Characterization of Digitalis-like Factors in Human Plasma
INTERACTIONS WITH NaK-ATPase AND CROSS-REACTIVITY WITH CARDIAC GLYCOSIDE-SPECIFIC ANTIBODIES*

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Much of the evidence for a physiologically important endogenous inhibitor of the sodium pump has been either contradictory or indirect. We have identified three discrete fractions in desalted deproteinized plasma from normal humans that resemble the activity of digitalis glycosides in that they: 1) are of low molecular weight; 2) are resistant to acid and enzymatic proteolysis; 3) inhibit NaK-ATPase activity; 4) inhibit Na+ pump activity in human erythrocytes; 5) displace [3H]ouabain bound to the enzyme; and 6) cross-react with high-affinity polyclonal and monoclonal digoxin-specific antibodies but not with anti-ouabain or anti-digitoxin antibodies. An additional fraction cross-reacted with digoxin-specific antibodies but had no detectable activity against NaK-ATPase. The three inhibitory fractions differed from cardiac glycosides in that their concentration-effect curves in a NaK-ATPase inhibition and [3H]ouabain radioreceptor assays were steeper than unlabeled ouabain. This suggests that these inhibitors are not simple competitive ligands for binding to NaK-ATPase. In the presence of sodium, no fraction required ATP for binding to NaK-ATPase, and in the presence of potassium, only one fraction had the reduced affinity for the enzyme that is characteristic of cardiac glycosides. Unlike digitalis, all three NaK-ATPase inhibitory fractions had reduced affinity for skeletal muscle sarcoplasmic reticulum Ca-ATPase.

The presence of at least three fractions in human plasma that inhibit NaK-ATPase and cross-react to a variable degree with different digoxin-specific antibody populations could explain much of the conflicting evidence for the existence of endogenous digitalis-like compounds in plasma. These inhibitors with NaK-ATPase are unknown (2–6). This topic is potentially important because an endogenous inhibitor of the sodium pump could affect regulation of vascular smooth muscle tone, sympathetic nervous system activity, and sodium homeostasis (7–10). Although inhibitors of NaK-ATPase such as vanadate and certain fatty acids are present in the blood and tissues of humans and animals, these compounds are not specific for the sodium pump and are not high affinity ligands for the cardiac glycoside binding site of NaK-ATPase (1, 11). Also, there are substances in human and animal plasma that interact with antibodies to digoxin, but it is controversial whether these substances also inhibit NaK-ATPase activity (3, 5, 12–15). Many of the discrepancies among these findings could be due to differences in methodology. In fact, the validity of some conclusions has been questioned solely because of differences in techniques (16, 17).

In this report, we describe the presence of NaK-ATPase inhibitory activity in deproteinized desalted plasma from normal humans. Using several chromatographic systems, we resolved the activity into three discrete fractions. Our criteria for the presence of an endogenous digitalis-like substance included: 1) the capacity to inhibit NaK-ATPase activity; 2) the ability to inhibit [3H]Rb+ influx into human erythrocytes; 3) the capacity to displace ouabain from NaK-ATPase; 4) the change in binding of the substance(s) to NaK-ATPase when the concentrations of ATP and potassium were varied; and 5) immunoreactivity with polyclonal and monoclonal antibodies to digitalis glycosides.

MATERIALS AND METHODS

Preparation of Plasma Extract
Plasma of normal humans who were not receiving cardiac glycosides was heated for 20 min in a boiling water bath, and the coagulated protein was removed by centrifuging for 60 min at 35,000 × g. 600–700 ml of the supernatant was applied to a 2 × 7-cm column of preparative C18 resin (Waters) that had previously been washed with 100 ml of acetonitrile and 300 ml of water. The loaded column was washed with 200 ml of water at a rate of 50 ml/h and then eluted successively with 30 ml each of 10, 25, 50, 80, and 100% acetonitrile. The eluates were pooled, lyophilized, resuspended in 6 ml of water, and centrifuged for 60 min at 45,000 × g. The supernatant was filtered through a 0.2-μm nylon filter, and the filtrate (designated as plasma extract) was used for different assay and chromatographic procedures.

Gel Filtration Chromatography
A 1.5-ml aliquot of the plasma extract was applied to a 1.5 × 35-cm Sephadex LH-20 column which had been equilibrated with 70% acetonitrile. Fractions were eluted at a rate of 0.5 ml/min with 70% acetonitrile and then lyophilized, reconstituted in water, and assayed.
We used two types of reverse-phase columns in a Waters HPLC system with a model 600 solvent programmer: an octadecylsilane column (μBondapak C18, 3.9 × 30 mm, 3 μm particle size) and another octadecylsilane column (μBondapak phenyl). Two ml of the plasma extract (a 200-fold concentration of the original plasma) were injected into one of these columns and eluted at 0.5 ml/min with either a linear 0-100% water-acetonitrile gradient or with different nonlinear gradient elution programs as specified below. UV detection was performed with a Beckman model 165 variable wavelength UV detector. One-ml fractions were collected and lyophilized in order to remove the acetonitrile; the fractions were reconstituted in 1 ml of water before being assayed.

**Assay Techniques**

(a) Na-K-ATPase Inhibition—Serial dilutions of the plasma extract were preincubated for 2 h at 37°C with 700 μM of incubation buffer I consisting of 100 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.25 mM Na₂EDTA, 5 mM MgCl₂, and 5 mM ATP plus 100 μl of a 0.6 mg/ml preparation of canine kidney cortex Na-K-ATPase (Sigma). Subsequently, KCl (final concentration, 20 mM) and 0.3 μCi of [γ-32P]ATP (New England Nuclear) were added to yield a final volume of 1 ml. This mixture was incubated for 30 min at 37°C, and the reaction was terminated by diluting 1 ml of ice-cold charcoal (Norit A) in 0.1 M HCl, 1 mM NaH₂PO₄, and 1 mM Na₂HPO₄. After 5 min at room temperature, the samples were centrifuged at 2000 × g for 10 min, and the 32P released was determined by liquid scintillation counting.

