Role of the Na\(^{+}, K^{+}\)-Adenosine Triphosphatase in the Accumulation of cis-Diaminedichloroplatinum(II) in Human Ovarian Carcinoma Cells

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ABSTRACT

We examined the importance of the Na\(^{+}, K^{+}\)-ATPase in the accumulation of cisplatin (DDP) accumulation in 2008 human ovarian carcinoma cells and describe changes in the Na\(^{+}, K^{+}\)-ATPase in DDP-resistant cells with DDP accumulation defects. Approximately 50% of DDP accumulation was inhibitable by ouabain. DDP accumulation into 2008 cells could be maximally inhibited when cells were preincubated with ouabain for 1 h prior to DDP exposure. The half-maximal inhibition was obtained with 0.13 \(\mu\)M ouabain. Similar inhibition of DDP accumulation was obtained when the Na\(^{+}, K^{+}\)-ATPase was blocked by ATP depletion or by incubating cells in K\(^{-}\)-free medium. This same percentage of DDP accumulation was Na\(^{+}\)-dependent and varied directly with Na\(^{+}\) concentration. These effects on DDP accumulation could be detected as early as 1 min after the imposition of 0\(-\)trans conditions, strongly suggesting that the inhibition was due to modulation of a drug influx step. The Na\(^{+}, K^{+}\)-ATPase in DDP-resistant cells and their DDP accumulation defect is not yet known.

INTRODUCTION

A variety of cell types with acquired resistance to DDP exhibit decreased accumulation of DDP as part of their resistant phenotype (1). The appearance of decreased DDP accumulation seems to happen at an early stage in the development of resistance and in many cell types can account for the low levels of resistance found after selection with DDP begins (1-4). It has been assumed for many years that DDP enters cells by a passive diffusion process; however, the exact mechanisms involved have not been completely defined and may differ between functionally different cell types. Considering the prevalence of decreased DDP accumulation as part of the DDP-resistant phenotype, and the need to define DDP accumulation mechanisms in order to understand better the cellular pharmacology of DDP, we have been studying the mechanisms whereby DDP enters 2008 human ovarian carcinoma cells and how these mechanisms have been altered in accumulation-deficient and resistant sublines (2, 5-7).

We previously reported differences in the accumulation of DDP in DDP-sensitive and resistant human ovarian carcinoma cells (2). As part of those studies, we discovered that a portion of DDP accumulation was energy dependent and ouabain inhibitable. These findings are intriguing in light of strong evidence that DDP accumulation was not carrier mediated, i.e., accumulation was not saturable or competitively inhibited with analogues (6, 7). To explain these results, our objective in these studies was to define the role of the Na\(^{+}, K^{+}\)-ATPase (EC 3.6.1.37), the specific target of ouabain, on DDP accumulation in 2008 cells.

MATERIALS AND METHODS

Drugs and Chemicals. DDP (clinical formulation) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). DDP pure powder was obtained from Alfa Products (Danvers, MA). \(^{[195mPt]}\)DDP was obtained from Oak Ridge National Laboratories (Oak Ridge, TN) (8). 3-O-[methyl-\(^3\)H]-glucose (79 Ci/mmol), \(^{[1]}\)H]ouabain (15.7 Ci/mmol), and 22NaCl (0.37 Ci/mmol) were obtained from NEN Research Products (Boston, MA). The \(^{22}\)NaCl (90 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Ouabain octahydrate, sodium azide, 3-O-methyl-d-glucose, choline chloride, and choline bicarbonate were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium without glutamine, glucose, NaHCO\(_3\), NaCl, and KCl was obtained from Flow Laboratories, Inc. (McLean, VA).

Cell Lines. The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary, was used in these studies (9). The DDP-resistant cells were generated as previously described by monthly selection with 1 \(\mu\)M DDP (9). These cells are designated 2008/DDP. Cells were grown as monolayers in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mm freshly added glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Cultures were equilibrated with humidified 5% CO\(_2\) in air at 37°C. Cells were routinely tested at 6-week intervals with a Gen-probe Mycoplasma detection kit (Fisher, Tustin, CA). All studies were done with Mycoplasma-negative cells. Clonogenic assays on plastic were conducted as previously described (9).

Cell Volume Determinations. The intracellular water space was determined in parent and resistant cells with 3-O-[methyl-\(^3\)H]-glucose by the method of Klotzkin et al. (10).

