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Purification and Characterization of a Na^+,K^+ ATPase Inhibitor Found in an Endotoxin of *Leptospira interrogans*

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We showed previously that the glycolipoprotein fraction prepared from *Leptospira interrogans* inhibited the Na^+,K^+ ATPase enzyme purified from brain or kidney and in isolated nephron segments (M. Younes-Ibrahim, P. Burth, M. V. Castro Faria, B. Buffin-Meyer, S. Marsy, C. Barlet-Bas, L. Cheval, and A. Doucet, C. R. Acad. Sci. Paris Ser. III 318:619–625, 1995). In the present communication, we have demonstrated that unsaturated fatty acids such as oleic and palmitoleic acids, which are adsorbed to this fraction, are effective inhibitors of the enzyme.

The severe form of leptospirosis induces characteristic kidney, liver, and lung lesions (8). Several leptospiral factors have been implicated as toxins or endotoxins (1, 4, 7, 22, 27). Vinh et al. (26) had shown that the glycolipoprotein (GLP) fraction from *Leptospira interrogans* was toxic to cultured fibroblasts, an effect which would involve GLP lipids. In a previous communication (28), we investigated the possible biochemical target of this leptospiral endotoxin, showing that *L. interrogans* GLP inhibited Na^+,K^+ ATPase activity either in purified preparations or in isolated nephron segments from rabbit kidney. We now describe the purification and chemical characterization of the leptospiral Na^+,K^+ ATPase inhibitor found in the GLP fraction.

L. interrogans serovar canicola strain RU10 and avirulent *Leptospira biflexa* serovar patoc were grown in Ellinghausen-McCullough-Johnson-Harris medium at Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, and were kindly supplied by Marta Pereira and Emilson D. Silva. *Escherichia coli* (ATCC 25922) was grown in Mueller-Hinton medium (Difco).

L. interrogans GLP was prepared exactly as previously described (26).

Lipid extractions from GLP preparations or from whole bacteria were based on the method of Bligh and Dyer (5). The three phases (chloroform, water-methanol, and the white interface between chloroform and water-methanol) were collected and dried. The Na^+K^+ ATPase inhibitory capacity of these extracts was evaluated after the residues were suspended in 0.2 ml of 100 mM Tris-HCl, pH 7.6.

A lyophilized rat brain Na^+,K^+ ATPase-enriched fraction, prepared by an adaptation of the procedure of Jørgensen (10), was used throughout this study as a detector for leptospira-produced inhibitors. The Na^+,K^+ ATPase and Mg^{2+} ATPase specific activities of this preparation were 105 and 3 μmol of P_i/h/mg of protein, respectively.

Total ATPase activity was measured in microtiter plates in the presence or absence (controls) of 10 to 20 μl of putative inhibitory fractions. The incubation mixture (final volume of 120 μl) contained the following: 110 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6), Na^+,K^+ ATPase preparation, and 5 mM ATP (disodium salt, vanadate free). The mixture without ATP was preincubated for 10 min at 37°C, and incubation was started by the addition of ATP. Mg^{2+} ATPase activity was determined in the presence of 3.6 mM ouabain. After a 10-min incubation at 37°C, the reaction was stopped by the addition of 100 μl of an activated charcoal suspension (20% in 0.3 M nitric acid). The plate was centrifuged, and 100-μl aliquots of supernatant were transferred to other wells. P_i formed was spectrophotometrically determined by adding 100 μl of the Lin and Morales reagent (13). Appropriate P_i standards and blanks were run simultaneously. Na^+,K^+ ATPase activity was obtained by calculating the difference between the total activity and the Mg^{2+} ATPase activity. The amount of the Na^+,K^+ ATPase preparation used in all experiments corresponded to an activity of 0.19 to 0.21 μmol of P_i/h per assay.

