Purification and Characterization of Digitalislike Factors from Human Plasma

JOHN M. HAMLYN, JANET A. SCHENDEN, JUANA ZYREN, AND LUBOMIR BACZYNSKYJ

SUMMARY Increased levels of a humoral inhibitor of active sodium transport have been associated with the response to acute and chronic hypervolemia and various forms of experimental as well as human essential hypertension. In this report, we describe the purification of inhibitors of Na⁺,K⁺-adenosine triphosphatase (ATPase) from the plasma of volume-expanded individuals. Of the two amphipathic materials obtained, only one of the factors when present in high concentrations showed the slow time-dependent component of inactivation similar to that of the cardiac glycosides. Inhibition was reduced in the presence of plasma proteins and was freely reversible. Both factors inhibited potassium-dependent p-nitrophenylphosphatase activity and specific [³H]ouabain binding in a manner similar to the cardiac glycosides. In contrast to ouabain and vanadate, however, high concentrations of potassium or norepinephrine, respectively, did not affect the magnitude or kinetic characteristics of inhibition. Structural analysis by mass spectroscopy showed a mass of 444 for factor 1, whereas factor 2 was conclusively identified as lysophosphatidylcholine-γ-palmitoyl. These factors probably inhibit Na⁺,K⁺-ATPase by a nonspecific mechanism involving reversible perturbation of lipid-enzyme interactions required for normal catalytic activity. The significance of these factors as modulators of sodium transport may be limited to pathological states associated with abnormalities in plasma protein binding or lipid metabolism. They do not appear to be directly related to the humorally mediated disturbance of cellular sodium transport in hypertension. (Hypertension 10 [Suppl I]: I-71-I-77, 1987)

KEY WORDS • humoral inhibitors • digitalislike material

INTEREST in the mechanisms involved in human essential hypertension has been stimulated repeatedly by numerous observations of abnormal cellular sodium homeostasis. While the exact nature of the abnormality has remained obscure, two general mechanistic possibilities are an intrinsic alteration of membrane structure and function, and an acquired functional defect mediated by humoral agents. A growing body of evidence supports the latter mechanism in association with an endogenous digitalislike factor. The plasma levels of this agent are increased in states of acute and chronic hypervolemia, in various animal models of hypertension, and in human essential hypertension, and the existence of this factor constitutes a key link in a hypothesis that relates interactive changes in cellular ion homeostasis with gross cardiovascular function in hypertension.

Additional precedent for the existence of such a factor is supported by the presence on the sodium pump of a conserved binding site of high affinity for a number of cardiotonic steroids. An outline of the converging ideas relating various aspects of the cardiac glycosides to the search for their endogenous counterparts has been presented elsewhere. The chemical nature of mammalian digitalis is under intensive study in many laboratories. In many instances, the same techniques used clinically for estimating the cardiac glycosides, such as assays for adenosine triphosphatase (ATPase) activity, ouabain binding, sodium pump activity, and specific radioimmunoassays, are currently employed to probe for the mammalian compound.

In this report we describe the purification, characterization, and identification of two putative digitalislike
materials obtained from the plasma of normotensive humans after acute expansion of blood volume. Both materials encouraged structural changes in the lipid environment of the sodium pump, and they may affect cellular ion metabolism by promoting changes in membrane structure and function relatively nonspecifically. One of these materials has been identified as a lysophospholipid. These volume-sensitive materials seem unlikely to be involved in the abnormal cellular ion metabolism seen in essential hypertension, but they may play a role in other pathological states. A preliminary account of this work has been published.15

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**Materials and Methods**

**Plasma Source**

Plasma was obtained by venipuncture of normotensive individuals aged 40 to 60 years after infusion of 500 ml of isotonic saline over 30 minutes. Plasma was stored frozen at −70°C under sterile conditions until used. Aliquots (250-ml batches collected on four different occasions) were obtained from each individual. No individuals had received cardiac glycosides or other medications.

