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Kinetics of Cu^{2+} inhibition of Na^+/K^+-ATPase

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Abstract

The interaction of Cu^{2+} with enzymatic activity of rabbit kidney Na^+/K^+-ATPase was studied in media with buffered, defined free Cu^{2+} levels. The IC_{50}-values are 0.1 μmol/l for Na^+/K^+-ATPase and 1 μmol/l for K^+-pNPPase. Dithiothreitol (DTT) reverses the inhibitory effect of Cu^{2+} in vitro. Cu^{2+} exerts non-competitive effects on the enzyme with respect to Na^+, K^+, ATP or pNPP, but has a mixed-type inhibitory effect with respect to Mg^{2+}. It is concluded that the appreciation of the inhibitory effect of Cu^{2+} on this enzyme requires carefully composed assay media that include a buffer for Cu^{2+}, and that the IC_{50}-values calculated according to this model indicate that Cu^{2+} may be more toxic than previously anticipated.

Keywords: Copper; Na^+/K^+-ATPase (EC 3.6.1.3); K^+-pNPPase; Kinetics

1. Introduction

In animal cells, the main sodium pump is the ouabain-sensitive Na^+/K^+-ATPase, a membrane-bound enzyme which frees energy from ATP to extrude Na^+ in exchange for K^+ [1–3]. This pump is the pivotal mechanism in the physiology of the cell, as it is involved, directly or indirectly, in cell volume regulation, the control of intracellular pH, the maintenance of free Ca^{2+} concentration and membrane potential. It is regarded as the major 'housekeeping' enzyme, but it also plays an important role in epithelial ion transport [4,5]. As a consequence, inhibition of this pump seriously affects cell physiological functions [6].

According to the extended Albers-Post reaction scheme [6], the Na^+/K^+-ATPase undergoes a sequence of transitions between the E_1 conformation with inward-facing cation binding sides and high affinity for Na^+ and an E_2 conformation with outward-facing cation binding sites and high affinity for K^+. Transitions between these two conformations are induced by phosphorylation–dephosphorylation reactions characteristic of P-type ATPases. Under physiological conditions, three intracellular Na^+ ions bind to the E_1ATP form. Hydrolysis of ATP and phosphorylation of the protein lead to the occlusion of three Na^+ ions followed by a transition to the E_2P form. Na^+ can

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now be released externally and two K^+ ions are bound; this leads to dephosphorylation and occlusion of the K^+ ions. Stimulated by ATP, a conformational change back to the E_1 form is induced, the K^+ ions are liberated to the cytoplasm, and the transport cycle is completed. Na^+/K^+-ATPase contains 34 functional SH-groups in its α-subunits, which play an important role in activation of the enzyme [7].

The oligoelement copper serves a pivotal role in the cell physiology of vertebrates and is required for bone formation, development of connective tissue, tissue pigmentation and cardiac function [8]. In the extracellular fluid, copper is bound to amino acids or albumins. About the actual transport across the plasma membrane very little is known. Copper is taken up by cells and incorporated into (metallo-)proteins (e.g. ceruloplasmin, metallothionein, glutathion) and metalloenzymes (e.g. superoxide dismutase, cytochrome c oxidase, tyrosinase, monoamine oxidase). The function of copper in enzymes often concerns the transfer of electrons and the binding of oxygen molecules. Cu^{2+} generally appears to be strongly chelated as a protective mechanism to circumvent its toxic actions.

Free Cu^{2+} inhibits Na^+/K^+-ATPase activity in a variety of cell types and in a variety of species [9–13]. Although the toxic action of Cu^{2+} is mainly attributed to covalent binding of Cu^{2+} to functional sulfhydryl (-SH) groups of membrane proteins and enzymes [14–17], including Na^+/K^+-ATPase [18–20], the kinetics of Cu^{2+} interaction with Na^+/K^+-ATPase are flawed by the fact that, in these studies, the total concentration of copper rather than the free Cu^{2+} concentrations was considered. It is generally believed that the ionic form of copper, Cu^{2+}, represents the toxic form of this metal [16]. The higher affinity of ATP for Cu^{2+} over Mg^{2+} [21] means that little Mg-ATP remains in an assay medium when Cu^{2+} is added (see Discussion). For a proper in-vitro evaluation of the enzymatic activity, Mg-ATP is required as substrate and cannot be replaced by Cu-ATP [22]. Therefore, an accurate control of the levels of Mg-ATP, Cu-ATP, Mg^{2+} and Cu^{2+} is essential for a kinetic analysis of Cu^{2+} inhibition of Na^+/K^+-ATPase.

