Neutral Lipid from Proteinuric Rat Urine Is a Novel Inhibitor of the Red Blood Cell Calcium Pump

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Abstract. Proteinuria may be associated with hypertension and progression of renal insufficiency, which in turn may accompany abnormalities in cell calcium homeostasis. Therefore, urine from rats made proteinuric by puromycin aminoglycoside administration was analyzed, in a search for factors affecting cellular calcium transport. Proteinuric urine was fractionated by thin-layer chromatography and HPLC, and the effects of the fractions on the plasma membrane calcium pump in human red blood cells were assessed. Proteinuric urine contained a powerful specific inhibitor of the calcium pump that had little or no effect on the Na+/K+- or Mg2+-ATPases. The inhibitor was characterized as a neutral lipid, migrating as a single band, that inhibited 45Ca2+ efflux. To confirm the presence of an inhibitor in other proteinuric states, the urine from two patients with proteinuria was examined and subjected to chromatography as in the rat studies. These thin-layer chromatographic fractions contained a very strong inhibitor of the red blood cell calcium pump, suggesting that this substance may have relevance for the pathogenesis of proteinuric renal disease in human patients. Rat proximal tubule cells in tissue culture, when challenged with lipid-replete albumin, secreted an inhibitor of the calcium pump that migrated in the same chromatographic band as the urine factor. Therefore, the processing of fatty acids borne by albumin into endocytosing proximal tubular epithelium results in the synthesis and release of a previously unknown lipid modulator of the calcium pump, an effect that may predispose kidney tissue toward elevations in cytosolic calcium levels in target cells.

Proteinuria in the nephrotic range is correlated with interstitial inflammation and fibrosis (1,2), with hypertension (3), and with progression to end-stage renal disease (4). Factors released by tubular epithelial cells in response to excessive or novel proteins in the tubular lumen may contribute to some of these complications (5). For example, proximal tubule uptake of albumin results in the production and urinary excretion of a novel factor that is chemotactic for macrophages and thus may underlie some of the tubulointerstitial inflammation associated with the proteinuric state (6,7). This factor is also produced by tubular epithelial cells in tissue culture, as a result of endocytosis of albumin-borne free fatty acids and their subsequent metabolism into a neutral lipid of unknown structure (6). Closely related lipid factors found in the cyst fluid of human patients with autosomal dominant polycystic kidney disease, the urine of rats with albuminuria, and the medium of rat proximal tubule cells (PTC) exposed to albumin-carrying fatty acids have been found to stimulate transepithelial fluid secretion and cellular proliferation of renal epithelial cells in vitro (8). Other conditions associated with proteinuria, such as hypertension (9,10) and chronic renal insufficiency (11), in turn may be associated with altered cell calcium homeostasis. Therefore, we have postulated that proteinuria may also result in the secretion into the urine of a new factor, an inhibitor of cell membrane calcium transport. In support of this hypothesis, we previously showed that a circulating factor in hypertensive patients increases cytosolic calcium levels in platelets (surrogates for vascular smooth muscle cells) and thus may result in vasoconstriction and high BP (12). Similarly, we have shown in patients with chronic renal insufficiency that HPLC fractions of plasma contain a factor that inhibits the red blood cell (RBC) membrane calcium pump (13,14). This may lead to higher cellular free calcium levels and the activation of a variety of possible target cells. This study describes our finding of a novel endogenous lipid inhibitor of the RBC membrane calcium pump in the urine in an animal model of proteinuria, namely the aminonucleoside model of nephrosis (15), and the confirmation that a similar factor was present in the urine from two patients with proteinuria. This substance, similar to the “macrophage chemottractant factor” (6), is excreted in proteinuric urine and is generated by tubular epithelial cells in culture. In addition to possible local effects on the kidney, it may gain access to the circulation and exert systemic effects on cell calcium homeostasis.

Materials and Methods

Experimental Design

We searched for a possible inhibitor of cell membrane calcium transport in three different types of biologic samples: (1) urine from proteinuric rats; (2) conditioned medium from renal tubular epithelial
cells in culture, challenged with lipid-replete albumin; and (3) urine from two patients with proteinuria. Briefly, rats were made proteinuric by the administration of puromycin aminonucleoside (PAN), as described previously (16). Urine obtained at the time of maximal proteinuria was extracted with organic solvent, initially fractionated by thin-layer chromatography (TLC), and further analyzed by HPLC. The resulting fractions were incubated either with isolated RBC membranes or with intact RBC to evaluate their effects on the RBC membrane calcium pump, using two different but complementary assays, i.e., assay of the Ca\(^{2+}\)-ATPase activity (the enzymatic counterpart of cation transport by the calcium pump) in isolated RBC membranes, which was used to screen for a possible inhibitor, and measurement of ATP-dependent \(^{45}\text{Ca}^{2+}\) efflux (a more direct measure of calcium transport) in intact RBC, which was used for confirmation of an inhibitory effect.

To determine the origin of the inhibitory lipid fractions, renal proximal tubule segments were isolated from 6-wk-old normal male Lewis rats, placed in short-term tissue culture (6 h), and challenged with lipid-replete albumin, as described previously (8). The conditioned media were extracted with solvent and fractionated by TLC and HPLC, similarly to the urine samples. The resulting fractions were then tested in the calcium pump assay. Finally, we looked for a possible systemic effect of the urinary inhibitor by comparing the cell membrane Ca\(^{2+}\)-ATPase activity in circulating RBC of proteinuric versus normal rats.

