal extracellular solution, the current was increased over the whole-voltage range without significant change in the reversal potential (control: -66.2 ± 3.3 mV; 1 μM CDDP: -67.1 ± 2.7 mV; $P>0.5$) (Figures 4a and b). The increased current at $+20$ mV ($513.3\pm92.5\%$, $P<0.05$) demonstrated an increased conductance, resulting from the opening of ion channels. When the extracellular solution had no Ca^{2+} added, cisplatin caused only a small increase in membrane current/conductance, which did not change significantly over the whole time of the experiments ($110.1\pm117.3\%$; Figure 4c, time courses in Figure 4d). These results indicate that the increased conductance triggered by cisplatin was due to the opening of calcium-activated channels. The elevated $[Ca^{2+}]_i$ most likely opens K_{Ca} channels, as adding charybdotoxin (3.3 nM) – a blocker of the large conductance of the K_{Ca} channels – prevented the activation of the K_{Ca} channels at $+20$ mV (Figures 4e and f).

IP_3 receptors are found at the cell membrane in HeLa-S3 but not in U2-OS cells

Because the increase in $[Ca^{2+}]_i$ clearly depended on the extracellular calcium concentration and was prevented by the blockade of the IP_3 receptor, we tested the hypothesis that IP_3 receptors are expressed in the cell membrane. Dellis *et al*. (2006) have shown in DT40 chicken cells that most IP_3 receptors are expressed in intracellular stores, but a small fraction is reliably directed to the plasma membrane. This fraction of receptors is substantially involved in the Ca^{2+} entry.

Using immunostaining and confocal fluorescence microscopy, we tested two different antibodies, one for all three subtypes of IP_3 receptors (Calbiochem) and one for the type 1 subtype (Alexis), both with affinity for the cytoplasmic C-terminus side of the receptor. The staining was identical for both antibodies. The results with the staining of all three subtypes are shown in Figure 4. Immunostaining for IP_3 receptors in HeLa-S3 cells resulted in fluorescence at the nuclear envelope and at the cell membrane (Figure 5a), while in U2-OS cells the immunostaining detected IP_3 receptors only at the nuclei and at cell organelles (Figure 5b). Z-stacks