**Reverse Phase Chromatography of Plasma Extracts**

One-liter amounts of plasma were dialysed for 12 to 16 hours against 10 volumes of 10 mM NH₄Ac, pH 6.8, using hollow fiber cartridges (Amicon, Danvers, MA, USA) with a molecular weight exclusion of 10,000. The dialysate was lyophilized, extracted with methanol, and dried under N₂. The residue was dissolved in 2.5 ml of water. Aliquots were typically chromatographed using 5-μm octadecylsilane columns (Altex, Beckman Instruments, San Ramon, CA, USA) in an CH₃CN/H₂O system at a flow rate of 0.2 ml/min. Absorbance at 254 nm was continuously monitored. Fractions of 500 μl were collected into silanized tubes and were dried under N₂, reconstituted in 500 μl water, and assayed for the presence of inhibitors of Na⁺,K⁺-ATPase by the method of Fujita et al.18

**Preparation of Na⁺,K⁺-ATPase**

Enzyme was prepared from the outer medulla of dog kidney by modification of published procedures.16 Briefly, microsomal membranes were obtained by differential centrifugation of a Teflon pestle homogenate. Membranes were extracted twice with 0.1% (wt/vol) sodium deoxycholate and dialysed for 24 hours at 0 to 4°C. The dialysed material was lyophilized in small individual aliquots and stored at −70°C.

**Assays for Determination of Na⁺,K⁺-ATPase and p-Nitrophenylphosphatase**

Enzyme activity was determined according to procedures published elsewhere,8 with the following modifications: 100 mM N-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid (TES-Tris), pH 7.4, 5 U lactate dehydrogenase, 5 U pyruvate kinase, 1.2 mM phosphoenolpyruvate tricyclohexylammonium, and 0.3 mM reduced nicotinamide adenine dinucleotide (NADH). The maximal activity of the adenosine 5'-triphosphate (ATP)-regenerating linked enzyme system was more than 250-fold the Na⁺,K⁺-ATPase activity. Assay was initiated by adding 2 to 3 μg of enzyme, and the decrease in absorbance at 340 nm due to NADH oxidation was continuously recorded for appropriate periods of time. One unit of inhibitor is defined as the amount of material required to inhibit 2 to 3 μg (0.6–1.0 pmol [¹H]ouabain-binding sites) of partially purified Na⁺, K⁺-ATPase by 50% under the assay conditions described above. In all cases, an action of inhibitory fractions from column chromatographic procedures on the ATP-regenerating system itself was excluded by inspection of the rate of NADH oxidation after the addition of 10 μM of adenosine 5'-diphosphate (ADP). Protein was estimated by dye binding17 using dried bovine albumin as standard. Approximately 98% of the total ATPase activity was inhibitable by ouabain. Typical Na⁺,K⁺-ATPase activities were 5 to 8 μmol/min/mg.

[¹H]Ouabain-Binding Studies

Fractions obtained from column chromatographic procedures were tested for ability to inhibit [¹H]ouabain binding to Na⁺, K⁺-ATPase. For specific binding, 10 μg of enzyme protein and various fractions from the column chromatographic procedures were incubated in 0.35 ml of a medium containing 0.5 mM EDTA and 10 mM TES-Tris, pH 7.4 for 1 hour at 23°C. Subsequently, 6.65 ml of a solution containing 10 mM MgCl₂, 1 mM Na₂HPO₄, 1 mM HEPES-Tris, pH 7.4, 1 X 10⁻⁷M ouabain (containing 0.62 μCi G-[¹H]ouabain, Amersham, Arlington Heights, IL, USA) was added, and incubation was continued for 2 hours at 37°C. Incubations were quenched by cooling to 4°C, rapidly filtered through 0.45-μm glass fiber filters and washed with ice-cold buffer solution lacking ouabain. Trapped counts were solubilized by soaking filters in 0.5 ml 10% (wt/vol) sodium dodecyl sulfate overnight followed by scintillation counting. In each case, compensation was made for the radiochemical purity of ouabain as determined by thin-layer chromatography. Nonspecific binding was estimated by incubation of excess unlabeled ouabain (10⁻⁴ M) in the original incubation medium. Nonspecific binding was typically less than 1% of the total counts that were bound.

**Results**

**Reverse Phase Chromatography**

Reverse phase chromatography of a partially purified methanol extract of a lyophilized dialysate of plasma is shown in Figure 1A. Several major peaks of ultraviolet-absorbing material were eluted from the column by the initial mobile phase. At 60 minutes the acetonitrile content of the mobile phase was rapidly increased over 12.5 minutes to 100%. The first peak of
Characterization of Inhibitory Actions of Peak 1 and 2 Materials