**Bioassays of Calcium Pump Activity**

**\(\text{Ca}^{2+}/\text{Mg}^{2+}\)-ATPase Activity in Isolated RBC Membranes.** This method was used as described previously in our laboratory (17). Briefly, multiple membrane-bound ATPases (\(\text{Mg}^{2+}\)-ATPase, \(\text{Na}^{+}/\text{K}^{+}\)-ATPase, basal \(\text{Ca}^{2+}\)-ATPase, and calmodulin [CaM]-activated \(\text{Ca}^{2+}\)-ATPase) were measured simultaneously in 96-well microtiter plates.

**Membrane Preparation.** RBC membranes were prepared from outdated human cells, as described (17). RBC were washed repeatedly in ice-cold 154 mM NaCl (saline solution) and then hemolyzed by the addition of imidazole buffer (20 mM, pH 7.4) containing ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,\(\text{N},\text{N},\text{N}'\)-tetraacetic acid (EGTA) (0.5 mM). Membranes were then washed three times in imidazole buffer without EGTA and separated by centrifugation at 33,000 \(\times\) g for 10 min. Membranes were resuspended in histidine/imidazole buffer (40 mM/40 mM, pH 7.1) and used for the ATPase assay. Membrane protein contents were determined by the bicinchoninic acid method (18).

**ATPase Assay.** The ATPase incubation media contained combinations of several of the following components, in a final volume of 100 \(\mu\)l. Standard assay buffer contained 18 mM histidine, 18 mM imidazole, 3 mM MgCl\(_2\), 80 mM NaCl, 15 mM KCl, and 0.1 mM EGTA (with or without 0.2 mM CaCl\(_2\), pH 7.1). The membrane suspension (7.5 \(\mu\)g of protein) contained 0.1 mM ouabain and 30 mM CaM. Reagents, membranes, and test TLC or HPLC fractions were added to the wells, and the reaction was started by the rapid addition of 3.0 mM ATP (pH 7.1) and was continued for 60 min at 37°C. The enzymatic activity was stopped by the addition of 25 \(\mu\)l of sodium dodecyl sulfate solution (5%, vol/vol). Finally, release of inorganic phosphate was measured by a modification of the method of Fiske and Subbarow (19), with ascorbate as the reducing reagent. This rapid nonisotopic method is very reproducible and has a coefficient of variation of <5% for within-run and between-run observations (17). All measurements were performed in triplicate. In some assays, the test substance was added to the wells and the solvent was evaporated with a stream of \(\text{N}_2\). The test substance was allowed to dissolve into the membrane suspension for 30 min at 37°C before the initiation of the assay.

**\(^{45}\text{Ca}^{2+}\) Efflux in Intact RBC.** Preparations of RBC for the \(\text{Ca}^{2+}\) Transport Assay. This method was used as described previously (14). Briefly, venous blood from healthy control subjects was collected in heparinized tubes and centrifuged at 1750 \(\times\) g for 10 min at 4°C, to separate the plasma and the buffy coat. The RBC pellet was immediately washed twice at 4°C with isotonic NaCl and was resuspended to a hematocrit of 10% in a Na\(^+/\text{K}^{+}\)-loading medium containing 75 mM NaCl, 75 mM KCl, 10 mM 3-(\(\text{N},\text{N},\text{N},\text{N}'\)-morpholino)propanesulfonic acid (MOPS)-Tris buffer (pH 7.4 at 37°C), 10 mM glucose, and 0.1 mM MgCl\(_2\). The washed RBC were loaded with 1 mM CaCl\(_2\) and \(^{45}\text{Ca}^{2+}\), using the calcium ionophore A23187 (final concentration, 4 \(\mu\)M) added to the loading medium. The specific activity was 5.27 \(\times\) 10\(^{11}\) cpm/mol. RBC were incubated for 20 min at 37°C, with continuous mixing. The ionophore was then removed by centrifugation for 5 min at 4°C and resuspension in loading medium with added bovine serum albumin (BSA) (final concentration, 1 g/L) and CaCl\(_2\) (1 mM). Cells were incubated twice at 4°C for 10 min and washed, and CaCl\(_2\) was omitted in the last incubation. To restore their normal ATP content, the RBC were washed and incubated twice for 10 min at room temperature in loading medium containing 5 mM inosine, 2 mM adenine, 5 mM Na\(_2\)HPO\(_4\), 10 mM MOPS-Tris buffer (pH 7.4 at 37°C), 10 mM glucose, and 0.1 mM MgCl\(_2\). Finally, cells were washed three times in cold loading medium containing 1 mM MgCl\(_2\). These calcium-loaded cells, with a full complement of ATP, were then kept on ice for measurements of calcium efflux.

