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Covalent binding of cisplatin impairs the function of Na^+/K^+-ATPase by binding to its cytoplasmic part

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ABSTRACT

This study was aimed at verifying the hypothesis that acute kidney failure accompanying cisplatin administration in the cancer therapy could be due to cisplatin interaction with the cytoplasmic part of Na^+/K^+-ATPase. Our results demonstrated that cisplatin-binding caused inhibition of Na^+/K^+-ATPase, in contrast to other platinated chemotherapeutics such as carboplatin and oxaliplatin, which are known to be much less nephrotoxic. To acquire more detailed structural information, we performed a series of experiments with the isolated large cytoplasmic segment connecting transmembrane helices 4 and 5 (C45 loop) of Na^+/K^+-ATPase. Electrochemistry showed that cisplatin is bound to the cysteine residues of the C45 loop, mass spectrometry revealed a modification of the C45 peptide fragment GSHMASLEA-VETLGSTSTICSDK, which contains the conserved phosphorylated residue Asp369. Hence, we hypothesize that binding of cisplatin to Cys367 can cause sterical obstruction during the phosphorylation or dephosphorylation step of the Na^+/K^+-ATPase catalytic cycle.

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)) is the most widely used chemotherapeutics in cancer treatment. Its biological action resides in its ability to form bifunctional adducts with cell nucleus DNA and thus, impair DNA replication and cell division [1]. Cisplatin can overcome cellular membranes by utilizing membrane copper transporters [2], or organic cation transporter OCT2 [3]. The transport into cytoplasm is significant for cisplatin activity. In the extracellular milieu with high chloride concentration, a relatively unreactive form with chlorides prevails, while the more reactive aquated form is favored in the cytoplasm with low chloride concentration [4].

However, patients receiving the cisplatin suffer from a large number of inevitable side-effects, such as neuropathies and hearing loss [1,5]. One of the most serious adverse effects is the acute renal failure that affects roughly 20–30% of patients during the first week of a cisplatin-based chemotherapy [6–8]. Other clinically approved platinum-based chemotherapeutics, the oxaliplatin and carboplatin, are much less nephrotoxic, however, they are efficient against different types of tumors.

The primary role of kidney is to filter metabolic waste products from blood to urine, and at the same time conserve metabolically valuable ions and biomolecules, such as glucose. While the first step of cleansing that occurs in glomeruli can be considered a relatively simple filtration, the reuptake of water, sodium and other valuable solutes is driven by a sophisticated system of specific membrane transporters. Apparently, the fine control of Na^+ concentration is crucial in this process. The Na^+ gradient is used by a variety of secondary active transporters, e.g. glucose, Ca^{2+} and H^+ transporters [9]. In contrast to many transporters that utilize the Na^+ gradient (and reduce it in this way), there is only one transporter that creates the gradient itself – the Na^+/K^+-ATPase (also called the sodium pump). Hence, while failure of any other transporter will decrease the reuptake efficiency of only one specific solute, failure of Na^+/K^+-ATPase will result in collapse of the whole machinery.

The human kidney daily filters around 180 L of fluid [9], and this tremendous metabolic turnover is probably the reason, why Na^+/K^+-ATPase expression is highest just in the kidney [10]. For this reason, we hypothesized that cisplatin binding may impair Na^+/K^+-ATPase function and that this has the most serious consequences in the kidney.

Na^+/K^+-ATPase structure has been recently determined by X-ray crystallography [11,12]. The minimal functional unit of the enzyme is formed by the catalytic α-subunit (110 kDa) that is responsible for both transport of cations and ATP-hydrolysis and

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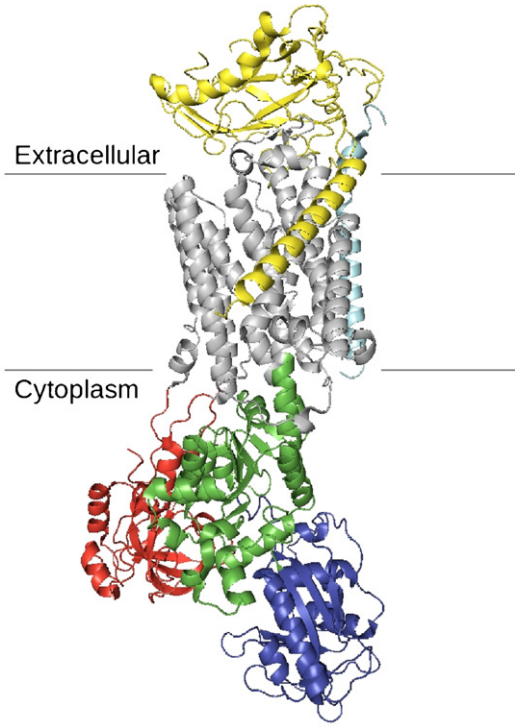

Fig. 1. Structure of Na^+/K^+-ATPase as determined by X-ray crystallography (PDB code 2zxe). Cytoplasmic domains N (blue) and P (green) are formed by the large segment connecting transmebrane helices M4 and M5 (C45 loop), domain A (red) is formed by N-terminus and C23. The β-subunit is colored yellow, the transmembrane helix of γ-subunit is in cyan.

the β-subunit (~55 kDa) that is necessary for proper maturation of the enzyme in the plasma membrane and probably also participates in the K^+-transport [12,13]. The transmembrane part is formed by ten α-helices (M1–M10) that form the cation-binding sites, which alternately open toward the cytoplasm and the extracellular space. In the cytoplasm, three well-separated domains can be found. The small domain A (actuator) comprises the N-terminus and a cytoplasmic loop connecting M2 and M3 (C23), while the other two domains designated as N (nucleotide-binding) and P (phosphorylation) constitute the large C45 loop (Fig. 1).

