In previous reports, we described the isolation and characterization of an endogenous digitalislike factor (EDLF). In this report, we describe a unique combination of bioassay and large-scale purification methodology that made possible the purification of sufficient quantities of this inhibitor of Na⁺,K⁺-ATPase for structural analysis. Using an initial XAD-2 extraction and preparative high-performance liquid chromatography followed by a batch enzyme affinity extraction and two subsequent semipreparative chromatographic steps, 300 l of human plasma was processed, yielding 31 μg (53 nmol) of pure EDLF and representing purification on a dry weight basis in excess of 0.6 billionfold. Four divergent pieces of evidence, including chromatographic, mass spectrometric, immunoreactive, and binding characteristics, suggested that the EDLF purified in the present study was either ouabain or an isomer of ouabain. This material may represent a plasma-borne, naturally occurring, selective, high-affinity ligand for the digitalis binding site that may play a significant role in the modulation of the sodium pump and thereby cellular electrolyte homeostasis in humans.

In those previous reports, plasma was dialyzed, and the dialysate was subjected to six preparative and semipreparative high-performance liquid chromatography (HPLC) steps. This process resulted in the isolation of an apparently homogeneous EDLF, but the amount of material that could be amassed for structural analysis was limited by the amount of dialysate that could be applied with reproducible results to the initial preparative column as well as by the high cost in material required for the bioassay to track inhibitory activity at each step in the purification process. In the present purification scheme, an initial XAD-2 extraction was introduced so that larger amounts of plasma could be processed, and a batch affinity extraction step was used that allowed purification to homogeneity with only three chromatographic steps, hence three bioassays instead of six. This resulted in sufficient quantities of EDLF for structural analysis.

Methods

Plasma Collection

Approximately 300 l human plasma were obtained by plasmapheresis from approximately 75 donors.
irrespective of sex or blood pressure status. The patients had an assortment of peripheral neuropathies but had not taken cardiotonic steroids, and the majority had not received other medications immediately before becoming donors. The use of plasma for this work was approved by the Human Volunteer Research Committee of the University of Maryland, Baltimore. Typically, 2.5–3.5 l plasma was exchanged each visit, and the plasma was stored at −20°C for 1–6 months before use.

**Enzyme Affinity Extraction**

Plasma was divided into batches of 12–15 l, thawed, and dialyzed for 24 hours at 23°C against approximately 3 volumes of medium containing 10 mM ammonium acetate (pH 6.8) using cellulose acetate membranes (Spectrapor, Spectrum Medical Industries, Inc., Los Angeles). The exclusion limit of the membranes was a molecular weight of 6,000–8,000 Da. Using a gravity-fed system, the dialysate from 24–30 l plasma was passed over an Amberlite XAD-2 column (Aldrich Chemical Co., Milwaukee, Wis.) previously washed with 10 l water (bed volume, 3.5 l). After application, the column was washed with approximately 3 volumes (10 l) water and eluted with 1 volume (3.5 l) methanol. The eluate was dried under vacuum at 50°C, and the combined solids from two columns, that is, material from 48–60 l plasma, were reconstituted in water. Preparative HPLC of the reconstituted XAD-2 extract was performed on a PrepLC 500A system (Waters Chromatography Div., Millipore Corp., Milford, Mass.) using a 5.7x30 cm column packed with 15–20 μm Vydac C18 (Millipore). The column was preequilibrated with water containing 0.1% trifluoroacetic acid before the sample was applied. The column was washed for 60 minutes under preequilibration conditions and then eluted with acetonitrile containing 0.1% trifluoroacetic acid using a two-step linear gradient: 0–10% acetonitrile in 10 l water and eluted with 1 volume (3.5 l) methanol. The eluate was dried under vacuum at 50°C, and the combined solids from two columns, that is, material from 48–60 l plasma, were reconstituted in water. Preparative HPLC of the reconstituted XAD-2 extract was performed on a PrepLC 500A system (Waters Chromatography Div., Millipore Corp., Milford, Mass.) using a 5.7x30 cm column packed with 15–20 μm Vydac C18 (Millipore). The column was preequilibrated with water containing 0.1% trifluoroacetic acid before the sample was applied. The column was washed for 60 minutes under preequilibration conditions and then eluted with acetonitrile containing 0.1% trifluoroacetic acid using a two-step linear gradient: 0–10% acetonitrile in 10 l water and 10–30% over the next 50 minutes. The flow rate throughout was 50 ml/min, and 2-minute fractions were collected. Inhibitory materials eluting between 84 and 88 minutes (<20% acetonitrile) from two preparative runs were combined and subjected to batch enzyme affinity extraction.