In this study we have evaluated the effect of buffered free Cu^{2+} levels on the kinetics of Na^+/K^+-ATPase isolated from rabbit kidney. The concentrations of ATP, Na^+, Mg^{2+}, Ca^{2+} and K^+ were varied under strictly buffered conditions and free ion levels were calculated using the CHELATOR program [23]. To study the specific interaction of Cu^{2+} with the K^+-dependent dephosphorylation, we assayed the K^+-dependent *p*-nitrophenylphosphatase (pNPPase) activity, which forms an integral part of the overall Na^+/K^+-ATPase reaction.

2. Materials and methods

2.1. Enzyme preparation

Na^+/K^+-ATPase from rabbit kidney outer medulla was purified according to Jørgensen [24] by zonal centrifugation of a microsomal fraction treated with sodium dodecylsulfate. Removal of contaminating ATP and washing of the preparation followed the method of Schoot et al. [25]. The Na^+/K^+-ATPase activity was predominantly ouabain-sensitive (>98%) and averaged around 850 μmol Pi/h per mg protein at 37°C, in our assay (see below). The enzyme was kept in 250 mmol/l sucrose, buffered at pH 7.4 with 50 mmol/l imidazole/HCl and stored at −20°C up to 1 month without significant loss of activity. Protein was determined using a Coomassie Brilliant Blue assay (Biorad), with bovine serum albumin (BSA) used as reference.

2.2. Enzyme assays

Na^+/K^+-ATPase activity was determined as described by Bonting et al. [26]. In short, the specific activity was measured as the difference in ATP hydrolysis in two media, A and E. Medium A contained (mmol/l): NaCl (100), KCl (10), $MgCl_2$ (5), Na_2ATP (5), oxalic acid (1) and imidazole/HEPES (30; pH 7.4). In medium E, KCl was omitted and ouabain (G-strophanthin, Boehringer; 0.1 mmol/l) was added. Oxalic acid was added to serve as buffer for Cu^{2+} (see below), and its addition per se did not affect the enzymatic activity. Five microliters of an enzyme suspension, equivalent to

250–400 ng protein, was mixed with 400 μl assay medium on ice and incubated at 37°C for 10 min. All incubations were carried out in triplicate. The reaction was stopped by the addition of 1 ml ice-cold trichloro-acetic acid (TCA, 8.6% w/v). The phosphate released was quantified after addition of 1 ml freshly prepared color reagent, containing 9.6 % (w/v) $FeSO_4 \cdot 6H_2O$, 1.15% (w/v) ammonium heptamolybdate in 0.66 M H_2SO_4 by assessing the ΔA700 with a PU 8620 spectrophotometer (Philips). Combined calcium/phosphorus standards (Sigma, 360-11) were used as reference. The specific activity was expressed in μmol Pi/h per mg protein.

The K^+-pNPPase activity was determined as the difference in *p*-nitrophenol (pNP) released in medium A and medium E [25]. Medium A contained (mmol/l): KCl (10), $MgCl_2$ (3), pNPP (10), oxalic acid (1) and imidazole/HEPES (30; pH 7.4). In medium E, potassium was omitted and ouabain (0.1 mmol/l) added. Incubation was carried out as described for the Na^+/K^+-ATPase assay above. The reaction was stopped by addition of 1 ml ice-cold NaOH (1 mol/l). The release of pNP was quantified colorimetrically at 420 nm. A commercially available pNP-standard (Sigma, 104-8) was used as reference. The enzymatic specific activity was expressed as μmol pNP/h per mg protein.