**Measurement of \(^{45}\text{Ca}^{2+}\) Efflux.** The initial rate of \(^{45}\text{Ca}^{2+}\) efflux was measured for 10 min at 37°C under control conditions and during exposure to different TLC or HPLC fractions of proteinuric urine or conditioned medium from renal tubular epithelial cells in culture. For this step, Ca\(^{2+}\)-loaded cells (approximately 2 ml) were resuspended in 5 ml of Na\(^+/\text{K}^{+}\)-efflux medium containing 20 mM NaCl, 130 mM KCl, 10 mM MOPS-Tris buffer (pH 7.4 at 37°C), 1 mM MgCl\(_2\), and 10 mM glucose. This medium was previously shown to prevent a “Gardos effect” and to maintain a stable cell volume and hemoglobin concentration during the efflux step (13). One hundred twenty-five microliters of the cell suspension were added to 0.5 ml of the efflux medium for duplicate measurements of Ca\(^{2+}\) efflux. After incubation, the tubes were rapidly chilled at 4°C for 2 min and centrifuged at 1750 \(\times\) g for 5 min at 4°C, and the supernatants were transferred into tubes for scintillation counting. A portion of the cell suspension was used for measurements of hematocrit and intracellular Ca\(^{2+}\) concentrations. Initial intracellular calcium concentrations were 2.5 to 3.0 mM, as measured in RBC hemolyzed with 20% perchloroacetic acid. All measurements were performed in duplicate. Control experiments (not shown) demonstrated that Ca\(^{2+}\) efflux was linear for 20 min at 37°C, decreased to near zero at 4°C, and was suppressed >90% by 1 mM vanadate or lanthanum (known inhibitors of the RBC membrane calcium pump). The coefficient of variation was <5% within runs and between runs.

**Measurement of Native \(\text{Ca}^{2+}\)-ATPase Activity in RBC Membranes.** Possible systemic effects of the urinary inhibitor were evaluated by measuring the cell membrane ATPase activity in circulating RBC from both proteinuric and normal rats. Heparinized blood samples (approximately 2 to 3 ml) were obtained from each rat in both groups on day 19, after urine collections were completed, at the time of maximal proteinuric effect. Blood was rapidly centrifuged, and the rat
RBC membranes were prepared as described above for measurement of multiple ATPase activities in normal human RBC.

Animals

Male Lewis rats (200 to 350 g, 8 to 16 wk of age; Charles River, Wilmington, MA) were used in all experiments involving proteinuria. They were fed normal rat chow ad libitum. Urine was collected by placing rats in metabolic cages overnight, with no access to food and unlimited access to water. Urine was collected overnight, and the rats were then returned to their normal housing. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of Proteinuria

Proteinuria was induced with the single administration of PAN (10 mg/100 g body wt, ip) on day 1 of these studies. Control rats received injections of normal saline solution (n = 5 for each group). Urine was collected from the rats during two time periods. The first was days 9 to 13 after the administration of PAN, which was previously shown to be the peak onset of proteinuria in this model (16). The second period was days 15 to 19, which was shown to be the plateau phase of protein excretion before resolution of proteinuria on day 21. As noted below, during period 1 the urine samples were pooled for protein measurements and for extraction. During period 2, for statistical confirmation of the findings in a series of experiments, urine protein levels were measured and the urine samples were extracted on an individual basis.

Extraction and Chromatographic Purification of Urinary Lipids

Extraction and separation of urinary lipids were conducted according to a modification of our previously described methods (8). Samples of urine were sequentially extracted with equal volumes of HPLC-grade ethyl acetate, and the organic phase was pooled. The extracts were evaporated under nitrogen gas, redissolved in ethyl acetate, and centrifuged at 3000 rpm for 30 min at 4°C, to remove insoluble precipitates. The supernatant was again dried under nitrogen and was resuspended in 0.1 ml of ethyl acetate. This solution was applied to the bottom of a TLC silica gel plate (LKB-4865-820, with preadsorption phase, 250-μm layer, 20 × 20 cm; Whatman International Ltd., Maidstone, Kent, United Kingdom) and developed in conjunction with lipid standards, i.e., sphingosine (SPH), 1-monooleoylglycerol (MOG), 1,2-dioleoylglycerol (DOG), 1,2,3-trioleoylglycerol (TOG), and cholesterol oleate. The fractions were separated by two-step TLC. The first developing solvent consisted of chloroform/methanol/glacial acetic acid (60:40:1, vol/vol/vol). The solvent was developed to an Rf of 0.33, after which the plate was removed, briefly air-dried, and placed in the second developing solvent system, consisting of petroleum ether/diethyl ether/glacial acetic acid (80:20:1, vol/vol/vol). This was allowed to advance to the top of the plate, after which the plate was dried and the lane containing the standards was observed using iodine vapor. The plate was divided into five zones, according to the migration of the standards (see below). The silica layer from each of the five zones was scraped into a glass vial containing 1 ml of isopropanol. Each sample was vortex-mixed, pelleted in a microfuge, and washed two additional times with isopropanol. The combined extracts of each fraction were evaporated to dryness under nitrogen.