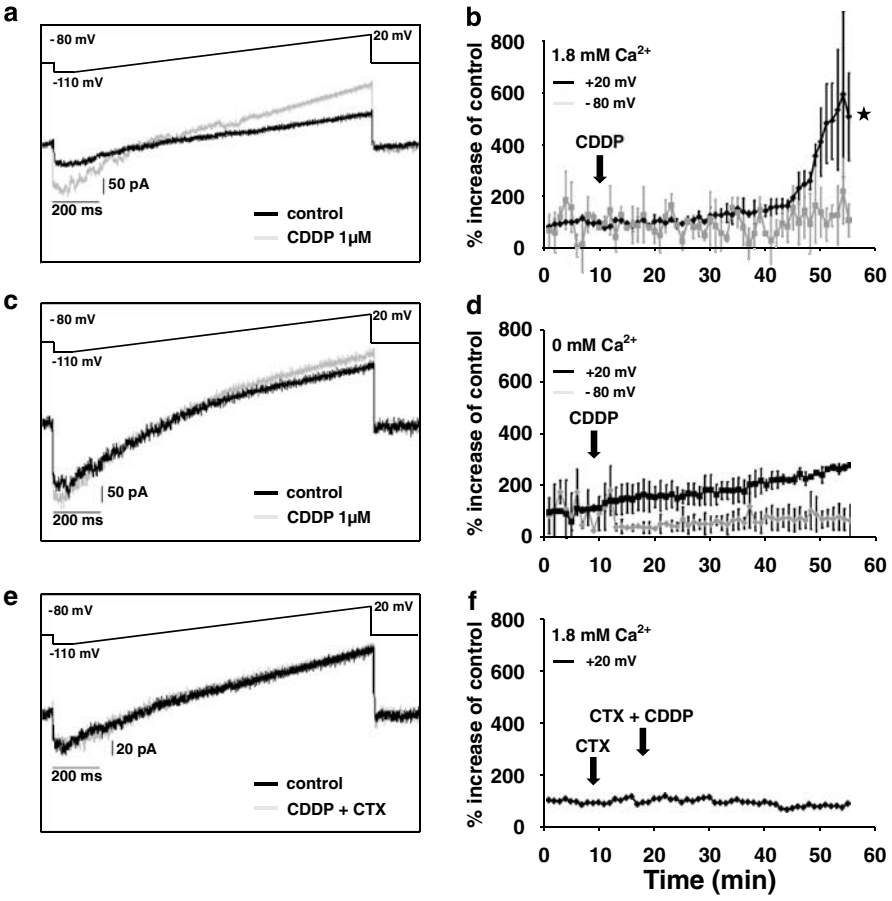

Figure 4 Electrophysiological recordings of membrane currents in HeLa-S3 cells before and after the application of cisplatin (CDDP). (**a**) Membrane currents of HeLa-S3 cells, elicited by a voltage ramp from −110 mV to +20 mV over 130 ms, before and with cisplatin (1 μM, $n=5$). (**b**) Time course of membrane currents (black line = measured at +20 mV; grey line = measured at −80 mV)) of HeLa-S3 cells using 1.8 mM calcium in the extracellular buffer. There was a clear increase in the currents at +20 mV after ~40 min of application. After 55 min, membrane currents elicited at +20 mV were significantly different compared to the experiments where no calcium was added (compare panel d; $^*P<0.05$; $n=5$). (**c** and **d**) Same procedure as in (**a**) and (**b**) but with no Ca^{2+} added to the external buffer. Note that after the application of CDDP, there was only a small increase in the current ($n=5$) over the time of the experiment at +20 mV and no change at −80 mV. (**e** and **f**) Same procedure as in (**a**) and (**b**) but using the K_{Ca}-channel blocker charybdotoxin (CTX; 3.3 nM). The examples shown illustrate that there is no change in the membrane current when the calcium-activated potassium channels are blocked, an indication that this is the main conductance activated by the entry of Ca^{2+}.

of both cell types illustrate these findings even more clearly than two-dimensional confocal images (data provided on request). No nonspecific binding of the secondary antibody was found (data not shown).

The cisplatin-induced elevation of $[Ca^{2+}]_i$ activates calpain but not caspase-8

Although cisplatin induces apoptosis and increases $[Ca^{2+}]_i$ in different cancer cell lines, the role of $[Ca^{2+}]_i$ in the initiation of apoptosis remains unclear. Recently, in a mouse photoreceptor cell line it was demonstrated that the calcium-induced activation of calpain results in apoptosis (Sharma and Rohrer, 2004), but it is known that calpain can also be activated by cisplatin (Mandic *et al*., 2002). To address whether this is also an issue in our cell lines, we investigated the expression of calpain and caspase-8 after the application of cisplatin (1 μM) for 15 min and 1 h. Both substances are key proteins involved in triggering apoptotic death. We tested their expression in HeLa-S3 as well as in U2-OS cells. While there were no changes after 15 min and 1 h after application (a time at which cisplatin increases $[Ca^{2+}]_i$ in HeLa-S3 cells but not in U2-OS cells; compare Figures 1a and 2b), cisplatin significantly activated calpain in HeLa-S3 cells, while this effects was diminished by 2-APB. In U2-OS cells, cisplatin did not increase the active form of calpain significantly. Like all caspases, caspase-8 is activated by cleavage of a single-chain zymogen into two subunits. Neither the small nor large catalytic subunit increased following cisplatin application in HeLa-S3 cells (Figure 6b). To test whether we are able to trigger an increase in caspase-8 under the conditions used, we applied TRAIL (160 ng ml^{-1}) to our HeLa-S3 cell line and induced a clear increase in the caspase-8 (Figure 6b).

