Identification of Na\(^{+}\),K\(^{+}\)-ATPase Inhibitors of Volume-Expanded Hog Plasma

TADASHI INAGAMI AND MASAAKI TAMURA

SUMMARY Na\(^{+}\),K\(^{+}\)-adenosine triphosphatase (ATPase) inhibitors possessing inhibitory activities against the specific binding of ouabain to Na\(^{+}\),K\(^{+}\)-ATPase and \(^{86}\)Rb uptake into hog erythrocytes have been purified from the plasma of hog acutely infused with saline. The purifications were performed by a combination of Amberlite XAD-2 adsorption chromatography and several steps of high performance liquid chromatography using four different types of columns. Inhibitors purified to homogeneity were identified as linoleic and oleic acids, \(\gamma\)-stearoyllysophosphatidylcholine, \(\gamma\)-arachidoyllysophosphatidylcholine, \(\gamma\)-linoleoyllysophosphatidylcholine, and \(\gamma\)-oleoyllysophosphatidylcholine. Small amounts of \(\beta\)-arachidoyllysophosphatidylcholine, \(\gamma\)-docosapentaenoyllysophosphatidylcholine, \(\gamma\)-elcoatrienoyllysophosphatidylcholine, and \(\gamma\)-palmitoyllysophosphatidylcholine were also detected by both fast atom bombardment mass and proton nuclear magnetic resonance spectrometric studies. Only \(\gamma\)-acyllysophosphatidylcholines showed inhibitory activities on Na\(^{+}\),K\(^{+}\)-ATPase and ouabain-binding activities. These lysophosphatidylcholines and unsaturated free fatty acids were effective at 100 \(\mu\)M levels in attaining 50% inhibition of the enzyme activity. The ouabain-displacing activity in plasma caused by these compounds increased with time during saline infusion. The maximal plasma levels of these components were approximately 10 times higher than that in the preinfusion plasma sample. (Hypertension 10 [Suppl I]: 1-000-1-000, 1987)

KEY WORDS • Na\(^{+}\),K\(^{+}\)-ATPase inhibitor • volume-expanded plasma • ouabain-displacing activity • lysophosphatidylcholines • unsaturated free fatty acids

MANY investigators have reported the existence of putative Na\(^{+}\),K\(^{+}\)-adenosine triphosphatase (ATPase) inhibitors in plasma of both clinically hypertensive subjects\(^{1,2}\) and experimentally hypertensive animals\(^{3-6}\) and implicated the inhibitors in the pathogenesis of essential hypertension.\(^{7,8}\) However, neither the structure nor the nature of the interaction of these inhibitors with Na\(^{+}\),K\(^{+}\)-ATPase is clear. Some investigators attributed the inhibitory action to an acidic peptide\(^{9}\) while others proposed the activity to be due to nonpeptidic substances.\(^{10,11}\) It has also been postulated that this inhibitor interacts with an antibody to digoxin.\(^{9-11}\) Studies in this field have been exceedingly complicated because different methodologies for extraction and purification have been used, and most of the studies are not complete in identifying the inhibitors. We attempted to contribute to the clarification of this complicated problem by the complete purification and structural analysis of the inhibitors.

Materials and Methods
Preparation of Plasma
Plasma of volume-expanded hogs was prepared by infusing 10% body weight of isotonic saline to crossbred hogs under pentobarbital anesthesia over 60 minutes as described previously.\(^{12}\) The infusion was continued thereafter at 40% of the initial rate for an additional 60 to 90 minutes. Plasma was prepared from blood collected into heparin from the femoral artery.

Assays
Inhibition of Na\(^{+}\),K\(^{+}\)-ATPase activity and inhibition of \(\left[{\text{H}}\right]\)ouabain binding to this enzyme were determined as described previously using partially purified rat brain Na\(^{+}\),K\(^{+}\)-ATPase.\(^{12}\) Inhibition of \(^{86}\)Rb influx into hog erythrocytes was measured by the method of Diamandis et al.\(^{13}\) Digoxinlike immunoreactivity was measured by radioimmunoassay according to the method of Gruber et al.\(^{9}\) using antidigoxin antibodies.

From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee.
Supported by Grants HL 14192 and HL 35323 from the National Institutes of Health.
Address for reprints: Tadashi Inagami, Ph.D., Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, TN 37232.
Extraction of Na⁺,K⁺-ATPase Inhibitors

Plasma of a volume-expanded hog (600 ml) was treated with 5.4 L of acetone/methanol (1:1, vol/vol) at −20°C for 16 hours. The mixture was filtered through Whatman (Clinton, NJ, USA) No. 1 filter paper and concentrated by a rotary evaporator. The resultant water phase was lyophilized.

