J. Membrane Biol. 102, 11–19 (1988)

The Journal of
Membrane Biology
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Silver Ion (Ag^+)-Induced Increases in Cell Membrane K^+ and Na^+ Permeability in the Renal Proximal Tubule: Reversal by Thiol Reagents

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Summary. The initial mechanisms of injury to the proximal tubule following exposure to nephrotoxic heavy metals are not well established. We studied the immediate effects of silver (Ag^+) on K^+ transport and respiration with extracellular K^+ and O_2 electrodes in suspensions of renal cortical tubules. Addition of silver nitrate ($AgNO_3$) to tubules suspended in bicarbonate Ringer's solution caused a rapid, dose-dependent net K^+ efflux ($K_m = 10^{-4}$ M, $V_{max} = 379$ nmol K^+/min/mg protein) which was not inhibited by furosemide, barium chloride, quinine, tetraethylammonium, or tolbutamide. An increase in the ouabain-sensitive oxygen consumption rate (QO_2) (13.9 ± 1.1 to 25.7 ± 4.4 nmol O_2/min/mg, $P < 0.001$), was observed 19 sec after the K^+ efflux induced by $AgNO_3$ (10^{-4} M), suggesting a delayed increase in Na^+ entry into the cell. Ouabain-insensitive QO_2, nystatin-stimulated QO_2, and CCCP-uncoupled QO_2 were not significantly affected, indicating preserved function of the Na^+,K^+-ATPase and mitochondria. External addition of the thiol reagents dithiothreitol (1 mM) and reduced glutathione (1 mM) prevented and/or immediately reversed the effects on K^+ transport and QO_2. We conclude that Ag^+ causes early changes in the permeability of the cell membrane to K^+ and then to Na^+ at concentrations that do not limit Na^+,K^+-ATPase activity or mitochondrial function. These alterations are likely the result of a reversible interaction of Ag^+ with sulfhydryl groups of cell membrane proteins and may represent initial cytotoxic effects common to other sulfhydryl-reactive heavy metals on the proximal tubule.

Key Words silver ion · epithelial transport · K^+ channels · sulfhydryl groups · oxygen consumption · proximal tubule · glutathione

Introduction

Many heavy metals are potent nephrotoxins, but the initial sites and mechanisms of their cytotoxicity remain incompletely defined. Pathological and physiological studies of heavy metal-induced nephropathy have identified the proximal tubule as the principal locus of renal injury, and disruption of mitochondrial function has often been cited as the predominant cytotoxic event (Passow, Rothstein & Clarkson, 1961; Gritzka & Trump, 1968; Weinberg, Harding & Humes, 1982, 1983; Humes & Weinberg, 1986). Unfortunately, previous studies have not resolved whether mitochondrial dysfunction is a primary or a secondary mechanism of cell injury. In other tissues, altered cell membrane cation permeability and oxidation of the sulfhydryl (SH) groups of important cell membrane and cytosolic enzymes have been implicated as critical toxic effects of heavy metals (Passow et al., 1961). However, the temporal relationships and relative contributions among these pathophysiological responses have not been adequately investigated. This study was designed to identify early cytotoxic effects of the SH-reactive heavy metal Ag^+ on the proximal tubule. Ag^+ was chosen because it is thought to be acutely nephrotoxic, has been shown to inhibit renal Na^+, K^+-ATPase activity in vitro (Nechay & Saunders, 1984), and has well-described adverse effects on the cell membrane morphology (Rangachari & Matthews, 1985) and ionic permeability of several biological systems.

To characterize the earliest mechanisms of Ag^+-mediated cell injury, as well as the time course, and interrelationships of these cytotoxic responses, we studied the acute, in vitro effects of Ag^+ on the major ion transport and metabolic functions of the proximal tubule. In particular, continuous measurements of net K^+ fluxes, an index of cell membrane and Na^+,K^+-ATPase integrity, and respiration (QO_2), an index of cellular oxidative metabolism, were made to identify the cytotoxic mechanisms of Ag^+. Since the proximal tubule relies almost exclusively on oxidative metabolism to supply its energy, measurement of QO_2 provides a direct assessment of the balance of ATP production and utilization by the cell. With the majority of ATP produced in the proximal tubule used to support the Na^+,K^+-ATPase, QO_2 can be used to monitor not only mitochondrial function, but also changes in

Na^+ transport. Thus, the combined measurement of K^+ transport and QO_2 provides a direct method for distinguishing initial sites of cell injury, and for assessing subsequent physiologic and pathophysiologic responses of the damaged cell. In addition, the thiol reagents dithiothreitol (DTT) and reduced glutathione (GSH) were used to investigate the role of SH-bearing ligands in the cellular response to Ag^+. Our results indicate that Ag^+ causes dramatic, early changes in the permeability of the cell membrane, first to K^+ and then to Na^+, at concentrations that do not limit Na^+,K^+-ATPase activity or mitochondrial function. These disturbances in cell function appear to be mediated by a reaction of Ag^+ with SH groups of cell membrane proteins, and likely represent the initial toxic effect of Ag^+ and perhaps other SH-reactive heavy metals on the proximal tubule.