The C45 loop represents approximately 40% of the total enzyme mass, dominates the cytoplasmic part and it is the most likely target for the putative interaction with cisplatin. It was demonstrated in several laboratories that the C45 loop can be isolated from the rest of the enzyme [14–16], retaining its structure, dynamic properties [16,17] and ability to bind nucleotides [18,19]. The solubility of the C45 loop enables its heterologous expression in *Escherichia coli* in high quantities and this greatly facilitates further experimental work.

There are numerous studies demonstrating that cisplatin can bind to proteins found either in blood or in cytosol [20–24]. In this study, we focused on the hypothesis that kidney damage is related to cisplatin interactions with the cytoplasmic part (C45 loop) of the Na^+/K^+-ATPase at the plasma-membrane/cytosol interface. Hence, besides estimation of cisplatin influence on the whole Na^+/K^+-ATPase activity, we also tested its interaction with the isolated C45 loop in order to get more detailed information.

2. Material and methods

2.1. Reagents

Unless otherwise stated, all chemicals were from Sigma–Aldrich Chemie (Steinheim, Germany). Cisplatin, oxaliplatin and carboplatin were dissolved in ddH_2O to a concentration of 1 mM. For further experiments, this solution was used as a 'non-activated cisplatin'. Activation by a 2-fold molar excess of $AgNO_3$ (24-h incubation), which irreversibly removes the chloride ligands as an AgCl precipitate, provided 'activated cisplatin'.

The Na^+/K^+-ATPase from porcine cerebral cortex was isolated by the method of Nakao et al. [25], pipetted into small aliquots, and stored in 20 mM Tris buffer, pH 7.4, containing the non-ionic detergent $C_{12}E_8$ at −20 °C. The molar concentration of Na^+/K^+-ATPase was estimated from the total protein concentration, considering MW (α + β) = 165 000 and a protein purity of 70%, as revealed by SDS-PAGE.

The large cytoplasmic segment connecting the transmembrane helices 4 and 5 (C45 loop, residues Leu^{354}-Ile^{777} of the mouse brain sequence) with a $(His)_6$-tag at the N-terminus was expressed in *E. coli* Rosetta cells (Promega, USA) and purified using a Co^{2+}-based affinity resin (Clontech, USA) as described previously [16]. Immediately after elution, the protein was dialyzed into TBS buffer (20 mM Tris, 140 mM NaCl, pH 7.6) and stored at −20 °C.

Protein concentration was determined using the Bradford assay [26] with BSA as a standard.

2.2. Gel electrophoresis of C45 loop

Samples of C45 loop were incubated with a denaturing buffer containing SDS, boiled for 5 min at 95 °C and separated by SDS PAGE. Staining and destaining of the gel was carried out by a standard CBB staining procedure [27].

2.3. Na^+/K^+-ATPase activity assay

A fluorimetric method for inorganic phosphate detection relying on the monitoring of m^7guanosine conversion to m^7guanine by nucleoside phosphorylase was proposed by Banik and Roy [28]. This has the advantage of relatively low demand on sample amount and concentration. However, we discovered that the original procedure, which proposed continuous detection of fluorescence intensity time-evolution, yielded only poorly reproducible results due to the substantial photobleaching of m^7guanosine. The reproducibility greatly improved when only short pulses of excitation light were used to monitor the fluorescence intensity at selected time intervals.

The assay medium contained 0.1 unit nucleoside phosphorylase, 3 mM ATP, 250 μM m^7guanosine (m^7Guo), 140 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 50 mM Tris, pH 7.4. After equilibration in a cuvette for 5 min, the reaction was started by the addition of 0.5 μM Na^+/K^+-ATPase.

The excitation wavelength was 310 nm and the emission wavelength was 390 nm; excitation and emission bandpasses were set to 10 nm and 20 nm, respectively. Intensity of fluorescence was integrated for 3 s every 7 min, meanwhile the sample was kept in darkness. Activity was calculated from the slope of the linear fit.

In the presence of 10 mM ouabain (a specific inhibitor of the Na^+/K^+-ATPase), the ATPase activity of the sample decreased to <8%. Hence, essentially all phoshatase activity of the sample could have been assigned to the activity of the Na^+/K^+-ATPase, and therefore ouabain was not introduced in further studies. The inhibitory effect of platinum-based drugs on the Na^+/K^+-ATPase activity was measured after 1 h incubation of these drugs at 37 °C in a buffer containing 20 mM NaCl. In the experiment, where we examined the protective effect of a thiol group-containing species against enzyme inhibition by cisplatin, 0.5 mM dithiothreitol (DTT) was introduced into the incubation buffer. Each point is represented as the mean of five to ten replicates. The statistical significance of the differences was evaluated using the two-tailed Student's *t*-test.