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Na,K-ATPase: a molecular target for *Leptospira interrogans* endotoxin

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Abstract

On the basis of our report that a glycolipoprotein fraction (GLP) extracted from *Leptospira interrogans* contains a potent inhibitor of renal Na,K-ATPase, we proposed that GLP-induced inhibition of Na,K-ATPase might be the primary cellular defect in the physiopathology of leptospirosis. The present study was designed to test this hypothesis by determining whether or not 1) GLP inhibits all the isoforms of Na,K-ATPase which are expressed in the tissues affected by leptospirosis, 2) Na,K-ATPase from leptospirosis-resistant species, such as the rat, is sensitive to GLP, 3) GLP inhibits Na,K-ATPase from intact cells, and 4) GLP inhibits ouabain-sensitive H,K-ATPase. The results indicate that in the rabbit, a leptospirosis-sensitive species, GLP inhibits with similar efficiency (apparent IC_{50}: 120-220 µg protein GLP/ml) all isoforms of Na,K-ATPase known to be expressed in target tissues for the disease. Na,K-ATPase from rat kidney displays a sensitivity to GLP similar to that of the rabbit kidney enzyme (apparent IC_{50}: 25-80 and 50-150 µg protein GLP/ml for rat and rabbit, respectively), indicating that resistance to the disease does not result from the resistance of Na,K-ATPase to GLP. GLP also reduces ouabain-sensitive rubidium uptake in rat thick ascending limbs (pmol mm^{-1} min^{-1} ± SEM; control: 23.8 ± 1.8; GLP, 88 µg protein/ml: 8.2 ± 0.9), demonstrating that it is active in intact cells. Finally, GLP had no demonstrable effect on renal H,K-ATPase activity, even on the ouabain-sensitive form, indicating that the active principle of GLP is more specific for Na,K-ATPase than ouabain itself. Although the hypothesis remains to be demonstrated *in vivo*, the present findings are compatible with the putative role of GLP-induced inhibition of Na,K-ATPase as an initial mechanism in the physiopathology of leptospirosis.

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- Leptospirosis
- Ouabain

Introduction

Although *Leptospira interrogans* has been incriminated in the origin of leptospirosis since the beginning of this century (1), the cellular disorders involved in the multiorganic dysfunctions during this infection remain unknown. We have previously reported that a crude preparation of glycolipoprotein endotoxin (GLP) extracted from *Leptospira interrogans* is a potent inhibitor of Na,K-ATPase from rabbit kidney (2). Based on this observation, it was also proposed that GLP-induced inhibition of Na,K-ATPase might be the initial cellular defect responsible for the leptospirotic physiopathology in the different organs. Indeed, many of the clinical manifestations of this disease may be viewed as a consequence of a primary defect in transmembrane transport of cations, the motor of which is Na,K-ATPase. The clinical manifestations include cardiac arrhythmia, hypovolemia, neurological disorders, myalgia, diarrhea, and disorders of kidney electrolyte handling. The present study was designed to further elaborate this physiopathological hypothesis.

First, we investigated whether or not GLP was able to inhibit Na,K-ATPase from sources other than the kidney. Although a direct interaction between the renal pump and the endotoxin was previously established (2), it remained to be determined whether GLP would equally inhibit the activity of all the isoforms of Na,K-ATPase, in particular the α_2 and/or α_3 isoforms which predominate in leptospirosis target tissues and cells other than kidney (heart, muscle, nervous system, etc.). For this purpose, the effect of GLP was compared on purified Na,K-ATPase from brain (which contains mainly the α_2 and α_3 isoforms, Ref. 3) and kidney. Because in the kidney itself the successive segments constituting the nephrons express forms of Na,K-ATPase displaying different functional and pharmacological properties (4,5), the effect of GLP was also evaluated at the level of the different segments of the nephron.

Second, because the rat is the main vector of leptospirosis (6) and is also a species resistant to ouabain (7,8), another inhibitor of Na,K-ATPase, we determined whether the resistance of rats to leptospirosis might be related to the resistance of their Na,K-ATPase to the inhibitory action of GLP. For this purpose, the sensitivity of renal Na,K-ATPase to GLP was compared in rat and rabbit nephrons.

Third, since GLP is likely to interfere with Na,K-ATPase on the cytosolic side of the membrane (since it modulates the affinity of Na,K-ATPase for sodium, Ref. 2), we addressed the question of whether or not it may alter Na,K-ATPase in intact, non-permeabilized cells, as expected if it were involved in the pathogenicity of *Leptospira*. For this purpose, the inhibitory action of GLP was investigated on the Na,K-pump activity determined by ouabain-sensitive rubidium intake into intact renal cells.

Finally, it is now well established that ouabain not only inhibits Na,K-ATPase but also some specific types of H,K-ATPase (9,10). We therefore determined whether GLP might be more specific for Na,K-ATPase than ouabain by evaluating its effect on ouabain-sensitive H,K-ATPase activity.