The eluted zones on the TLC plate were labeled fractions 1 to 5 and were defined according to the migration of standards, as follows: fraction 1 (Rf of 0 to 0.2), from the origin to and including SPH; fraction 2 (Rf of 0.2 to 0.4), from above SPH to and including MOG; fraction 3 (Rf of 0.4 to 0.6), from above MOG to and including DOG; fraction 4 (Rf of 0.6 to 0.8), from above DOG to and including TOG; fraction 5 (Rf of 0.8 to 1.0), from above TOG to the top of the plate, including cholesterol oleate. As a control, a blank TLC plate was subjected to the same procedure without the addition of urinary extracts, with subsequent purification of the same fractions.

In some experiments, further purification was performed via reverse-phase HPLC, with a Waters 600E pump and controller, a Waters 996 photodiode-array detector (Waters Associates, Milford, MA), and a Supelcosil LC-CDP column (5 μm, 4.6 × 25 cm). The flow rate was 1 ml/min. The mobile phase was as follows: 0 to 10 ml (isocratic), 25% acetonitrile/75% 50 mM ammonium acetate; 10 to 15 ml (linear), 70% acetonitrile/30% 50 mM ammonium acetate; 15 to 25 ml (linear), 100% acetonitrile; 30 ml (isocratic), 100% acetonitrile; 60 ml (isocratic), 25% acetonitrile/75% 50 mM ammonium acetate. Fractions (0.5 ml) were collected at 0.5-min intervals. Eluted peaks were tested for biologic activity. Concentrations of lipid extracts were defined as dilutions of 1×, with 1× being the lipid equivalent purified from 1 ml of urine or conditioned medium.

Culture of Proximal Tubules

To determine a potential source of the biologically active lipid isolated from urine, proximal tubule segments were isolated by enzymatic digestion of cortical fragments from the kidneys of normal male Lewis rats, separated by density sedimentation, and placed in short-term tissue culture, as recently described (8). Only tubular suspensions exceeding 90% purity, as determined by morphologic analysis and histochemical staining, were used. As reported previously (6,8), tubules were incubated for 6 h in medium containing albumin (2.4 mg/ml; Sigma Chemical Co.) onto which oleate had been absorbed. Oleate was previously shown to be the albumin-borne fatty acid precursor of complex neutral lipids released by proximal tubules after the endocytosis of albumin. The biologic activities of these lipids include the induction of monocyte chemotaxis and the modulation of chloride secretion in renal epithelial cells (6,8). In preliminary experiments, fatty acid-free albumin bearing oleate was found to be equivalent as a source of ATPase-modulating activity, compared with normal, lipid-replete albumin. Control experiments used lipid-depleted albumin (6,8). The conditioned medium was then fractionated by TLC and HPLC, as described above for urine, and tested in the calcium pump bioassay.

Confirmation in Patients with Proteinuria

To evaluate the relevance of our findings in rats to human disease, urine was obtained from two patients with significant proteinuria. Patient 1 had nephrotic syndrome and proteinuria of >20 g/24 h, resulting from membranoproliferative glomerulonephritis associated with cryoglobulinemia and chronic hepatitis C. The serum creatinine level for this patient was 2.2 mg/dl at the time of sampling. Patient 2 had diabetes with normal renal function, creatinine level of 1.2 mg/dl, and proteinuria of >1.2 g/24 h. These samples were compared with urine from a control subject with normal renal function and no proteinuria. Urine was extracted and fractionated by TLC as described above for the rat model, and the fractions were then tested with the bioassay for Ca2+/Mg2+-ATPase-inhibitory activity.

Results

Effects of Urinary TLC and HPLC Fractions on Calcium Pump Activity

The urine of animals made proteinuric by injections of PAN was collected from day 9 to day 14, pooled, extracted, and...
separated by TLC as described above. The five fractions were incubated with RBC membranes for 60 min, to test for inhibitory effects in the Ca\(^{2+}\)/Mg\(^{2+}\)-ATPase bioassay. At least two experiments were performed in each case at every step in the chromatographic separation, and different eluents were tested to maximize the inhibitor yield and specificity. A typical example is shown in Figure 1. Using the RBC membrane bioassay, we found that TLC fraction 2, containing monoglycerides, exhibited powerful inhibition of the ATPase. In this early example, the inhibition affected the pump primarily in its CaM-dependent configuration (75% decrease). However, the basal calcium pump activity was subsequently reduced in most experiments (see the group data in Figure 4). Other fractions showed only a weaker, less specific inhibition of the several ATPases tested.

The inhibitory activity found in TLC fraction 2 was further purified by elution from the silica from the TLC plate with ethyl acetate, dried under nitrogen, and resuspended in acetonitrile. Fraction 2 was then subjected to reverse-phase HPLC, and the new subfractions were evaluated for inhibitory effects in the ATPase assay. A single peak eluting at 18 min, with monoglyceride standards, contained all of the inhibitory activity. It was defined as peak 1 to distinguish it from a peak containing a recently described monoglyceride chemoattractant for macrophages (peak 2), which eluted in the next 0.5-ml fraction (6,8). Peaks were defined by bioactivity contained in that fraction; there was insufficient mass to manifest ultraviolet absorption for either the ATPase-modulating activity or the chemotactic activity.

