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ISOLATION AND IDENTIFICATION OF HEMIN AS AN ENDOGENOUS Na^+/K^+-ATPASE INHIBITOR FROM PORCINE BLOOD CELLS

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Summary: A substance which is a potent inhibitor of Na^+/K^+-ATPase activity and competitively displaces [^{3}H]ouabain binding to this enzyme was isolated from porcine blood cells. From its chemical and physicochemical properties, this activity was identified as hemin (chloroprotohemin IX). Hemin showed a dose dependent curve for Na^+/K^+-ATPase inhibitory activity similar to that of ouabain and displaced [^{3}H]ouabain binding as potent as 1/100 of ouabain itself.

The Na^+/K^+-ATPase (E.C. 3.6.1.3), responsible for maintaining Na^+/K^+ gradients in animal cells, is the receptor for digitalis compounds, plant-derived cardiotonic glycosides. The idea that an endogenous ligand for the digitalis receptor exists has been demonstrated (1,2). Such a ligand may play a physiological role in regulating water and electrolyte balance and be, therefore, relevant to the development of essential hypertension. Several attempts to characterize the endogenous digitalis-like substance (Na^+/K^+-ATPase inhibitor) have been made and many substances have been purified from a variety of mammalian sources such as plasma (3,4), urine (5), adrenal gland (6), brain (7), hypothalamus (8), amniotic fluid (9), heart (10) and cerebrospinal fluid (11,12). Although a lot of candidate compounds having various chemical properties were reported, the complete structural elucidation of an active compound remained unsolved. Araki *et al.* (13) isolated three peptidal Na^+/K^+-ATPase inhibitors, SPAI-1, -2 and -3, from porcine intestine and determined their structures as polypeptides consisting of 49 (SPAI-1) or 61 (SPAI-2 and -3) amino acid residues. These peptides seemed not to be the real endogenous digitalis substances, however, since the inhibitory pattern was different from that of ouabain - a typical, potent digitalis substance. Quite recently, Goto *et al.* (14) reported the isolation of a digitalis-like factor which was indistinguishable from digoxin, another digitalis substance originally isolated from *Digitalis* plants, from 3 x 10^4 L of human urine but whether this substance was produced in human body remained to be determined.

We screened the extracts from the various organs and plasma to assay for the inhibition of Na^+/K^+-ATPase and found a common activity showing the same HPLC behavior in all the extracts tested. This activity was further revealed to be most abundant in the blood cell fraction. Accordingly, we isolated the active substance from porcine blood cells and identified hemin (chloroprotohemin IX) as a potent mammalian Na^+/K^+-ATPase inhibitor with [^{3}H]ouabain

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displacing activity. We report here the structural elucidation of hemin as an endogenous Na^+/K^+-ATPase inhibitor.

MATERIALS AND METHODS

Purification of Na^+/K^+-ATPase inhibitor from blood cell fraction. Porcine blood (2.0 L) was centrifuged (2000 x g, 30 min) and the blood cell fraction (1.0 L) was mixed with 2.0 L of Milli Q (Millipore, Bedford, USA) water and lysed. After boiling for 10 min, the precipitation obtained by centrifugation (10,000 rpm, 30 min) was extracted with 700 ml of 70 % CH_3CN/0.1 % TFA for 24 hr at 4 °C. After centrifugation (10,000 rpm, 30 min), the supernatant was mixed with 1.0 L of Milli Q water to make the concentration of CH_3CN up to 30 % and loaded on a C_{18} column (Inertsil ODS, ϕ20 x 50 mm, Gasukuro Kogyo, Tokyo, Japan) at a flow rate of 10 ml/min. The column was then eluted with 100 ml each of 40 %, 50 % and 95 % CH_3CN/0.05 % TFA, successively, and the eluate was collected by 10 ml fractions, which were assayed for inhibitory activity. The main activity was eluted with 50 % CH_3CN/0.05 % TFA and the active fractions were combined and evaporated to dryness. The active material was reconstituted with 10 ml of 35 % CH_3CN/0.05 % TFA and then applied to the reverse phase HPLC on a preparative C_{18} column (YMC-Pack ODS, ϕ20 x 250 mm, Yamamura Kagaku, Kyoto, Japan), which was eluted with a gradient of 35 % to 95 % CH_3CN/0.05 % TFA at a flow rate of 6.0 ml/min. The inhibitory activity which appeared when the CH_3CN concentration reached about 50 % was subjected to the final purification, after evaporation to dryness followed by dissolving into 1.0 ml of 55 % CH_3CN, with a C_{18} column (TSKgel ODS-80T_M, ϕ4.6 x 250 mm, Tosoh, Tokyo, Japan) and an isocratic elution with 55 % CH_3CN/0.05 % TFA at a flow rate of 0.7 ml/min, with injecting the 100 µl of the reconstituted solution for one cycle, which was repeated for ten times.