Material and Methods

Preparation of leptospirotic glycolipoprotein

The glycolipoprotein endotoxin was extracted from *Leptospira icterohaemorrhagiae copenhageni* (a gift from Prof. J. Andrade, Fiocruz, Rio de Janeiro, Brazil) according to the procedure of Braun and Wolff (11) modified by Vinh et al. (12). After lyophilization, GLP was dissolved in distilled water and the protein content of the preparation was determined by the Bradford method (13) using Bio-Rad reagents and bovine serum albumin (BSA) as standard. A stock solution was

prepared at a concentration of 350 µg GLP protein/ml, which corresponds to approximately 1 mg GLP/ml since the protein fraction of GLP accounts for about 35% of its whole mass (Adler B, Monash University, Clayton, Australia, personal communication). The stock solution was aliquoted and stored at -20ºC. The protein content of GLP was used as reference for concentrations.

Preparation of purified Na,K-ATPase from rabbit brain and kidney

Na,K-ATPase was purified from whole rabbit brain (except meninges) and kidney medulla according to the procedure of Jorgensen (14). After anesthesia, the organs were removed and homogenized in a Potter apparatus (5 strokes at 1,000 rpm) in homogenization buffer (10 ml per g of tissue) containing 250 mM sucrose and 30 mM histidine, pH 7.2. After 15 min of centrifugation at 6,000 *g*, the supernatant was centrifuged for 30 min at 48,000 *g* and the pellet was resuspended (5-6 mg protein/ml) in the homogenization buffer. This preparation was then diluted to 1.35-1.40 mg protein/ml in a medium containing 3 mM Na_2-ATP, 2 mM EDTA, 50 mM imidazole and 0.58 mg/ml sodium dodecyl sulfate (SDS), pH 7.5, and incubated for 30 min at 20ºC. After this treatment with SDS, the preparation was layered onto a sucrose gradient (12.5 ml at 29.4%, 7.5 ml at 15% and 5 ml at 10% in 25 mM imidazole and 1 mM EDTA, pH 7.5) and centrifuged for 90 min at 60,000 rpm, and the pellet was resuspended in the imidazole/EDTA solution (2 mg protein/ml), lyophilized and stored at -20ºC. Before use, the enzyme was resuspended in 10 mM Tris (hydroxymethyl) aminomethane (Tris)-HCl. Purification was only partial since the specific activity of the enzyme was 1.38 and 2.73 µmol mg^{-1} min^{-1} for brain and renal Na,K-ATPase, respectively. Dose-inhibition curves of the enzyme preparations with ouabain confirmed that they consisted of the α_1 isoform for the kidney enzyme and the α_2 and α_3 isoforms for the brain Na,K-ATPase (data not shown).

Tubule microdissection

Kidneys were obtained from male New Zealand rabbits (1.5-2.0 kg body weight) or male Wistar rats (180-200 g body weight) fed the usual laboratory diet with free access to tap water. Except for the slight variations dictated by the difference in kidney size, the procedure for kidney perfusion and microdissection described below was similar for the two species.

After anesthesia with pentobarbital (50 mg/kg body weight, *ip* for rats or *iv* for rabbits), the left rabbit kidneys were excised and perfused *in vitro* via the renal artery (20 ml, 1.5 ml/min) while the rat kidneys were perfused *in situ* via the aorta (4 ml, 5 ml/min) with cold microdissection solution (see composition below) containing 0.16% (w/v) collagenase (from *Clostridium histolyticum*, 0.452 U/mg, Boehringer Mannheim, Germany). After perfusion, the kidney was immediately sliced into small pyramids, which were incubated in aerated dissection solution containing 0.08% (w/v) collagenase at either 35ºC for 30 min or at 30ºC for 20 min, for rabbit and rat, respectively. Pyramids were then thoroughly rinsed in ice-cold dissection solution and stored in the cold until use.

The composition of the microdissection solution varied as a function of the experimental protocol. For measurement of ATPase activities, the solution (A) contained 137 mM NaCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 0.44 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM Tris-HCl, 1 mM $CaCl_2$ and 0.1% (w/v) BSA, pH 7.4. For the measurement of ^{86}Rb uptake the solution (B) derived from Eagle's minimal essential medium contained 120 mM NaCl, 5 mM RbCl, 1 mM $MgSO_4$, 0.15 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 5 mM glucose, 2

mM lactate, about 4 mM essential and non-essential amino acids, 0.03 mM vitamins, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), 0.3% (w/v) dextran (Mr 40,000), and 0.1% BSA.