DDP Accumulation. For DDP accumulation studies, parental and resistant cells were seeded into 6-well tissue culture plates or 100-mm tissue culture dishes, depending on the specific activity of the \(^{[195mPt]}\)DDP. After 3-4 days, when the plates approached confluency, the medium was aspirated and replaced with RPMI 1640 medium containing the indicated concentrations of \(^{[195mPt]}\)DDP at 37°C. The dishes were immediately returned to the incubator. At appropriate time points, the plates were removed from the incubator, the medium was aspirated, and the cells were washed 4 times with PBS at 4°C (Oxoid, Columbia, MD). For 100-mm plates, 3-ml of 1 N NaOH was added, and the cells were allowed to digest overnight. An aliquot was removed for the determination of protein content by the method of Bradford (11), and 2.5 ml was either placed in test tubes and counted with a Trak 1191 gamma counter (Tecator Analytic, Elk Grove Village, IL) or mixed with

Received 10/25/90; accepted 5/6/91.

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1 Supported by Grant CA-23100 from the National Cancer Institute, Grant CH-417 from the American Cancer Society, and Grant 100-R107 from Bristol-Myers, Co. This work was conducted in part by the Clayton Foundation for Research, California Division. P. A. A. is a Clayton Foundation Investigator.

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* Abbreviations used: DDP, cis-diaminedichloroplatinum(II); PBS, phosphate-buffered saline consisting of, per liter: 8 g NaCl, 0.2 g KCl, 1.15 g Na\(_2\)HPO\(_4\), and 0.2 g KH\(_2\)PO\(_4\); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
8 ml of 3a708 scintillation fluid (RPI Corp., Mount Prospect, IL) and 1 ml of 3.5 M acetic acid and counted on a Beckman LS-230 beta counter (Beckman, Fullerton, CA) with the channel windows set to wide open.

When [195mPt]DDP was not available, accumulation studies were conducted with unlabeled DDP. Cells were seeded into 100-mm tissue culture plates, and when the cells approached confluency, the medium was replaced with 5 ml of RPMI 1640 containing 100 μM DDP diluted from clinical vials. After a 1-h incubation, the medium was aspirated, and the monolayers were washed once with 0.05% trypsin/0.53 mM EDTA at 4°C and then trypsinized with 5 ml of trypsin/EDTA for 6 min at 37°C. The trypsinized cells were added to 10 ml of complete medium at 4°C and centrifuged for 3 min at 500 × g. The cell pellet was resuspended in 10 ml of RPMI 1640 at 37°C containing 500 μM DDP dissolved directly in the medium from pure powder and then centrifuged again. The cell pellet was then resuspended in 1.0 ml of 0.9% saline and sonicated for 30 s at a power setting of 3 and a 30% duty cycle (5 W pulsed) (Sonifier 450; Branson, Danbury, CT). One hundred μl was removed and mixed with 100 μl of 1 N NaOH for protein determination. The remaining cell lysate was then analyzed for protein by atomic absorption spectrophotometry using a Perkin Elmer 373 atomic absorption spectrophotometer equipped with a 2200 graphite furnace (Perkin Elmer, Norwalk, CT). The following heating program was used: 90°C for 50 s, ramp to 1300°C in 10 s and hold for 30 s, 2500°C under maximum power for 7 s. No differences in the results were noted between accumulation studies conducted with radiolabeled DDP versus unlabeled DDP; the method used is indicated in the figure legends.

Short-term accumulation experiments were conducted as described previously (6). Briefly, subconfluent monolayers of cells were generated in 60-mm tissue culture plates. Cells were washed twice with RPMI 1640 or the modified RPMI 1640. RPMI 1640 at 37°C containing 500 μM DDP dissolved directly in the medium from pure powder was then added to the plates and incubated for 1–10 min. The media were then aspirated and the cells rapidly washed 4 times with PBS at 4°C. The cells were scraped with a rubber policeman into the residual PBS (approximately 0.2 ml). The sample was sonicated, an aliquot removed and mixed with an equal volume of 1 N NaOH for protein determination, and the platinum content of the remainder was determined by atomic absorption spectrometry.

Ouabain Binding. For ouabain-binding studies, two 100-mm plates of subconfluent cells were washed with PBS and then scraped into 1.25 ml of a K+-free buffer at 37°C consisting of 140 mM NaCl, 1.5 mM MgCl2, 3.0 mM CaCl2, 10 mM glucose, 10 mM Tris-HCl, pH 7.4. Aliquots (0.225 ml) of this cell suspension were mixed with an equal volume of 1 N NaOH for protein determination.