Materials and Methods

Preparation of Cortical Tubules

Female New Zealand White rabbits (2 to 3 kg) were anesthetized by the intramuscular injection of ketamine (35 mg/kg), atropine (0.04 mg/kg), and xylazine (5 mg/kg) and inhalation of ether. The ear vein was injected with 1000 units of sodium heparin to provide systemic anticoagulation. Through a midline abdominal incision, the aorta cephalad to the renal arteries was ligated, the vascular supply to the gut clamped, and the inferior vena cava incised. The distal aorta was cannulated with PE-50 tubing, and the kidneys were perfused (25 ml/min) free of blood with a hypertonic, bicarbonate Ringer's buffer bubbled with 95% O_2/5% CO_2 at 37°C. The perfusion was continued for another 15 min with an identical solution containing 50 mg/dl collagenase (Type V, collagenase activity 1000 units/mg, Sigma). In some experiments, when collagenase with sufficient caseinase activity was unavailable, neutral protease (Type XIV, Sigma) (2 mg/dl) was added to the solution to obtain adequate tubule disaggregation. The net K^+ fluxes and QO_2 of tubules prepared by either digestion procedure were identical. After collagenase perfusion, the kidneys were perfused again with the hypertonic buffer for 1 min at 37°C and then with 50 ml of ice-cold hypertonic buffer. The kidneys were then excised into ice-cold, isosmotic bicarbonate buffer. The tubule fragments were dispersed with gentle stirring on a magnetic stir plate at 4°C, sieved through gauze, and centrifuged and washed three times (300 × g, 4°C) to remove cellular debris and most glomeruli in the supernatant. This preparation yielded a nearly homogeneous (greater than 90% by light microscopic morphology) suspension of proximal tubule fragments with open lumina. Previous studies, using a similar preparation prepared in a slightly different manner (Balaban et al., 1980), have demonstrated that these tubules are capable of normal transepithelial ion transport and metabolic function, and that net K^+ fluxes and QO_2 represent almost exclusively proximal tubule processes (Soltoff & Mandel, 1986).

The tubules were suspended in the bicarbonate buffer solution to yield a final concentration of 3 to 5 mg tubular protein/ml and stored at 4°C. Before measurement of QO_2 or K^+ fluxes, 3-ml aliquots of the tubule suspension were placed in capped, 50-ml polycarbonate flasks, gassed with 95% O_2/5% CO_2, and incubated for 30 min at 37°C in a shaking water bath (60 rpm). This preincubation allows for restoration of normal intracellular Na^+,K^+, and ATP content. The buffer solution used in this preparation and for all subsequent experiments was bubbled with 95% O_2/5% CO_2, titrated to a pH of 7.40, and contained (in mM) sodium chloride (115), sodium bicarbonate (25), potassium chloride (5), magnesium sulfate (1), monosodium phosphate (0.4), disodium phosphate (1.6), calcium chloride (1.2), sodium lactate (4), D-glucose (5), L-alanine (1), and 0.6% (wt/vol) dextran (T40, Pharmacia). The dextran was dialyzed (12,000 to 14,000 mw cutoff Spectrapor membrane tubing, Fisher Scientific) for 24 hr against distilled water. In experiments using barium chloride, an equimolar substitution of magnesium chloride for magnesium sulfate was made to prevent the precipitation of Ba^{2+} as the sulfate salt. The kidney perfusion was performed with a hypertonic solution of identical composition except for the addition of 25 mM mannitol.

K^+ Fluxes

Net K^+ fluxes were measured with an extracellular, solid-state K^+ electrode (WPI Model POT-1, World Precision Instruments, New Haven, Conn.) and an ultrawick glass reference electrode (MERE-1, WPI) filled with 1 M n-methyl-D-glucamine chloride, pH 8.0. The electrode had a slope of 53 to 59 mV per decade K^+ concentration with a "linear" response between $10^{-4.5}$ M and 10^{-2} M K^+. The selectivity of K^+/Na^+ was 10,000 : 1, with a response time (including mixing) of 1 to 2 sec. The K^+ and reference electrodes were sealed into a thermoregulated 2-ml glass chamber and connected to a high-impedance electrometer (Model VF-2, WPI). The resulting voltage difference was fed into a lowpass Bessel filter (Model 902LPF, Frequency Devices, Inc., Haverhill, Mass.) with a cutoff frequency of 1 Hz, amplified (10×), and converted to a digital signal at 2 Hz with a 12-bit A-D converter (Model #DT2801, Data Translation, Marlboro, Mass.). The electrodes, electrometer, and filter were located inside a grounded Faraday cage. *DT Notebook* software (Data Translation) was used for data collection. A computer program was written to convert the voltage reading to K^+ concentration. Net K^+ fluxes were measured during the linear phase of the digital tracing following an experimental addition using a computer program written for this purpose. This system allowed continuous measurement of net K^+ fluxes with a resolution 20 to 30 μM K^+. An increase or decrease in extracellular K^+ concentration was interpreted as the net release or uptake, respectively, of K^+ by the tubules. An experiment was initiated by placing 2.0 ml of preincubated tubule suspension into the chamber. The suspensions were bubbled gently with 95% O_2/5% CO_2, stirred with a magnetic bar, and maintained at 37°C. At the end of each experiment, digitonin (200 μg/mg protein) was added to equilibrate intracellular and extracellular K^+ and allow for estimation of total cellular K^+ content. Five μl of antifoam B emulsion, diluted 1 : 10 with buffer, was added to the chamber before each experiment to prevent excessive foaming caused by the bubbling of the tubules.