Single pieces of nephron were dissected in microdissection solution at 0-4°C under stereomicroscopic observation and were identified by morphological and topographical criteria as previously described (15). In the rabbit kidney, segments of proximal convoluted and straight tubules (PCT and PST) were dissected in the superficial cortex next to their attachment to the glomeruli and in the outer stripe of the outer medulla next to their attachment to the thin descending limb, respectively. Medullary (MTAL) and cortical portions (CTAL) of the thick ascending limb of Henle's loop were obtained from the inner stripe of the outer medulla and from the cortex, respectively. The initial bright portion of distal convoluted tubules (DCT) and the late portion, or connecting tubule (CNT), were dissected separately. Collecting ducts were dissected either from the cortex below the last branching point (cortical collecting duct, CCD) or from the inner stripe of the outer medulla (outer medullary collecting duct, OMCD). In the rat kidney, the study was restricted to the PCT, MTAL, CTAL, CCD and OMCD which were characterized by the same criteria as in the rabbit. The length (0.4-1.0 mm) of each segment was determined by computerized image analysis.

ATPase assay

Pretreatment of the samples before measurement of ATPase activity varied as a function of the sample. To increase the cell permeability of nephron segments to reagents, samples were individually rinsed with distilled water and, after sucking away the surrounding water, 0.2 µl of either GLP (at various concentrations) or distilled water was added. Samples were frozen on dry-ice and, after thawing, they were preincubated for 5 min at 37°C. For purified enzymes, 0.2 µl of either distilled water or GLP (at various concentrations) was added to 0.2 µl of enzyme (about 20 µg protein/ml). Then, samples were preincubated for 5 min at 37°C.

ATPase activities were measured as previously described (10,16). Briefly, 1 µl of ATPase assay medium (see below) was added to each sample and the samples were incubated for 15 min at 37°C. The reaction was then stopped by the addition of 5 µl of 5% (w/v) ice-cold trichloroacetic acid and the samples were transferred individually to 2 ml of a suspension of 10% (w/v) activated charcoal. After mixing and centrifugation, the radioactivity of 500 µl of supernatant, which contained P_i formed from ATP, was determined by liquid scintillation. For technical reasons, it was not possible to maintain the same concentration of GLP during ATPase measurement and preincubation. Thus, the concentrations of endotoxin indicated in the results and Figures correspond to those present during the preincubation. Those effectively present during the ATPase assay were only 1/6 (nephron segments) or 1/7 (purified enzymes) of it.

The distinction between the different ATPases was based on their cation-specific stimulation and on their sensitivity to specific inhibitors. Thus, for each structure 10-14 samples were distributed randomly into two groups, one for measuring basal Mg-ATPase activity and the other for measuring stimulated ATPase activity. In addition, samples without nephron segments were treated in parallel in each experiment to determine the spontaneous breakdown of ATP.

For determination of Na,K-ATPase activity, stimulated ATPase was determined in an assay medium containing 50 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 1 mM ethylene glycol-bis(ß-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 100 mM Tris-HCl, and 10 mM Na_2-ATP as well as tracer amounts of [γ ^{32}P]-ATP, pH 7.4. The assay

medium for determination of basal ATPase was similar except that KCl and NaCl were omitted and 1 mM ouabain was added. Na,K-ATPase activity was calculated for each structure as the difference between the mean stimulated ATPase activity and the mean basal ATPase activity.

For determination of H,K-ATPase activity, stimulated ATPase was measured in a medium containing 2.5 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris-HCl, 5 mM Tris-ATP and tracer amounts of [γ ^{32}P]-ATP, pH 7.4. Basal ATPase was determined in a similar medium except that KCl was omitted, and H,K-ATPase activity was calculated as the difference between the mean stimulated ATPase activity and the mean basal ATPase activity.

Because the measurement of tubular H,K-ATPase is based on the nominal absence of Na^+, we determined the actual concentration of this cation during the assay. For this purpose, nephron segments were treated as done in a usual ATPase assay (permeabilization, preincubation with GLP and incubation) except that [^{32}P]-ATP was omitted. At the end of the incubation, the concentration of Na^+ was measured in ≈30-nl aliquots of the assay medium by flame microspectrophotometry, as described previously (17). The Na^+ concentration ranged from 50 to 70 µM, which, based on the activation curves of Na,K-ATPase by Na^+ previously reported for nephron segments (18), would account for Na,K-ATPase activities <1 pmol mm^{-1} h^{-1}, i.e., 100-fold lower than K-ATPase activities measured in this study (see Figure 5).

ATPase activities were reported either as a function of protein content (purified ATPase) or of tubular length (nephron segments).

Measurement of $^{86}Rb^+$ uptake

$^{86}Rb^+$ uptake was measured according to a method previously developed in our laboratory (19) and modified slightly (20). Pools of 10-12 segments of MTAL were transferred with 0.7 µl microdissection solution B into the concavity of a sunken bacteriological slide. After addition of another 0.7 µl of solution B supplemented or not with 5 mM ouabain and/or GLP, segments of the nephron were preincubated for 10 min at 37°C to allow the regeneration of transmembrane ion gradients. Incubation was then initiated by adding 0.5 µl of prewarmed solution B supplemented with ^{86}RbCl (100 nCi/sample, Amersham) and stopped after 30 sec by adding 20 µl of an ice-cold rinsing solution containing 150 mM choline chloride, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mM $BaCl_2$ and 5 mM HEPES, pH 7.4, the osmolarity being adjusted to 500 mOsmol/kg. The segments of the nephron on each slide were then rapidly rinsed in three successive baths and individually transferred with 0.2 µl of rinsing solution to a small microscopic coverslip. After photography for the determination of its length, each sample was dropped into a counting vial containing 500 µl of 1% (w/v) deoxycholic acid, and its radioactivity was measured. In each experiment, the blank which was substracted from all data was determined by measuring the mean radioactivity of 8-12 replicate samples consisting of 0.2 µl of the last rinsing solution.