Concentrated chloroform extracts obtained from leptospiral GLP or whole bacteria and fatty acid standards were spotted onto thin-layer chromatography (TLC) plates (25 by 75 mm) covered with 0.25-mm-thick layers of Silica Gel 60 H (Merck). The mobile phase was *n*-hexane–ethyl ether–acetic acid (80:30:1). Some plates were stained with iodine vapors. Unstained plates were used in the identification of Na^+,K^+ ATPase inhibitory fractions of bacterial origin.

Separation of nonesterified fatty acids (NEFA) by high-pressure liquid chromatography (HPLC) was performed in a Waters model 996 apparatus equipped with a photodiode array detector and a μBondapak C_{18} column (7.8 by 300 mm; Waters) equilibrated in tetrahydrofurane (THF)-acetonitrile-water (25:35:45) containing 0.1% acetic acid. Portions (1 to 2 mg) of NEFA fractions were dissolved in this solvent mixture (300 to 500 μl) and injected into the column. Elution was performed at a flux of 1.0 ml/min (25°C) and started with the same solvent mixture. After 10 min, a THF linear gradient was established until the proportion of THF over the initial mixture reached 60% (in 90 min). UV absorbance at 227 nm of the eluate was recorded, and 1.0-ml fractions were collected, dried, and homogenized in 200-μl portions of 100 mM Tris-HCl, pH 7.6. Aliquots of 10 μl were tested for Na^+,K^+ ATPase inhibitory activity.

Portions of about 50 to 100 μg of HPLC-separated NEFA

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TABLE 1. Inhibition of Na^+,K^+ ATPase activity by chloroform-methanol extracts

Extracted material[a]	% Inhibition of Na^+, K^+ ATPase activity[b] in phase		
	Chloroform	Water-methanol	Interface
L. interrogans (GLP fraction)	71.2 ± 6.4	8.1 ± 2.3	<5.0
L. interrogans (whole cells)	65.3 ± 7.2	9.0 ± 1.0	<5.0
L. biflexa (whole cells)	74.1 ± 7.7	11.3 ± 3.7	6.4 ± 2.1
E. coli (whole cells)	<5.0	<5.0	<5.0

[a] Samples of about 2 mg of GLP fractions suspended in 1.0 ml of water and 2 to 3 g (wet weight) of sedimented cells suspended in 10 ml of water were homogenized with chloroform-methanol.
[b] Means of at least three experiments ± SDs.

fractions, taken from the tube in which the maximal inhibition of the Na^+,K^+ ATPase activity was attained, were methylated with diazomethane (21) and analyzed by gas chromatography-mass spectrometry. A Hewlett-Packard 5890 Series II gas chromatograph equipped with a fused silica HP1 column (0.2 mm by 25 m) and coupled to a Hewlett-Packard 5972 electron impact mass spectrometer were used. Chromatographic conditions were as follows: helium flux, 38 cm/s; pressure, 19 lbs/in^2; starting column temperature, 40°C (1 min), increasing 12°C per min until 300°C.

Protein was determined by the method of Peterson (20). Quantification of NEFA was based on the method of Bragdon (6). Adequate NEFA standards were run simultaneously.

Reagents were obtained from Sigma Chemical Company. Solvents were distilled twice before use.

We initially tested the enzyme inhibitory properties of chloroform-methanol-water extracts from *L. interrogans*, *L. biflexa*, and *E. coli*. The enzyme inhibitor was found only in the chloroform phase of leptospiral extracts (Table 1). The inhibitory effect of the intact GLP (corresponding to 10 μg of GLP protein) was also compared with the effect of 15 μg of the chloroform-extracted GLP lipid. The lipid-to-protein ratio of the GLP fraction (1.5) was determined experimentally. The percent inhibition of the Na^+,K^+ ATPase (mean of three different experiments ± standard deviation [SD]) were 32.2 ± 3.6 (intact GLP) and 30.9 ± 4.1 (GLP lipid).