Chemical properties of Na^+/K^+-ATPase inhibitor. The molecular weight was determined by measuring the fast atom bombardment mass spectrum with a JMS-HX110 mass spectrometry (JEOL, Akishima, Japan) using *m*-nitrobenzyl alcohol as a matrix. The UV-VIS absorption spectra were obtained with an SPD-M6A photodiode array UV-VIS detector (Shimadzu, Kyoto, Japan) coupled to a Shimadzu HPLC system (LC-6A liquid chromatograph). The material was chromatographed on the TSKgel ODS-80T_M column with an isocratic elution of 55 % CH_3CN/0.05 % TFA and the spectrum of the peak on chromatogram was measured with the photodiode array detector. The retention time and spectrum of the purified material was compared with those of authentic hemin (Wako Pure Chemical, Osaka, Japan). The 500 MHz ^1H NMR spectra were measured with a Bruker AM-500 NMR spectrometer (Bruker, Karlsruhe, Germany) using dimethylsulfoxide as the solvent.

Na^+/K^+-ATPase inhibitory activity. Seventy microliters of 32 mM Tris-HCl buffer (pH 7.4) supplemented with 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl and 5 mM EDTA was added to 10 µl of sample solution in a 1.5 ml centrifuge tube and the resulting mixture was kept at 37 °C for 10 min. The Na^+/K^+-ATPase (0.002 units, from dog kidney, Sigma, St. Louis, USA) dissolved in 10 µl of Tris-mannitol buffer (250 mM mannitol, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4) was added to the sample solution and after preincubation for 8 min at 37 °C, 10 µl of 10 mM ATP (Oriental Yeast, Tokyo, Japan) was added and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 55 % trichloroacetic acid and cooling on ice. After centrifugation at 10,000 rpm for 5 min, the supernatant was applied to an ion exchange HPLC to quantify the content of ADP and ATP. ADP and ATP were separated by a HPLC system employing a TSKgel DEAE-5PW column (ϕ7.0 x 70 mm, Tosoh) with an isocratic elution of 0.28 M sodium phosphate buffer (pH 7.0) in 20 % CH_3CN (flow rate, 1.0 ml/min) at 40 °C and quantified by absorbance measured at 254 nm. The inhibitory rate of ADP production from ATP was determined by comparing the amounts of ADP and ATP. Porphyrin-related compounds, hematoporphyrin, protoporphyrin, biliverdin and bilirubin were purchased from Sigma. In each assay, the sample solution of hemin (Wako Pure Chemicals) was prepared by diluting its 0.1 M $NaHCO_3$ solution of an appropriate concentration with buffer employed up to the desired concentration.

Inhibitory activity for the binding of [^{3}H]ouabain to Na^+/K^+-ATPase. To 10 µl of sample solution in a 1.5 ml centrifuge tube, 10 µl of 10 nM [^{3}H]ouabain (Amersham Japan, Tokyo, Japan), 10 µl of ATPase (0.004 units), 70 µl of 0.5 M Tris-HCl (pH 7.7, supplemented with 50 mM $MgCl_2$, 1.0 M NaCl and 50 mM KCl) and 10 µl of 10 mM ATP were added and incubated for 2 hr. The reaction was stopped by the filtration through Whatman GF/B glass microfiber filter (Whatman, Maidstone, UK) which was then punched out. The resulting discs were put into scintillation vials to which the universal liquid scintillation cocktail AQUASOL-2 (duPont, Wilmington, USA) was poured in order to measure the radioactivity remained on the glass filter

discs employing an LS 5801 liquid scintillation counter (Beckman, Berkeley, USA). The values for 100 % or 0 % binding were measured using H_2O or 1.0 mM ouabain (Wako Pure Chemical) instead of samples, respectively.