2.3. Buffering of Cu^{2+}

In all assays, free Cu^{2+} concentrations were calculated using the computer algorithm Chelator [23]. This algorithm recomputes stability constants obtained from the literature [21] to reflect the effects of ionic strength, pH, and temperature of the medium used, and calculates the contribution of ionized species of metals, chelator, complexes and

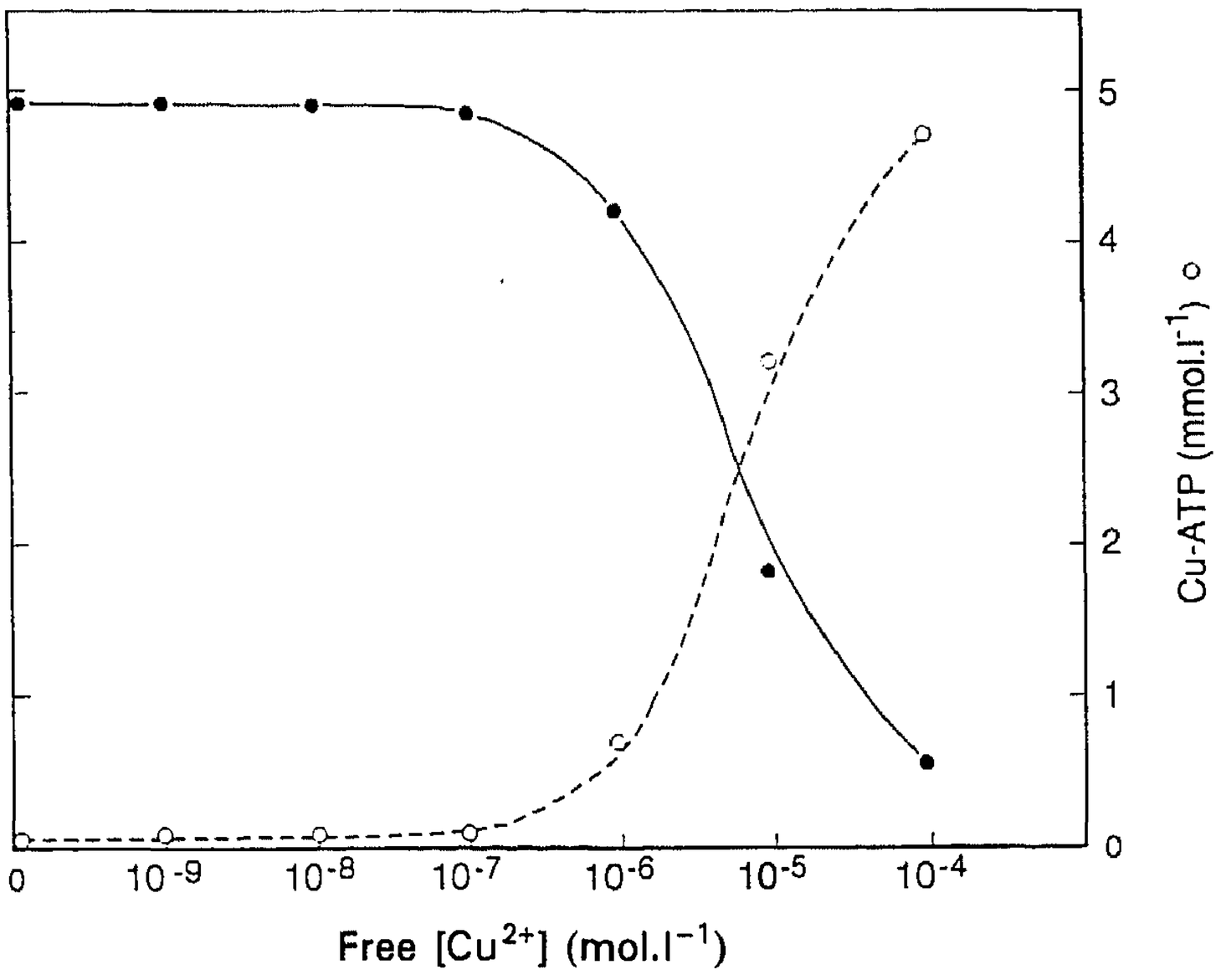

Fig. 1. The occurrence of Cu-ATP and Mg-ATP salts in an assay medium containing 1 mmol/l oxalic acid, 5 mmol/l ATP and 5 mmol/l free Mg^{2+}. Calculated free Cu^{2+} (*X*-axis) was varied and the occurrence of Mg-ATP (left *Y*-axis) and Cu-ATP (right *Y*-axis) chelates were calculated according to [23]. At 10^{-5} and 10^{-4} mol/l free Cu^{2+}, the Mg-ATP level drops and the Cu-ATP level increases significantly. At 10^{-5} mol/l Cu^{2+} the concentration of Mg-ATP drops to 1.8 mmol/l, which still allows determination of enzyme activity under apparent V_{max} conditions. At 10^{-4} mmol/l free Cu^{2+}, 0.6 mmol/l Mg-ATP is formed and this concentration is suboptimal for Na^+/K^+-ATPase activity ($K_{0.5}$ for ATP: 0.44 mmol/l). Increasing the oxalic acid concentration does not improve the composition of the assay medium with respect to Mg-ATP at high Cu^{2+} levels.

pH buffers to net ionic strength. A 1 mmol/l capacity of oxalic acid buffer was used to define the free Cu^{2+} levels and control the levels of Mg-ATP and Cu-ATP. The stability constants K_d of the ligands (HEPES, ATP and oxalic acid) for the metal ions (Mg^{2+}, Cu^{2+}) were taken from Sillén and Martèll [21].