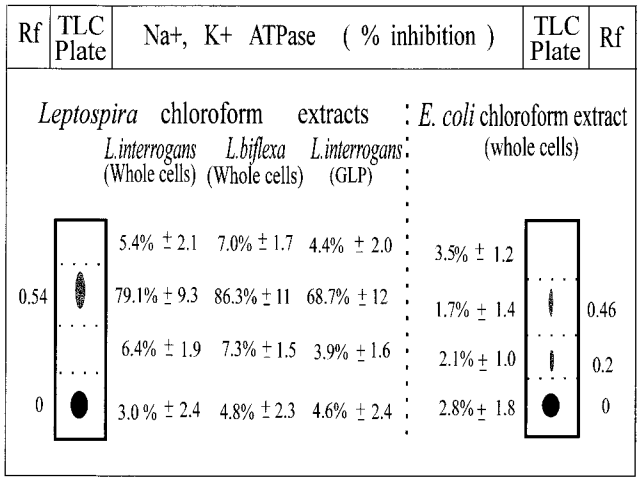

FIG. 1. Inhibition of Na^+,K^+ ATPase by bacterial lipid fractions separated by TLC. Samples of approximately 2 mg of chloroform-extracted lipids from *L. interrogans*, *L. biflexa*, *L. interrogans* GLP, and *E. coli* were spotted onto TLC plates. After the run, silica layers from four regions of unstained plates (delimited by the broken lines in the TLC plate inserts) were scraped off and extracted twice with chloroform. Extracts were evaporated, suspended in 200-μl portions of 100 mM Tris-buffer, pH 7.6, and 20-μl aliquots were tested in the Na^+,K^+ ATPase assay. A schematic representation indicating regions on plates and R_fs of main spots (as revealed by iodine vapor staining) is shown. Results are means of three different experiments ± SDs. Corresponding silica scraps from control plates inhibited enzyme activity from 2 to 8%.

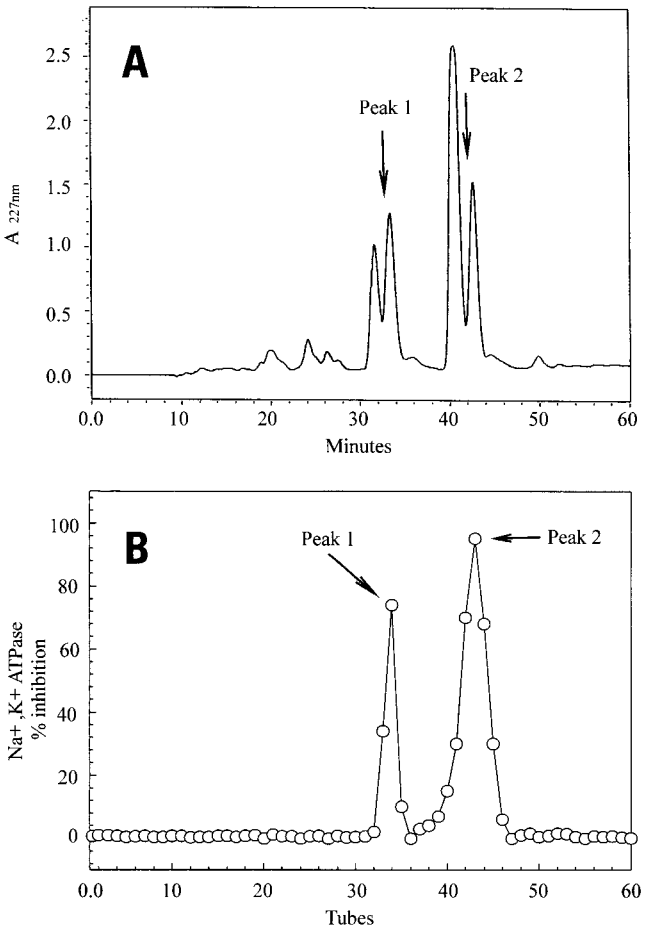

FIG. 2. Identification of HPLC-fractionated NEFA peaks inhibiting Na^+,K^+ ATPase activity. The NEFA fraction of *L. interrogans* cells, extracted from TLC plates, was separated by HPLC. The solvent flux was kept at 1 ml/min, and 1-ml fractions of the eluate were taken. The direct UV scanning of the column eluate at 227 nm is shown in panel A. Collected tubes were evaporated, and the residues were suspended in 200 μl of Tris buffer; three 10-μl aliquots were then tested in the Na^+,K^+ ATPase assay (B). Controls (100% of the enzyme activity) contained 10 μl of Tris buffer. Two peaks (labeled peak 1 and peak 2) presented inhibitory properties. This figure shows the results of a representative experiment.