Effect on rat blood pressure. The sample dissolved in 250 µl of physiological saline was injected through the femoral vein to a male Wistar rat (250 g) anesthetized with pentobarbital and the blood pressure of the femoral artery was measured.

RESULTS

In preliminary experiments, we had found that the Na^+/K^+-ATPase inhibitory activity could be detected in almost all the extracts from various tissues tested and shared similar chromatographic behavior on reverse phase HPLC. Among these tissues, blood cell fraction contained the most abundant Na^+/K^+-ATPase inhibitory activity. Therefore, we carried out the isolation of the inhibitor using the porcine blood cells as our starting material. The purification procedure described in the **MATERIAL AND METHODS** afforded the partially purified material, which was subjected to the final step of purification using an analytical C_{18} column with an isocratic elution of 55 % acetonitrile in 0.05 % TFA yielding the active substance as a single peak (Fig. 1). About 20 mg of the active substance was obtained from 2.0 L of porcine blood. The active fraction was rechromatographed using the separation system employed in the final step to confirm the homogeneity as well as to measure a UV-VIS absorption spectrum of

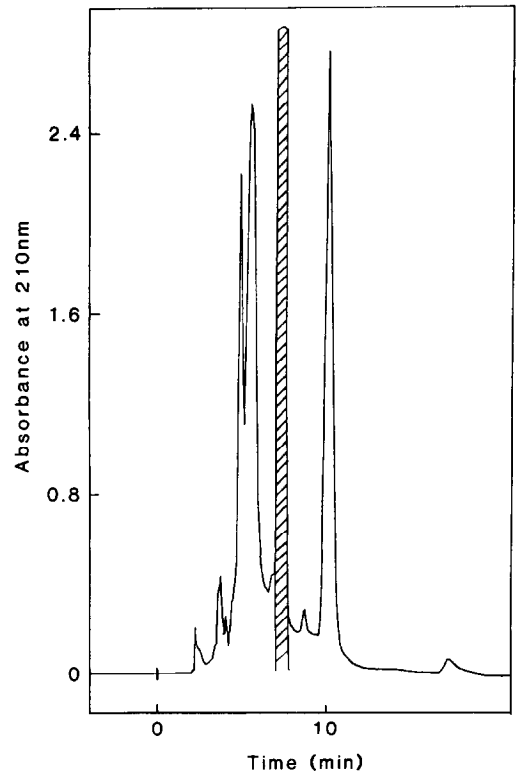

Fig. 1. HPLC profile of the final purification step on a TSK ODS-80T_M column. The inhibitory activity obtained in the preceding step was subjected to an analytical C_{18} column (TSKgel ODS-80T_M, ϕ4.6 x 250 mm) using an isocratic elution with 55 % CH_3CN/0.05 % TFA at a flow rate of 0.7 ml/min. The activity was recovered with the hatched peak.