RESULTS

We originally observed that a 30-min exposure to 200 μM ouabain caused an approximate 25% decrease in the DDP accumulated over the following hour (2). We have subsequently examined the time course of this inhibition. Fig. 1 shows that the inhibition was maximized when 2008 cells were incubated with ouabain for approximately 1 h prior to DDP exposure. The accumulation in ouabain-treated cells was 53 ± 7% of control (n = 8) after a 1-h ouabain pretreatment (Table 1). Ouabain was maintained in the medium during the course of the DDP exposure. Longer preincubation times actually reversed the inhibition and led to a slight stimulation of DDP accumulation. To determine whether the inhibition was due to an effect on cell volume, we measured the changes in the intracellular water space during the course of the experiment. No significant changes in cell volume were found during ouabain treatment (Fig. 1). The reason for the increasing accumulation after 1.5 h was unknown but was probably a result of ouabain toxicity. However, even though no cells survived this brief exposure to ouabain as assessed by clonogenic assay, trypan blue was excluded from cells after 2.5- to 4-h exposure.
to 200 μM ouabain, indicating that this increase was not due to generalized permeabilization of the cells (data not shown).

With a 1 h preincubation, the effect of ouabain on DDP accumulation was linear with ouabain concentrations up to at least 0.100 μM (Fig. 2). The maximum inhibition of approximately 45% was achieved at 0.500 μM; only a slight additional increase in inhibition was found up to 200 μM. The half-maximal inhibition occurred at 0.130 μM.

The effect of ouabain on the accumulation of platinum in cells exposed to DDP for 1 h does not provide information concerning whether the result was due to changes in drug transport, drug metabolism, or intracellular sequestration. Accordingly, short-term DDP accumulation experiments were conducted after a 1-h preincubation of cells in 200 mM ouabain. Fig. 3A shows that ouabain decreased DDP accumulation as early as 1 min after exposure to DDP. Accumulation at 1 min declined to 60 ± 15% of controls (n = 3), suggesting that the effect was on a drug influx step (Table 1). For comparison, we confirmed that the Na+,K+-ATPase was the locus of the Na+ gradient with the associated electrochemical potential across plasma membranes. Inhibition of the Na+,K+-ATPase with a K+-deficient medium which prevented the pump from cycling. As with ouabain, a K+-free medium decreased DDP accumulation to 58 ± 7% (n = 3) of controls (Table 1).

The Na+,K+-ATPase maintains the potassium and sodium gradients and the associated electrochemical potential across plasma membranes. Inhibition of the Na+,K+-ATPase with ouabain destroys these gradients. To determine whether the Na+ gradient was necessary for DDP accumulation, we conducted studies in RPMI 1640 medium in which the NaCl was replaced with equimolar choline chloride, and the NaHCO3 was replaced with equimolar choline bicarbonate. DDP accumulation in low-Na+ medium was 46 ± 13% (n = 5) of controls at 1 h and 44 ± 8% (n = 3) of controls at 1 min (Table 1). Fig. 4 shows the effect of Na+ concentration on DDP accumulation. DDP accumulation increased steadily with Na+ concentration.

Since a functioning Na+,K+-ATPase affects a significant portion of DDP accumulation and since our DDP-resistant cells are deficient in DDP accumulation, we asked whether 2008/DPP cells had an alteration in their Na+,K+-ATPase. The rate of [3H]ouabain binding to 2008 and 2008/DDP cells is shown in Fig. 5A. The 2008/DDP cells bound ouabain more slowly than 2008 cells, and the maximal binding at saturation was less. Scatchard analysis of these data (Fig. 5B) indicated that the KD was 27 nM in 2008 cells and 29 nM in 2008/DDP cells. There was 0.72 pmol/mg protein of the Na+,K+-ATPase on the surface of 2008 cells, and this decreased by 36% to 0.46 pmol/mg protein in 2008/DDP cells. The 2008/DDP cells were found to be cross-resistant to ouabain as indicated by continuous exposure clonogenic assay (Fig. 6). 2008/DDP cells that were approximately 3-fold resistant to DDP were 2.3 ± 0.2 (n = 3) cross-resistant to ouabain as determined by the concentration causing 50% inhibition of colony formation.

The basal activity of the Na+,K+-ATPase was compared in 2008 and 2008/DDP cells by measuring the influx of 86Rb+. No significant difference in total, ouabain-inhibitable, or bumetanide-inhibitable 86Rb+ influx was observed between the two cell types (Table 2). To determine whether the altered Na+,K+-ATPase generated an altered Na+ gradient in 2008/DDP cells, we measured Na+ levels normalized to concomitant measurements of intracellular water space. No significant difference was found under basal conditions. The 2008 and 2008/DDP cells maintained intracellular concentrations of 15.1 ± 1.7 and 12.9 ± 2.3 mM Na+, respectively. Treatment with 200 μM ouabain...
ouabain for 1 h increased these values 8-fold to 121.8 ± 5.5 and 99.2 ± 13.4 nM.