We also investigated whether the plasma extract inhibited a partially purified Na-K-ATPase obtained from the supraorbital salt glands of ducks. The isolation of this enzyme was described previously (18). Briefly, supraorbital salt-secreting glands obtained from ducks which had been consuming a high-salt diet were removed and frozen in liquid nitrogen. The glands were thawed, minced, and the tissue was homogenized at 0°C in 10 volumes of buffer I consisting of 0.25 M sucrose, 10 mM Tris and 1 mM EDTA (pH 7.5). After straining the homogenate through gauze, it was centrifuged for 15 min at 5000 × g, and the resulting pellet was resuspended in buffer II and recentrifuged for 15 min at 5000 × g. The combined supernatants were then centrifuged at 48,000 × g for 1 h, and the pellet was resuspended in buffer II to yield a final protein concentration of 10 to 15 mg/ml. This microsomal fraction of the avian salt gland contained Na-K-ATPase with a specific activity of 400 μCi of P/mg of protein/h; it did not contain Mg²⁺-ATPase activity. The inhibitory capacity of the HPLC fractions of the plasma extract was examined using the methods described above, using this Na-K-ATPase preparation or the canine kidney Na-K-ATPase.

The effects of potassium or ATP on the inhibitory capacity of each HPLC fraction from the plasma extract were tested using the following assay. Either 0 or 5.0 mM KCl with or without 5 mM ATP was added to a mixture of Na-K-ATPase, the HPLC fraction being tested, and incubation buffer I. After preincubating for 5 min at 37°C, 0.5 μCi of [γ-32P]ATP and 20 μM of potassium were added, and 10 min later, the reaction was stopped by adding 3 ml of ice-cold charcoal suspension in phosphate buffer.

(b) Radioreceptor Assay—In this assay, 25 μl of the 0.6 mg/ml canine kidney Na-K-ATPase (Sigma) in buffer I was preincubated for 2 h at 37°C with ouabain or aliquots of HPLC fractions of the plasma extract. 0.4 μCi of [3H]ouabain (New England Nuclear, 18 Ci/mmol) were added (final volume, 1 ml) and incubated for another 20 min. The reaction was stopped by adding 3 ml of ice-cold buffer I and filtering through Whatman GF/C glass fiber filters. After washing the filters with 6 ml of ice-cold buffer I, the [3H]ouabain-enzyme complex retained on the filter was measured by liquid scintillation counting. Specific binding was calculated by measuring the [3H]ouabain bound in the presence and absence of 1 mM ouabain in the incubation medium.

(c) Sodium Pump Activity in Human Erythrocyte—Erythrocyte sodium pump activity was determined using a modification of the Na⁺-loaded red cell technique described by Cooper et al. (19). Blood from a normal donor was drawn into heparinized tubes, centrifuged, and the red cells were separated and washed four times in a choline washing solution containing 149 mM choline, 1 mM Mg²⁺, 10 mM Tris-MOPS (pH 7.4) at 4°C. To load the cells with sodium, 2 ml of packed red cells were added to 5 ml of a nystatin solution containing 70 mM NaCl, 70 mM KCl, 10 mM Tris-MOPS (pH 7.4), 55 mM sucrose, and 156 μg/ml nystatin (Sigma). The mixture was kept at 4°C for 20 min with periodic mixing. The erythrocytes were then centrifuged and resuspended in 5 ml of the above solution containing 7.5 mM sodium nystatin and incubated for another 20 min at 4°C. Bound nystatin was removed by washing four times at 33°C with a solution of 70 mM NaCl, 70 mM KCl, 10 mM Tris-MOPS (pH 7.4), 55 mM sucrose, 10 mM glucose, and 1.0 mg/ml albumin (Sigma, Type V). The cells were subsequently washed five times at 4°C with the choline washing solution. Aliquots of these Na⁺-loaded cells were used to determine the activities of the hemoglobin, hematocrit, and the internal sodium concentration. Sodium-loaded cells were added to chilled tubes containing 150 mM NaCl, 1 mM MgCl₂, 10 mM Tris-MOPS, pH 7.4, at 37°C, 10 mM glucose, and 4 mM KCl plus 100 μCi of [32P]ATP, to which different HPLC fractions with activity had been added. The final volume in each tube was approximately 1 ml. All determinations were performed in triplicate with 5- and 25-min incubations at 37°C in a shaking water bath. In experiment 2 (Table II), 1 mM furosemide was added to a parallel set of tubes containing aliquots of each of the HPLC peaks plus 10⁻⁴ M ouabain. This was done to the plasma extract. This inhibitor was not attributable to the Na⁺-K⁺-ATPase activity.

(d) Radioimmunoassay—Polyclonal and monoclonal digoxin-specific antibodies were tested for reactivity with each HPLC fraction of the plasma extract. A digoxin-specific rat monoclonal antibody (kindly provided by Dr. Frank Shand of Burroughs Wellcome Laboratories, United Kingdom), rabbit polyclonal anti-digoxin antibodies (New England Nuclear), and sheep polyclonal anti-digoxin antibodies (20) were used in these experiments. To obtain the sheep antibodies, the animals were injected intramuscularly each week with 3 mg of digoxin-human serum albumin conjugate in 1 ml of Freund’s adjuvant containing 10 mg of killed tubercle bacillus. After 20 weeks, sera were collected and stored at −20°C. This serum had less than 0.1% cross-reactivity with the following steroids: cholesterol, cortisol, 17β-estradiol, progesterone, and testosterone.