Cisplatin induces $[Ca^{2+}]_i$-dependent apoptosis

Exposure of HeLa-S3 cells to 1 μM cisplatin significantly reduced cell viability when the trypan blue cytotoxicity test

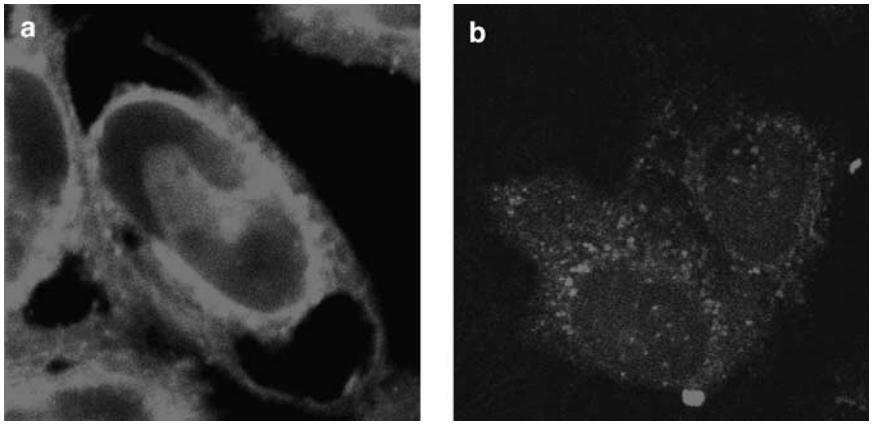

Figure 5 Distribution of IP_3-receptor antibody in (**a**) HeLa-S3 cells ($n=9$) and (**b**) U2-OS cells ($n=6$). Notice in HeLa-S3 cells IP_3 receptor distribution at the cellular membrane, the nuclear envelope and at organelles, while in U2-OS cells the receptor is present only in organelles. Staining with the secondary antibody did not reveal any nonspecific staining in any of the two cell lines (data not shown). IP_3 receptor, inositol-1,4,5-trisphosphate receptor.

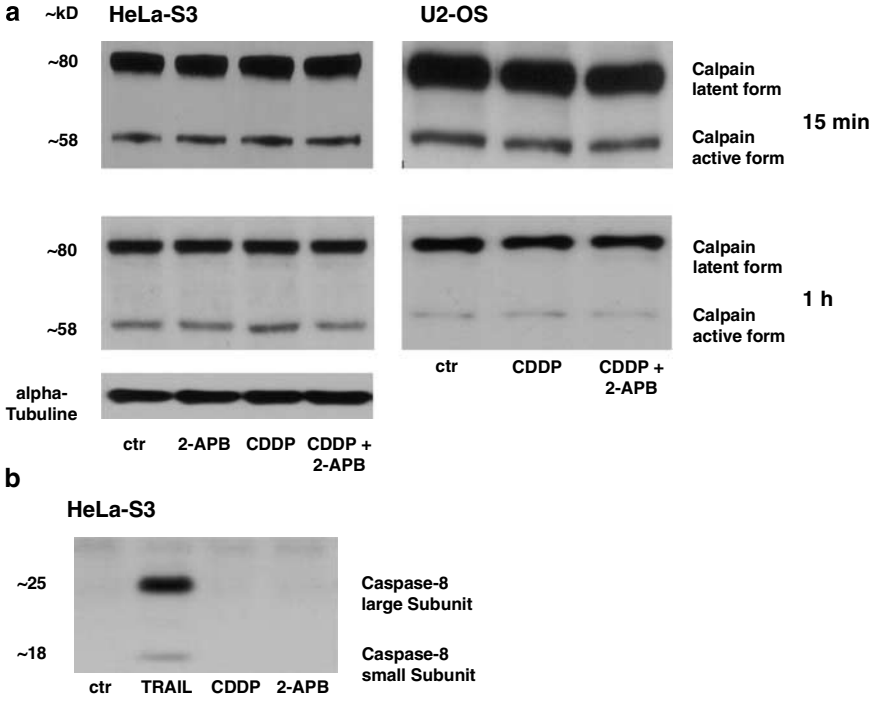

Figure 6 Calpain, but not caspase-8, is increased in HeLa-S3 cells, while calpain is not elevated in U2-OS cells, after exposure to cisplatin (CDDP). (**a**) Left panel: while there was no change after 15 min of incubation, after 1 h of exposure to 1 μM cisplatin, active calpain was highly increased in HeLa-S3 cells and this increase was blocked by 50 μM 2-APB ($n=13$). Right panel: this effect was not observed in U2-OS cells ($n=6$; 1 μM cisplatinfor 15 min and 1 h of exposure). (**b**) Cisplatin did not increase the large or small subunit of caspase-8 in HeLa-S3 cells after 1 h of exposure, while the activation of the TRAIL receptor showed that the caspase-8 antibody was effective ($n=9$). 2-APB, 2-aminoethoxydiphenyl borate; TRAIL, TNF-related apoptosis-inducing ligand.