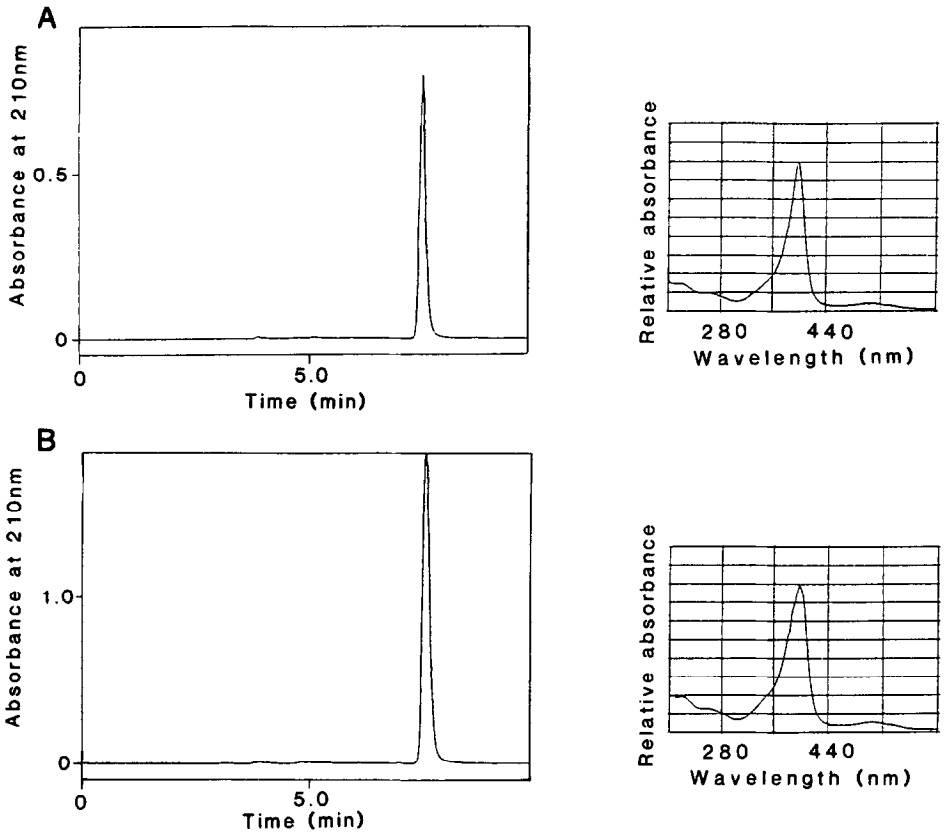

Fig. 2. HPLC profiles (left panel) and UV-VIS absorption spectra (right panel) of hemin (A) and the inhibitor of Na^+/K^+-ATPase purified from blood cells (B). Hemin and the active fraction purified from blood cells were rechromatographed using the separation system employed in the final step and the UV-VIS absorption spectra of the peaks were measured with a Shimadzu SPD-M6A photodiode array UV-VIS detector.

the isolated active substance (Fig. 2 (A)). The spectrum showed a maximal absorption peak at around 400 nm, which was characteristic of hemin-related compounds. The absorption spectrum of authentic hemin was measured under the same conditions as the active substance (Fig. 2 (B)) and revealed that the retention time and absorption spectrum of the authentic hemin were fully identical to those of the Na^+/K^+-ATPase inhibitor obtained from porcine blood cells. Mass spectroscopy utilizing the fast atom bombardment method revealed that the active substance showed a molecular ion peak at m/z 616.1 (Fig. 3), which was assignable to that of dechlorinated hemin (616.18, calculated as $C_{34}H_{32}N_4O_4Fe^+$). The ^1H NMR spectrum of the inhibitor showed a unique spectrum in which the signals spread for extraordinarily wide resonance region, resulted from the paramagnetic effect of iron atom, and there was no difference between the spectrum of the inhibitor and that of authentic hemin (Fig. 4). From these results the isolated inhibitor was identified as hemin (chloroprotohemin IX).

The isolated inhibitor and authentic hemin showed coincident dose dependent curves for the inhibition of Na^+/K^+-ATPase and this curve paralleled that of ouabain (Fig. 5). The IC_{50} was about 10^{-6} M, which was comparable to the value of ouabain. Porphyrin-related compounds,