The elution of polyclonal antisera or monoclonal antibody that would bind 50% of 10 nCi of a [3H]labeled histamine derivative of digoxin (Corning) was determined and used to construct binding curves in the presence of graded concentrations of digoxin. Free ligand was separated from the bound fraction by using dextran-coated charcoal or a second antibody technique. Both procedures yielded equivalent results. In the charcoal technique, plasma extract or HPLC fractions were preincubated with 1.0 ml of 100 μCi of [3H]digoxin (specific activity 60 Ci/mmol) and monoclonal or rat monoclonal antibody in tubes containing 4 mg/ml nonspecific IgG (bovine, Sigma) and sufficient 0.1 M Tris-Cl buffer, pH 7.4, to yield a final reaction volume of 0.95 ml. IgG was used as a carrier protein because preliminary studies showed that the inhibitory activity in the HPLC fractions was adsorbed by bovine or human serum albumin. After the preincubation, 10 nCi of [3H]digoxin (50 μl) were added, and the tubes were incubated at room temperature for 1 h. The reaction was terminated by adding 0.3 ml of 10% Norit A charcoal in the HCl-phosphate buffer, and after 5 min, the tubes were centrifuged at 2000 × g for 10 min. The radioactivity in 1 ml of each tube was determined.

The lowest limit of detection for digoxin in this assay was 1 pg/ml; the assay was log linear from 1 to 10 pg/ml when the rat monoclonal antibody was used and from 10 to 100 pg/ml with the sheep polyclonal antibody. In the double antibody technique, the HPLC fractions from plasma extract were preincubated for 2 h at room temperature with a 1:2 dilution of rabbit polyclonal anti-digoxin antibody that had been previously absorbed against anti-rabbit IgG (New England Nuclear). The preincubation tubes also contained 4 mg/ml bovine IgG (Sigma) and sufficient 0.1 M Tris-Cl buffer (pH 7.4) to yield a final reaction volume of 0.95 ml. Subsequently, 10 nCi (50 μl) of [3H]digoxin were added, and the mixture was incubated at 37°C for 20 min. The reaction was stopped by adding 2 ml of a solution containing 500 mM Tris and 1 mg/ml EDTA (pH 7.5). The samples were centrifuged at 2000 × g for 10 min in the cold. Radioactivity in the pellet was measured. With these modifications of the New England Nuclear kit (RIANEN), we could detect 5 pg/ml digoxin and the assay was log linear between 5 and 10 ng/ml.

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1 The abbreviations used are: HPLC, high performance liquid chromatography; MOPS, 4-morpholinopropansulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.
In addition to the digoxin radioimmunoassay, ouabain and digi-
toxin radioimmunoassays were developed using rabbit anti-digitoxin
antibodies (East Acres Biologicals, Southbridge, MA) and anti-oua-
bain antibodies that were raised in rabbits as previously described
(21). Briefly, rabbits were immunized with ouabain bound to human
serum albumin using a poly-DL-alanine bridge to the mannosese moiety
of ouabain. The antigen was injected weekly for 4 weeks. Subse-
sequently, the amount of ouabain-specific antigen that would bind
50% of 0.04 μCi of [3H]ouabain (New England Nuclear, 18 Ci/mmol)
or digitoxin-specific antigen that would bind 0.04 μCi of [3H]
digitoxin was determined, and this amount of serum was preincubated
for 12 h at 4 °C with unlabeled ouabain or digitoxin, plus oua-
bain, or aliquots from HPLC fractions of plasma extract (final reaction vol-
ume, 0.95 ml). After this preincubation, 0.04 μCi of [3H]ouabain or
[3H]digitoxin (50 μl) were added and incubated for 1 h at room
temperature. Free ligand was separated from the bound fraction by
adding 0.3 ml of a 10% dextran-coated charcoal suspension. Radio-
activity in the supernatant of the centrifuged sample was counted.
The limit of ouabain detection in the ouabain radioimmunoassay was
5 × 10⁻¹⁰ M, and the limit of digitoxin detection in the digitoxin
radioimmunoassay was 5 × 10⁻¹¹ M. There was less than 1% cross-
reactivity between these antibodies and the various endogenous ste-
nols listed above.

Aliquots of the plasma extract were reconstituted in 0.1 M Tris-
HCl (pH 7.3) and incubated for 60 min at 37 °C with either 1 mg/ml
trypsin or 3 mg/ml Pronase (Sigma Type IV). After boiling for 5 min
and centrifuging for 30 min at 40,000 × g, the supernatants were
assayed for NaK-ATPase inhibitory activity and digitoxin-like immu-
noreactivity.

(c) Calcium ATPase Inhibition Assay—Ca-ATPase was prepared
from rabbit skeletal muscle sarcoplasmic reticulum by the method of
Harigaya and Schwartz (22). The enzyme was assayed in a buffer
solution containing 5 mM MgSO₄, 5 mM ATP, 80 mM KCl, 50 mM
imidazole (pH 6.8), and either 0.06 mM CaSO₄ or 0.5 mM EGTA. Ali-
quots of the HPLC fractions were preincubated with the Ca-
ATPase preparation for 10 min at 25 °C. Buffer solution was then
to added to yield a volume of 1 ml and a final enzyme concentration
of 0.11 mg/ml. This mixture was incubated for another 10 min at 25 °C
until the reaction was stopped by adding 1.0 ml of ice-cold 10% trichloroacetic acid. After centrifuging for 15 min at 2500 × g in the
cold, the inorganic phosphorus in 1 ml of supernatant was determined
by the Fiske-SubbaRow method (23). The rates of ATP hydrolysis
occurring in the presence and absence of calcium were measured, and the difference between the rates was calculated to yield the net activ-
yty.

RESULTS

In developing a method for extracting NaK-ATPase inhibi-
tory activity and digitoxin-like immunoreactivity, we evaluated
several reported techniques for deproteinizing, desalting, and
concentrating plasma. To deproteinize, plasma was heated at
neutral pH in a boiling water bath for 10 min. We found that
if the plasma were acidified to pH 5.5 using acetic acid before
heating (3, 24, 25), most of the NaK-ATPase inhibitory and
digitoxin-like immunologic activity precipitated with plasma
proteins. When unacidified plasma was heated, extraction of
the resulting protein precipitate with either ether or acetoni-trile
yielded only the amount of enzyme inhibitory activity or
digitoxin-like immunoreactivity expected to be found in the
aqueous phase of the pellet. Thus, deproteinizing plasma by
boiling at neutral pH simplifies the extraction procedure and
enhances the yield of NaK-ATPase inhibitory activity.