Chloroform extracts from whole *Leptospira* cells or from GLP fractions were resolved into two main spots by TLC: one remained in the origin, and the other migrated with an R_f of 0.54, corresponding to the R_f of NEFA. The inhibitory capacity of the Na^+,K^+ ATPase was found in the region corresponding to leptospiral NEFA (Fig. 1).

When NEFA from *L. interrogans* were separated by HPLC (Fig. 2), two peaks with retention times of 33.4 min (peak 1) and 42.7 min (peak 2) presented inhibitory properties. Peak 1 was resolved by gas chromatography-mass spectrometry analysis into three other peaks. Major peaks (retention times, 14.941 and 15.030 min) were identified by their mass spectra as hexadecenoic acid methyl esters (molecular ion 268), and the minor peak (retention time, 15.119 min; molecular ion 270) was characterized as the hexadecanoic acid methyl ester

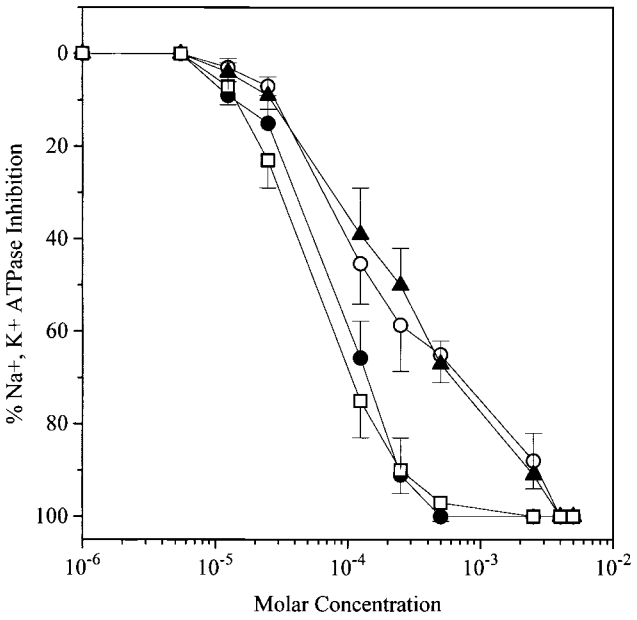

FIG. 3. Inhibition of Na^+,K^+ ATPase by fatty acids of HPLC peaks 1 and 2 compared with commercial oleic and palmitoleic acids. Fatty acids from peaks 1 and 2, dissolved in chloroform, were quantified. Dried preparations were then suspended in 100 mM Tris-HCl, pH 7.6. Commercial fatty acids were weighed and directly suspended in this same buffer. Appropriate aliquots of these suspensions were tested in the enzyme assay. Control samples (100% activity) contained the same volume of Tris buffer as the experimental samples. Results are means ± SDs of three to five different experiments. Symbols: ●, fatty acids from peak 1; ○, fatty acids from peak 2; ▲, commercial oleic acid; □, commercial palmitoleic acid.

(palmitic acid). HPLC peak 2 was defined as the *cis*-oleic acid (retention time, 16.649 min; molecular ion 296). The *cis*-monounsaturated fatty acids Δ^9-octadecenoic (oleic), Δ^9-hexadecenoic (palmitoleic), and Δ^{11}-hexadecenoic (palmitovaccenic) acids are the predominant unsaturated fatty acids in leptospira (24). Thus, the HPLC peak 1 contains mainly palmitoleic and palmitovaccenic acids with a minor contamination of palmitic acid (about 4%).