The half-maximum inhibitory concentration of Cu^{2+}, the IC_{50} value, was assessed by varying the Cu^{2+} levels from 10^{-9} to 10^{-4} mol/l by addition of calculated amounts of $Cu(NO_3)_2$.

In a control experiment it was shown that the addition of 1 mmol/l NO_3^- (as $NaNO_3$; the highest concentration that could result from the addition of $Cu(NO_3)_2$) did not affect Na^+/K^+-ATPase activity (data not shown).

The use of oxalic acid as Cu^{2+} buffer guaranteed that the addition of copper to the assay media had negligible effects on the concentration of Mg-ATP. For instance, at the IC_{50} of Cu^{2+} for overall Na^+/K^+-ATPase activity (0.1 μmol/l; Fig. 2), 4.8 mmol/l Mg-ATP was present, exceeding the calculated level of Cu-ATP more than 50 times. Under our experimental conditions, therefore, saturating levels of Mg-ATP were always present in the assay media.

2.4. Calculations

Kinetic parameters ($K_{0.5}$ and V_{max}) were derived after fitting the data to the Michaelis–Menten equation, using non-linear regression data analysis. Substrate concentrations were varied around the apparent $K_{0.5}$-concentration. Experiments were repeated at least 6 times with different enzyme preparations, unless otherwise stated.

3. Results

3.1. Inhibition of Na^+/K^+-ATPase and K^+-pNPPase

As shown in Fig. 2, increasing concentrations of Cu^{2+} result in a sigmoidal inhibition curve for the