In examining methods for desalting and concentrating the
plasma supernatant, we found that preparative C₁₈ resin
(Waters) was effective and yielded as much as a 200-fold
concentration of the inhibitory factor. The sodium and potas-
sium concentrations of the plasma extract prepared in this
way were 10 and 0.5 mM, respectively; vanadate was unde-
tectable by atomic absorption spectrometry. In contrast, when
we attempted to desalt by gel filtration using Sephadex, NaK-
ATPase inhibitory activity adsorbed to the gel, even when the
columns were eluted with high concentrations of ammonium
acetate. Although ion-exchange chromatography of the inhibi-
tory activity from deproteinized plasma yielded similar quan-
tities of activity, this technique offered no advantage over the
C₁₈ resin (26).

The usefulness of ultrafiltering the heated plasma super-
natant as a preparative step (13) also was examined. Using
plasma, the plasma extract, or the HPLC fractions of plasma
extract, we found that the NaK-ATPase inhibitory activity
and much of the digitoxin-like immunoreactivity adsorbed to
ultrafiltration membranes (Amicon YM or UM series). This
nonspecific adsorption could be reduced substantially if
ethanol were added to a final concentration of 40%. Conse-
quently, ultrafiltration was not used routinely because it did
not simplify the preparative procedure and it decreased the
yield of inhibitory activity recovered.

To determine whether the initial plasma extract contained
digitalis-like activity, aliquots of the extract were assayed in
the enzyme inhibition assay, the radioimmunoassay, and
a radioimmunoassay with sheep polyclonal digoxin-specific anti-
bodies. Dilution of the factor(s) in the plasma extract yielded
NaK-ATPase inhibition and [3H]ouabain displacement curves that
were appreciably steeper than the corresponding curves for ouabain (Fig. 1, A and B). However, serial dilutions
of the plasma extract in the digoxin radioimmunoassay yielded a displacement curve that was slightly shallower than
that of digoxin itself (Fig. 2). It is possible that the difference in
these curves was due to recognition of the plasma factor(s) by
only a subset of the anti-digoxin antibodies in the poly-
clonal mixture.

Three types of experiments were used to identify charac-
teristics of the factor(s): stability during acid hydrolysis or
protease digestion, solubility in polar and nonpolar solvents,
and estimation of molecular size using ultrafiltration. Heating
the plasma extract to 110 °C in 6 N HCl for 24 h or incubating
it with Pronase (Sigma Type IV) or trypsin did not change
either the ability of the factor(s) to inhibit NaK-ATPase or
to inhibit labeled digoxin binding in the radioimmunoassay.
The inhibitory activity partitioned equally between ether and
water, and both the inhibitory and immunoreactive activity
in the plasma extract were ultrafilterable through an Amicon
YM-2 membrane that had a 2000 molecular weight exclusion
limit. The exclusion limit of the Amicon ultrafiltration mem-
brane was validated by showing that it excluded (>90%) [3H]
inulin (molecular mass, 5000 daltons) but passed completely
L-[^U-¹⁴C]phenylalanine (165 daltons) and [3H]digoxin (751
daltons).

To separate the factor(s) in the plasma extract, two types
of chromatography techniques were used: gel filtration
through an LH-20 (Sephadex) column and reverse-phase
HPLC. We found that the elution pattern of the plasma
extract from LH-20 hydrophobic Sephadex revealed a peak of activity that inhibited NaK-ATPase and displaced [3H]
ouabain from the enzyme. This peak corresponded to the
inclusion volume of the column (Fig. 3A). There were two
other peaks that exhibited digoxin-like immunoreactivity; the
first partially overlapped the peak containing the inhibitor
of NaK-ATPase while the second peak did not inhibit the en-
zyme.

Factors with enzyme inhibitory activity in the plasma ex-
tract also were separated using a C₁₈ HPLC column and a
linear 0–100% water-acetonitrile gradient. As shown in Fig.
3B, there were two peaks containing immunoreactivity when
assayed with the sheep polyclonal digoxin-specific antibodies.
Only the peak that eluted at the higher acetonitrile concen-
tration also contained NaK-ATPase inhibitory activity. To
examine whether acidifying the solvents or ion pairing
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FIG. 1. A, effect of aliquots of plasma extract on canine kidney cortex NaK-ATPase activity. Serial dilutions of the desalted, deproteinized plasma extract (Δ—Δ) were assayed for NaK-ATPase inhibitory activity as described under “Materials and Methods” and contrasted here with the concentration-effect curve of ouabain (A—A). One hundred per cent NaK-ATPase activity was approximately 90 pmol Pi/mg protein/h. B, effect of aliquots of plasma extract on binding of [3H]ouabain to NaK-ATPase. Serial dilutions of the desalted, deproteinized plasma extract (Δ—Δ) were assayed in a [3H]ouabain radioreceptor assay as described under “Materials and Methods” and are contrasted here with graded concentrations of ouabain (A—A).

FIG. 2. Effect of aliquots of plasma extract on %digoxin binding to a polyclonal digoxin-specific antibody. Serial dilutions of the desalted deproteinized plasma extract (O—O) were compared with graded concentrations of unlabeled digoxin (●—●) in a digoxin radioimmunoassay using sheep polyclonal anti-digoxin antibodies (see “Materials and Methods”). Each point represents the mean of three replicate determinations.

changed the retention time on this HPLC column, the solvents were acidified with 0.1 M acetic acid or treated with 0.1% trifluoroacetic acid. Neither technique influenced the retention time of the peaks. The fractions that inhibited NaK-ATPase or reacted with the antibody did not exhibit measurable UV absorption at 210 or 260 nm.

Higher resolution of the enzyme inhibitory and the immunoreactive activity in the plasma extract was achieved using the gradient elution program shown in Fig. 4. Three peaks containing NaK-ATPase inhibitory activity were eluted from the C18 column at approximately 50, 80, and 85% acetonitrile. There also was a shoulder of activity associated with the peak eluting at 50% acetonitrile. The three fractions that displaced [3H]ouabain from the enzyme coincided exactly with the enzyme inhibitory activity. Each of the peaks containing the inhibitory activity also contained substances that cross-reacted with digoxin-specific sheep or rabbit polyclonal antibodies. There was one additional fraction eluting at 40% acetonitrile that contained only digoxin-like immunoreactivity (IR1). Interestingly, the activity in this peak did not adsorb to ultrafiltration membranes, as did the enzyme inhibitory activity of the other peaks.