For all experimental conditions, Rb^+ influx was measured on 6-8 replicate samples. Na,K-ATPase-dependent Rb^+ influx was taken as the difference between the means of Rb^+ influx measured in the absence or presence of ouabain, and is reported as pmol of Rb^+ transported per ml of tubule length per min.

Statistical analysis

Statistical comparisons between groups were performed by the Student *t*-test or, when necessary, by analysis of variance according to Dunnett (21). P values less than 0.05 were considered to be significant.

Results

Effect of GLP on purified Na,K-ATPase

Figure 1 shows that GLP completely inhibited Na,K-ATPase purified from both rabbit brain and rabbit kidney in a dose-dependent manner. Half maximum inhibition was observed in the presence of GLP in the range of 120 to 220 µg protein/ml for brain and kidney enzyme, respectively. It is worth noting that complete inhibition of Na,K-ATPase activity occurred within a very narrow range of variation of GLP concentration (50-400 µg protein/ml for the kidney enzyme), which suggests some cooperativity in the interaction between GLP and Na,K-ATPase.

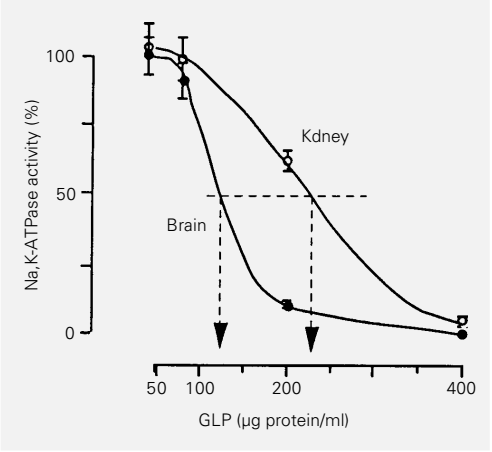

Figure 1 - Inhibition of Na,K-ATPase purified from rabbit brain and kidney by GLP. Na,K-ATPase activity was measured in enzyme purified from rabbit brain and rabbit kidney after preincubation for 5 min at 37ºC in the absence or presence of different concentrations of GLP. Data are reported as percent of the activity determined in the absence of GLP (1.38 and 2.73 µmol mg^{-1} min^{-1} for brain and kidney enzymes, respectively), and are means ± SEM of 4 experiments.

Table 1 - Inhibition of Na,K-ATPase activity by GLP along the rabbit nephron.

Na,K-ATPase activity was determined in rabbit nephron segments preincubated for 5 min at 37ºC in the absence or presence of 35, 175 or 350 µg GLP protein/ml. Data (in pmol mm^{-1} h^{-1}) are reported as means ± SEM for the number of animals given in parentheses. *P<0.001 compared to control (analysis of variance).

	Control	GLP (µg protein/ml)		
		35	175	350
PCT	2588 ± 157 (6)	2657 ± 226 (4)	1060 ± 139* (4)	214 ± 67* (4)
PST	1212 ± 113 (4)			206 ± 119* (4)
MTAL	3010 ± 126 (9)	1736 ± 176* (6)	339 ± 98* (7)	139 ± 116* (4)
CTAL	1324 ± 91 (3)			168 ± 77* (3)
DCT	3939 ± 300 (4)			675 ± 154* (4)
CNT	3014 ± 92 (4)			971 ± 77* (4)
CCD	1129 ± 69 (10)	968 ± 120 (5)	454 ± 60* (7)	190 ± 22* (4)
OMCD	640 ± 88 (4)			205 ± 91* (4)

Effect of GLP on tubular Na,K-ATPase

The data (Table 1 and Figure 2, top panel) show that GLP (350 µg protein/ml) almost abolished Na,K-ATPase activity in all segments of the rabbit nephron, including those which express the α_1 isoform of the catalytic subunit of Na,K-ATPase (proximal tubule, thick ascending limb) and the α_3-like form (collecting duct) (4). This inhibition was dose dependent (Table 1 and Figure 2, bottom panel) and half-maximal inhibition was observed with GLP in the range of 50-150 µg protein/ml in the thick ascending limbs, proximal tubules and collecting ducts.

In the nephron of the rat, a species resistant to leptospirosis, Na,K-ATPase activity was also markedly reduced by GLP (190 µg protein/ml) in the proximal tubule, thick ascending limb and collecting tubule (Table 2 and Figure 3, top panel). Rat kidney Na,K-ATPase appeared to be slightly more sensitive to GLP than the rabbit enzyme since half-maximal inhibition was observed in the range of 25-80 µg protein/ml for the thick ascending limbs, proximal tubules and collecting ducts (Table 2 and Figure 3, bottom panel).