was applied. The effect was time dependent. To further test if cell death occurred by apoptosis, Hoechst staining was carried out and nuclei with morphology typical of apoptosis were scored in relative to healthy nuclei. Counts of apoptotic nuclei showed that cisplatin increased cell death by apoptosis. The effect was reduced when the rise of $[Ca^{2+}]_i$ was diminished either by 2-APB or a nominal calcium-free extracellular solution (Figure 7). In addition, the apoptotic rate was unchanged in U2-OS cells (Figure 7).

Discussion

In clinical practice, cisplatin is the first-choice chemotherapy for epithelial malignancies and the standard treatment for cervical cancer treatment (Boulikas and Vougiouka, 2004). Extensive work on cisplatin cytotoxicity in tumour cells has been carried out, but very little is known how cisplatin interacts with calcium homeostasis in tumour cells. Recently, Kawai *et al*. (2006) have shown that in a nontumour model

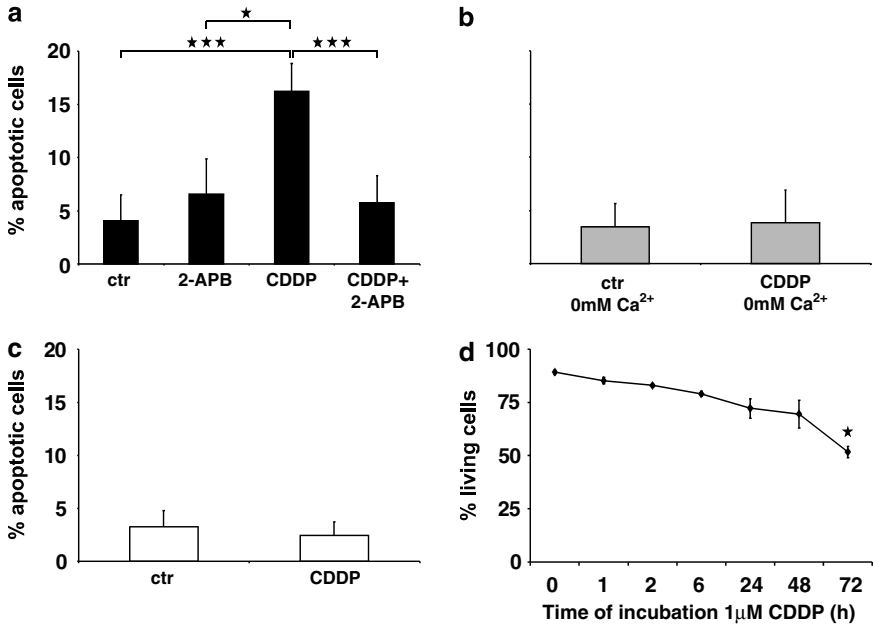

Figure 7 Cisplatin (CDDP)-induced increase of apoptotic rate is reduced in HeLa-S3 but not in U2-OS cells, when no calcium is added or IP_3 receptors are blocked. (**a**) Apoptotic rate under different conditions in normal calcium (1.8 mM) containing medium. In HeLa-S3 cells, cisplatin increased apoptotic rate (***$P<0.001$ compared to control) and this increase was significantly reduced by 2-APB (***$P<0.001$; means $\pm$ s.d.). (**b**) Apoptotic rates with calcium-free incubation medium are not significantly changed by cisplatin. (**c**) Apoptotic rate in U2-OS cells incubated in normal calcium medium is also unchanged after cisplatin. (**d**) Cell viability test. The number of living cells is decreased significantly (*$P<0.05$; means $\pm$ s.d.) beginning at 72 h of exposure to 1 μM cisplatin. Note time dependence of cytotoxicity. IP_3 receptors, inositol-1,4,5-trisphosphate receptors; 2-APB, 2-aminoethoxydiphenyl borate.