**DISCUSSION**

Our studies have shown that DDP accumulation in 2008 human ovarian carcinoma cells is neither saturable nor competitively inhibited by structural analogues (6, 7). These data strongly suggest that DDP transport is not carrier mediated in these cells. However, DDP accumulation is modulated by cAMP, dependent on the membrane potential, partially ouabain inhibitable, partially energy dependent, and, as now shown, partially sodium dependent (2, 13, 14). Many of these observations point to a central role for the Na+,K+-ATPase in DDP accumulation. The present studies were undertaken to examine this role in more detail. Our data suggest that the ouabain effect was due to a direct inhibition of the Na+,K+-ATPase and dissipation of the Na+ gradient which then affects DDP influx.

The inhibition of DDP accumulation by ouabain was maximized by increasing the preincubation time to 1 h. This was consistent with the slow association of ouabain with the Na+,K+-ATPase in K+-free media (15, 16). The effect of ouabain was linear with concentrations up to approximately 0.2 μM, which was consistent with the range of the published KD for ouabain binding to the Na+,K+-ATPase and the KD found by Scatchard analysis of ouabain binding to 2008 cells (16, 17). The effects of energy depletion, ouabain inhibition, and Na+ depletion on DDP accumulation were detectable as early as 1 min after 500 μM DDP exposure. The detection of decreased accumulation 1 min after the imposition of 0-trans conditions provides strong evidence that these effects on drug accumulation were a result of inhibition of initial drug influx and not due to an effect on DDP metabolism, subcellular distribution, or DDP efflux.

Since the Na+,K+-ATPase appeared to play a pivotal role in DDP accumulation, we hypothesized that resistant cells with decreased DDP accumulation may have alterations in their Na+,K+-ATPase. 2008/DDP cells were found to be cross-resistant to ouabain, suggesting that this was the case. DDP-resistant R1.1 murine T-lymphoma cells with accumulation defects have also been reported to be cross-resistant to ouabain, implying that this observation is not unique to 2008 cells (18, 19). Scatchard analysis of ouabain binding indicated that the KD did not change in 2008/DDP cells, but the number of Na+,K+-ATPase molecules had decreased 36%. To determine Na+,K+-ATPase activity, we measured K+ influx with 86Rb+. No change was found in either total K+ influx or K+ influx through the Na+,K+-ATPase. This indicated that the Na+,K+-ATPase basal activity was the same in parent and resistant cells. This was not unexpected in that HeLa cells with 10-fold differences in Na+,K+-ATPase expression nonetheless have similar Na+,K+-ATPase activity (12). At present, we do not know whether the change in the Na+,K+-ATPase in resistant cells is directly linked to the accumulation defect or why a change in Na+,K+-ATPase number without a change in activity should affect DDP accumulation. The alteration in the Na+,K+-ATPase in 2008/DDP cells does not appear to result in a Na+ gradient that is any less steep than in 2008 cells. An obvious explanation for our data is that a Na+-dependent DDP transporter exists and that destruction of the Na+ gradient either by incubation in low-Na+ medium or inhibition of the Na+,K+-ATPase (by ouabain, ATP depletion, or low-K+ medium) compromises this DDP transport pathway. However, this explanation is contradicted by the inability to demonstrate the existence of a carrier for DDP by saturation kinetics or analogue competition (6, 7). This Na+-dependent pathway appears to account for approximately half of the total DDP accumulation *in vitro* at all concentrations studied. Whether Na+ dependent transport also makes a significant contribution to DDP accumulation *in vivo* remains to be determined.

We and others have not failed to notice that tissues with the highest levels of Na+,K+-ATPase are also the ones associated with the primary toxicities of DDP, *i.e.*, nephro-, neuro-, and otoxicity. Recent findings strengthen this correlation in that the distribution of Na+,K+-ATPase along the nephron correlates with the foci of DDP toxicity (20), *i.e.*, the proximal convoluted tubule, the medullary and cortical ascending limbs, and the distal tubule all have higher levels of Na+,K+-ATPase than other regions of the nephron (21). In addition, the locus of DDP damage in the inner ear is the outer hair cells in the cochlea (22). The cochlea is rich in Na+,K+-ATPase which functions to maintain the steep ion gradients in the endolymph (22–25). The specificity of DDP toxicity for peripheral nerves and not the brain can be explained by the reasoning that, because of the blood-brain barrier, this is the only Na+,K+-ATPase-rich nerve tissue that is accessible to DDP. The


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