Effect of GLP on Rb^+ uptake

In order to determine whether GLP inhibits Na,K-ATPase in intact cells, we evaluated its effect on the Na,K-pump activity, as determined by ouabain-sensitive Rb^+ uptake in intact rat nephron segments. The results in Figure 4 indicate that at a concentration of 88 µg protein/ml, GLP inhibited ouabain-sensitive Rb^+ uptake by more than 65% in the rat MTAL, i.e., to an almost similar extent as Na,K-ATPase activity (see Figure 3, bottom).

Effect of GLP on ouabain-sensitive and -insensitive tubular H,K-ATPases

The effect of GLP was evaluated on both the ouabain-sensitive and the ouabain-insensitive H,K-ATPase activities present in the rat thick ascending limb and collecting duct, respectively (10). At a concentration which induces a maximal inhibition of Na,K-ATPase activity in these two nephron segments (190 µg protein/ml, Figure 3), GLP had no effect on H,K-ATPase activities (Figure 5).

Discussion

The present data confirm the previous observation (2) that a glycolipoprotein fraction (GLP) extracted from *Leptospira interrogans* inhibits the Na,K-ATPase α_1 isoform from rabbit kidney, and extend this observation to other isoforms of Na,K-ATPase from rabbit and rat. These results also indicate that GLP has no effect on Na, K-ATPase-related proteins such as renal H,K-ATPases and finally demonstrate that GLP inhibits the transport activity of Na,K-ATPase.

Although the active principle of GLP responsible for the inhibition of Na,K-ATPase has not yet been purified or characterized, the present study was undertaken to further elucidate the putative role of Na,K-ATPase inhibition by GLP in the physiopathological mechanism of leptospirosis. Indeed, several studies on animals (22-25) as well as humans (26-28) have demonstrated the presence of leptospirae and/or leptospira antigens in diseased tissues. The presence of GLP itself in these tissues was also detected immunologically during experimental leptospirosis (23). However, because leptospirosis is a pleiotropic disease which alters the function of many tissues and organs, we determined whether or not GLP inhibits Na,K-ATPase from these distinct tissues.

Indeed, it is now well established that different tissues express different isoforms of Na,K-ATPase which display distinct properties, in particular towards inhibitors such as ouabain (reviewed in Ref. 3). The results in Figure 1 demonstrate that GLP inhibited not