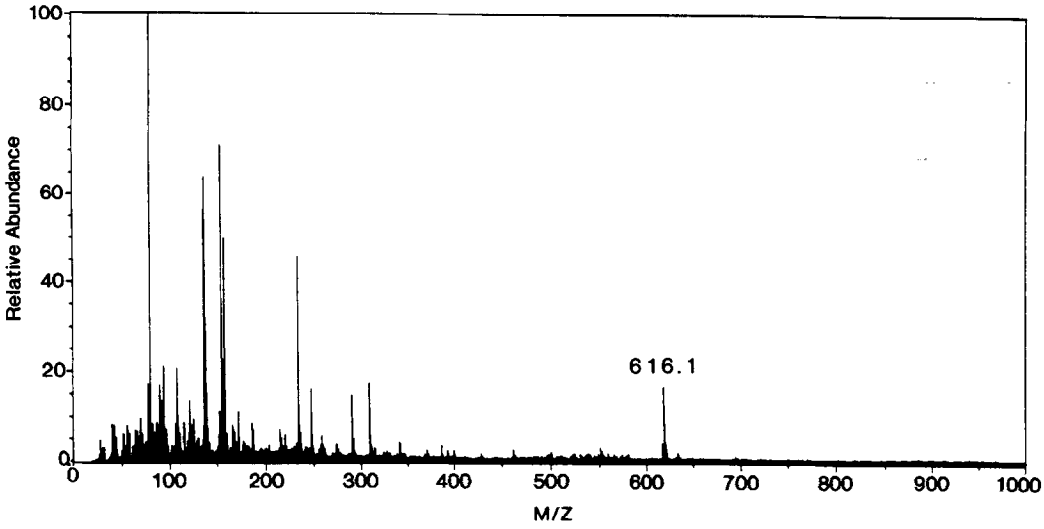

Fig. 3. Fast atom bombardment mass spectrum of the active substance.

hematoporphyrin, protoporphyrin and biliverdin showed a mild inhibitory activity at a concentration of 10^{-4} M but bilirubin had no activity (Fig. 5). Displacement activity of hemin for the binding of [^{3}H]ouabain to Na^+/K^+–ATPase was as potent as 1/100 of ouabain itself and the dose

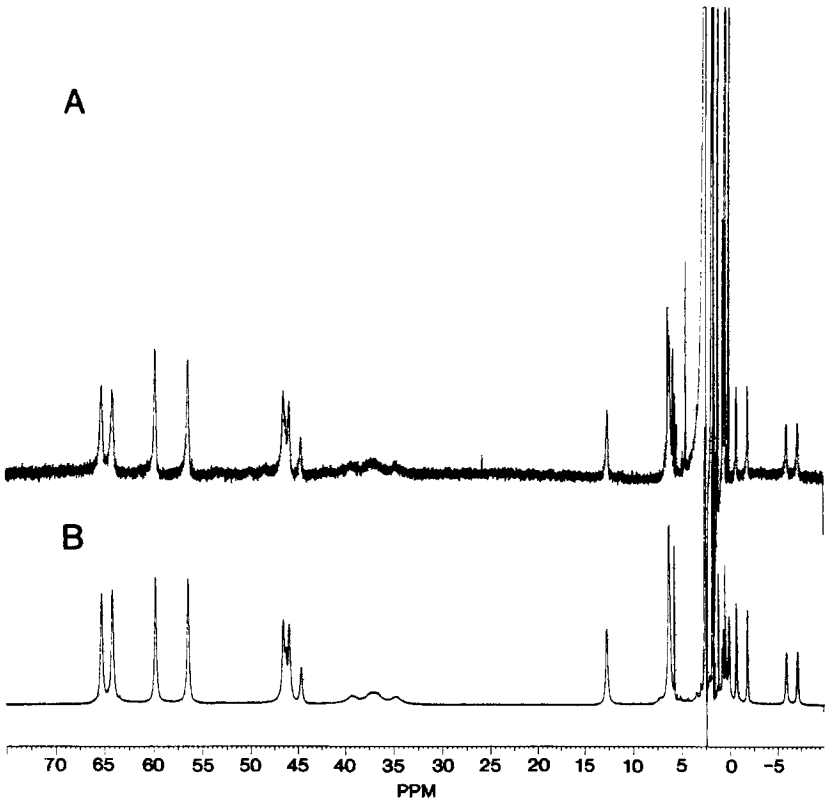

Fig. 4. The 500 MHz ^{1}H NMR spectra in dimethyl sulfoxide solution of hemin (A) and the active substance (B).