**Enzyme Affinity Extraction**

Combined materials were incubated for 3 hours at 37°C in 420 ml buffer containing (mM) Tris-Cl 200 (pH 7.2), MgCl2 5, and Na3HPO4/Na2HPO4 5 to which an approximate twofold molar excess of partially purified lamb kidney Na+,K+-ATPase3 was added. Under such conditions, a variety of cardenolides bind to high-affinity binding sites on Na+,K+-ATPase.4 After incubation, the solution was centrifuged at 150,000g for 2 hours at 4°C to separate enzyme from the supernatant. The supernatant was lyophilized, and the solids were taken up in water and, after filtration through a 5-μm filter (Acrodisc, Gelman Sciences Inc., Ann Arbor, Mich.), were subjected to two sequential HPLC steps.

**Post Affinity Chromatography**

In the first post affinity extraction step, a Waters µbondapak phenyl semiprep column (7.8 mm×30 cm) was preequilibrated with water containing 0.1% heptafluorobutyric acid. Sample was applied, and the column was washed for 5 minutes under preequilibration conditions and then eluted with isopropanol containing 0.1% heptafluorobutyric acid using a two-step linear gradient: 0–10% isopropanol in 5 minutes and 10–30% over the next 50 minutes. The flow rate was 3 ml/min throughout, and 0.5-minute fractions were collected. Inhibitory materials eluting between 24 and 25.5 minutes (near 16% isopropanol) were pooled, dried, and taken to the next step. In the second post affinity extraction and final step, a C18 semiprep column (Beckman Instruments, Fullerton, Calif.) (10 mm×25 cm) was preequilibrated with water containing 0.1% trifluoroacetic acid. Sample was applied, and the column was washed for 5 minutes under preequilibration conditions and then eluted with acetonitrile containing 0.1% trifluoroacetic acid using a two-step linear gradient: 0–10% acetonitrile in 5 minutes and 10–30% over the next 50 minutes. The flow rate was 3 ml/min throughout, and 0.5-minute fractions were collected.

**Assay of Endogenous Digitalislike Factor**

Inhibitory activity was determined using modifications to methods described elsewhere.5 All chromatographic fractions were assayed using 150 μl of solution containing (mM) NaCl 150, RbCl 2 with tracer amounts of 86Rb (New England Nuclear, Boston), MgSO4 1, Na2HPO4 1, CaCl2 2, glucose 5, and HEPES-Tris 20 (pH 7.4) in microtiter plates. Incubation periods lasting 2 hours at 37°C were initiated by addition of washed human red blood cells (final hematocrit 16%) and quenched by adding 125 μl incubation medium to 400 μl ice-cold medium lacking 86Rb. The quenched samples were layered over an equal volume of silicone oil (specific gravity, 1.01–1.02) and centrifuged at 800g for 10 minutes at 4°C. Medium with free 86Rb layered on top of the oil was removed by aspiration, and 86Rb associated with the red blood cell pellet beneath the oil layer was determined by gamma spectrometry. Using these procedures, 56 fractions could be assayed routinely in triplicate in the absence and presence of 1 mM ouabain. The ouabain-sensitive component of uptake by the red blood cells was determined from total 86Rb uptake minus uptake in the presence of ouabain. In all instances, inhibition of ouabain-sensitive uptake was expressed as percent inhibition determined from
ouabain-sensitive uptake in respective control cells in the absence of added sample.

Column Calibration

The C18 semiprep column was calibrated for ouabain under the exact conditions of the final purification step by applying six varying amounts of ouabain ranging from 1.4 to 20.5 nmol. In this manner, a quasi-extinction coefficient for ouabain based on peak absorbance at 200 nm was determined to be approximately 30 nmol/OD unit, and the retention time of ouabain was 31.6 (±0.1) minutes.