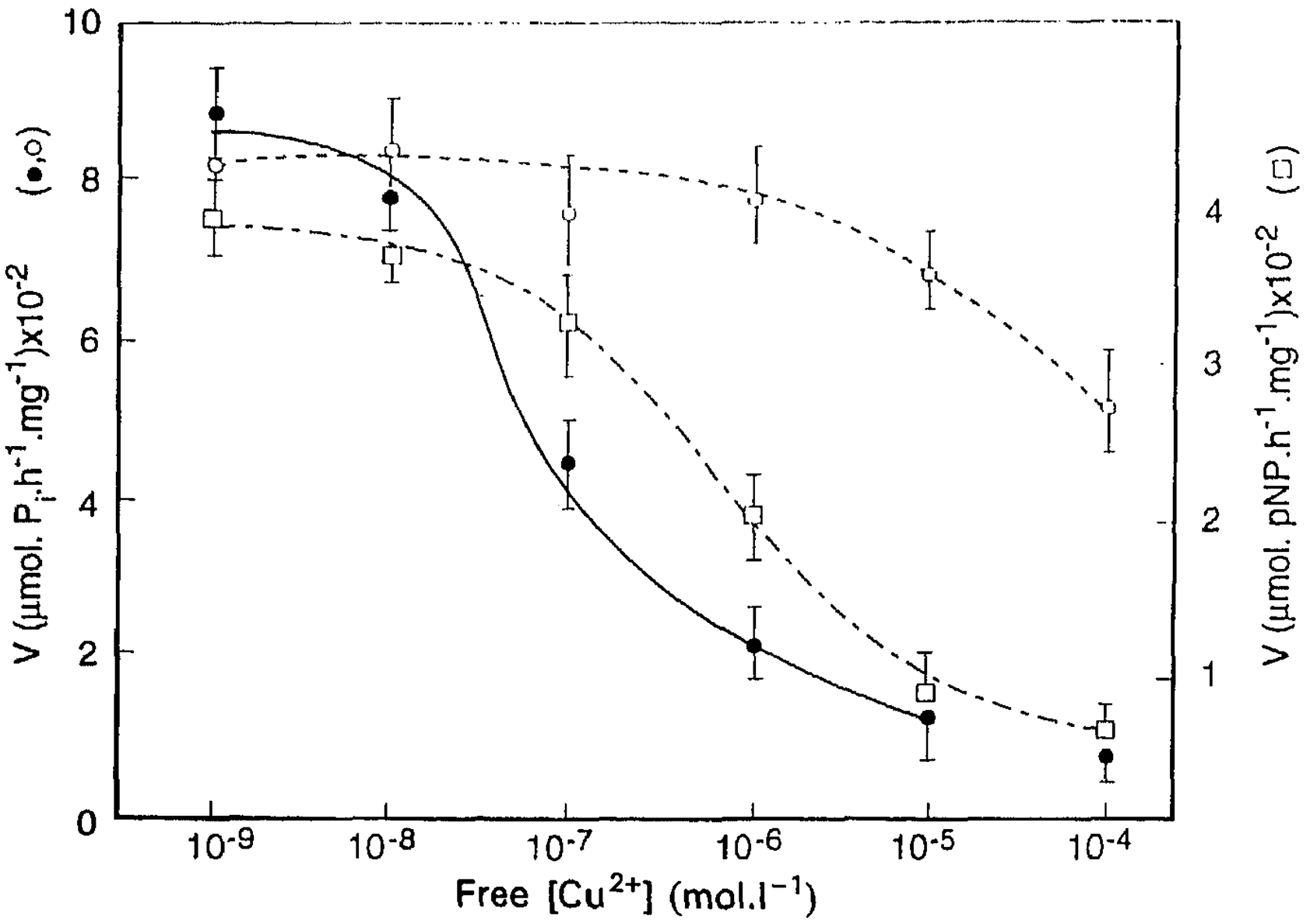

Fig. 2. Inhibition of rabbit kidney Na^+/K^+-ATPase activity and K^+-pNPPase activity by free Cu^{2+}. The experimental conditions are described in Materials and methods. Na^+/K^+-ATPase (●) is half-maximally inhibited at 0.1 ± 0.01 μmol/l Cu^{2+}, K^+-pNPPase (□) at 1.0 ± 0.1 μmol/l Cu^{2+}. DTT (0.1 mmol/l) has no effect on the enzyme at 'no-effect' levels of Cu and reverses the inhibitory effect of free Cu^{2+} on Na^+/K^+-ATPase over the total range of Cu^{2+} concentrations tested (○). The IC_{50}-values of Na^+/K^+-ATPase and pNPPase are significantly different ($N = 6–10$; $P < 0.01$). Mean values are given, bars indicate standard deviation.