To confirm that active fractions inhibited calcium transport and to test whether the inhibitor could interact with the calcium pump from the external side, we incubated the TLC fractions and HPLC peak 1 with \(^{45}\)Ca\(^{2+}\)-loaded intact RBC. As shown in Figure 2, HPLC peak 1 markedly inhibited calcium efflux. The inhibition was stronger than that observed with the less well-purified TLC fraction 2. Other fractions were inactive or displayed significantly less activity. Furthermore, HPLC peak 1 produced a clear dose-dependent response (data not shown).

![Figure 1](image1.png)

**Figure 1.** Inhibitory effects of thin-layer chromatographic (TLC) fractions. TLC fractions of proteinuric rat urine were incubated for 60 min with isolated red blood cell (RBC) membranes. Compared with the control buffer, TLC fraction 2 displayed a strong inhibitory effect on the Ca\(^{2+}\)-ATPase. In this experiment, the pump was inhibited primarily in the calmodulin (CaM)-activated configuration (top) and not in its basal state (bottom). Other fractions had much weaker activity or were not inhibitory (a downward direction means no effect or minor stimulation). These are typical early findings from individual experiments. Group findings, in which fraction 2 inhibited the ATPase in both configurations, are shown in Figure 4.

![Figure 2](image2.png)

**Figure 2.** Direct measurement of \(^{45}\)Ca\(^{2+}\) efflux in intact human RBC, for confirmation that TLC fraction 2 inhibited the RBC cell membrane calcium pump after a 30-min preincubation. Further separation of the most active TLC fraction (fraction 2) by HPLC revealed that the maximal inhibitory activity was concentrated in HPLC peak 1. Note that a downward direction indicates no effect or minor stimulation.
Finally, we excluded possible nonspecific effects of BSA (which was initially used to resuspend the lipid fractions) on any of the transport ATPases, as shown in Figure 3. Neither BSA nor BSA plus a TLC blank had any effect on ATPase activities, in contrast to BSA-containing lipid fractions. The latter strongly inhibited the CaM-activated Ca\(^{2+}\)-ATPase, inhibited the Ca\(^{2+}\)-ATPase less in its basal form, and only weakly affected the Na\(^{+}/K\(^{+}\)-ATPase.

To extend these early findings in individual animals, PAN-induced nephrosis was induced in a group of five rats, which were matched by five control animals. After induction with PAN, proteinuria was rapidly detected, reaching very high levels by day 9. The average proteinuria was 268.3 ± 97 mg/d (mean ± SD) by day 13 for the PAN-treated group and 31.4 ± 18.3 mg/d for the control animals \((P < 0.001)\). Proteinuria continued to increase and was maximal on days 15 to 19 (period 2); on day 19, it was equal to 548 ± 187.5 and 66.7 ± 12.3 mg/d in the PAN-treated rats and the controls, respectively \((P < 0.001)\).

Our first objective was to confirm that the inhibitor was present in this new group of PAN-treated rats and that it was expressed in TLC fraction 2, as seen in earlier experiments. To simplify this step, urine samples from all animals \((n = 5\) in each group) for days 9 and 13 (period 1) were pooled and separated by TLC as described. As observed earlier for individual animals, only fraction 2 from pooled urine contained an active inhibitor of the calcium pump (data not shown). Other ATPases were minimally inhibited. Our principal objective was to provide statistical confirmation of these findings. Therefore, urine from each PAN-treated rat was collected separately during period 2 (the time of maximal proteinuria) and separated chromatographically, and fraction 2 samples were incubated with RBC membranes in a series of individual experiments (Figure 4). Again, the TLC fraction 2 sample from each rat had a strong inhibitory effect on the CaM-activated Ca\(^{2+}\)-ATPase \((47.6 ± 1.95 \text{ versus } 19.0 ± 8.5 \text{ nmol/min per mg protein, } P < 0.001\text{ for PAN-treated versus control rats})\). It had a similar effect on the basal calcium pump activity \((18.8 ± 0.4 \text{ versus } 6.42 ± 2.5 \text{ nmol/min per mg protein, } P < 0.001)\). This is consistent with an effect on the enzyme itself, rather than on CaM-binding sites. Other ATPases either were inhibited very weakly, such as the Na\(^{+}/K\(^{+}\)-ATPases \((6.42 ± 2.5 \text{ versus } 5.44 ± 0.5 \text{ nmol/min per mg protein, } P < 0.05)\), or were unaffected, such as the Mg\(^{2+}\)-ATPase. This suggests that the inhibitory effects were specific for the calcium pump and did not reflect generalized membrane dysfunction. Finally, as seen in preliminary observations, the fraction 2 inhibitor in this group of PAN-treated rats exhibited a clear dose-dependent effect on the Ca\(^{2+}\)-ATPase in both its CaM-activated and basal configurations (Figure 5).

**Effects of TLC Fractions of PTC-Conditioned Medium on Calcium Pump Activity**

To determine the potential cellular source of the lipid inhibitor, PTC were collected and cultured as described above, in medium containing either oleate-replete albumin or lipid-depleted albumin (8). Conditioned medium was separated by TLC as described for the urine samples, and the fractions were tested for inhibitory activity in the RBC membrane ATPase bioassay. As observed previously with the urine samples, TLC fraction 2 of PTC-conditioned medium contained a very strong inhibitor of the Ca\(^{2+}\)-ATPase. In a typical example (Figure 6),
the CaM-activated ATPase was completely inhibited by this fraction. Some evidence of inhibition was noted to spill over into fractions 3 and 4. As seen with the urine factor, these effects were dose-dependent and linear, with a steeper slope for the PTC supernatants, compared with urine samples (data not shown).