Oxygen Consumption Measurements

The O_2 tension of the extracellular medium was measured polarographically using an O_2 electrode inserted into a closed 1.8-ml glass chamber (Gilson, Middleton, Wis.) maintained at 37°C by a circulating water jacket. Additions of chemicals were made from a microliter syringe (Hamilton, Reno, Nev.) through a port in the

chamber. The electrode was connected to an O_2 meter (Yellow Springs Instruments, Yellow Springs, Ohio), and the reading of O_2 tension was plotted as a function of time on a chart recorder (Kipp and Zonen, Holland). The slope of the recording thus represented the QO_2. The response time of the electrode, measured by the addition of sodium hydrosulfite to previously oxygenated medium, was routinely less than 2 sec. Changes in QO_2 after experimental maneuvers were measured as initial, steady-state changes in the recording during the first minute of exposure. Sodium butyrate (1 mM) was added for experiments involving nystatin or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In the presence of this fatty acid, mitochondrial oxidative metabolism can be stimulated to maximal ADP-coupled and -uncoupled rates (Harris et al., 1981), and thus toxin-induced mitochondrial dysfunction may be detected with greater sensitivity. Respiration studies performed in the absence of butyrate produced comparable results.

Chemicals

All reagents were of analytical grade and obtained from standard commercial sources. A stock solution of silver nitrate ($AgNO_3$) was prepared in distilled water daily and protected from light. Stock solutions of ouabain (also protected from light), dithiothreitol, and reduced glutathione were made in the bicarbonate Ringer's solution on the day of experiments. Nystatin (10 mg/100 μl), furosemide, and digitonin (25 mg/100 μl) were prepared daily in dimethylsulfoxide, whereas CCCP was prepared in methanol. A stock solution of sodium butyrate (0.5 M, pH 7.40) was stored frozen until used. All experimental additions represented an increase in suspension volume of less than 0.2%. Neither the reagents nor the solvents had any effect on the performance of the electrode; furthermore, the solvents did not measurably affect cell function.

Data Analysis

Tubular protein was used to normalize QO_2 and K^+ fluxes and was determined on perchloric acid (6% PCA/1 mM ethylenediaminetetraacetic acid) precipitates dissolved in 0.1 N sodium hydroxide/5% deoxycholate by the Lowry method (Lowry et al., 1951); bovine serum albumin was used as the standard. Data are expressed as the mean ± SEM and were analyzed for significance by the paired or unpaired Student's *t*-test, as appropriate. A value of $P < 0.05$ was taken to represent a statistically significant difference between group means.

Results

K^+ Fluxes

Addition of $AgNO_3$ to the suspensions caused, within seconds, a rapid net K^+ efflux from the tubules (Fig. 1). The threshold concentration of $AgNO_3$ for this effect was 3×10^{-5} M. The delay in onset of the K^+ efflux appeared to be related to the $AgNO_3$ concentration; at increasing concentrations of $AgNO_3$, K^+ efflux was detected earlier. At 10^{-4} M (K_m) $AgNO_3$, the mean delay before the onset of K^+ release was 7 ± 2 sec from the time of chemical addition. Using a "windowing" function of the computer program, the rate of K^+ efflux could be resolved into two linear phases separated by a curvilinear transition phase. As seen in the inset of Fig. 1, the initial rate of efflux persisted for only 8 to 10 sec, after which (approximately 20 sec after $AgNO_3$ addition) the rate of efflux rapidly increased. The rate of this later period of efflux was also measured during the maximal linear phase. Comparison of the initial and maximal rates of K^+ efflux at various concentrations of $AgNO_3$ yielded hyperbolic curves. Eadie-Hofstee transformations of these data (Fig. 2) showed both rates to be saturable functions with identical K_m values (1.1×10^{-4} M). The V_{max} of the initial rate was 379 nmol K^+/min/mg protein, whereas that of the maximal rate was 741 nmol/min/mg prot. Thus, at concentrations of 10^{-4} M or greater $AgNO_3$, greater than 50% of the intracellular content of K^+ was extruded within less than 1 min.

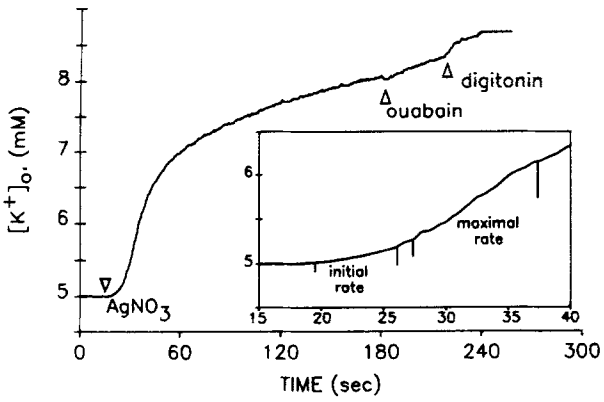

Fig. 1. Digital output of the extracellular K^+ electrode from a representative experiment showing the rapid release of K^+ from the tubules which occurred within seconds of the addition of $AgNO_3$ (10^{-4} M). Note the increase in the K^+ efflux rate caused by ouabain (10^{-4} M), indicating preserved Na^+,K^+-ATPase function. Digitonin released the remaining intracellular K^+. To highlight the brief delay in onset of the K^+ efflux, and the distinction between the initial and maximal rates of K^+ efflux, the inset displays the electrode tracing, with identical units for the ordinate and abscissa, from 15 sec (the time of $AgNO_3$ addition) to 45 sec

The cellular mechanisms of this K^+ efflux were then studied using known inhibitors of ion transport (Table 1). Neither furosemide (1 mM), an inhibitor of both $Na^+/K^+/2Cl^-$ and KCl cotransport in several epithelia (*see* O'Grady, Palfrey & Field, 1987, for review), nor amiloride (1 mM) affected the rate of Ag^+-induced K^+ release. Barium has been shown to block K^+ permeability pathways in the rabbit proximal tubule (Soltoff & Mandel, 1986) and K^+ channels in the apical and basolateral membranes of