(renal cells), relatively high concentrations of cisplatin (250–750 μM) induced a rise in $[Ca^{2+}]_i$. In our work we investigated the effect of clinically relevant concentrations of cisplatin on calcium homeostasis of different tumour cells and we have correlated these effects with apoptotic cell death. Low concentrations of cisplatin (0.001–10 μM) increased $[Ca^{2+}]_i$ in HeLa-S3 but not in U2-OS cells. $[Ca^{2+}]_i$ rise was concentration dependent and mediated by IP_3 receptors. Overall, $[Ca^{2+}]_i$ increase in HeLa cells was associated with cisplatin-induced apoptotic death with the involvement of calpain. Our results are summarised in Figure 8. Our findings show that cisplatin has a higher activity on some specific cell types, in this case to tumour cells (see also Pai and Sodhi, 1992). These authors have also shown selectivity of cisplatin for certain cell lines (mononuclear cells from human peripheral blood).

Platinum complexes are probably highly specific as cisplatin but not carboplatin was able to increase $[Ca^{2+}]_i$ in the same HeLa-S3 cell line. This finding is compatible with earlier work (Koivusalo *et al.*, 2002; Carland *et al.*, 2005) showing that, in cervix carcinoma cells, different platinum compounds have different cytotoxic potency: cisplatin > oxaliplatin > carboplatin. Differences were also described in comparison to transplatin (Dalla Via *et al.*, 1998), but only limited work was carried out in tumour cell models. Different effects on $[Ca^{2+}]_i$ in two human lung adenocarcinoma cell lines (A549, cisplatin sensitive and A549/DDP, cisplatin resistant) were documented by Liang and Huang (2000). These authors found that in the cisplatin-resistant cell line, $[Ca^{2+}]_i$ increased less and faster than in the sensitive cell line.

In our experiments, the increase in $[Ca^{2+}]_i$ depended on the presence of extracellular Ca^{2+}, while the intracellular Ca^{2+} stores were filled when cisplatin was applied. These observations differ from those of Kawai *et al.* (2006). These authors found that neither the extracellular calcium chelator EGTA nor the calcium channel antagonist nicardipine inhibited the rise of $[Ca^{2+}]_i$, and therefore concluded that cisplatin depleted the calcium stores. However, the cisplatin concentrations these authors used are not physiological (up to 750 μM) and therefore an additional depletion of the intracellular stores cannot be excluded. Furthermore, Tomaszewski and Büsselberg (2007) demonstrated that currents through voltage-activated calcium channels are reduced by cisplatin, but the effective concentration was higher (IC_{50} of peak current: 23.9 $\pm$ 4.5 μM) than in the experiments presented in this study.

While the increased $[Ca^{2+}]_i$ depended on the presence of extracellular Ca^{2+}, a block of IP_3 receptors with 2-APB abolished this effect in our experiments. A connection between $[Ca^{2+}]_i$ rise and IP_3 receptors (type 1) has been demonstrated in cisplatin-resistant bladder cancer cells, where this IP_3 receptor was markedly reduced, as was $[Ca^{2+}]_i$, compared to a parental nontumour cell line (Tsunoda *et al.*, 2005). A lower expression of IP_3 receptors using small-interfering RNA, in parental cells, prevented apoptosis and resulted in decreased sensitivity to cisplatin. However, overexpression of IP_3 receptors in cisplatin-resistant cells induced apoptosis and increased sensitivity to cisplatin. These results suggest that cisplatin-induced down-regulation of IP_3 receptor expression is closely associated with the acquisition of cisplatin resistance in bladder cancer cells (Tsunoda *et al.*, 2005).