ATPase Activity in Circulating RBC from PAN-Treated Rats

Next, we evaluated the possibility that the calcium pump inhibitor, a monoglyceride secreted into the urine by PTC, might also have access to the circulation and thus be responsible for a systemic effect on other cells. Therefore, on day 19 (the time of maximal proteinuria) all rats in both groups were euthanized and 2 to 3 ml of blood was collected from each animal, in heparinized tubes. Sufficient blood could be collected from only four PAN-treated and three control rats. Blood was centrifuged and the RBC membranes were isolated as described above for the preparation of human RBC. Although blood samples were small in this experiment, sufficient amounts of RBC protein were obtained for determination of all native ATPase activities. Moreover, less material was required because rat RBC have relatively higher ATPase activities than do human cells.

Comparison of native ATPase activities between PAN-treated and control rats revealed a pattern similar to that observed during incubation of normal RBC membranes with TLC fraction 2. These effects are illustrated in Figure 7. In circulating RBC, CaM-activated Ca$^{2+}$-ATPase activity was significantly inhibited (72.04 ± 12.5 versus 98.7 ± 3.6 nmol/min per mg protein in PAN-treated and control rats, respectively; $P < 0.01$). A lesser degree of inhibition was observed for the Ca$^{2+}$-ATPase in its basal form (8.07 ± 1.4 versus 10.4 ± 0.7 nmol/min per mg protein, $P < 0.5$) and for the Na$^+$/K$^+$-ATPase (18.0 ± 2.6 versus 23.4 ± 1.9 nmol/min per mg protein, $P < 0.02$). It is very likely that some of the bound inhibitor was lost during membrane preparation or because of interactions with plasma proteins or binding sites, thus reducing the effects on the rat RBC enzymes we tested.

Findings in Patients with Proteinuria

TLC fractions from patients with proteinuria and from a normal control subject were incubated with the RBC membranes as described for the rat studies. To confirm that human urine may contain an inhibitor of the Ca$^{2+}$-ATPase, we measured only the activity of Ca$^{2+}$/Mg$^{2+}$-ATPase, in its basal and CaM-activated configurations. Other ATPases were not evaluated in these cases. Our findings are summarized in Table 1.

As in the rat studies, a powerful inhibitor of the calcium pump (in either its basal or CaM-activated state) was present in urine from the two patients with severe proteinuria and was absent from the control urine. The inhibition seemed to occur independently of renal function, because patient 2 showed normal creatinine levels. The effect was observed primarily in TLC fractions 2 and 3, but lesser degrees of inhibition seemed to spill over into other fractions (not shown in Table 1). Because the only purpose of these measurements in human
More recently, proximal tubules metabolizing albumin the urine by PTC when challenged with lipid-replete albumin characterized as a nonpolar lipid that is synthesized and released into accumulation of inflammatory cells. This factor was characterized after 3 d of albumin loading and preceded the interstitial was highly chemotactic for monocytes; this factor was present in the protein overload model in rats. In these animals, urine was found to contain a factor that was recently demonstrated in the protein overload model in rats proteinuria may lead to interstitial inflammation and fibrosis hypertension (3). A potential mechanism by which extensive proteinuria is correlated with interstitial inflammation and fibrosis has not been defined, because the factor could also cross outside of the cell, and it suggests that the inhibitor may play a role in the transport enzymes when applied to the cytoplasmic side of the cell membrane and be operative on the cytoplasmic side of the cell membrane, as described previously (14). Again, fraction 2 demonstrated powerful inhibition of the RBC membrane calcium pump, with dose-dependent activity (Figure 2). This shows that the inhibitor can interact with the transport enzymes when applied to the outside of the cell, and it suggests that the inhibitor may play a physiologic (or pathologic) role. However, the site of interaction has not been defined, because the factor could also cross the cell membrane and be operative on the cytoplasmic side of the enzyme.

In the second stage of these studies, we provided statistical confirmation of the preliminary findings. A new group of five PAN-treated rats was compared with a control group. All PAN-treated rats developed extensive proteinuria. In period 1 (days 9 to 13), we confirmed the preliminary finding that, in

Table 1. Effects of urine TLC fractions in patients with severe proteinuria

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<tr>
<th>TLC Fraction and Activity</th>
<th>Inhibition (%)</th>
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<tr>
<td></td>
<td>Control Urine</td>
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<tr>
<td>Fraction 2</td>
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<tr>
<td>basal $\mathrm{Ca}^{2+}$-ATPase</td>
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<tr>
<td>CaM-activated</td>
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* Values are percentage inhibition compared with a solvent blank. + indicates no inhibitory effect or slight stimulation of the pump.

Figure 7. Evidence that circulating RBC in PAN-treated rats ($n = 4$) exhibited statistically lower activity for their own membrane $\mathrm{Ca}^{2+}$-ATPase, compared with normal nonproteinuric controls. The pattern of inhibition (especially affecting the CaM-activated state) was the same as we found when urine or conditioned medium TLC fractions from the same animals were incubated with normal RBC membranes. This suggests a possible systemic effect of the lipid inhibitor.