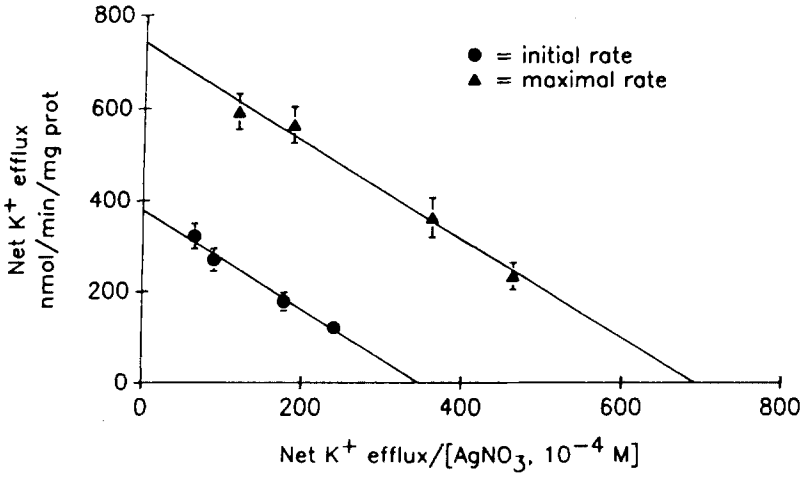

Fig. 2. Eadie-Hofstee analysis of the initial and maximal rates of net K^+ efflux. The y-intercept represents V_{max}, and the slope is equivalent to $-K_m$. Data points represent means ± SEM, $n = 6$. The y-intercepts and slopes of the linear regression lines were 379 nmol/min/mg and -1.1×10^{-4} M ($r = 0.991$) for the initial rates and 740 nmol/min/mg and -1.1×10^{-4} M ($r = 0.993$) for the maximal rates of K^+ efflux, respectively

Table 1. Lack of effect of K^+ transport inhibitors on the maximal rate of $AgNO_3$-induced net K^+ release[a]

Inhibitor	Ag^+-induced K^+ efflux (nmol/min/mg prot)
Control ($n = 6$)	362 ± 24
Furosemide (1 mM, $n = 4$)	363 ± 19
Ba^{2+} (5 mM, $n = 6$)	349 ± 27
Quinine (1 mM, $n = 4$)	377 ± 38
Tolbutamide (1 mM, $n = 4$)	352 ± 22

[a] Inhibitors of various K^+ transport pathways were given before 10^{-4} M $AgNO_3$, and the maximal rate of Ag^+-induced net K^+ flux was measured. The response to 10^{-4} M $AgNO_3$ (control, $n = 6$) is shown for comparison. Each value represents the mean ± SEM. No statistical differences were found between experimental and control groups.

the *Necturus* proximal tubule (Kawahara, Hunter & Giebisch, 1987), but did not inhibit the Ag^+-induced K^+ efflux. Tetraethylammonium (TEA) and quinine, inhibitors of K^+ channels in the proximal tubule (Gogelein & Greger, 1984) and cortical collecting tubule (Hunter et al., 1986), also were without effect. In addition, tolbutamide, an inhibitor of the ATP-sensitive K^+ channels of pancreatic B cells (Gillis et al., 1987) and cardiac myocytes (Misler, 1987) did not decrease the rate of K^+ release. To test whether an influx of Ca^{2+} mediated the K^+ efflux (i.e., Ca^{2+}-activated K^+ channels), extracellular Ca^{2+} was depleted by pretreatment of the suspension with 2 mM ethylene glycol bis-(β-aminoethyl ether) N,N,N′N′-tetraacetic acid (EGTA). Though EGTA might be expected to chelate some free Ag^+, this intervention did not prevent the Ag^+-mediated effects, emphasizing the dramatic effects of this metal on K^+ transport. Because inhibition of Na^+,K^+-ATPase activity, by allowing expression of passive K^+ transport pathways, could also cause a net K^+ efflux, ouabain was administered to the tubule suspensions after $AgNO_3$ to test for residual Na^+,K^+-ATPase activity. As seen in Fig. 1, ouabain (10^{-4} M) increased the rate of net K^+ efflux induced by prior treatment with $AgNO_3$. This result suggests preserved Na^+,K^+-ATPase activity. Thus the Ag^+-mediated K^+ efflux does not represent simply the inhibition of the Na^+/K^+ pump, but appears to involve transport via a pathway which is insensitive, or rendered insensitive, to the various known inhibitors of K^+ transport.

O_2 Consumption Measurements

To elucidate further the cellular mechanisms of the Ag^+-mediated K^+ efflux, parallel experiments of net K^+ transport and respiration were performed. In paired experiments, in which K^+ and O_2 electrode measurements were made in separate chambers on tubules taken from the same suspension ($n = 7$), $AgNO_3$ (10^{-4} M) caused a stimulation of QO_2 at 26 ± 3 sec, nearly 20 sec after the K^+ efflux began (7 ± 2 sec after $AgNO_3$ addition). Figure 3 shows the time course of changes in K^+ transport and QO_2 caused by 10^{-4} M $AgNO_3$ in such an experiment. The stimulation of QO_2 by $AgNO_3$ was dose dependent (Fig. 4), with a threshold concentration of 3×10^{-5} M $AgNO_3$ (identical to the threshold concentration for the K^+ efflux). At concentrations greater than 5×10^{-4} M, $AgNO_3$ caused a biphasic response, with an initial stimulation for approximately 30 sec, followed by an inhibition of QO_2. 10^{-4} M $AgNO_3$, the K_m for the observed K^+ efflux, was used in subsequent experiments to study the stimulation of QO_2 and its relationship to the altered K^+ transport.