The active fractions contained only trace amounts (<20 μM each) of K⁺, Na⁺, Ca²⁺, and Mg²⁺ as determined by flame and atomic absorption spectroscopy. These materials were further characterized in a number of different assays. Three different functional properties of the Na⁺,K⁺-ATPase were investigated: 1) ability to inhibit Na⁺,K⁺-ATPase activity, 2) ability to inhibit p-nitrophenylphosphatase (p-NPPase) activity, and 3) ability to inhibit specific binding of radiolabeled ouabain to the enzyme. The effects of standard quantities of Peaks 1 and 2 are shown contrasted with ouabain in each of the three assay systems in Figure 2. The column fractions were found to be equipotent in all three assay systems, whereas the same assays showed the distinct and well-known differential sensitivity to ouabain. For instance, binding was reduced 50% by 10 nM unlabelled ouabain in Figure 2C, whereas 10 and 2 μM were required to inhibit p-NPPase (see Figure 2B) and Na⁺,K⁺-ATPase (see Figure 2A), respectively. These data offered the first evidence that the mechanism of inhibition of Na⁺,K⁺-ATPase by these column fractions was not fully ouabain-like.

The sensitivity of inhibition by ouabain and vanadate to alteration of various assay conditions was compared to results obtained with the two chromatographic fractions. The results are illustrated in Figure 3. Inclusion of increased amounts of potassium in the assay greatly antagonized the inhibition produced by 1 μM ouabain and potentiated the inhibition produced by 1 μM vanadate. In contrast, raising the potassium content had little effect on the inhibition produced by Peak 1 or 2 material. Furthermore, inclusion of norepinephrine almost completely reversed the inhibition by vanadate, whereas no effect on inhibitory potency of ouabain or the column fractions was seen. These criteria offered preliminary evidence that neither of these inhibitory plasma factors behaves like a cardiac glycoside or vanadate.

The ability of various preincubation conditions to affect the magnitude of inhibition of Na⁺,K⁺-ATPase with the same column fractions is presented in Table 1. In contrast to ouabain, phosphorylating (Na⁺ + ATP + Mg²⁺) conditions had little effect whereas dephosphorylating conditions (K⁺) showed a small degree of antagonism confined to Peak 2 material. The lack of response to Mg²⁺ also suggests that these plasma-derived materials are different from the Na⁺,K⁺-ATPase inhibitor from bovine hypothalamus.19, 20

Kinetic Aspects of Inhibition

The two inhibitors showed characteristically different behavior in the coupled optical assay. Peak 1 material rapidly inhibited the Na⁺,K⁺-ATPase, achieving pseudosteady-state levels within 5 to 10 seconds of enzyme addition (Figure 4A). Peak 2 material showed two inhibitory components (see Figure 4B). At low doses, inhibition rapidly reached steady state, whereas higher concentrations were associated with the appearance of an additional slow phase of inhibition.

Absorbance eluting with a characteristic retention time of 87 to 90 minutes was found to contain the Na⁺,K⁺-ATPase inhibitory material. The inhibitory fractions were subsequently rechromatographed using a different gradient program (see Figure 1B). Two major peaks (1 and 2) of inhibitory activity were obtained with retention times of 50 and 70 minutes. Peak 1 material was associated with three small UV peaks, whereas Peak 2 material showed little or no apparent absorption at 254 nm.
FIGURE 2. Characterization of Peaks 1 and 2 inhibitory fractions from reverse phase chromatography: comparison with ouabain. All assays were performed under identical conditions with respect to enzyme protein and amounts of inhibitors. Means ± SE of four experiments are shown. A. Inhibition of Na\(^+\),K\(^+\)-ATPase activity. B. Inhibition of p-nitrophenylphosphatase (p-NPPase) activity. C. Inhibition of radiolabeled ouabain binding to Na\(^+\),K\(^+\)-ATPase. The ouabain concentrations refer to unlabeled ouabain.

FIGURE 3. Effects of potassium ion (K\(^+\)) and norepinephrine (NE) on Peaks 1 and 2 from reverse phase chromatography: comparison with vanadate and ouabain. In each case, normalized activity has been presented with respect to the appropriate controls lacking inhibitors. The designation C refers to the presence of inhibitor in the absence of other modifying factors (i.e., K\(^+\) or NE). K\(^+\) = 100 mM, NE = 0.5 mM.

