The Structure of the Digitalislike and Natriuretic Factors Identified As Macrocyclic Derivatives of the Inorganic Carbon Suboxide

Franz KEREK

The Natriuretic and Endogenous DigitalisLike Factors (EDLFs) are disclosed to be cyclomeric and macro-ring closed derivatives of the inorganic carbon suboxide. The macrocyclic cyclohexamer with six carbon suboxide units has a molar mass of 408.2 Da, as previously been found for the EDLF of animal origin. The anhydrous cyclohexameric factor is lipophilic but is transformed into more hydrophilic derivatives by the stepwise addition of water. Based on the present findings, it appears that EDLFs exist in solution as an equilibrium mixture of lipophilic and hydrophilic forms and not as a single chemical substance. This structural assumption better accounts for the earlier observed highly anomalous properties of EDLFs. The simultaneously found higher molar mass (4,100 and 4,900 Da) macrocyclic carbon suboxide derivatives are tentatively identified as the Natriuretic factors. (Hypertens Res 2000; 23 Suppl: S33-S38)

Key Words: Na,K-ATPase, digitalislike factors, natriuretic factors, carbon suboxide

Introduction

Na⁺/K⁺-ATPase is a plasma membrane enzyme found in all higher eukaryotic cells that is responsible for the outward/inward pumping of the Na/K ions using ATP bond energy as driving force (1). Through its tissue- and isoform-specificity, this sodium pump controls essential cellular functions like cell volume, heat production, membrane potential, free Ca²⁺ concentrations, and pH values (2). Plenty of experimental data, as recently reviewed (3-6), confirm that the endogenous ligands of the sodium pump, called Endogenous DigitalisLike Factors (EDLFs), are ubiquitously present in animal tissues and fluids. Their role in natriuresis suggests that they even may function as natriuretic hormones (7). The significantly increased EDLF assay values in the urine or plasma of patients with hypertension, diabete, mellitus, renal failure or myocardial infarction as compared to healthy subjects, support the probable involvement of EDLFs in these pathologies (8). Despite tremendous research efforts, the structure of EDLFs has until recently been unknown, which has created some confusions with other substance classes, particularly with cardiac glycosides. Cardiac steroids of plant origin like ouabain or digoxin are strong and specific inhibitors (Kᵢ=10⁻⁶-10⁻⁸ M) of the sodium pump (6). This high affinity suggests cardiac glycosides as candidate physiological regulators of the sodium pump, but their endogenous presence is restricted to only a few plants and toads; in addition, their high toxicity in mammals excludes a priori such a ubiquitous function. Findings to date confirm only the first assumption of Szent-Györgyi (9), that cardiac steroids are substitutes for endogenous ligands and nothing more.

The 585-Da molar mass ion of ouabain found in earlier EDLF preparations (10, 11) provides the primary evidence of its presumed endogenous presence in animals. But in all studies where this 585-Da mass ion has been found, plant ouabain has been applied in parallel as a standard; thus possible contamination can not be excluded definitively. The strong tendency of ouabain to adhere to glass vessels by complexing with borate groups of the...
Based on the identical sodium pump-inhibiting properties, of a simple inorganic gas, the carbon suboxide (C₃O₂), identifying them to be not organic substances, but derivatives have been successful in elucidating their structures, identifying HPLC fractions were assayed for their biologic activity. Up until now, the primary cause of the failed structural elucidation. In our opinion, however, disclosure of the chemical structure has been hindered first of all by the highly anomalous properties of the EDLFs. Contrary to most organic substances, EDLFs gave neither sharp HPLC elution peaks with reproducible retention time values nor significant UV absorption and NMR spectra. If the preparative HPLC fractions were assayed for their biologic activity, the elution range of the active substances was unusually broad. The observation that EDLF elute from the reversed phase column both in the hydrophilic and in the lipophilic range has often been reported. Uncontrolled variations in the biological activity of EDLFs have often been noticed, but the cause of the changes remains unknown. The reported strong decrease in activity in response to storage at −70°C is highly unusual for organic compounds. The very low yields of preparation (i.e. 10 μg from 100 l urine) could be explained if the EDLFs were volatile, but organic substances with a molar mass of ca. 400 Da are not so.

The molar mass of the EDLF of animal origin is 408.2 Da (21, 22), and this value can be identified in some earlier published mass spectra of the EDLF (10, 23) although this was not specified at that time.