Results

Purification of Endogenous Digitalislike Factor

Figure 1 shows results obtained with C18 preparative-scale, reversed-phase chromatography of XAD-2 extract of dialysate from 50 l plasma. A series of peaks with strong absorption at 220 nm and one major inhibitory peak (\(^{85}\)Rb uptake into human red blood cells) plus other smaller inhibitory peaks were usually apparent. In some other chromatographs, the small peaks relative to the major one were larger. In either event, material eluting between 84 and 88 minutes was selected for further purification, because inhibition associated with the major peak displayed time-dependent binding that appeared to be sensitive to extracellular potassium ions; that is, inhibition was digitalislike.

In the purification of EDLF, typically all of the inhibitory material eluted from the preparative column was taken directly to the batch enzyme affinity extraction step. In one instance, however, to assess the effectiveness of the affinity step, preparative column material equivalent to 5 l plasma before and after affinity extraction was chromatographed on the C18 semiprep column. Absorbance and inhibitory activity are shown in Figures 2A and 2B. In this particular example, 99% of the absorption at 220 nm was eliminated, whereas more than 50% of the inhibitory activity was retained.

Enzyme affinity-extracted material, when subjected to sequential chromatography as illustrated in Figures 3 and 4, yielded apparently homogeneous EDLF. Numerous peaks of 220 nm absorption were apparent with the phenyl column (Figure 3). Some contamination was undoubtedly a carryover from the preparative stage, whereas other was probably introduced by the buffer or enzyme preparation during the affinity extraction step. Inhibitory activity appeared as a single peak. However, discrimination at this point was not sufficient to determine if activity was or was not associated with a single absorbance peak. In contrast, inhibitory activity after additional chromatography of the material eluting between 24 and 25.5 minutes from the phenyl column on a C18 semiprep column (Figure 4) appeared to be associated with a single, symmetrical absorbance peak devoid of leading or trailing edges.

Yield of Purified Endogenous Digitalislike Factor

Three hundred liters of human plasma was processed, yielding 31 \(\mu\)g (53 nmol) of pure EDLF and representing purification on a dry weight basis in excess of 0.6 billionfold. Pure EDLF was quantitated using a quasi-extinction coefficient that was determined for EDLF and found to be, within experimental error, the same as that determined for ouabain, that is, approximately 30 nmol/OD. For EDLF, this was determined from previous estimates of the concentration of EDLF\(^{1,2}\) using enzyme titration techniques modified from Josephson and Cantley\(^5\) and peak absorbance at 220 nm in fractions from the final purification step. Based on total absorbance units remaining at selected points in the purification, more than 99% of the impurities were removed by the initial purification steps—that is, the dialysis and the XAD-2 and preparative
FIGURE 2. C$_{18}$ semipreparative-scale, reversed-phase chromatography before (panel A) and after (panel B) affinity extraction. In each case, material equivalent to 5 l plasma (PEQ) was applied to columns. Broken line in both panels shows linear acetonitrile gradient of 10% at 10 minutes to 30% at 60 minutes with which columns were developed. Flow rate was 3 ml/min; 0.5-minute fractions were collected. Inhibitory activity (inhibition of $^{86}$Rb uptake in human red blood cells) was determined using 2% (100 ml PEQ) of fractions.

columns—with the remaining approximately 1% of the impurities removed by the final steps—that is, the affinity extraction and the phenyl and C$_{18}$ columns. Overall yield of the purification scheme requires, at a minimum, correction for a theoretical 25% loss during dialysis and, in addition, further correction for the 40% loss of inhibitory activity during the affinity extractions (Figure 2). On a molar basis, this 40% loss of activity equates to a 61% loss of EDLF. Accordingly, yield was, at the most, 30%.

Properties of Purified Endogenous Digitalislike Factor

Purified EDLF was subjected to fast atom bombardment mass spectrometry and tested for cross-reactivity with rabbit antibodies to ouabain. Over the mass range m/z 100 to m/z 2,500 Da, only one peak was apparent, a protonated molecular ion at m/z 585.2, in the biologically active fraction that was absent in the fraction immediately preceding or following. A detailed description of an analysis of EDLF by fast atom bombardment mass spectrometry is provided elsewhere. Analysis of EDLF using antibodies to ouabain revealed a high degree of cross-reactivity, a displacement curve parallel with ouabain, and tightly linked biological (inhibition of rubidium uptake) and immunologic activity at all steps in the purification sequence. A detailed description of the interaction of EDLF with antibodies to ouabain also is provided elsewhere.