Table 1
Kinetic analyses of Na^+/K^+-ATPase and K^+-pNPPase activities in the absence (control) and presence of Cu (0.1 and 1 μmol/l for Na/K-ATPase and K-pNPPase, respectively) ($N = 4$)

Substrate	Control		Cu	
	V_{max} (μmol/h per mg)	$K_{0.5}$ (mmol/l)	V_{max} (μmol/h per mg)	$K_{0.5}$ (mmol/l)
ATP	857	0.44	429* (50)	0.48
Na^+	780	21.8	340* (44)	23.5
pNPP	408	0.70	229* (56)	0.55
K^+	375	1.85	224* (60)	1.86
Mg^{2+}	832	0.005	385* (46)	0.064*

Values in parentheses indicate the % inhibition. ATP-, Na- and Mg-dependence were assayed as the Na- and K-dependent, ouabain-sensitive ATPase activity; the pNPP- and K-dependence were assayed as the K-dependent, ouabain-sensitive pNPPase activity. Mean values for 6–10 experiments are given; in all cases the standard deviation was less than 10% of the mean value.
*$P < 0.05$.

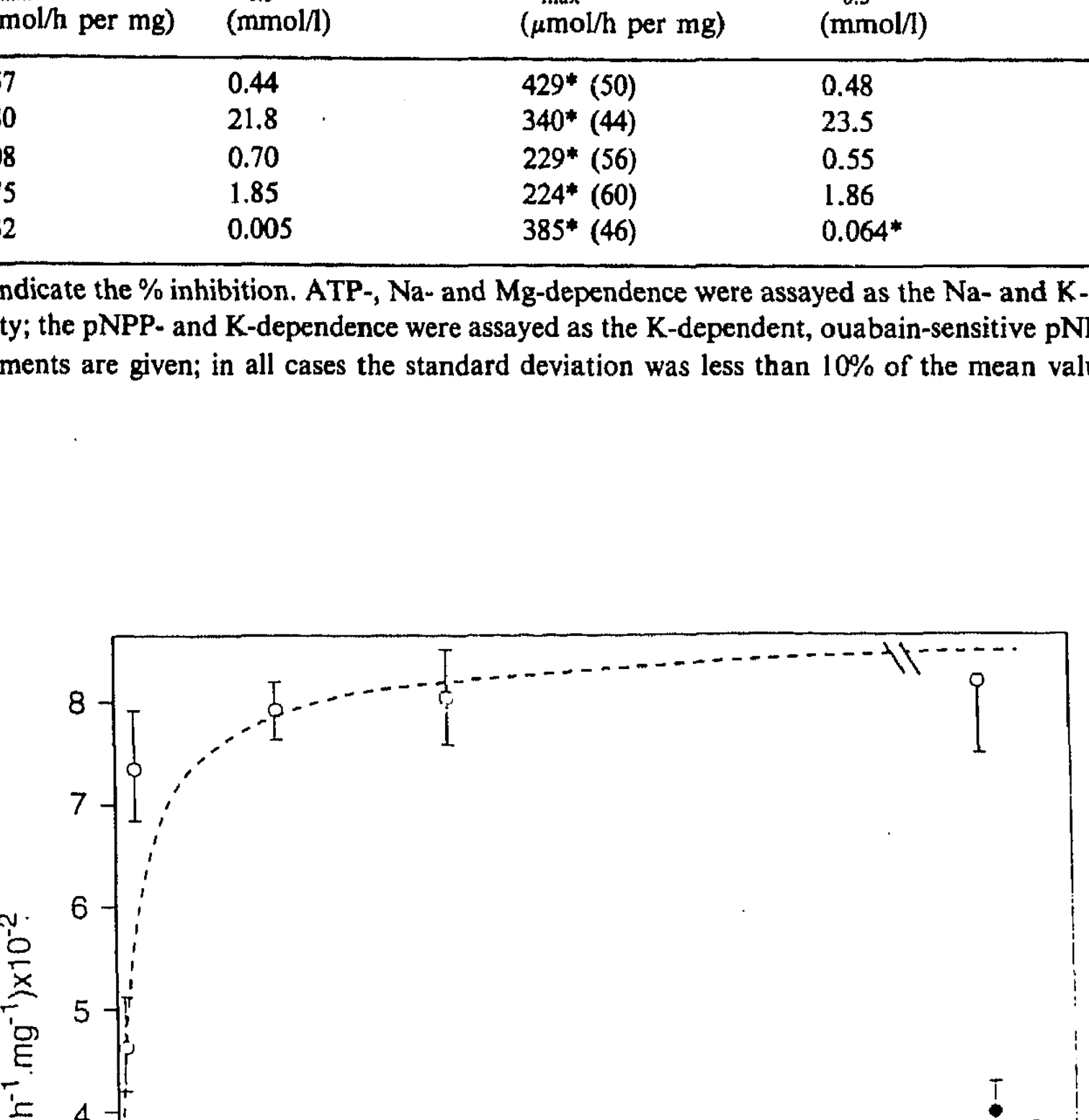

Fig. 3. Kinetics of Mg^{2+} dependence of Na^+/K^+-ATPase activity in the presence or absence of Cu^{2+}. Cu^{2+} significantly decreases the V_{max} and increases the $K_{0.5}$ for Mg^{2+} (dashed line, controls; solid line, presence of Cu^{2+}). The Eadie–Hofstee transformation of the data (inset) indicates mixed-type inhibition. For apparent $K_{0.5}$ and V_{max} values see Table 1. $N = 6–10$; mean values are given, bars indicate standard deviation.

Na^+/K^+-ATPase as well as the K^+-pNPPase activity. At 10^{-7} mol/l Cu^{2+}, significant inhibition of Na^+/K^+-ATPase (50%) and K^+-pNPPase activity (15%) was observed. The IC_{50}-value for Na^+/K^+-ATPase (0.1 ± 0.01 μmol/l) is 10 times lower than that for the pNPPase (1.0 ± 0.1 μmol/l). In all subsequent kinetic studies we applied 0.1 μmol/l Cu^{2+} in Na^+/K^+-ATPase and 1.0 μM Cu^{2+} in pNPPase determinations. Dithiothreitol (0.1 mmol/l) almost fully reversed the inhibition of Na^+/K^+-ATPase activity by Cu^{2+}.

3.2. Interaction with substrates

Table 1 summarizes the effects of Cu on the kinetics of Na^+/K^+-ATPase activities. Cu^{2+} (at the IC_{50}-value of 0.1 μmol/l) non-competitively inhibited the enzyme when tested for its ATP-dependence, its Na^+-dependence, its pNPP-dependence or its K^+-dependence: the calculated V_{max}-values were reduced to the predicted 50% based on the application of IC_{50} concentrations of Cu^{2+}. The $K_{0.5}$ values for the respective substrates were not influenced by Cu^{2+}. However, 0.1 μmol/l Cu^{2+} induced a mixed-type inhibition of Na^+/K^+-ATPase with regard to Mg^{2+}-activation: a significant decrease in the V_{max} (from 832 to 385 μmol Pi/h per mg protein) is accompanied by a more than 12-fold increase in the $K_{0.5}$ (Table 1); this phenomenon is illustrated in Fig. 3.

4. Discussion

Our data show that the IC_{50}-value of Cu^{2+} for rabbit kidney Na^+/K^+-ATPase was up to 3 orders of magnitude lower than IC_{50} values reported, for instance Na^+/K^+-ATPase from rat brain [9], rat renal cortex [11], pigeon brain [27] and erythrocyte ghosts [28]. This discrepancy is obviously based on the differences in experimental design and approach. In our study we have calculated free copper (Cu^{2+}) concentrations, whereas the total, nominal Cu concentration was considered in the studies cited.