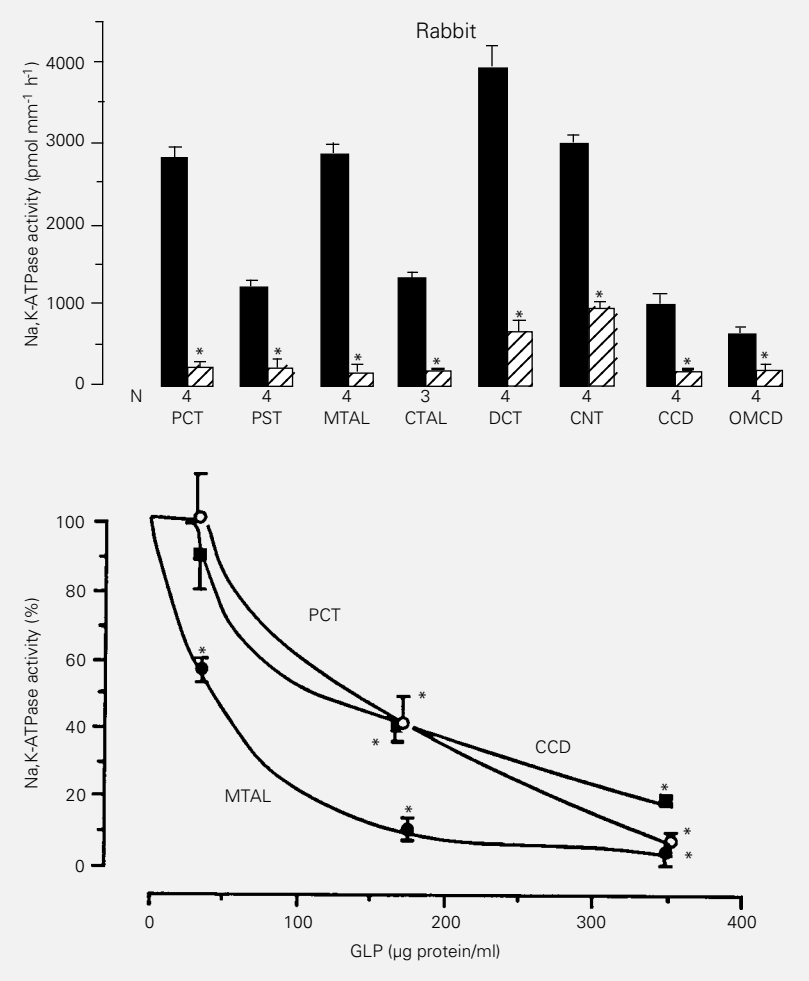

Figure 2 - Inhibition of Na,K-ATPase activity in the rabbit nephron by GLP. *Top*, Na,K-ATPase activity was measured in nephron segments dissected from rabbit kidneys and preincubated for 5 min at 37ºC in the absence (■) or presence (▨) of 350 µg GLP protein/ml. Data are reported as means ± SEM for N animals. PCT and PST, Proximal convoluted and straight tubules; MTAL and CTAL, medullary and cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCD and OMCD, cortical and outer medullary collecting duct. *P<0.025 compared to respective control (Student *t*-test). *Bottom*, Segments from rabbit PCT (○), MTAL(●), and CCD (■) were preincubated for 5 min at 37ºC in the presence of different concentrations of GLP prior to the measurement of Na,K-ATPase activity. Data are reported as percent of the activity measured in the absence of GLP in the corresponding segments of the nephron and are means ± SEM for 4-7 animals. Control activities (100%) were similar to those shown in the top of Figure 2. *P<0.001 compared to control (no GLP) (analysis of variance).

Table 2 - Inhibition of Na,K-ATPase activity by GLP along the rat nephron.

Na,K-ATPase activity was determined in rat nephron segments preincubated for 5 min at 37ºC in the absence or presence of 19, 95 or 190 µg GLP protein/ml. Data (in pmol mm^{-1} h^{-1}) are reported as means ± SEM for the number of animals given in parentheses. *P<0.001 compared to control (analysis of variance).

	Control	GLP (µg protein/ml)		
		19	95	190
PCT	2284 ± 162 (7)	2120 ± 204 (5)	1012 ± 69* (4)	780 ± 177* (4)
MTAL	3434 ± 189 (8)	2216 ± 54* (4)	495 ± 41* (4)	381 ± 121* (4)
CTAL	3167 ± 255 (4)			94 ± 94* (4)
CCD	754 ± 79 (4)	698 ± 99 (3)	329 ± 42* (3)	142 ± 71* (3)
OMCD	613 ± 172 (3)			35 ± 19* (3)

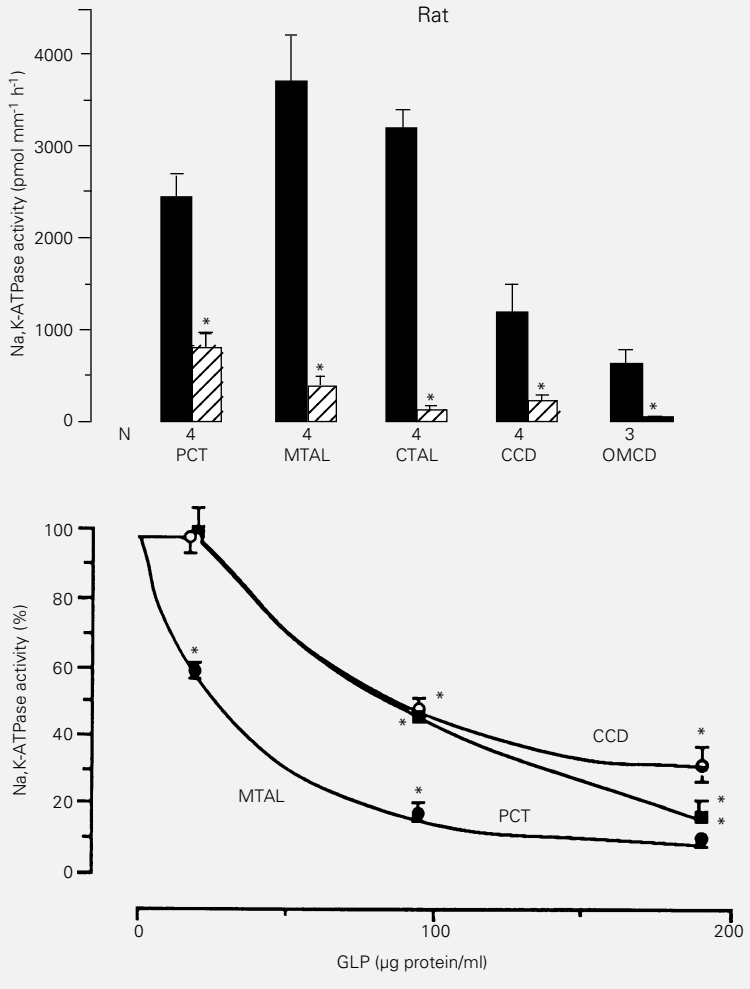

Figure 3 - Inhibition of Na,K-ATPase activity in the rat nephron by GLP. *Top*, Na,K-ATPase activity was measured in nephron segments dissected from rat kidneys and preincubated for 5 min at 37ºC in the absence (■) or presence (▨) of 190 µg GLP protein/ml. Data are reported as means ± SEM for N animals. For abbreviations, see the legend to Figure 2, top. *P<0.025 compared to control (Student *t*-test). *Bottom*, Segments from rat proximal convoluted tubule (■, PCT), medullary thick ascending limb (●, MTAL), and cortical collecting tubule (○, CCD) were preincubated for 5 min at 37ºC in the presence of different concentrations of GLP prior to the measurement of Na,K-ATPase activity. Data are reported as percent of the activity measured in the absence of GLP in the corresponding segments of nephron and are means ± SEM for 3-5 animals. Control activities (100%) were similar to those shown in the top of Figure 3. *P<0.001 compared to controls (analysis of variance).