Discussion

Recently, there has been a resurgence of interest in circulating inhibitors of the sodium pump (i.e.,

FIGURE 3. Phenyl semipreparative-scale, reversed-phase chromatography of affinity-extracted sample. Material equivalent to 99 l plasma (PEQ) was applied to column. Broken line (top panel) shows linear isopropanol gradient of 10% at 10 minutes to 30% at 60 minutes with which column was developed. Flow rate was 3 ml/min; 0.5-minute fractions were collected. Inhibitory activity (inhibition of $^{86}$Rb uptake in human red blood cells) was determined using 0.12% (120 ml PEQ) of fractions.
FIGURE 4. Final purification step of $C_18$ semipreparative-scale, reversed-phase chromatography. Material equivalent to 99 l plasma (PEQ) was applied to column. Broken line (top panel) shows linear acetonitrile gradient of 10% at 10 minutes to 30% at 60 minutes with which column was developed. Flow rate was 3 ml/min; 0.5-minute fractions were collected. Inhibitory activity (inhibition of $^{86}$Rb uptake in human red blood cells) was determined using 0.12% (120 ml PEQ) of fractions.

EDLFs). One reason for this renewed interest is recent evidence linking circulating sodium pump inhibitors to essential hypertension in humans. These factors may or may not (see reviews by de Wardener and Clarkson and Wechter and Benefin) be the same as those postulated to promote the natriuresis frequently associated with volume-expanded states. In any event, before now, structural information concerning endogenous sodium pump inhibitors was lacking. Two problems may be partly responsible for the previous lack of progress. One problem may be that the assays often used were highly susceptible to false-positive results. The Na$^+$ pump (i.e., Na$^+$,K$^+$-ATPase) is a very complex system with multiple substrates (such as Na$^+$, K$^+$, Mg$^{2+}$, ATP), and accordingly, slight changes in assay conditions can produce artifactual inhibition or stimulation of the Na$^+$ pump. In both previous and present purification efforts, we used a bioassay (i.e., $^{86}$Rb uptake in human red blood cells) throughout as opposed to biochemical techniques favored by others. This assay seemed to be less susceptible to nonspecific effects and allowed us to eliminate many nonspecific pump inhibitors and to focus on substances more likely to be physiologically relevant. In addition, present modifications of this assay allowed us to assay all chromatographic fractions from a given run simultaneous, thereby eliminating any variability introduced by the need for multiple assays on a particular run. Another problem may be that insufficient starting material frequently was used, such that the probability of obtaining sufficient quantities of pure material for structural analysis was negligible. In the present work, methods were modified to process hundreds of liters of plasma.

Although the present purification scheme and the one used previously have some common steps, important differences do exist, namely, introduction of XAD-2 chromatography and the enzyme affinity step. In the previous scheme, purification was not limited to any particular type of material. In contrast, in the present scheme, the affinity step excluded all materials except those that bound specifically to Na$^+$,K$^+$-ATPase under the reaction conditions known to support high-affinity binding of cardenolides. In addition, only bound materials subsequently released from the enzyme under non-binding conditions were used for further purification. Although previous studies, binding studies in particular, suggested that the affinity of EDLF for the cardiac glycoside binding site under a variety of conditions may be greater than that of ouabain, differences between ouabain and EDLF are not apparent in more recent reports. In this context, it should be noted that the EDLF used in the earlier work was substantially less pure than that used in later studies, and the impurities may have interfered nonspecifically with EDLF binding to the cardiac glycoside binding site, thereby giving falsely high affinities. We believe that this is more likely than the possibility that the previous purification procedures and the present ones isolated different substances, because in other respects, the inhibitors purified by the past and present schemes appeared to be the same. That is, at each chromatographic step in the present purification scheme — the $C_{18}$ preparative column eluted with acetonitrile, the phenyl semiprep column eluted with isopropanol, and the $C_{18}$ semiprep column eluted with acetonitrile — retention times were identical to those found previously with comparable systems. In addition, inhibitory material from both schemes inhibited the ion transport and hydrolytic functions of the Na$^+$,K$^+$-ATPase with similar EC$_{50}$s. Thus, the XAD-2 chromatography and the enzyme affinity step were significant developments that improved both the selectivity of purification and the scale of the
process, thereby enabling sufficient quantities of inhibitor to be obtained for structural analysis.