The three peaks containing enzyme inhibitory activity were separated most consistently using a phenylpropylsilane column and a water-acetonitrile step-gradient elution program (Fig. 5). Three distinct peaks with enzyme inhibitory activity (EI1, EI2, EI3) were eluted at approximately 35, 50, and 80% concentrations of acetonitrile. Each peak also had digoxin-like immunoreactivity when assayed with polyclonal digoxin-specific antibodies (Fig. 5). However, this chromatographic technique did not separate the factor(s) with digoxin-like immunoreactivity alone (i.e. IR1, see Fig. 4) from peak EI1 (Fig. 5).

To determine the specificity of the immunoreactivity in these different HPLC peaks, each of the fractions were assayed with rat monoclonal anti-digoxin antibody (Fig. 6A), sheep polyclonal anti-digoxin antibodies (Fig. 6B), and rabbit polyclonal anti-ouabain antibodies or anti-digitoxin antibodies (Fig. 7). The rat monoclonal digoxin-specific antibody cross-reacted with factors in the IR1 and EI2 peaks but did not cross-react with factors in peak EI1 or EI3 (Fig. 6A). Neither rabbit polyclonal ouabain-specific antibodies nor digitoxin-specific antibodies cross-reacted with any of the fractions shown in Figs. 4 and 5 at the concentrations tested (Fig. 7).

For each peak containing NaK-ATPase inhibitory activity, a [3H]ouabain displacement curve was determined (Fig. 8). Low concentrations of the inhibitory substance in peaks EI1, EI2, and EI3 caused a small but significant increase in [3H] ouabain binding to the enzyme (Fig. 8). In Fig. 9 are shown the concentration-effect curves for dilutions of each peak containing enzyme inhibitory activity against the canine kidney cortex NaK-ATPase (Sigma) and the NaK-ATPase prepared from the avian salt gland. In general, the avian enzyme appeared to be more sensitive to the inhibitory activity obtained from human plasma. Although preincubations of 2 h were used to ensure equilibrium inhibition of NaK-ATPase
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Fig. 3. A, fractionation of plasma extract by hydrophobic gel filtration (Sephadex LH-20) chromatography. An aliquot of the plasma extract was layered onto Sephadex LH-20 and eluted with 70% acetonitrile in water (v/v) at a flow rate of 0.5 ml/min. NaK-ATPase inhibitory activity (●—●), assayed as described under "Materials and Methods," eluted within the included volume (fractions 8-18, determined by the elution of bovine serum albumin and acetone). Digoxin-like immunoreactivity (∆—∆), assayed with sheep polyclonal digoxin-specific antibodies, eluted in two separate peaks, one of which overlapped the enzyme inhibitory activity. B, fractionation of plasma extract by reverse phase (C18) HPLC. An aliquot of plasma extract, representing approximately 400 ml of plasma, was injected into a 3.9 × 30-cm C18 reverse phase column, 10-μm particle size, and eluted with a 0-100% linear water-acetonitrile gradient at a flow rate of 0.5 ml/min. NaK-ATPase inhibitory activity (●—●) eluted only at relatively high acetonitrile concentrations, while digoxin-like immunoreactivity (∆—∆) eluted in two discrete peaks at approximately 50 and 80% acetonitrile. RIA, radioimmunoassay.

Fig. 4. Fractionation of plasma extract by reverse phase (C18) HPLC using a step-gradient elution program. An aliquot of plasma extract, representing approximately 400 ml of plasma, was injected onto a C18 reverse phase HPLC column in 20% acetonitrile and eluted at 0.5 ml/min according to the gradient program (—). NaK-ATPase inhibitory activity could be separated into three peaks (●—●); these corresponded exactly to the fractions containing [3H]ouabain displacing activity in the radioreceptor assay (described under "Materials and Methods"; data not shown). Digoxin-like immunoreactivity was assayed with polyclonal sheep and rabbit digoxin-specific antibodies (∆—∆) and digoxin-specific monoclonal antibodies from rat (∆——∆). The peak identified as IRI, which exhibited no enzyme inhibitory activity, did contain factors which cross-reacted with all digoxin-specific antibody populations. RIA, radioimmunoassay.

Fig. 5. Fractionation of plasma extract by reverse phase (phenylpropylsilane) HPLC using a step-gradient program. A 2-ml aliquot of the plasma extract, representing a 200-fold concentration of the original plasma volume, was injected into a phenylpropylsilane reverse phase HPLC column and eluted with the water-acetonitrile step-gradient elution program (—). NaK-ATPase inhibitory activity (●—●) eluted in three discrete peaks labeled EI1, EI2, and EI3. These peaks corresponded exactly with displacing activity as detected in the [3H]ouabain radioreceptor assay (data not shown). Digoxin-like immunoreactivity was assayed with both polyclonal sheep and rabbit anti-digoxin antibodies (∆—∆) and with rat digoxin-specific monoclonal antibodies (∆——∆). RIA, radioimmunoassay.

activity, the time course of inhibition was found to be rapid. At 37 °C, inhibition of NaK-ATPase activity by each peak was apparent within 15 s and appeared to be complete after 10 min. These experiments were done using an amount of each peak sufficient to inhibit at least 70% of NaK-ATPase activity. By comparison, equilibrium inhibition of NaK-atpase activity by 10⁻⁷ M ouabain in our assay required a 90-min incubation at 37 °C.