We have recently reported (24) isolating some new, very strong and specific inhibitors of the sodium pump from various plant and bacterial sources. Moreover, we have been successful in elucidating their structures, identifying them to be not organic substances, but derivatives of a simple inorganic gas, the carbon suboxide (C₃O₂). Based on the identical sodium pump-inhibiting properties, the same molar mass values, and the similar spectral and chemical properties, it was assumed that the earlier-identified EDLFs of animal origin belong to the same class of structured carbon suboxide derivatives.

**Materials and Methods**

The isolation and purification of the new sodium pump-inhibiting factors from plant or bacterial sources as been described previously (24). The yellowish-brown lipophilic raw product was further purified by semipreparative HPLC with an 250/10 mm Nucleosil 100-5 C-18 HD column from Macherey-Nagel (Düren-Germany), with the acetonitrile/water gradient increasing from 5 to 90% in 60 min. The purification procedure was repeated until the obtained symmetrical peak was homogenous in terms of its spectral characteristics and the biological activity. The purified “lipopholic factor,” which elutes from the RP-18 HD column at high acetonitrile concentrations, is soluble in organic solvents like alcohols or acetone. It was watersolubilized by treatment with diluted bases, producing the hydrophilic form of the active substance. The HPLC analyses were been performed on a Bio-Tek Kontron Kromasystem-2000 (Neufahrn-Germany) with a diodearray detector using 25/4 mm Nucleosil 300-5 C18 HD columns (Macherey-Nagel). For the LC-MS analyses 12.5/2 mm Nucleosil 100-5 C-18 columns and an acetonitrile/water 0.1% TFA gradient from 5-90% in 20 min were used. For the LC-MS measurements the HPLC with multilane detector was coupled to an API 150 MS device with ESI and heated nebulizer mass detectors from Perkin-Elmer-Sciex Biosystems (Weiterstadt-Germany).

The higher molar mass homologues were isolated from the concentrated aqueous solution through acetone or ethanol precipitation and purification with AMICON ultrafiltration membranes and Gel-Permeation Chromatography (GPC) performed with 60-cm PW-2000 and SW-3000 columns from Tosohaa (Stuttgart-Germany). By analytical GPC the tₚ values were compared to those of polyethylene glycol standards. Polyacrylamide-based gel electrophoresis (SDS-PAGE) was performed using Proteogel (National Diagnostics, Atlanta) with a 37.5: 1 acrylamide: bisacrylamide ratio, with the content of the gel being 15%. Rainbow™ low molecular weight protein markers (2,350-46,000 Da) of Amersham (Freiburg-Germany) were used as standards. The MALDI-TOF mass spectra were recorded with a Bruker Biflex T (Bremen-Germany) and dihydroxycinnamic acid being used as a matrix.

The natriuretic effect was assayed according to knock (25) by slow injection of the test solutions into the jugular vein of the rat after a stable baseline value was obtained. The rate of the sodium excretion measured for 6 consecutive 30 min collection periods is expressed in μE min⁻¹ units.
The Na+/K+-ATPase was prepared from the outer medulla of rabbit and rat kidneys (17) and the inhibitory activity was determined by the pyruvate kinase/lactate dehydrogenase assay (26). The lipophilic form was applied by the corresponding dilution of a 0.1-1.0 mg/ml stock solution in 1, 2-propanediol ACS (Sigma, St. Louis) while the hydrophilic substance was diluted directly with the standard medium (Tris-EDTA 1 mM pH=7.2, NaCl 100 mM; KCl 10 mM; MgCl₂ 5 mM, Imidazole 25 mM and Na₂ATP 1.5 mM, PEP 2 mM). Base line correction with the correspondingly diluted 1, 2-propanediol solution was applied accordingly.

Results and Discussion

The purified yellowish-brown lipophilic inhibitor was obtained from plant sources with yields of up to 0.001-0.040% from the dried raw material. By conversion of the lipophilic into the hydrophilic form, a significant change in the UV absorbance spectra could be observed, as the spectra became less intense and exhibited a continuous decrease from 200-400 nm with two very subtle shoulders at 220 and 260 nm. The FAB and ESI mass spectrometric analyses of the active factor revealed the presence of the 408.2-Da molar mass ion together with mass ions corresponding to the adducts with Na⁺ (431), K⁺ (447) as well as NaCl (466), KCl (482), and TFA (523). The MS obtained with a heated nebulizer detector shows signs of hydrated adducts of the 408.2-Da basic unit with anywhere from one to six water molecules added, giving mass ion values at 426, 444, 462, 480, 498, and 516 Da, respectively.

The LC-MS with a selected ion mass (SIM) detector at 409 Da shows that the molecular ion eluted as a sharp peak in the hydrophilic range and as a broad one in the lipophilic region. Similar observations have been made if the Na⁺ adduct (431 Da) of the molar mass ion is selected for detection.