To ensure that the source of the purified EDLF was the plasma, two control studies were conducted. In one, 25 l of water substituted for plasma was taken through the preparative HPLC step. No inhibitory activity (rubidium uptake into human red blood cells) was apparent. In the secondary study, semipreparative HPLC fractions minus those containing EDLF were combined and resubjected to the affinity extraction process, with no apparent introduction of inhibitory activity. Thus, components of the purification process (XAD-2, dialysis membranes, Na⁺,K⁺-ATPase) were excluded as the source of the purified EDLF.

Three hundred liters of human plasma was processed, yielding 53 nmol (31 µg) of pure EDLF. An estimate of the minimal plasma concentration for EDLF requires correction for a theoretical 25% loss during dialysis and, in addition, further correction for the 40% loss of inhibitory activity during the affinity extractions (Figure 2). On a molar basis, this 40% loss of activity equates to a 61% loss of EDLF. Thus, the final calculated minimal concentration is 0.60 nM. This is the average minimal concentration for the entire 300 l. Undoubtedly, individual donors had levels substantially higher and lower than the average. The potential physiological and pathophysiological significance of circulating concentrations of this level of a high-affinity inhibitor of the sodium pump as well as pertinent questions regarding the source of this EDLF have been addressed elsewhere.8–16

We have shown that plasma levels of EDLF determined by an enzyme-linked immunosorbant assay were lower than those estimated from yield of EDLF during purification.8 However, it should be emphasized that the measurements with the immunosorbent assay were conducted using plasma from euolemic, normotensive individuals, whereas the source of EDLF for purification was plasma from mildly volume-expanded patients irrespective of blood pressure status (i.e., most were normotensive, but some were hypertensive). Both volume expansion28 and hypertension16–18 may be associated with elevated levels of EDLF and, accordingly, may account for the apparent discrepancy in the estimates of circulating levels of EDLF.

At least four pieces of evidence suggest that the EDLF purified in the present study is either ouabain or an isomer of ouabain. First, in four different HPLC systems, EDLF and ouabain were found to have identical retention times or to comigrate. With the columns and conditions of the final two purification steps, ouabain had identical retention times as those observed for EDLF (data not shown). We also have shown that EDLF and ouabain comigrated on a different C₁₈ column eluted with acetone and on a cyclodextran column eluted with methanol/butanol.7 Second, fast atom bombardment mass spectrometry of EDLF revealed an exact mass of the protonated molecular ion of EDLF of 585.295 Da compared with the theoretical value of 585.291 Da for ouabain.7 Third, EDLF and ouabain were indistinguishable with a rabbit anti-ouabain antibody.8 Finally, binding of EDLF to Na⁺,K⁺-ATPase requires the same complement of ligands that supports the binding of ouabain to the Na⁺ pump.2 Until now, we have referred to this inhibitor of the sodium pump isolated from human plasma as EDLF. This general nomenclature was used because the inhibitor had many of the properties of the cardiac glycosides. More recent findings that this substance is chromatographically, biochemically, and immunologically similar to ouabain and has an exact mass identical to ouabain suggest that more specific nomenclature such as ouabainlike compound now should be used.

In summary, in this report we describe a unique combination of bioassay and large-scale purification methodology that made possible the purification of sufficient quantities of an inhibitor of Na⁺,K⁺-ATPase for structural analysis. We believe this is the first endogenous Na⁺ pump inhibitor of mammalian origin to be structurally determined and one of the first biologically active compounds present in subnanomolar concentrations to be purified and identified entirely from plasma as the source. It remains to be determined whether this material represents a naturally occurring ligand for the digitalis binding site that, by virtue of its presence in plasma and its selectivity and high affinity, plays a significant role in the modulation of the sodium pump and thereby cellular electrolyte homeostasis in humans.

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