The inhibitory response of the Na^+,K^+ ATPase preparation to these leptospiral fatty acids (HPLC peaks 1 and 2) was then compared with the inhibition curves of *cis*-oleic and *cis*-palmitoleic standards. The results shown in Fig. 3 suggest that the brain enzyme preparation is more efficiently inhibited by monounsaturated C_{16} fatty acids (peak 1) than by oleic acid (peak 2).

When an aqueous suspension of *L. interrogans* was mechanically disrupted (by shaking with glass beads during 6 h at 4°C) and centrifuged (30,000 × *g*, 30 min), about 46% of the inhibitory capacity was detected in the supernatant and the rest remained in the sediment. Repeated extractions of the sediment with water (four times, under the same conditions described above) solubilized most of the inhibitory factor. The inhibitor was neither dialyzed nor filtered through a 10-kDa membrane (Centriprep 10; Amicon Corp.) and was precipitated as GLP from this extract by the same procedure used for GLP preparation from extracts of lysozyme-treated cells (26). These findings point out that unsaturated NEFA (NEUFA) are mainly bound to the GLP fraction in these bacteria.

By using different approaches, other researchers had already pointed out the general toxicity of some leptospiral lipids such as oleic acid (9, 26). Otherwise, taking impairment of active transport Na^+ and K^+ as a promising biochemical target for the leptospiral endotoxin, we have already shown that the leptospiral GLP fraction was a potent inhibitor of the Na^+,K^+ ATPase activity (28). In the present communication, we conclude that NEUFA are the basic GLP components involved in this inhibitory effect.

Commercial oleic acid was considered less toxic than the one obtained from *Leptospira* (26). However, our data indicate that Δ^9-octadecenoic acid from *Leptospira* and the commercial *cis*-oleic acid exert similar inhibitory effects on the Na^+,K^+ ATPase preparation, but hexadecenoic acids seem to be more potent inhibitors.

The inhibition of Na^+,K^+ ATPase by oleic acid and other NEUFA has been described, but the mechanism of inhibition remains unclear (11, 12, 18). Some other NEUFA effects have also been described, such as activation of protein kinase C (14), activation of skeletal muscle and erythrocyte Ca^{2+} ATPase (15, 17), and inhibition of gastric H^+,K^+ ATPase (25).

We have shown that GLP did not alter membrane-associated Mg^{2+} ATPase and adenylate cyclase activities (28). The fact that the Na^+,K^+ ATPase inhibitory property of GLP is associated with its NEUFA content does not necessarily imply that GLP exerts exactly the same inhibitory effects as unbound NEUFA in this or other enzyme systems. In fact, we are now comparing properties of GLP and unbound NEUFA in several enzyme systems.

The proposal that the release of GLP-associated NEUFA by lysed bacteria and the consequent inhibition of the Na^+ and K^+ pump in these target tissues may play crucial roles in the pathogenesis of leptospirosis is, therefore, worthy of further investigation. The local liberation of free NEUFA from GLP may also occur due to the metabolic activity in colonized tissues. Interestingly, the adult respiratory distress syndrome, a pathology that frequently leads to death in leptospiral infection, can be induced experimentally in animals by intravenously injected oleic acid (16). Moreover, this kind of lung injury seen in other pathologies such as trauma, sepsis, pancreatitis, and fat embolism was associated with aberrations of fatty acid metabolism, leading to increased oleic acid levels in the bloodstream (3). Physiopathological studies aimed at strengthening the correlation among leptospiral NEUFA (free or GLP adsorbed), active transport of Na^+ and K^+, and other biochemical, physiological, or histopathological changes associated to leptospirosis are now under way.

We have shown that the saprophytic *L. biflexa* is indistinguishable from the pathogenic *L. interrogans* serovar canicola with respect to NEUFA accumulation. Virulence factors in spirochetes are not well established. Other features besides toxin production may be involved in pathogenicity (2, 19, 23).

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