To determine whether the Ag^+-mediated stimu-

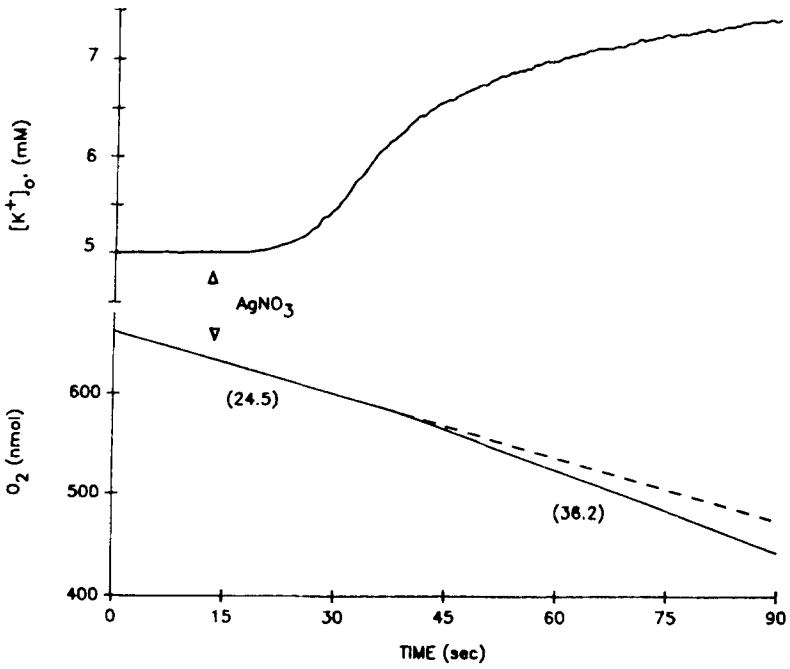

Fig. 3. Net K^+ efflux and QO_2 by suspensions of cortical tubules treated with 10^{-4} M $AgNO_3$. The top tracing depicts the output from the K^+ electrode, whereas the bottom tracing represents the output of the O_2 electrode. The Figure shows a representative, paired experiment in which both electrode measurements were obtained from tubules taken from the same suspension. Note that spontaneous QO_2 remained unchanged until most of the K^+ had been released, at which time QO_2 increased. The values of the basal and $AgNO_3$-stimulated QO_2 are in parentheses

lation of QO_2 was the primary result of increased Na^+ transport or of other, Na^+ transport-independent ATP-consuming processes, specific, well-characterized perturbations were made and the effects on QO_2 monitored. Ouabain (10^{-4} M) distinguished the Na^+ transport-dependent and -independent QO_2, whereas nystatin (>240 units/mg prot) resolved the Na^+,K^+-ATPase-mediated, ADP-dependent respiration. Under basal, control conditions, nystatin raises cytosolic Na^+ concentration, and thereby maximally stimulates Na^+,K^+-ATPase activity; the resultant change in cytosolic ATP/ADP drives ADP-mediated, mitochondrial ATP production to maximal capacity (Harris et al., 1982). Maximal rates of nystatin-stimulated QO_2 are achieved only when the Na^+,K^+-ATPase and mitochondria are fully functional. CCCP (10^{-6} M), an uncoupler of mitochondrial oxidative phosphorylation, causes maximal rates of QO_2 independent of Na^+,K^+-ATPase activity or ADP. The uncoupled QO_2 is diminished only when mitochondrial function is directly disturbed, or when the supply of reducing equivalents derived from metabolic substrates is altered. Therefore, altered Na^+ entry will affect only ouabain-sensitive QO_2, whereas direct changes in Na^+,K^+-ATPase turnover will also affect nystatin-stimulated QO_2. Direct effects on mitochondrial oxidative metabolism will affect all respiration parameters.

The effects of 10^{-4} M $AgNO_3$ on basal, ouabain-sensitive, and ouabain-insensitive QO_2 are presented in Fig. 5. Under basal conditions, the QO_2 was 24.5 ± 1.1 nmol O_2/min/mg for the control tubules and 24.1 ± 1.3 nmol O_2/min/mg for the tubules subsequently treated with $AgNO_3$. $AgNO_3$ stimulated basal QO_2 by approximately 50% (from 24.1 ± 1.3 to 36.2 ± 2.5 nmol O_2/min/mg). This stimulation was almost entirely ouabain sensitive, indicating that the Na^+,K^+-ATPase was functional and activated. Interestingly, amiloride, an inhibitor of Na^+ entry pathways, did not inhibit these changes in QO_2. In the presence of sodium butyrate (Table 2), nystatin-stimulated basal QO_2 by 52 ± 4%, whereas CCCP increased basal QO_2 by 148 ± 10%. Pretreatment with $AgNO_3$ did not significantly change the nystatin-stimulated QO_2 or CCCP-uncoupled QO_2. Taken together, these data indicate that $AgNO_3$ stimulates primarily transport-dependent QO_2 by increasing the entry of Na^+ into the cell. Given the

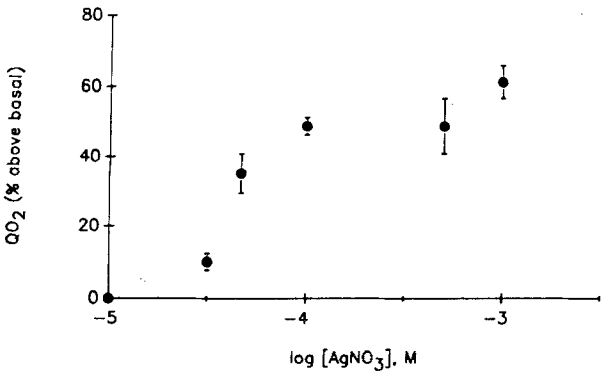

Fig. 4. Dose-response of $AgNO_3$-induced stimulation of QO_2. Data are expressed as the means ± SEM, $n = 6$