TABLE 1. Effects of Preincubation Conditions on Inhibition by Plasma Factors: Comparison with Ouabain

<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Ouabain (10(^{-5}) M)</th>
<th>Peak 1 (1 U)</th>
<th>Peak 2 (1 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ K(^+)</td>
<td>20</td>
<td>51</td>
<td>34</td>
</tr>
<tr>
<td>+ Mg(^{2+})</td>
<td>86</td>
<td>56</td>
<td>48</td>
</tr>
<tr>
<td>+ Na(^+)</td>
<td>30</td>
<td>53</td>
<td>51</td>
</tr>
<tr>
<td>+ Na(^+), ATP</td>
<td>4</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td>+ Na(^+), ATP, Mg(^{2+})</td>
<td>100</td>
<td>55</td>
<td>41</td>
</tr>
</tbody>
</table>

Na\(^+\),K\(^+\)-ATPase (5 \(\mu\)g) was incubated in 950 \(\mu\)l of buffer containing 0.5 mM EGTA, 50 mM TES-Tris, pH 7.4, at 37°C for 30 minutes in the presence of the inhibitors above. Final concentrations of the preincubation substrates were (in mM): K\(^+\), 20; Mg\(^{2+}\), 5; Na\(^+\), 100; ATP-Tris, 3. After preincubation, 50 \(\mu\)l of 20× concentrated reaction medium used in the coupled optical assay was added and the reaction monitored spectrophotometrically for 2 minutes. Results represent the means of four to five individual experiments.

Dose-Response Relationships

The dose-inhibition relationships for Peak 1 and 2 materials are illustrated in Figure 5. In contrast to ouabain, the slope of the response for both chromatographic fractions was much steeper with Hill coefficients of greater than 3 for Peak 1 and greater than 1.7 for Peak 2 as compared with 0.9 for ouabain (Hill plots not shown). These data indicate the presence of several binding sites for the plasma factors on Na\(^+\),K\(^+\)-ATPase. The IC\(_{50}\) for ouabain was 1 to 2 \(\mu\)M and for Peak 2 was approximately 6 \(\mu\)M based on a molecular weight of 500. Similarly, the IC\(_{50}\) for Peak 1 material appeared to be about 10 \(\mu\)M based on a molecular weight of 450. The Ca\(^{2+}\)-ATPase prepared from rabbit skeletal muscle sarcoplasmic reticulum\(^{21}\) was inhibited...
SODIUM PUMP INHIBITORS IN PLASMA/Hamlyn et al.

1.5—1
1.3—
1.1—
0.9—
0.7—

> 4
minutes

FIGURE 4. Characteristic inactivation kinetics of the Na⁺,K⁺-ATPase by inhibitors obtained from reverse phase chromatography. The inactivation kinetics for Peak 1 and 2 materials are shown in A and B, respectively. In each case the reaction was initiated by addition of enzyme at zero time. The numbers adjacent to the traces represent the dose used in microliters. The dashed line represents results obtained in the presence of strophanthidin (10⁻³ M).

50% by Peak 2 at calculated concentrations greater than 250 μM. Thus, Peak 2 is a relatively specific inhibitor of Na⁺,K⁺-ATPase as compared to the sarcoplasmic reticulum Ca²⁺-ATPase. Peak 1 material was not tested for specificity due to insufficient quantity of material remaining after mass spectroscopy.

The total extractable plasma content of Peaks 1 and 2 were estimated to be 5000 and 6000 U/L, respectively, using direct water/methanol extractions of intact plasma. Relative amounts were 450 and 560 U/L, respectively, when dialysates were used, suggesting extensive binding to components unable to penetrate the dialysis membrane. In support of this contention, inclusion of fatty acid–free bovine serum albumin in the coupled optical assay (0.5 mg/ml) with 10 U of either Peak 1 or 2 material reduced inhibition to insignificant levels. These results indicate the capacity (at least 100-fold reduction of free concentration) for extensive protein binding of these particular inhibitors.

Reversibility of Inhibition

Table 2 summarizes results from reversibility studies. Preincubation of Na⁺,K⁺-ATPase with the indicated inhibitors in the doses shown produced complete inhibition of enzyme activity. When the inhibited enzyme was diluted 100-fold into the coupled optical assay, both the vanadate- and ouabain-pretreated enzyme remained completely inhibited for at least 3 to 5 minutes, whereas the enzyme preincubated with Peak 1 and 2 materials showed greater than 90% recovery of activity within the first 5 to 10 seconds. Thus the inhibition associated with these plasma-derived materials is rapidly reversible, in contrast to both ouabain and vanadate.

FIGURE 5. Dose-inhibition curves for ouabain (○), Peak 1 material (▲), Peak 2 material (●), and authentic lysophosphatidyl cholined-γ-palmitoyl (●). The dose scale for Peak 1 is presented in microliters and located arbitrarily on the x axis.
Inhibitors were preincubated with 30 μg of enzyme in 1.0 ml of reaction buffer containing 3 mM ATP, 5 mM MgSO₄, 100 mM NaCl, and 40 mM TES-Tris, pH 7.4, at 37°C for 30 minutes. Aliquots of 10 μl were then diluted (100-fold) into the coupled optical assay containing the appropriate inhibitor at the same concentration as during preincubation conditions or into nominally inhibitor-free assay.