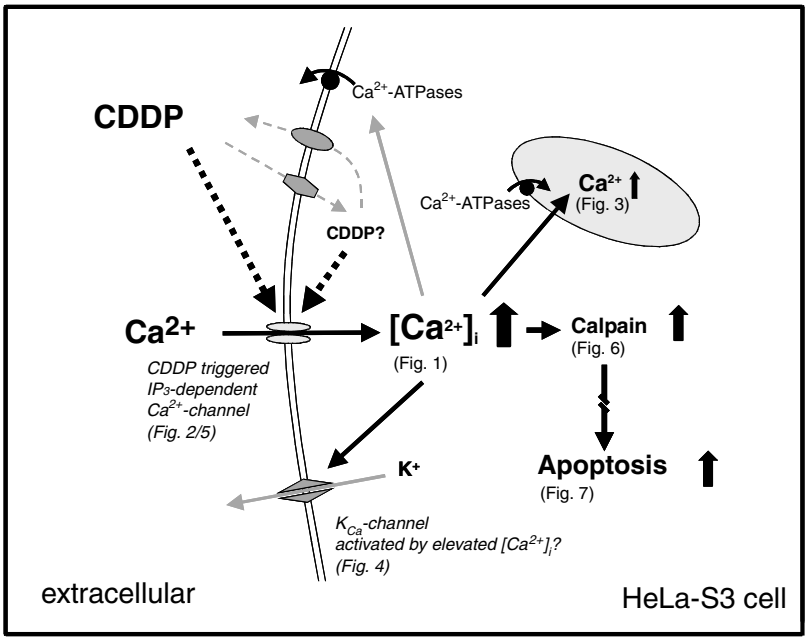

Figure 8 An interlocking hypothesis of the interaction of cisplatin with HeLa-S3 cells, which combines the results found by our different approaches, is illustrated in this scheme. Cisplatin might be able to enter and leave the cell by transporters. Intracellular and/or extracellular cisplatin (or its metabolites) increase $[Ca^{2+}]_i$ concentration dependently by IP_3 receptor-controlled calcium channels that are located at the cellular membrane. When extracellular calcium enters the cytosol, $[Ca^{2+}]_i$ increases after 20–30 min, which activates extruding transport mechanisms (Ca-ATPases), resulting in a consequent Ca^{2+} increase in the calcium stores after about 60 min and most likely by the activation of mechanisms that transport Ca^{2+} to the extracellular side. When the amount of Ca^{2+} entering the cell and the extruding mechanisms are balanced, the $[Ca^{2+}]_i$ does not further increase (plateau phase) but the cytosolic concentration is higher than under control conditions. An elevated $[Ca^{2+}]_i$ activates the calcium-dependent calpain (elevated after 1 h), a key protein for triggering the apoptotic pathway. When Ca^{2+} enter through the IP_3-dependent calcium channels is blocked by the IP_3 receptor antagonist 2-APB, the $[Ca^{2+}]_i$ does not increase nor are the apoptotic pathways triggered. $[Ca^{2+}]_i$, intracellular calcium; IP_3 receptor, inositol-1,4,5-trisphosphate receptor.

Unfortunately, these results neither indicate the location of the IP_3 receptors (at the cellular membrane or at the calcium stores) nor their site of action (IP_3 receptors located at the calcium stores might influence the opening of membrane channels by an intracellular pathway). To distinguish a possible effect of cisplatin on Ca^{2+} release from the stores, from extracellular Ca^{2+} entry, calcium stores were depleted by caffeine (Thayer *et al*., 1988) before cisplatin was given additionally. The identical increase in $[Ca^{2+}]_i$ after the application of cisplatin, compared to the experiments where the calcium stores were not depleted before, is another indication that cisplatin-induced elevation of $[Ca^{2+}]_i$ did not depend on an IP_3 receptor-mediated Ca^{2+} release from the stores. In addition, the significant increase in Ca^{2+} in the stores as shown with the store-specific dye rhod-2 provides strong evidence that the majority of the calcium stores were not depleted. Furthermore, our immunostaining experiments for different types of IP_3 receptors showed a clear staining at the cellular membrane of HeLa-S3 but not in U2-OS cells, an indication that this receptor complex could directly modify calcium entry from the extracellular space.