Discussion

It has been suggested that the severity of proteinuria may be a factor in the progression of renal disease (20). For instance, proteinuria is correlated with interstitial inflammation and fibrosis (1,2,6), with renal disease progression (4), and with hypertension (3). A potential mechanism by which extensive proteinuria may lead to interstitial inflammation and fibrosis was recently demonstrated in the protein overload model in rats (6). In these animals, urine was found to contain a factor that was highly chemotactic for monocytes; this factor was present after 3 d of albumin loading and preceded the interstitial accumulation of inflammatory cells. This factor was characterized as a nonpolar lipid that is synthesized and released into the urine by PTC when challenged with lipid-replete albumin (6). More recently, proximal tubules metabolizing albumin were found to release closely related neutral lipid factors that stimulate transepithelial fluid secretion and cellular proliferation; biochemically, these factors are identical to those that have been found in cyst fluid from active cysts in polycystic kidney disease (8). On the basis of these findings, we have postulated that lipid factors present in proteinuric urine may be linked to other complications of proteinuria, such as renal interstitial damage and hypertension, by means of another mechanism, i.e., an abnormality in cell membrane calcium transport. This hypothesis is an extension of our previous findings in closely related conditions, such as chronic renal failure (13,14). We have shown that a humoral factor that can be chromatographically separated from fractions of uremic plasma is a strong inhibitor of the RBC membrane calcium pump (13,14). Similarly, we have found in patients with hypertension a circulating factor that increases cytosolic calcium levels in normal platelets (12). Therefore, the demonstration of similar factors in proteinuric urine may suggest a new mechanism, i.e., alteration of cellular calcium transport, as a mediator of some complications of extensive proteinuria.

The primary finding from these studies is that a novel lipid factor, an endogenous inhibitor of the RBC membrane calcium pump, is secreted in the urine of rats with PAN-induced extensive proteinuria. Urine was collected at the time of maximal proteinuria, which usually reached a peak 1 wk after PAN exposure. In preliminary studies, we collected urine from individual rats, separated it by TLC, and resuspended it in BSA-containing buffer. Five TLC fractions were incubated with isolated RBC membranes. We found that fraction 2, containing monoglycerides, exhibited a strong inhibitory effect on the $\mathrm{Ca}^{2+}$-ATPase, primarily in its CaM-activated configuration, and had only a weak effect on the basal pump activity and on the $\mathrm{Na}^{+}/\mathrm{K}^{+}$-ATPase (Figure 1). Possible nonspecific effects of the solvent or the fatty acid-free BSA used to resuspend the lipid fraction were excluded (Figure 3). In addition, experience with other circulating factors suggests that more than one complementary bioassay should be used for confirmation of an inhibitory effect. This was emphasized by Woolfson et al. (21) in the case of digitalis-like immunoreactivity, which does not always indicate the presence of a true inhibitor of the $\mathrm{Na}^{+}/\mathrm{K}^{+}$-ATPase. Therefore, we confirmed that the lipid fractions inhibited $^{45}\mathrm{Ca}$ efflux from intact RBC (an indication of cation transport by the cell membrane), as described previously (14). Again, fraction 2 demonstrated powerful inhibition of the RBC membrane calcium pump, with dose-dependent activity (Figure 2). This shows that the inhibitor can interact with the transport enzymes when applied to the outside of the cell, and it suggests that the inhibitor may play a physiologic (or pathologic) role. However, the site of interaction has not been defined, because the factor could also cross the cell membrane and be operative on the cytoplasmic side of the enzyme.

In the second stage of these studies, we provided statistical confirmation of the preliminary findings. A new group of five PAN-treated rats was compared with a control group. All PAN-treated rats developed extensive proteinuria. In period 1 (days 9 to 13), we confirmed the preliminary finding that, in
PAN-treated rats, only fraction 2 from pooled urine had a strong inhibitory effect over the Ca\(^{2+}\)-ATPase (the enzymatic counterpart of cation transport by the calcium pump). Therefore, during period 2 (days 15 to 19) we collected all urine samples from individual PAN-treated and control rats, fractionated them by TLC, and measured the inhibitory activity of fraction 2 in five separate experiments. As shown in Figure 4, under these conditions, the CaM-activated and basal activities were similarly inhibited. Although the effect on the Na\(^+/K^+\)-ATPase was statistically significant, it was minor and possibly of no biologic importance. Again, there was a linear dose–response curve for the inhibitor, with a steeper slope for the Ca\(^{2+}\)-ATPase in its activated state (Figure 5). These measurements were based on pooled extracts from the five PAN-treated rats, because the amounts of fraction 2 were limited.