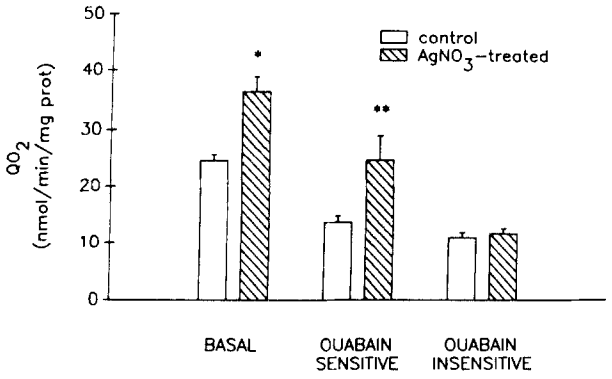

Fig. 5. Bar graph depicting the effect of 10^{-4} M $AgNO_3$ on basal, ouabain-sensitive, and ouabain-insensitive QO_2 by cortical tubule suspensions. Ouabain was added at a concentration of 10^{-4} M. Each bar represents the mean ± SEM, $n = 6$. * denotes $P < 0.01$, ** $P < 0.001$

Table 2. Effect of $AgNO_3$ on nystatin-stimulated and CCCP-uncoupled O_2 consumption in rabbit cortical tubule suspensions[a]

QO_2 (% above basal)	+ Nystatin	+ CCCP
Control ($n = 7$)	51 ± 4%	148 ± 10%
$AgNO_3$-treated ($n = 7$)	52 ± 3%	124 ± 13%

[a] Values are means ± SEM. Tubule suspensions were equilibrated with sodium butyrate (1 mM) prior to measurement of QO_2. In control tubules, nystatin or CCCP was added after a 30-sec measurement of basal QO_2. In the experimental group, $AgNO_3$ (10^{-4} M) was added after the basal measurement. After 20 sec of $AgNO_3$ treatment, nystatin or CCCP was given, and the resultant steady-state QO_2 measured, for comparison with control tubules.

normal responses to nystatin and CCCP, ADP-coupled and -uncoupled respiration appeared to respond normally to this concentration of Ag^+.

Effect of Thiol Reagents

Because Ag^+ reacts with SH groups to form hemisilver sulfides [in preference to reactions with amino, imidazole, carboxyl, and phosphoryl moieties (Gurd & Wilcox, 1956)], DTT and GSH were used to probe the involvement of membrane protein SH groups in the cellular response to Ag^+. These reagents maintain protein SH groups in their reduced form under oxidant stress (Cleland, 1964). Under basal conditions, neither DTT (1 mM) nor GSH (1 mM) affected the net K^+ transport or QO_2 of the tubule suspensions. Addition of DTT (1 mM) or GSH (1 mM) to the suspension, immediately before treatment with $AgNO_3$, prevented the changes in K^+ transport and QO_2 previously observed. Since this result could also represent an effect of DTT and

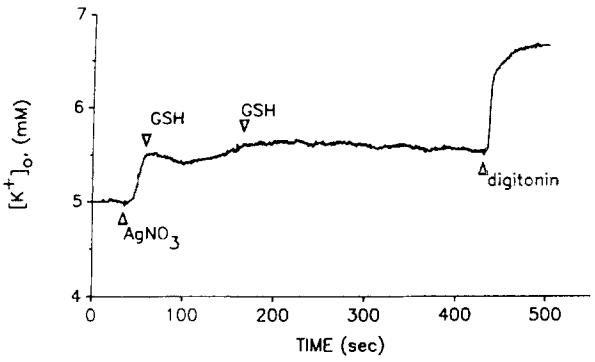

Fig. 6. Tracing of the K^+ electrode output demonstrating the effects of external GSH (1 mM) on the $AgNO_3$-induced K^+ efflux. Under steady-state conditions, $AgNO_3$ (10^{-4} M) was added to the tubule suspension. During the ensuing period of K^+ release, GSH was injected and immediately prevented further K^+ efflux. The brief period of K^+ uptake which follows likely represents Na^+,K^+-ATPase activity. Substitution of DTT (1 mM) for GSH produced similar responses

GSH to reduce Ag^+, thereby preventing binding of the ion with the cell, the effects of the thiol reagents on K^+ transport were measured during the period of maximal K^+ efflux induced by Ag^+. When DTT (1 mM) or GSH (1 mM) was given after $AgNO_3$, during the period of rapid K^+ efflux, the K^+ release abruptly stopped (Fig. 6). Furthermore, the brief period of K^+ influx which followed the addition of GSH (Fig. 6) suggests that the Na^+,K^+-ATPase remained functional.