Peaks EI1, EI2, and EI3, from the phenylpropylsilane HPLC column, and peak IR1 from C18 HPLC were subjected to acid
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FIG. 6. A, cross-reactivity of HPLC fractions of plasma extract with rat monoclonal digoxin-specific antibodies. Serial dilutions of aliquots of fractions from C18 and phenylpropylsilane reverse phase HPLC were tested in a digoxin radioimmunoassay using rat digoxin-specific monoclonal antibodies and a high specific activity iodinated derivative of digoxin (see "Materials and Methods"). Aliquots from peaks E11 (O-----O) and EI3 (O-----O) showed no cross-reactivity, while factors in peaks EI2 (■---■) and IE1 (■---■) demonstrated cross-reactivity. The concentration-effect curve of digoxin (■---■) is given for comparison. B, cross-reactivity of HPLC fractions of plasma extract with polyclonal sheep digoxin-specific antibodies. Serial dilutions of aliquots of fractions from C18 and phenylpropylsilane reverse phase HPLC were tested in a digoxin radioimmunoassay using sheep polyclonal anti-digoxin antibodies. With the polyclonal antibody, all HPLC fractions showed some cross-reactivity with digoxin (symbols same as in A), although peak EI3 (O-----O) was markedly less cross-reactive than the others.

FIG. 7. A, absence of cross-reactivity between HPLC fractions and ouabain-specific antibodies. Serial dilutions of each of the HPLC fractions were digoxin-like immunoreactivity (O-----O) were also assayed with rabbit ouabain-specific polyclonal antibodies using [3H]ouabain as the labeled hapten. None of the fractions showed any immunoreactivity. The concentration-effect curves of digoxin (■---■) and ouabain (■---■) are shown for comparison. B, absence of cross-reactivity between HPLC fractions and digitoxin-specific antibodies. Serial dilutions of each HPLC fraction (O-----O) failed to show any cross-reactivity with rabbit digitoxin-specific polyclonal antibodies, using [3H]digitoxin as the labeled hapten. The high degree of specificity of this antibody is reflected in the markedly reduced sensitivity of the assay for digoxin (■---■) compared to digitoxin (■---■). RIA, radioimmunoassay.

hydrolysis. The digoxin-like immunoreactivity was unaffected by acid hydrolysis, but the enzyme inhibitory activity in peak EI1 declined by about 80% during heating with 6 N HCl for 24 h. The inhibitory activity in peaks EI2 and EI1 declined by only 20%. Inhibitory activity and immunoreactivity in each peak was ultrafilterable through Amicon YM-2 membranes after adding 40% (v/v) ethanol; this indicates that the molecular mass is <2000 daltons.

The specificity of the interaction with NaK-ATPase was further tested by examining each peak with enzyme inhibitory activity to determine if it inhibited rabbit skeletal muscle sarcoplasmic reticulum Ca-ATPase (Table I). In contrast to their inhibitory effects on NaK-ATPase, fractions EI1 and EI2 stimulated Ca-ATPase activity. Fractions from peak EI2 stimulated Ca-ATPase activity when the concentration was <100% of that needed to inhibit NaK-ATPase activity; higher concentrations of this peak inhibited Ca-ATPase.

To determine whether the fractions with NaK-ATPase inhibitory activity also would inhibit monovalent cation transport in human erythrocytes, we examined the effects of peaks EI1, EI2, and EI3 on ouabain-sensitive [3H]Rb+ influx using the technique of Cooper et al. (19). To ensure that the rate of [3H]Rb+ influx was not influenced by variations in the internal Na+ concentration, erythrocytes were loaded with 70 mM NaCl. All three NaK-ATPase inhibitory fractions significantly inhibited Na+ pump activity (Table II). However, each fraction also increased a ouabain-insensitive "leak" of [3H]Rb+ into the cell that could not be attributed to an increase in furosemide-sensitive cotransport. Only higher concentrations of material from peak EI1 consistently reduced Na+ pump activity without causing obvious red cell lysis.

Potassium is known to inhibit binding of cardiac glycosides to NaK-ATPase and in the presence of Na+, ATP is required for binding of glycosides to the enzyme (27). To examine
whether the factors in peaks EI1, EI2, and EI3 also exhibit these characteristics, NaK-ATPase inhibition curves were constructed after preincubation with and without ATP, or with ATP plus or minus 5 mM KCl, and the results were compared to those obtained with ouabain. Inhibition of NaK-ATPase by peaks EI1, EI2, and EI3 showed no ATP sensitivity in contrast to the inhibition of NaK-ATPase by ouabain (Fig. 10). Peak EI3 exhibited reduced inhibitory activity when 5 mM K⁺ was present in the preincubation media (Fig. 10). Similar results were obtained in the [³H]ouabain binding assay (data not shown).

We tested whether the inhibitory activity was affected by incubating with albumin. All NaK-ATPase inhibitory activity in each of the peaks was removed by adding 0.5 mg/ml bovine serum albumin (Sigma) or human serum albumin (Pentax). Moreover, adding albumin to the assay tubes after aliquots had been preincubated with NaK-ATPase reversed the inhibitory activity of peak EI3. In contrast, enzyme inhibition by peaks EI1 and EI2 was not reversible by incubating for 4 h with 0.5 mg/ml human serum albumin.

**DISCUSSION**

In this report, we describe a method for resolving several plasma fractions that inhibit NaK-ATPase. The factors contained in these fractions resemble digitalis glycosides in that they are low molecular weight protease-resistant compounds that inhibit both isolated NaK-ATPase preparations and the Na⁺ pump in intact erythrocytes, displace [³H]ouabain from the enzyme, and cross-react with polyclonal digoxin-specific antibodies. The finding that all these factors were recognized by polyclonal anti-digoxin antibodies suggests that they have some degree of structural similarity to digoxin and consequently to each other.