With respect to the source of the urinary lipid inhibitor, we hypothesized that, similar to the macrophage chemotactant and lipid secretagogue recently described (6,8), it might be produced by PTC exposed to proteinuric concentrations of fatty acid-containing albumin. This phenomenon, which is difficult to study in situ, can be evaluated with in vitro experiments. Therefore, PTC in tissue culture were cultured for 24 h in the presence of fatty acid-replete BSA. Aliquots of conditioned medium were then extracted and chromatographically separated by TLC, as performed for the urine samples. As shown in Figure 6, supernatant fraction 2 completely blocked the CaM-activated Ca\(^{2+}\)-ATPase (100% inhibition) in RBC membranes. The inhibition was stronger than that observed with urinary fractions, although dilutions differed and precise comparison was not possible. The important point is that, on the basis of their similar chromatographic mobilities, the inhibitors present in urine and PTC supernatants probably represent the same lipid factor. Precise biochemical identification of this neutral monoglyceride will require further purification and analysis by mass spectrometry in future studies.

TLC fractions from two patients with severe proteinuria contained a similar inhibitor of the Ca\(^{2+}\)-ATPase in both its basal and CaM-activated states. As in the rat studies, the activity migrated with TLC fraction 2, extended to fraction 3, and to a much lesser degree spilled over into other fractions. In this preliminary confirmation we did not evaluate other ATPases, and the specificity of the human inhibitor for the Ca\(^{2+}\)-ATPase remains to be determined. More importantly, these findings confirm the presence of the inhibitor in other proteinuric states (membranoproliferative glomerulonephritis and diabetes in human patients) and its capacity to cross species lines. These observations strongly support the relevance of this new mechanism for the pathogenesis of human renal disease. More studies will be required to evaluate the presence of an inhibitor in other renal conditions, its relation to renal function and the progression of renal insufficiency, and its structural identity.

Tubular epithelial cells challenged by fatty acid-laden albumin seem to release a group of biologically active lipids, including a monocyte chemotactant (6), a chloride secretagogue (8), and now a novel calcium pump inhibitor. These substances share similar properties. They are complex neutral lipids, chromatographically behaving as monoglycerides, that are derived from oleic acid (6). Identification will require massive scale-up of the isolation, purification to near-homogeneity, and analysis by mass spectrometry. Although each of these activities isolated from proteinuric urine and albumin-challenged PTC behaves chromatographically as a monoglyceride, the activities are separable by HPLC, suggesting that they are similar but not identical. HPLC of the active fraction of the PAN-treated rat urine demonstrates a peak at 19 to 20 min in the chromatograph, in the same region as the macrophage chemotactic factor described previously (6). The latter substance was subjected to chemical treatments to explore structure-function relationships. Alkaline treatment with methanolic KOH, which hydrolyzes esters, did not blunt the chemotactic activity. In contrast, both borohydride and HCl treatment markedly reduced monocyte migration (8). This suggests that ketone and glycol groups, or comparable reactive groups, may mediate the biologic activity. In summary, we found the inhibitor to be a neutral lipid (according to its solvent extractability), with fatty acids as precursor molecules and with chromatographic characteristics and biochemical sensitivities similar to those of other members of this new group of biologically active lipids (6,8). These observations, in the context of proteinuria, suggest that tubules exposed to albumin may release a variety of lipid metabolites that could contribute to the interstitial leukocyte accumulation, blunted chloride absorption, and susceptibility to hypertension that can accompany progressive renal disease and can be correlated with the severity of proteinuria (5). There is growing evidence that neutral lipids can mediate paracrine cell signaling. Oleoyl amide has been identified as a member of a novel neurotransmitter class (22), and monobutyl glycerol, a monoglyceride, served as a potent inducer of angiogenesis when released by adipocytes (23).

Our findings provide direct evidence of an endogenously secreted lipid inhibitor of the plasma membrane calcium pump, which may lead to increased cytosolic calcium concentrations in target cells. No other endogenous inhibitors of the Ca\(^{2+}\)-ATPase have been identified (24). It is quite interesting that this inhibitor is a metabolite of oleate, because oleate itself is a known biologic activator of this enzyme (25). Because the calcium pump is ubiquitous, an inhibitor secreted by PTC into proteinuric urine may be associated with local effects, such as the activation of macrophages or other inflammatory cells, and may thus contribute to the progression of renal disease. Furthermore, access to the circulation may allow the factor to display systemic effects. We have found that circulating RBC in PAN-treated rats have a statistically lower activity of their own membrane Ca\(^{2+}\)-ATPase, compared with normal control rats (Figure 7). The pattern of inhibition was the same as we found when urine or conditioned medium TLC fractions from the same animals were incubated with normal human RBC membranes. A systemic effect of this lipid could lead to high cytosolic calcium levels in other cells, such as vascular smooth muscle cells, which may result in hypertension, which in turn has been correlated with extensive proteinuria (3). Finally, the finding of a calcium pump inhibitor in the urine from two
patients with proteinuria strongly suggests that this substance may have a significant role in the pathogenesis of renal disease in human patients. This is also supported by the recent description of similar factors in the plasma of patients with renal insufficiency (13,26) and by the high cytosolic calcium concentrations and the inhibition of the cell Ca\(^{2+}\)-ATPase that are common features of uremia in human patients and in animal models of renal disease (11). Previously, we characterized a small, hydrophilic, heat-stable inhibitor of the calcium pump in HPLC fractions of uremic plasma (13,14). In contrast, these findings demonstrate a neutral lipid molecule that migrates chromatographically with the monoglycerides, is possibly related to oleic acid, and may represent a new group of lipid inhibitors of cation transport by the membrane calcium pump.

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References