Adsorption Chromatography

Amberlite XAD-2 (Sigma Chemical, St. Louis, MO, USA) was prewashed with 20 volumes of methanol and distilled water and packed in a glass column (2.8 × 60 cm). Plasma extract obtained from 600 ml of volume-expanded plasma was suspended in an equal volume of distilled water and applied to the column preequilibrated with distilled water. After washing with 3 column volumes of distilled water, the inhibitory activity was eluted with 3 column volumes of methanol.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) on aminopropyl columns (0.46 × 25 cm; Alltech, Deerfield, IL and DuPont, Wilmington, DE, USA) and a cyanopropyl column (0.46 × 25 cm, DuPont) was carried out using a decreasing linear concentration gradient of acetonitrile at a flow rate of 1 ml/minute. The reverse phase HPLC on Zorbax octadecylsilane column (0.46 × 25 cm, DuPont) and SynChropak octyl column (0.41 × 25 cm, SynChrom, Linden, ID, USA) was run using linear gradients of acetonitrile or 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 1 ml/minute. The absorbance was monitored at 214 nm.

Structure Analysis

Proton nuclear magnetic resonance (1H-NMR) spectrometry and fast atom bombardment (FAB) mass spectrometry were used to determine the structures of purified substances.

Results

Purification of Unsaturated Fatty Acids as Components of Ouabain-Displacing Activities

A crude extract prepared by acetone/methanol extraction and prefractionated by adsorption chromatography on an Amberlite XAD-2 was subjected to normal phase HPLC on an aminopropyl column. Two activity peaks, Peaks 1 and 2, were obtained as outlined in Figure 1. Peak 1, consisting of unsaturated fatty acids, was purified to homogeneity by three additional steps of HPLC. Five active peak fractions were recognized and further purified to homogeneity. Two of them, which corresponded to over 70% of the total activity in Peak 1, were obtained in quantities sufficient for structural determination by 1H-NMR and FAB mass spectrometry and were identified as linoleic (18:2) and oleic acid (18:1). Peak 2 was separated to Peaks 2-1 and 2-2 (Figure 2). Peak 2-2 was purified to homogeneity by three additional steps of HPLC, and its structure was identified as γ-stearoyllysophosphatidylcholine (LPCS). Peak 2-1 required three to five steps of HPLC to yield four homogeneous peaks and three peaks containing two components. FAB mass spectrometry and 1H-NMR identified these compounds as γ-arachidoyllysophosphatidylcholine (LPCA [γ]), γ-linoleoyllysophosphatidylcholine (LPCL), γ-oleoyllysophosphatidylcholine (LPCO), γ-docosapentaenoyllysophosphatidylcholine (LPCD), γ-eicosatrienoyllysophosphatidylcholine (LPCE), and γ-palmitoyllysophosphatidylcholine (LPCP). β-arachidoyllysophosphatidylcholine (LPCA [β]) was also identified in one homogeneous peak.

![Diagram of purification scheme](http://hyper.ahajournals.org/)

**Figure 1.** Scheme of purification of ouabain-displacing activities from volume-expanded hog plasma. HPLC = high-performance liquid chromatography; CN = cyanopropyl; ODS = octadecylsilane; NH₂ = aminopropyl; LPC = lysophosphatidylcholine; LPCA(β) = β-arachidoyllysophosphatidylcholine; LPCA(γ) = γ-arachidoyllysophosphatidylcholine; LPCD = γ-docosapentaenoyllysophosphatidylcholine; LPCE = γ-eicosatrienoyllysophosphatidylcholine; LPCL = γ-linoleoyllysophosphatidylcholine; LPCO = γ-oleoyllysophosphatidylcholine; LPCP = γ-palmitoyllysophosphatidylcholine; LPCS = γ-stearoyllysophosphatidylcholine. (Reprinted with permission from Tamura et al. 1987. American Chemical Society.)
Discussion

These results indicate at least two groups of humoral Na\(^{+}\), K\(^{+}\) -ATPase inhibitors in acutely volume-expanded hog. One group consists of unsaturated free fatty acids\(^{12}\) and the others are LPCs with unsaturated and saturated fatty acids.\(^{14-16}\) The free fatty acids peak accounted for slightly more than half of the total inhibitory activity in plasma as shown in Figure 1.