only the α_1 isoform of the Na,K-ATPase catalytic subunit which predominates in the rabbit kidney but also the α_2 and α_3 isoforms present in the brain. In the rabbit kidney itself, the two different forms of Na,K-ATPase which were previously demonstrated (4,29) in the collecting duct (α_3-like form) and in more proximal nephron segments (α_1 form) proved to be sensitive to GLP (Figure 2). The dose-inhibition curves depicted in Figures 1 and 2 deserve three additional comments. First, they suggest a positive cooperativity in the inhibition of Na,K-ATPase by GLP. Second, they suggest that there might be some small differences in the sensitivity of the different isoforms of Na,K-ATPase to GLP. The brain enzyme (α_2 and α_3 isoforms) is 3-fold more sensitive than the kidney enzyme (α_1 isoform). However, it is not known whether such differences in sensitivity have functional consequences. Third, they indicate the range of GLP concentrations required for inhibition of Na,K-ATPase. Although the concentration of GLP achieved in infected tissues is not known, it is possible to estimate it on the basis of the following considerations. According to the procedure used in this study (see Material and Methods), approximately 10^{10} bacteria were needed to obtain 350 µg of GLP pro-

tein. Assuming that the release of GLP from leptospirae occurs *in vivo* with a similar efficiency, and based on the concentration of bacteria detected in kidneys of infected animals (10^{10} bacteria per g of tissue, Ref. 30), the tissue concentration of GLP could be as high as 350 µg protein/ml, i.e., sufficient to fully inhibit renal Na,K-ATPase. This is a rather rough estimate of the possible concentrations of GLP in infected tissues, but it should be stressed that partial inhibition of Na,K-ATPase promoted by lower concentrations of GLP would be sufficient to induce pathological alterations.

The finding (2) that GLP increases the apparent affinity of Na,K-ATPase for sodium (uncompetitive inhibition) suggested that it might interact with the sodium-binding sites of Na,K-ATPase, i.e., on the cytosolic surface of cell membranes. This raised the question of whether GLP might also be able to inhibit Na,K-ATPase in intact cells, as expected if such inhibition were to play a causal role in the physiopathology of leptospirosis. In fact, GLP inhibits to the same extent both Na,K-ATPase in permeabilized (ATPase activity, Figure 3) and in intact tubular cells (transport activity, Figure 4), which is consistent with a possible physiopathological role.

That GLP inhibits Na,K-ATPase-mediated transport in rat thick ascending limb cells may appear at variance with the report of Magaldi et al. (31) who showed that MTAL from normal and leptospirotic guinea pigs display similar transport capacity when microperfused *in vitro*. In fact, two explanations may account for this apparent discrepancy. First, it is possible that the concentration of GLP achieved *in vivo* at the level of MTAL from leptospirotic animals is too low to inhibit Na,K-ATPase. More likely, the interaction between Na,K-ATPase and GLP is too labile to persist under the *in vitro* microperfusion conditions. In particular, GLP is likely to be washed out of the bath.

Interspecies comparison revealed that Na,K-ATPase from rat and rabbit kidneys displays a similar sensitivity to inhibition by GLP (Figures 2 and 3), which may seem paradoxical since the rat is resistant to the disease (6). In fact, this finding suggests that the resistance of a given species to the disease does not result from the resistance of its cells to GLP but rather from the amount of toxin released at the level of its tissues. The latter would vary with the degree of bacterial colonization of the tissues, itself depending on species-specific immunological responses. Under these circumstances, the cellular sensitivity of a species naturally resist-

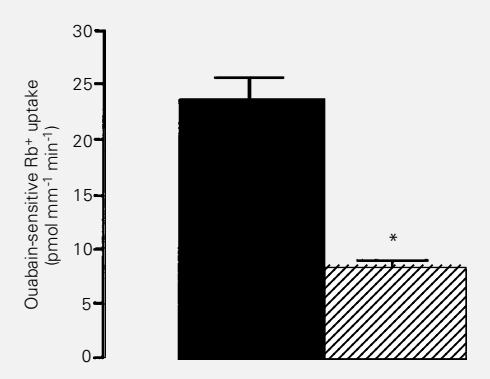

Figure 4 - Inhibition of ouabain-sensitive Rb^+ uptake by GLP. Ouabain-sensitive Rb^+ uptake was determined in rat MTAL preincubated for 10 min at 37°C in the absence (■) or presence (▨) of GLP (88 µg protein/ml). Data are reported as means ± SEM for 5 animals. *P<0.001 compared to control (Student *t*-test).