Discussion

The mechanisms by which various nephrotoxins disrupt normal, integrated cell function are incompletely understood. Under toxic stress, the proximal tubule must preserve both its intracellular ionic composition, through maintenance of cell membrane ionic permeabilities and Na^+,K^+-ATPase function, and energy production derived from oxidative metabolism. In this study, we demonstrated that Ag^+, a nephrotoxic heavy metal, caused an early, rapid efflux of K^+ and a delayed stimulation of ouabain-sensitive respiration in suspensions of renal cortical tubules. These responses indicate specific alterations in the permeability of the cell membrane to K^+ and then to Na^+, and were observed at concentrations of $AgNO_3$ which did not affect the functional capacity of the mitochondria or Na^+,K^+-ATPase, as evidenced by the normal QO_2 responses to CCCP and nystatin. Furthermore, since cellular respiration was intact during the initial period of K^+ efflux [in contrast to a significant inhibition of QO_2 caused by digitonin (*not shown*)],

this early net K^+ extrusion represented a selective alteration in the permeability of the cell membrane to K^+, rather than cell lysis, which would disrupt all cell function. The functional integrity of the cell was further documented by the appropriate, additional release of K^+ in response to ouabain, indicating preserved mitochondrial ATP production and Na^+,K^+-ATPase turnover, even after three min of exposure to $AgNO_3$. Thus, our results demonstrate an effect of Ag^+ to increase the permeability of the cell membrane to K^+ and then to Na^+ without disrupting Na^+,K^+-ATPase activity or mitochondrial function.

Although systemic silver poisoning generally causes limited organ injury, toxic renal effects have been attributed to silver (Lucke, 1946; Rosenman, Seixas & Jacobs, 1987). In addition, intraperitoneal injections of silver have been shown to cause renal tubule degeneration in the rabbit (LaTorraca, 1962). To our knowledge, no other investigations of the initial stages of Ag^+-mediated nephrotoxicity have been reported. In other epithelia, however, significant pathologic effects of Ag^+ have been demonstrated in vitro. Ag^+ has been shown to increase the conductance of the rat ileum (Clarkson & Toole, 1964), toad bladder (Walser, 1970), toad skin (Gerencer et al., 1977, 1983), frog skin (Curran, 1972), and rabbit corneal epithelium (Klyce & Marshall, 1982). In the gastric mucosa of the bullfrog, an increase in anion conductance following treatment with Ag^+ was reported (Rangachari & Matthews, 1985). Abnormalities of K^+ transport have also been observed. Using $^{42}K^+$ washout studies of frog skin bathed in Na_2SO_4-Ringer's solution. Curran (1972) demonstrated a marked K^+ efflux within minutes following Ag^+ addition. Similarly, a marked $^{86}Rb^+$ efflux from rabbit corneal epithelium was observed after treatment with $AgNO_3$ (Klyce & Marshall, 1982). The effects of Ag^+ on the cation transport of the cornea were prevented and reversed by DTT and GSH, indicating the toxicity of Ag^+ involved its SH reactivity. Most recently, a marked, direct inhibitory effect of Ag^+ on the Ca^{2+}, Mg^{2+}-ATPase of the sarcoplasmic reticulum of skeletal muscle, resulting in a rapid release of Ca^{2+}, has been demonstrated (Gould et al., 1987).

In the present study, cytotoxic effects of Ag^+ were even more dramatic. Within seconds, $AgNO_3$ caused a massive K^+ efflux from the tubules, releasing nearly the entire intracellular content of K^+ within 3 min. For comparison, the V_{max} for the initial rate of K^+ release, 379 nmol/min/mg, is more than twice that induced by ouabain in this preparation (157 nmol/min/mg, Kone and Gullans, *unpublished observations*), and far greater than the initial rate of release (105 ± 5 nmol/min/mg) reported by Soltoff and Mandel (1986) using ouabain (10^{-4} M) in a similar tubule preparation. Ouabain, by inhibiting cellular uptake of K^+ by the Na^+,K^+-ATPase, unmasks the passive permeability pathways of K^+ transport. Soltoff and Mandel (1986) have further demonstrated that these "leak" pathways can be blocked by 5 mM Ba^{2+}. Since the Ag^+-induced net K^+ efflux is far greater than the rate of K^+ release caused by simple inhibition of the Na^+,K^+-ATPase, the K^+ permeability of the cell membrane must be increased. The fact that Ba^{2+}, along with other known inhibitors of K^+ permeability pathways in epithelia such as TEA, quinine, and tolbutamide, did not prevent this effect, suggests that Ag^+ increases K^+ permeability through other pathways. Alternatively, Ag^+ may interfere with the inhibitory properties of these compounds, allowing release of K^+ through these channels. Of note, the rate and magnitude of the K^+ loss caused by Ag^+ is greater than that reported in the corneal (Klyce & Marshall, 1982) or toad bladder epithelium (Curran, 1972). These differences in the relative magnitude and time course of K^+ efflux induced by Ag^+ may reflect the higher density of specific K^+ leak pathways (i.e., channels and cotransporters) in the proximal tubule, or a greater susceptibility of these pathways to Ag^+-mediated injury. These factors may, in part, explain the susceptibility of the proximal tubule to injury by heavy metals.

In addition to an increase in the K^+ permeability of the cell membrane, an inhibition of the Na^+, K^+-ATPase by Ag^+ could contribute to the observed K^+ release. Such an effect might represent either a direct action of Ag^+ on this enzyme or a disruption of normal mitochondrial function, with subsequent metabolic inhibition of the Na^+/K^+ pump. An inhibition of Na^+, K^+-ATPase activity would be expected to decrease the ouabain-sensitive and nystatin-stimulated QO_2. Instead, Ag^+ stimulated ouabain-sensitive QO_2, indicating enhanced Na^+/K^+ pump activity and increased entry of Na^+ into the cell. The ability of ouabain to accelerate the Ag^+-mediated K^+ efflux (Fig. 1) confirms this notion. Given the relatively unchanged ouabain-insensitive, nystatin-stimulated, and CCCP-uncoupled QO_2, Ag^+ did not appear to increase significantly other ATP-consuming processes or impair mitochondrial ATP production. The fact that the stimulation of ouabain-sensitive QO_2 began 19 sec after the initiation of K^+ release, suggests that the K^+ efflux was mediated by an ion permeation pathway which was relatively selective for K^+. In contrast, nystatin, which exhibits nearly equal selectivity for Na^+ and K^+ (Cass & Dalmark, 1973), increased K^+ efflux and QO_2 almost simultaneously, within 10 sec of addition to the tubule sus-