When studying the mechanisms and kinetics of enzymes and the effects of heavy metal on these processes, accurately controlled concentrations including physiological ions, such as Mg^{2+} and Ca^{2+}, are required [23,28,29]. Proceeding from stability constants K_a = 4.18 l/mol for Mg-ATP and K_a = 5.92 l/mol for Cu-ATP, we calculate that in a medium containing 3 mmol/l ATP and 10 μmol/l Cu^{2+}, 99.9% of the Cu would be chelated by ATP to become Cu-ATP and the concentration of Cu-ATP would exceed three times that of Mg-ATP. A proper functioning of Na^+/K^+-ATPase requires Mg-ATP, and Cu-ATP cannot replace Mg-ATP [22]. Therefore, it is necessary to control strictly the concentrations of Cu^{2+}, Cu-ATP and Mg-ATP in the experimental system for a kinetic analysis of Cu^{2+} inhibition of this enzyme. The introduction of oxalic acid (K_a = 2.3 l/mol for Mg-oxalate and K_a = 4.8 l/mol for Cu-oxalate) in the assay medium provides an appropriate buffer, which allowed us to compose media with up to 10^{-5} mol/l Cu^{2+}, keeping the concentration of Mg-ATP far above its $K_{0.5}$-value (see Fig. 1): at a calculated 1 μmol/l free Cu^{2+} about 6 times more Mg-ATP relative to Cu-ATP was present; at 10 μmol/l Cu^{2+}, 1.8 mmol/l Mg-ATP was still present, which is over 4 times the $K_{0.5}$ (0.44 mmol/l) for the substrate. At 10^{-4} mmol/l Cu^{2+}, however, only approximately 10% Mg-ATP and 90% Cu-ATP are present. We conclude that the free Cu^{2+} exerted the inhibitory effect, as Cu-ATP appears to give an inert complex.

Since Na^+/K^+-ATPase (and K^+-pNPPase) activity depends on interaction with the ligands ATP, Mg^{2+}, Na^+, pNPP, and K^+, we kinetically analyzed the inhibition by Cu^{2+} to establish a key event in the inhibitory action. The results — non-competitive inhibition of the overall Na^+/K^+-ATPase activity by Cu^{2+} (IC_{50}, 0.1 μmol/l) with respect to ATP and Na^+, and non-competitive inhibition of the pNPPase activity by 1 μmol/l Cu^{2+} (IC_{50} for pNPPase) with respect to pNPP and K^+ — suggest that Cu^{2+} does not interfere with the specific binding of these ligands. The mixed inhibition by Cu^{2+} with respect to Mg^{2+} could be explained in at least two ways. First, Cu^{2+} may compete with free Mg^{2+} at a Mg^{2+} binding site and directly affect the Mg^{2+}-dependent steps, like the phosphorylation of the enzyme to the E_1P conformation, which is dependent on tightly bound Mg^{2+} [3]. Second, 'non-specific' binding of Cu^{2+} to functional catalytic groups, such as SH-

groups [10], may underlie inhibition. The integral Na^+/K^+-ATPase has two α-subunits [6], each containing at least 34 essential sulfhydryl groups in its catalytic center [7]. Cu^{2+} is a well-known potent reagent for thiol groups; its interaction with membrane and enzymatic SH-groups may therefore result in toxic effects [15,16]. The ability of the SH-group reducing agent, dithiothreitol (DTT), to protect against Cu^{2+} toxicity would confirm that such groups in the enzyme are a target for Cu^{2+}, in line with other studies [19,20,30]. However, DTT also strongly chelates Cu^{2+}, and evidence was given — comparing DTT with the chelator TPEN which lacks reducing properties — that such an effect could also explain the protective action of DTT on Na^+/K^+-ATPase [13]. Clearly, both effects of DTT will simultaneously occur in experiments as presented here, and we cannot discriminate one effect from the other.

Our results demonstrate that the toxic effect of copper may be attributed to the ionic species, Cu^{2+}. The low IC_{50} value for Cu^{2+} reported here is in line with the notion that in vivo copper is always tightly bound (e.g. to albumin, metallothionein or transcuprein [8]) and free Cu^{2+} levels are essentially zero, apparently to protect the organism from toxic effects of this element. Non-competitive inhibition with respect to the ligands (Na^+, K^+, ATP and pNPP) and mixed inhibition with Mg^{2+} for the enzyme by Cu^{2+} indicate that Cu^{2+} interferes directly with Mg^{2+} binding to the enzyme and utilization of Mg-ATP. When studying enzymatic reactions in vitro and the effects of heavy metals for their interaction(s), it appears necessary to calculate and control the free Cu^{2+}, and its interference with the other components of the assay medium.

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