The hydrophilic factor very efficiently inhibits the Na⁺/K⁺-ATPase both from rabbit and rat medulla. Its inhibition constant value of 1.4×10⁻⁸ M for the rabbit medulla enzyme confirms that the effect of the hydrophilic factor is 90-fold stronger than that of ouabain Kᵢ=1.3×10⁻⁶ M on the same enzyme preparation (Fig. 1).

The inhibitory effect of the carbon suboxide derivatives is similarly efficient on the Na⁺/K⁺-ATPase isolated from the rat kidney medulla while ouabain manifested on rat enzyme a 10³ fold lower activity. The inhibition curves obtained with these enzyme preparations showed a steep, dose dependent increase but usually not exceeding a maximal value of 84%. The comprehensive Na⁺/K⁺-ATPase investigations have been performed by H-J Apell and R. Stimac (Konstanz-Germany), and detailed results of the study will be reported elsewhere. The higher molar mass homologues (m.w. >4.0 kDa) showed only low inhibition of the sodium pump but enhanced significantly the natriuresis in rats. The excretion rate of the sodium ions has increased from the 6.7±1.8 μE⋅min⁻¹ base line value to: 17.3±3.9 μE⋅min⁻¹ mean value during the 90 min period after the injection of the 4-5 kDa molar mass fraction.

From the spectral and chromatographic data we deduced the chemical structure of these natural sodium pump inhibitors as: cyclomeric and macroring closed derivatives of the inorganic carbon suboxide C₃O₂. The gaseous carbon suboxide O=C=O=C=O is the doubly dehydrated derivative of malonic acid and in most reactions it gives derivatives of this acid (27). Small amounts of carbon suboxide always accompany carbon monoxide, from which it is easily formed by simple electric discharges. The presence of carbon suboxide in the archaic earth atmosphere, rich in CO, must be considered therefore as very probable (28).

The experimentally established molar mass of the very strong sodium pump inhibitor of plant origin is 408.2 Da, a value corresponding exactly to the six fold mass of the carbon suboxide i.e. 6×68.03 Da. The less intense mass ion value at 544.2 Da found in the same spectrum and in that of the EDLFs (22), corresponds exactly to the eightfold mass of the carbon suboxide (8×68.03 Da), this likely represents the macroring-closed cyclooctamer of C₃O₂. The general formula of these cyclooligomeric and macrocyclic carbon suboxide derivatives is:

$$\text{com-}(\text{C}_3\text{O}_2)_n$$

where com symbolizes the cyclo-oligomeric and macrocyclic structure and n corresponds to the degree of cyclomerization of the C₃O₂. The N size of the double-strained macroring is N=2n, i.e. the cyclohexamer with n=6 forms a 12-membered macroring.

From the theoretically infinite values of “n”, only a restricted number of homologues have been identified experimentally, like the here-mentioned n=6 or 8 as well as some multiples of 6 or 8. With the existence of only cer-
tain com-(C₃O₂)ₜ derivatives with preferred “n” values, the here described substances differ essentially from the earlier known, amorphously condensed carbon suboxide polymers (27). The latter compounds have no distinct polymerization degree values and are water-reactive due to the cumulated double bonds preserved at their edges. The com-(C₃O₂)ₜ derivatives presented in this study are, in contrast, water-stable, and the lack of IR bands at 2,100-2,200 cm⁻¹ proves the absence of cumulated O=C =C=C-bonds. From the various structural isomers (e.g. head-to-head or randomly condensed 2-, or 4-pyrene rings), compatible with the experimental molar mass of 408.2 Da, the structure with six head-to-tail condensed 4-pyrene rings additionally fused in a double-strained 12-membered macroring is shown in Fig. 2.

The lack of ¹H NMR signals and the presence of only two resonance peaks in the ¹³C NMR spectrum confirm the proposed structure with its alternately head-to-tail condensed 4-pyrene rings. This highly symmetric structure is achiral in accordance with the lack of chiroptical properties which has been observed previously for EDLFs of animal origin (11).

The higher molar mass homologues with significant natriuretic effect are by no means proteins, as proved by the lack of any protein-specific chemical reactions and hydrolysis products. The presumed proteic nature of some earlier identified higher molar mass natriuretic or digitalis-like factors (22, 29) must be revisited accordingly, and they belong probably to the same class of natural carbon suboxide derivatives. The existence of homologues with higher molar masses was well evidenced by the SDS-PAGE and GPC results. By the GPC method, the experimental mass values at 4-5 kDa fitted well the calibration curve obtained with polyethylene glycol standards of known molar mass. Only the molar mass values deduced from the SDS-PAGE are significantly higher (10-12.5 kDa). Either are the globular proteins not adequate standards for the new structure class or some higher forms of the factors are not well dissociated in the gel. The exact molar mass values measured by MALDI-TOF method are, in accordance with the GPC, at 4,100 and 4,900 Da which correspond surprisingly to some “magic” values for the degree n of cyclomerization in the general formula com-(C₃O₂)ₜ of the here-discovered carbon suboxide derivatives. The two most intense molar mass values at 4,100 and 4,900 Da correspond indeed to n=60 and n=72, respectively. (Fig. 3).