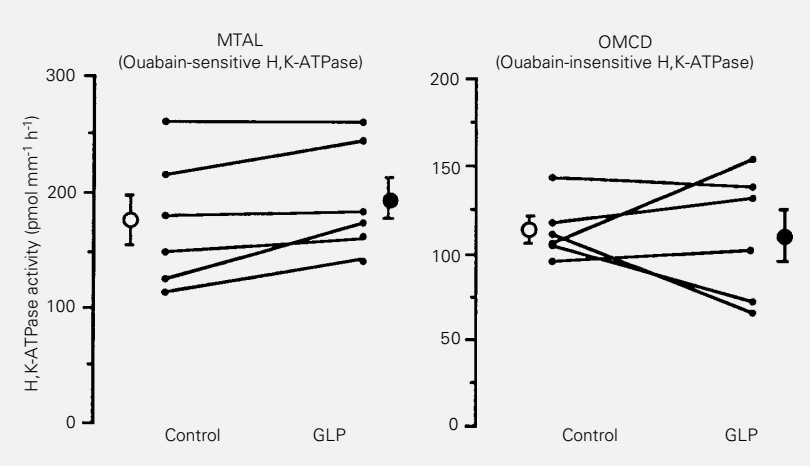

Figure 5 - Lack of effect of GLP on tubular H,K-ATPase activities. Ouabain-sensitive and -insensitive H,K-ATPase activities were determined in rat MTAL and OMCD, respectively. Nephron segments were preincubated for 5 min at 37°C in the absence (control) or presence of GLP (190 µg protein/ml) before determination of H,K-ATPase activity. Lines indicate ATPase activities determined in the same animals. Circles with error bars indicate means ± SEM for six rats in each group.

ant to leptospirosis is not inconsistent with the physiopathological potential of GLP. That immunosuppression turns resistant-species sensitive to the multiorganic alterations of leptospirosis (32) corroborates this hypothesis.

Because rabbit tubular Na,K-ATPase (α_1 form) is about 100-fold more sensitive to ouabain than its rat counterpart (5,29), the similar sensitivities of rat and rabbit renal Na,K-ATPase to GLP further demonstrate that GLP is not a ouabain-like substance. This conclusion is also supported by the fact that 1) in contrast to ouabain, GLP does not alter the apparent affinity of Na,K-ATPase for potassium (2), and 2) GLP does not alter the activity of renal ouabain-sensitive H,K-ATPase (Figure 5).

Taken together with the findings that GLP alters neither basal Mg-ATPase activity (Table 3) nor hormone-sensitive adenylate cyclase activity (2), this last result demonstrates the rather high specificity of GLP for Na,K-ATPase. In addition, it indicates that GLP, or better its active principle when it will become available, should be preferred to ouabain when attempts are made to specifically inhibit Na,K-ATPase.

In conclusion, the present results support the hypothesis that GLP released during lysis of *Leptospira interrogans* is able to inhibit Na,K-ATPase activity in epithelial as well as non-polarized cells. Cellular dysfunctions induced by this inhibition in infected tissues might account for many symptoms, in particular those associated with electrolytic disorders such as disturbances of renal electrolyte handling, cardiac arrhythmia or diarrhea. Depending on the intensity of Na,K-ATPase inhibition in colonized tissues, disorders may vary from slight metabolic dysfunction to organic failure. Further studies, in particular the demonstration that Na,K-ATPase activity is lower in tissues from diseased animals than in tissues from normal ones, will be needed to determine the role of GLP in the physiopathology of leptospirosis.

Table 3 - GLP has no effect on Mg-ATPase activity along the rabbit and the rat nephron.

Mg-ATPase activity was determined in rabbit and rat nephron segments preincubated for 5 min at 37ºC in the absence or presence of 350 µg GLP protein/ml (rabbit) or 190 µg GLP protein/ml (rat). Data (in pmol mm^{-1} h^{-1}) are reported as means ± SEM for the same animals as in Figures 2 and 3. GLP had no significant effect on Mg-ATPase activity in any nephron segment (Student *t*-test).

	Rabbit		Rat	
	Control	GLP	Control	GLP
PCT	1955 ± 250	1926 ± 298	2758 ± 193	2561 ± 136
PST	923 ± 81	840 ± 113		
MTAL	2263 ± 269	2095 ± 219	1405 ± 186	1598 ± 161
CTAL	567 ± 48	478 ± 131	1899 ± 165	1720 ± 137
DCT	2158 ± 97	2139 ± 234		
CNT	2184 ± 242	2077 ± 349		
CCD	2110 ± 172	1625 ± 149	714 ± 227	801 ± 178
OMCD	842 ± 145	871 ± 154	362 ± 92	392 ± 74

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