These results are in agreement with the observations by Bidard et al.\(^{17}\) that commercially obtained free fatty acids and mixtures of LPCs, mainly consisting of saturated fatty acids, inhibited ATPase. Kelly et al.\(^{18}\) reported fractionation of plasma ATPase inhibitors and found that inhibitory fractions show mass spectrometric signals for LPCP and several free fatty acids. That lysocephatidylethanolamine does not show the inhibition\(^{17}\) and the present observation that LPCA(\(\beta\)) lacks the inhibitory activity indicate the action of these compounds are dependent on certain specific structures. Also the inhibition of Na\(^{+}\), K\(^{+}\) -ATPase is selective; the ouabain-insensitive ATPase (Mg\(^2+\)-ATPase) is only slightly inhibited by LPCs (M. Tamura, unpublished result), which indicates that the inhibitor is preferential to Na\(^{+}\), K\(^{+}\) -ATPase.

A question remains to be clarified as to whether unsaturated free fatty acids and LPCs are physiological regulators of Na\(^{+}\), K\(^{+}\) -ATPase, causing diuresis and hypertension. These lipids bind to plasma albumin, and their effective concentrations may be reduced in plasma. Nevertheless, the observation that the ouabain-displacing activities under Peaks 1, 2-1, and 2-2 increase markedly during saline infusion is highly intriguing. The mechanism of the formation of free fatty acids and LPCs by volume expansion is not clear. It must be noted that the LPCs with polysaturated fatty acids found in the present studies are unusual forms that were not known to occur in nature, since those isolated to date usually contain saturated fatty acid in their \(\gamma\)-hydroxyl positions.

The Na\(^{+}\), K\(^{+}\) -ATPase inhibitors from various sources show immunological cross-reactivity with digoxin.\(^{9-11}\) The LPCs isolated in the present studies do not cross-react with antibodies to digoxin, while free fatty acids showed apparent cross-reactivity. It is possible that the cross-reactivity of the latter was due to interference with the radioimmunoassay. The Na\(^{+}\), K\(^{+}\) -ATPase inhibitory activities were found in several different tissues.\(^{19-22}\) Whether these inhibitors are different, similar, or identical substances requires complete purification and determination of structure.

Acknowledgments

The authors are indebted to Drs. Thomas M. Harris and Harumitsu Kuwano for NMR analyses; to Drs. Takeshi Kinoshita, Brian J. Sweetman, and Ian A. Blair for mass spectrometric analyses; and to Trinita Fitzgerald and Edward Price, Jr. for technical assistance.

References

Figure 3. Left. The inhibitory effect of purified linoleic (A) and oleic acids (Δ) and authentic linoleic (●) and oleic acids (○) on Na\(^+\),K\(^+\)-ATPase activity (A), on binding of \(^3\)Houabain to Na\(^+\),K\(^+\)-ATPase (B), and on binding of \(^3\)Hdigoxin to antidigoxin antibody (C). Right. The inhibitory effect of the purified γ-arachidoyl- (●), β-arachidoyl- (○), γ-linoleoyl- (Δ), and γ-oleoyl-hyposphatidylcholine (●) on Na\(^+\),K\(^+\)-ATPase (D), on binding of \(^3\)Houabain to Na\(^+\),K\(^+\)-ATPase (E), and on \(^86\)Rb uptake into hog erythrocytes (F). On left side of each panel, dose-response curves of these activities were determined in comparison to ouabain (○) and digoxin (○) under the same assay conditions. Each point is the mean of duplicate determinations in two separate experiments and expressed as the percentages of maximal Na\(^+\),K\(^+\)-ATPase activity, maximal binding of \(^3\)Houabain to Na\(^+\),K\(^+\)-ATPase, maximal binding of \(^3\)Hdigoxin to antidigoxin antibody, and maximal \(^86\)Rb uptake activity.

(Reprinted from Tamura et al. 16)


16. Tamura M, Harris TM, Higashimori K, Sweetman BJ, Blair IA, Inagami T. Lysophosphatidylcholines containing polyunsaturated fatty acids were found as Na\(^+\),K\(^+\)-ATPase inhibitor in acutely volume-expanded hog. Biochemistry 1987;26:2797-2806


Identification of Na+,K+-ATPase inhibitors of volume-expanded hog plasma.
T Inagami and M Tamura

Hypertension. 1987;10:I108
doi: 10.1161/01.HYP.10.5_Pt_2.I108
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/10/5_Pt_2/I108

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/