Nevertheless, there are important differences between the compounds we have studied and the digitalis glycosides. Each of the enzyme-inhibitory fractions also enhanced ouabain-independent influx of ⁶²Rb⁺ into Na⁺-loaded erythrocytes to variable degrees (Table II). Digitalis glycosides do not stimulate or inhibit Ca-ATPase activity directly, but two of the fractions (EI1 and EI3) stimulated Ca-ATPase activity. More...
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While a higher concentration inhibited it (Table I). In addition to this lack of specificity for NaK-ATPase, the concentration-effect curves of these inhibitors in the [3H]ouabain binding assay (Fig. 8) are much steeper than that of ouabain. These steeper concentration-effect curves could indicate that some form of positive cooperativity was occurring during binding. This suggests that the inhibitors do not behave as simple competitive ligands for the cardiac glycoside binding site on NaK-ATPase. Other differences include the findings that NaK-ATPase inhibition by two of the fractions (EI, EL) was unaffected by potassium and that no fraction required ATP to promote binding to and inhibition of the enzyme or to inhibit [3H]ouabain binding to NaK-ATPase.

There are well-characterized sodium pump inhibitors in plasma that are neither specific for NaK-ATPase nor digitalis-like in their interaction with the enzyme. Vanadate inhibits phosphorylation of several phosphate-transferring enzymes including NaK-ATPase and Ca-ATPase (1, 11). It is unlikely that the inhibitory activity within any of the plasma fractions was vanadate for the following reasons. First, plasma was desalted before being fractionated on HPLC. Second, vanadium was not detected by atomic absorption spectrometry in any of the fractions with NaK-ATPase inhibitory activity. Finally, vanadate promotes, rather than inhibits, ouabain binding under the conditions of our [3H]ouabain displacement assay.

Aside from vanadate, other compounds such as palmitylcarnitine and arachidonic and linoleic acids can inhibit the sodium pump and reduce binding of labeled ouabain to NaK-ATPase (28, 29). We have found that these three lipid compounds do not cross-react with anti-digoxin antibodies (data not shown). Nevertheless, there are compounds in plasma that, if concentrated by the preparative procedures we used, might yield positive results in a digoxin radioimmunoassay. For example, high concentrations of ions could interfere nonspecifically with hapten-antibody binding. We sought to minimize these confounding variables by using desalted plasma extracts or HPLC fractions and performing the radioimmunoassays at neutral pH. It seems unlikely that nonspecific inhibition of hapten-antibody binding could explain our results because only peaks EI, and IR inhibited [125I]digoxin binding to the monoclonal digoxin antibody using an assay technique that was identical to that using polyclonal antibodies. In addition, the same fractions that inhibited binding of labeled digoxin to the polyclonal digoxin-specific antibodies did not inhibit hapten binding to highly specific polyclonal ouabain- or digitoxin-specific antibodies, at least at the concentrations tested. The markedly reduced cross-reactivity between these antibodies and digoxin (Fig. 7) and the finding that the HPLC fractions only cross-reacted with digoxin antibodies suggest that the compounds in the inhibitory fractions have a greater structural similarity to digoxin than to either ouabain or digitoxin.

The absence of immunoreactivity of the HPLC fractions in

### Table I

**Effects of fractions with NaK-ATPase inhibitory activity on Ca-ATPase**

The NaK-ATPase inhibitory peaks from two different phenylpropylsilane reverse phase HPLC separations were assayed in a Ca-ATPase inhibition assay. For separation I, all samples were performed in quintuplicate and for separation II, in quadruplicate. Each sample with NaK-ATPase inhibitory activity had a significant (p < 0.05) effect on Ca-ATPase activity.

| Peak Separation | Inhibition of NaK-ATPase activity | Ca-ATPase | Net activity | Per cent of control |
|-----------------|-----------------------------------|-----------|--------------|--------------------|
| Control I       | %                                 | \( \mu \text{mol P/min/mg} \) | 0.406 ± 0.004 | 100                |
| II              | %                                 | \( \mu \text{mol P/min/mg} \) | 0.398 ± 0.006 | 100                |
| EI1             | 100                               | 0.584 ± 0.007 | 144 ± 1.6    | 75 ± 2.3           |
| EI2             | 100                               | 0.331 ± 0.002 | 133 ± 0.5    | 75 ± 2.3           |
| EI1             | 75                                | 0.449 ± 0.007 | 113 ± 2.0    | 75 ± 2.3           |
| EI2             | 100                               | 0.433 ± 0.004 | 106 ± 1.1    | 75 ± 2.3           |
|                | 95                                | 0.422 ± 0.007 | 106 ± 2.0    | 75 ± 2.3           |

### Table II

**Effects of fractions with NaK-ATPase inhibitory activity on Na+ pump activity**

The effects of NaK-ATPase inhibitory activity from two different phenylpropylsilane reverse phase HPLC separations were tested for their ability to inhibit \(^{86}\)Rb\(^+\) influx into human red cells. All assays were performed in triplicate. The media contained 140 mM NaCl, 4 mM K\(^+\), 1 mM MgCl\(_2\), 10 mM Tris-MOPS, pH 7.4, at 37°C and 100 \(\mu\)Ci \(^{86}\)Rb\(^+\). The amount of the inhibitor used in each assay is presented as per cent of inhibition of NaK-ATPase activity as measured in the enzyme inhibition assay (see "Materials and Methods"). Higher concentrations of peaks from EI1 and EI2 caused cell lysis.