### Structural Analyses

Fast atom bombardment mass spectrometry of Peak 1 material revealed one major mass of 444. This material could not be identified conclusively. The Peak 2 material was conclusively identified as lysophosphatidyl choline-γ-palmitoyl (LPC). All aspects of the chromatographic and inhibitory actions of the purified material identified by mass spectrometry were identical to commercial LPC.

### Discussion

This paper describes the isolation of two putative digitalislike materials from the plasma of volume-expanded individuals. These materials were of low molecular weight and freely passed through sieving membranes with molecular weight exclusion in the range of 10,000.

Both materials had some properties and actions characteristic of the cardiotonic steroids in that they inhibited the specific binding of [⁹⁻H]ouabain to ATPase, as well as ATPase and p-NPPase activities (see Figure 3). In spite of these similarities with the cardiac glycosides, however, a more detailed investigation showed that these factors were 1) essentially unaffected by alterations of assay conditions known to modify the inhibitory efficacy of ouabain and vanadate (see Table 1) and thus appear nonselective of either major conformational state (E₁/E₂) of Na⁺,K⁺-ATPase, 2) characterized by the rapid development of a freely reversible inhibition at low doses (see Figure 4, Table 2), and 3) in other studies (not shown) found to increase the dissociation of [⁹⁻H]ouabain from a preformed enzyme-ouabain complex. These actions suggest that these plasma factors are not fully digitalislike and probably do not interact directly with the ouabain-binding site.

The amount of inhibitory activity present in extracts of plasma was very high when compared to a digitalizing dose of the cardiac glycosides. The total plasma contents of Peak 1 and 2 materials were 5000 and 6000 U of inhibitory activity per liter respectively, whereas a digitalizing dose of ouabain would have been expected to increase the plasma potency by no more than 10 U/L. However, less than 1% of the plasma inhibitors we have isolated appear unbound in normal plasma due to interaction with plasma proteins. While the free concentrations of these inhibitors in vivo are unknown, under normal conditions they may be too low to play a major role in influencing sodium transport directly. They may be more important in pathological conditions associated with ischemia, abnormal lipid metabolism, or reduced plasma protein binding.

Both inhibitors obtained by reverse phase chromatography had amphipathic properties, and this was confirmed by the identification of the second material as a lysophospholipid. These amphipathic materials most likely inhibit Na⁺,K⁺-ATPase by virtue of their detergentlike properties. Under normal conditions, these agents probably do not modulate the activity of membrane transport proteins directly. They may play an indirect role by encouraging lipid phase transitions that lead to perturbations of lipid-protein interactions. The latter are considered important for the activity of various membrane-bound enzyme systems, including those involved in vascular smooth muscle contraction and ion transport systems.

Several laboratories are presently engaged in the search for endogenous inhibitors of Na⁺,K⁺-ATPase. These factors have been described in human plasma, urine, cerebrospinal fluid, and bovine brain. In most instances the preparative and assay methods are laboratory-specific, so that it is difficult to compare the factors characterized by bioassays and enzyme or transport techniques. At present, none of the mammal-derived factors described are completely digitalislike as determined by characteristic interactions with the sodium pump. Furthermore, the emphasis on whether a substance is digitalislike may be of secondary importance. As an example, the plasma from hypertensive dogs is equipotent in inhibiting the sodium pumps in the rat tail or dog mesenteric arteries, even though the dog is approximately 500-fold more sensitive to the cardiac glycosides than the rat. Thus, species-specific sensitivity to the cardiac glycosides is apparently not shared by the endogenous inhibitor. In addition to species differences, sensitivity to the cardiac glycosides is greater in tissues enriched with a unique form of the sodium pump. The existence of a similar phenomenon for endogenous material is unknown but appears likely. Furthermore, the distribution of tissue sensitivities may be different than that for the cardiac glycosides, suggesting that the role of the endogenous factor might not be anticipated by knowledge of the action of the cardiac glycosides alone.

In summary, the plasma obtained from volume-expanded humans contains elevated levels of two amphipathic materials that share some actions that are characteristic of the digitalis glycosides. One material has been identified as a lysophospholipid. While it is unlikely that either entity represents the physiologically relevant regulator of cellular sodium transport present in hypertension or hypervolemia, the role of these agents in selected pathophysiological states...
manner by which these materials are related to changes in extracellular fluid volume deserve further study.

Acknowledgments

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