Our results also indicate an increase in membrane conductance after the application of cisplatin but only when Ca^{2+} is present in the extracellular solution, another hint of Ca^{2+} entry. It is most likely that the Ca^{2+} entering the cell secondarily activates other membrane ion channels such as a calcium-dependent potassium channel (K_{Ca}) (North and Tokimasa, 1987). This conclusion is underlined by the fact that a block of the large conductance K_{Ca} channel by charybdotoxin did not change the membrane conductance even when CDDP was applied for more than 1 h. Since a remaining 'leak' current and the K_{Ca}-channel current might be relatively large compared to the expected Ca^{2+} entry, no change of the reversal potential was observed. Our findings are also in agreement with inside-out patch experiments of Negulyaev *et al*. (1993). They describe calcium permeable channels in HeLa cells, whose activities were increased when they applied IP_3 (5–10 μM) to the inner membrane surface. While they did not vary the extracellular calcium concentration, the cytosolic calcium concentration (0.01 and 10 μM) did not influence the channel conductance.

Our findings also show that $[Ca^{2+}]_i$ rises because Ca^{2+} is entering through the IP_3-receptor channel complex, since we were able to show the IP_3 receptor is located at the cell membrane of HeLa-S3 cells. This conclusion is fully confirmed by Dellis *et al*. (2006) in DT40 chicken cells. They also demonstrated a fraction of IP_3 receptors at the cellular membrane and a calcium entry after activation of these receptors (Dellis *et al*., 2006).

We suggest that the cisplatin-induced elevation of $[Ca^{2+}]_i$ is required for activation of calpain and the induction of apoptosis. Schloffer *et al*. (2003) showed in HeLa cells that a 3 h treatment with cisplatin triggers cell death by apoptosis (with chromatin fragmentation, strong cytoplasmic accumulation of cytochrome C and an activation of caspase-9).

This result was confirmed by Boehning *et al*. (2003) who in addition found the binding of cytochrome *c* to the IP_3 receptor amplified a calcium-dependent apoptosis. Here we show that a concentration of 1 μM cisplatin induces time-dependent cell death with clear nuclear morphology of apoptosis.

Several authors have considered that DNA damage is the main cause of the anti-proliferative effect of cisplatin (Mandic *et al*., 2003; Jeyapalan *et al*., 2005). These authors also discussed different apoptotic, cisplatin-triggered, pathways in inner ear hair cells: (1) reactive oxygen species and free radicals; (2) activation of caspases; and (3) activation of calpain. In our study, we have shown that cisplatin is able to elevate activated calpain, a protease with involvement in apoptosis. Mandic *et al*. (2002) have shown in human melanoma cells that cisplatin-induced calpain activation occurs early in apoptosis with the maximum activity of CDDP-induced calpain activity between 3 and 5 h. The same group also emphasises that calpain-mediated Bid cleavage is important in drug-induced apoptosis (Mandic *et al*., 2003). They have also demonstrated that calpain activation may be associated with endoplasmic reticulum (ER) stress, suggesting that the ER is a cytosolic target of cisplatin.

To summarise, in this study we found a cisplatin-induced rise in $[Ca^{2+}]_i$ in HeLa-S3 cells that depends on both extracellular calcium and IP_3 receptors. This $[Ca^{2+}]_i$ increase occurred within 30 min and triggered the activation of calpain (after 1 h), but not of caspase-8. Therefore, an IP_3 receptor-dependent calcium influx triggered by CDDP results in the activation of calpain and possibly ends with programmed cell death. This is at least one possible signal pathway required for cisplatin-induced apoptosis.

Our results suggest that the efficiency of cisplatin treatment could be enhanced if $[Ca^{2+}]_i$ could be increased pharmacologically, possibly by an independent activation of IP_3 receptors.

Acknowledgements

We thank Dr W Michael King for critical reading and valuable comments, Dr Stilla Freede for support in performing and analysing western blots and Dr Sinclair Cleveland for fitting the concentration dependence of CDDP to the $[Ca^{2+}]_i$ increase.

Conflict of interest

The authors state no conflict of interest.

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