pension (*personal observation*). Since the influx of Na^+ following $AgNO_3$ addition occurred after the release of a significant proportion of the intracellular K^+, the Na^+ entry may have represented an adaptive response to restore the depleted cell volume. Alternatively, Ag^+ may have directly altered cell membrane Na^+ permeability. Thus the delay in Na^+ influx (relative to the K^+ efflux) may have been the result only of differences in the accessibility or susceptibility to Ag^+ of the pathways governing cationic permeability.

The cellular mechanisms by which $AgNO_3$ mediates the extrusion of K^+ and the delayed entry of Na^+ are of interest. The nearly immediate onset of the $AgNO_3$-induced K^+ release suggests an avid interaction of Ag^+ with the cell membrane. Though we did not directly measure the concentration of Ag^+ in our experiments, the estimated concentration of Ag^+ in our suspensions would be on the order of 10^{-11} M [given the solubility product constant of 1.56×10^{-10} at 25°C for Ag^+ in a chloride-containing Ringer's solution (CRC Handbook, 1970)]. The remaining Ag^+ in the suspension would likely be in the form of the neutral complex of AgCl, which could conceivably act at the cell membrane and/or subcellular loci, but would not be expected to be reversed by the thiol reagents. The time-dependent increase in net K^+ release may reflect the different responses of certain subpopulations of tubules (for example S1 versus S2 proximal tubule segments), the interaction of Ag^+ with increasing numbers of tubules, the additive effects of AgCl neutral complex, or the progressive alteration in membrane permeability. Given the identical K_m values for the initial and maximal rates of K^+ efflux (Fig. 3), the two rates are likely sequential phases of the same underlying injury.

Ag^+ is known to react strongly with SH groups to form stable hemi-silver sulfides, but additionally could react with other chemical groups. However, the fact that the onset of the Ag^+-induced K^+ efflux is so rapid, and is totally prevented and immediately reversed by DTT or GSH, suggests this effect is the result of a reversible interaction of Ag^+ with SH-bearing ligands at the cell membrane. Furthermore, as Klyce and Marshall (1982) have observed, it is improbable that DTT, a large, polar molecule, could enter the cell so rapidly to reverse the K^+ release if $AgNO_3$ were acting intracellularly. That the cell membrane K^+ permeability in our preparation appears to be governed, in part, by the redox state of membrane protein SH groups suggests the presence of cysteine residues in these proteins. Interestingly, cysteine residues have recently been reported as constituents of the membrane-spanning regions of two complementary DNA clones from the Shaker locus of *Drosophila*, which is thought to encode a K^+ channel component (Tempel et al., 1987).

Although, to our knowledge, the effects of specific SH-reactive reagents on ion transport have not been investigated in the mammalian proximal tubule, studies in other tissues have documented significant functional alterations similar to those induced by Ag^+ in this study. The impermeable and purportedly SH-specific reagent *p*-choromercuriphenyl sulfonic acid (pCMBS) has been shown to increase the cation conductance of human erythrocytes (Knauf & Rothstein, 1971) and toad urinary bladder (Spooner & Edelman, 1976), and to stimulate short-circuit current (and by inference Na^+ uptake) across frog skin (Benos, Mandel & Simon, 1980). In addition, N-ethylmaleimide (NEM), a permeant SH reagent, has been shown to stimulate K^+ influx in low K^+ sheep and goat erythrocytes (Lauf & Theg, 1980) and human erythrocytes (Wiater & Dunham, 1983). Although we have observed a net K^+ efflux from renal cortical tubules induced by NEM (*unpublished observations*), a concomitant inhibition of respiration (in contrast to the effects of $AgNO_3$) was also discovered.

In conclusion, we have demonstrated early, dramatic changes in the permeability of the cell membrane to K^+ and Na^+ after exposure of renal cortical tubule suspensions to $AgNO_3$. Mitochondrial function and ATP-consuming processes unrelated to ion transport were unaffected by this metal. An important role for SH groups of cell membrane proteins in this cytotoxic response, and in the maintenance of cell membrane K^+ permeability, has been implicated. The disruption of cell membrane integrity by Ag^+ likely represents the initial and predominant mechanism of cell injury, and may be common to other nephrotoxic heavy metals. In addition, we have applied a protocol for the continuous measurement of K^+ transport and respiration in renal cortical tubule suspensions to identify the locations and mechanisms of cell damage by exogenous toxins. This system provides a direct and sensitive in vitro method for the study of cell injury, and may prove useful in research of other nephrotoxins.

We thank Drs. Peter Lauf and Brian Cohen for fruitful discussions of this project, and Dr. Barry Brenner for his careful review of the manuscript. Dr. Steven Hebert provided help in setting up the computer analysis. This work was supported in part by a National Institutes of Health Grant DK 36031 (Dr. Gullans) and in part by a Grant-In-Aid from the American Heart Association (Dr. Gullans) with funds contributed in part by the Massachusetts Affiliate of the American Heart Association. Dr. Kone was the recipient of National Research Service Award DK 07862. Portions of this work were presented at the 41st Annual Meeting of the Society of General Physiologists.

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Received 12 October 1987; revised 4 January 1988