The existence of these two particular molar mass homologues is tentatively explained by the self-associations between 10 or 12 cyclohexameric units presumably joined through multiple carbonyl-carbonyl additions. The particular significance of these two numbers may be that: 10

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**Fig. 2.** Cyclomerization of 6 carbon suboxide molecules forming the cyclohexameric and macroring closed derivative: com-(C₃O₂)₆ with molar mass of 408.2 Da.

**Fig. 3.** The molecular formula of the higher molar mass carbon suboxide derivatives.
or 12 cyclohexamers are needed to form a "supramolecular" sphere which affords the highest number of mutual carbonyl-carbonyl additions. Multiple intermolecular water elimination between the hydrated cyclohexamers is the second possible way to form the higher molar mass derivatives.

The addition of water molecules to the carbonyl groups in the 408.2-Da cyclohexamer restricts the conjugation between the C=C--C=O bonds, but due to the simultaneous reduction of the macroring tension the hydrated forms are more favored thermodynamically (Fig. 4). The lower-intensity UV absorbance of the "hydrated form" is explained by the conversion of the more strongly absorbing carbonyl groups into geminal-diols which have practically no absorbance above 200 nm. It is furthermore assumed that the lipophilic anhydrous pyrone cyclohexamer and its stepwise hydrated derivatives are present in solution as an equilibrium mixture. The composition of this equilibrium is expected to be strongly medium-dependent; thus it can be shifted by adequate solvents or solutes in one direction or another. The equilibrium between the free 408.2-Da cyclohexamer and its more hydrophilic forms is influenced by the concentrations of Na and K ions. This influence was investigated by HP gel permeation chromatography, with the NaCl and KCl concentrations ranging between 0-200 mM/l. The sensitive shift in the structure equilibrium with increases in the sodium ion concentrations toward a more inhibited Na+/K+-ATPase and vice versa may represent the key to a feedback mechanism by which the sodium pump may be regulated physiologically.

In summary, the structure elucidation showing the EDLFS to be macrocyclic derivatives of the inorganic carbon suboxide answers several questions in the field. The confusion between EDLFS and cardiac glycosides can be considered to be definitively clarified. The endogenous ligand of the Na+/K+-ATPase is a complex inorganic substance able to adopt medium-dependent structural forms. The broad HPLC peaks and the shift in EDLFS elution from the lipophilic to the hydrophilic region are explained by the complex equilibrium between the lipophilic and the more hydrophilic forms. The higher molar mass self-associates of the cyclohexameric units are tentatively identified as natriuretic factors. The wide distribution of carbon suboxide derivatives agrees with the ubiquitous nature of the Na+/K+-ATPase. Carbon suboxide, as a component of the archaic earth atmosphere, was present in the early phases of evolution to serve, in its structured forms, as endogenous ligand of the similarly long-time conserved ATPase receptor. For the biosynthesis of EDLFS a malonate-polyketide pathway is assumed. Malonyl coenzyme A is the probable building element for the biosynthesis of these structured carbon suboxide derivatives. The malonate-polyketide biosynthesis may be accomplished by various kinds of cells. Therefore, there is no reason to consider the hypothalamus or adrenal glands as particular sites of EDLFS biosynthesis. As compared to the biologically very important nitric oxide, the carbon suboxide derivatives appear to be essential novelties. Our results suggest for the first time that a simple inorganic gas is able to exert important physiological effects through its complex structured forms. The comprehensive investigation of these structures and of the bioregulatory mechanism of these carbon suboxide derivatives is a great challenge for chemistry and several areas of life science. Preliminary results (30) have revealed the potent immunoregulatory effect of the macrocyclic carbon suboxide derivatives, with related and highly promising therapeutic applications for rheumatic arthritis and related autoimmune pathologies. The existing data suggest the possible intervention of these derivatives at the level of the innate immune system, probably in the regulation of macrophage activity (31). An elucidation of the pharmacodynamic mechanism of these natural inorganic factors could open new and efficacious therapeutic approaches for a variety of autoimmune, cardiovascular and metabolic diseases.

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