| Peak Separation | Inhibition of NaK-ATPase activity | \(^{86}\)Rb\(^+\) influx | Oubain sensitive |
|-----------------|-----------------------------------|------------------------|-----------------|
| Control         | %                                 | mmol/L cells/h | Total | 10\(^{-4}\) M ouabain | 10\(^{-4}\) M ouabain + 10\(^{-4}\) M furosemide | Ouabain sensitive |
| Experiment 1    | 2.30 ± 0.06                       | 0.45 ± 0.07 | 1.85 ± 0.09 |
| Experiment 2    | 2.22 ± 0.04                       | 0.43 ± 0.01 | 1.79 ± 0.04 |
| EI1             | 90                                | 1.61 ± 0.04 | 3.95 ± 0.06 | 0.64 ± 0.07\(^a\) | 65.4 ± 2.8 |
| Experiment 1    | 10                                | 2.72 ± 0.03 | 0.52 ± 0.01 | 2.10 ± 0.03\(^a\) | -3.5 ± 1.6 |
| Experiment 2    | 50                                | 1.98 ± 0.07 | 0.37 ± 0.01 | 0.30 ± 0.01 | 1.61 ± 0.07\(^a\) | 10.1 ± 3.9 |
| EI2             | 25                                | 2.19 ± 0.01 | 0.51 ± 0.01 | 1.69 ± 0.01\(^a\) | 5.6 ± 0.6 |
| Experiment 1    | 10                                | 2.48 ± 0.10 | 0.72 ± 0.01 | 1.76 ± 0.10 | 4.9 ± 0.4 |
| Experiment 2    | 50                                | 1.85 ± 0.15 | 0.59 ± 0.01 | 0.73 ± 0.01 | 1.27 ± 0.02\(^a\) | 29.1 ± 1.1 |
| EI1             | 50                                | 2.48 ± 0.04 | 1.07 ± 0.02 | 1.42 ± 0.05\(^a\) | 23.2 ± 2.7 |
| Experiment 2    | 10                                | 2.32 ± 0.02 | 0.54 ± 0.01 | 0.29 ± 0.01 | 1.78 ± 0.02 | 0.6 ± 1.1 |

\(^a\) p < 0.001 compared to ouabain-sensitive flux in the absence of the NaK-ATPase inhibitors.

\(^b\) p < 0.05 compared to ouabain-sensitive flux in the absence of the NaK-ATPase inhibitors.
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FIG. 10. The effect of varying the concentrations of potassium and ATP on the NaK-ATPase inhibitory activity of the fractions from phenylpropylsilane HPLC. To contrast the inhibitory activity of each of the peaks shown in Fig. 5 with that of ouabain, aliquots from peaks E1 (upper right panel), E11 (lower left panel), and E13 (lower right panel) and increasing concentrations of ouabain (upper left panel) were added to the NaK-ATPase inhibition assay (see "Materials and Methods"). The buffer contained 100 mM Na+, 50 mM Tris (pH 7.4), 0.25 mM Na₂EDTA, 5 mM MgCl₂, 0.6 mg/ml canine kidney cortex NaK-ATPase, and 0 mM KCl and 5 mM ATP (O---O) or 5 mM KCl and 5 mM ATP (-----O), or 0 mM KCl and 0 mM ATP (O---O). Only the inhibitory activity of peak E13 was reduced by potassium, and none of the peaks required ATP to inhibit the enzyme. Similar results were obtained in the [³H]ouabain binding inhibition assay (data not shown).

The ouabain or digitoxin radioimmunoassays might be due in part to the lower sensitivity of these assays for ouabain or digitoxin, compared to the sensitivity of the digoxin radioimmunoassays for digoxin. Since the use of a high specific activity iodinated derivative of digoxin was responsible for this increased sensitivity, we also performed the radioimmunoassay with [¹H]digoxin and sheep polyclonal anti-digoxin antibodies. In this assay also, there was definite, although limited, cross-reactivity of aliquots from HPLC fractions IR1, E11, and E12 despite a much reduced sensitivity for digoxin itself (data not shown).

Apparently, deproteinization of plasma by boiling must release NaK-ATPase inhibitory factor(s) that are bound to plasma proteins in vivo. Protein binding could limit the access of these compounds to NaK-ATPase, but this may be necessary to assure that the unbound level in plasma of a potent sodium pump inhibitor(s) remains low because of the ubiquity and biological importance of NaK-ATPase. On the other hand, protein-bound sodium pump inhibitors could become physiologically important in conditions in which plasma protein binding is reduced, such as uremia. This would account for the increased plasma NaK-ATPase inhibitory activity and digoxin-like immunoreactivity that is reported to occur in patients with chronic renal failure (6, 14). Finally, these compounds may be physiologically important chiefly in those tissues in which they are produced; in this case, their presence in plasma would represent a spillover. This would be analogous to norepinephrine since plasma norepinephrine levels reflect net release of this neurotransmitter from all peripheral sympathetic nerve terminals and do not identify a specific site of production.

The chemical nature of these compounds is unknown, but our data on protease and acid sensitivity suggest that they are not polypeptides. Several investigators have reported that certain fatty acids, including arachidonic acid as well as some prostaglandins, can inhibit NaK-ATPase and displace [¹H]ouabain from the enzyme (28–32). These unsaturated long-chain fatty acids may not interact directly with the digitalis
binding site but could interact with membrane compounds and either facilitate or restrict the conformational changes that NaK-ATPase must undergo during transport of cations from one side of the lipid bilayer to the other (29). In fact, fatty acids can stimulate Ca-ATPase activity even in calmodulin-deficient membranes (33). This could explain why low concentrations of phosphatidylserine stimulate the NaK-ATPase activity of phospholipase- or detergent-treated membranes, while higher concentrations of the phospholipid inhibit the enzyme (29). Ahmed and Thomas (28) have reported that the reversible inhibition of NaK-ATPase seen during incubation with micromolar concentrations of several unsaturated fatty acids is reduced by adding K+ to the media. In their studies, varying the concentration of ATP did not influence the NaK-ATPase inhibitory activity of the fatty acids. These observations are similar to the results we obtained with the Ela peak.

Our results explain certain controversies regarding the existence of endogenous digitalis-like factors. We have shown that factors with digoxin-like immunoreactivity can be isolated from normal human plasma, but that these substances exhibit variable affinities for different anti-digitalis antibody populations. Moreover, some, but not all, of these immunoreactive substances co-elute with factors that inhibit NaK-ATPase activity. This could explain the reported lack of a relationship between levels of digoxin-like immunoreactivity and NaK-ATPase inhibitory activity in unfractionated plasma (3). In addition, the presence of multiple NaK-ATPase inhibitors in plasma will complicate the interpretation of how endogenous sodium pump inhibitory activity in unfractionated plasma is related to the level of blood pressure.

Whether or not the factors described in this report are biologically important sodium pump inhibitors or are true ligands for the cardiac glycoside binding site of NaK-ATPase has not been established. Nevertheless, these data provide evidence that compounds within human plasma have many properties of a putative endogenous digitalis-like substance.

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