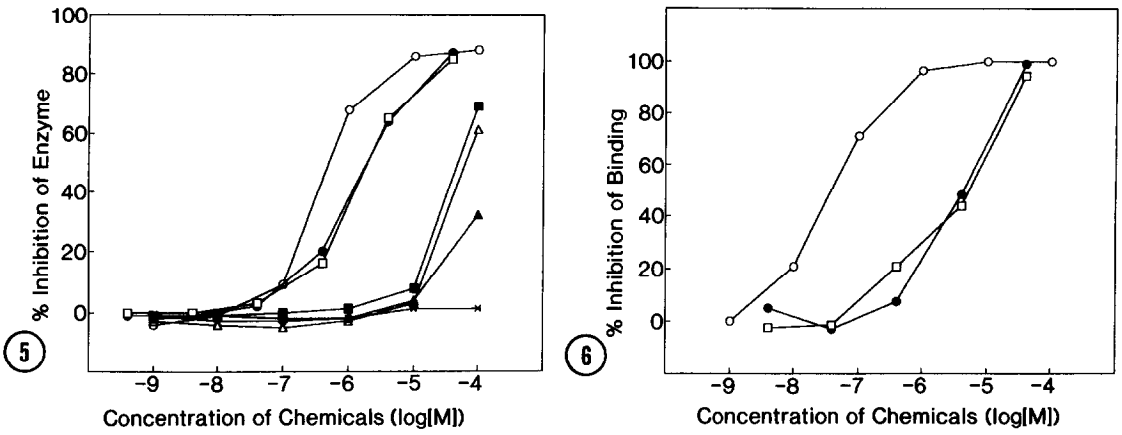

Fig. 5. Na^+/K^+-ATPase inhibitory activity of ouabain (○), hemin (●), the inhibitor purified from blood cells (□), hematoporphyrin (■), protoporphyrin (△), biliverdin (▲) and bilirubin (x). Each point is the mean of duplicate determinations in two separate experiments for ouabain, hemin and the inhibitor purified from blood cells and that of single determination in three separate experiments for others.

Fig. 6. Inhibitory activity for the binding of [^{3}H]ouabain to Na^+/K^+-ATPase of ouabain(○), hemin (●) and hemin purified from blood cells (□). Each point is the mean of duplicate determinations in two separate experiments.

dependent curve paralleled that of ouabain (Fig. 6). Ouabain ($\geq 10^{-6}$ M) and hemin ($\geq 10^{-5}$ M) evoked similar sustained increases in blood pressure in the rat (Fig. 7).

DISCUSSION

The endogenous, digitalis-like substance, which is believed to be involved in essential hypertension, has been explored by several investigators who have variously reported the identity of this substance to be a lipid, steroid or peptide (1,2). Recent results concentrated on the possibility that the endogenous compound is water soluble and nonpeptidic and has low molecular weight (3-8,15). The Na^+/K^+-ATPase inhibitor isolated here from porcine blood cells was identified as hemin from the following data, retention time on HPLC, UV-VIS absorption spectrum, molecular weight determined by fast atom bombardment mass spectrum, NMR spectrum, pattern of the inhibition for Na^+/K^+-ATPase and inhibitory activity against the binding of [^{3}H]ouabain to Na^+/K^+-ATPase. It is noteworthy that hemin showed the strongest inhibitory activity to Na^+/K^+-ATPase among the compounds so far reported. Nevertheless, hemin does not necessarily represent the exemplary digitalis substance described above since hemin is rather hydrophobic compound.

Administration of digitalis compounds, such as digoxin and digitoxin, induce the increase in blood pressure. Ouabain was also reported to show a hypertensive effect on the anesthetised dog by intravenous injection at a dose of 0.03 mg/kg (16). In our experiment, ouabain as well as hemin evoked sustained increase in blood pressure in the rat.

Hemin is insoluble in water at neutral or acidic conditions but solubilizes in water as a complex with proteins and is distributed in blood, lymph and various organs. This means that the digitalis activity derived from hemin must be found in the various mammalian sources but

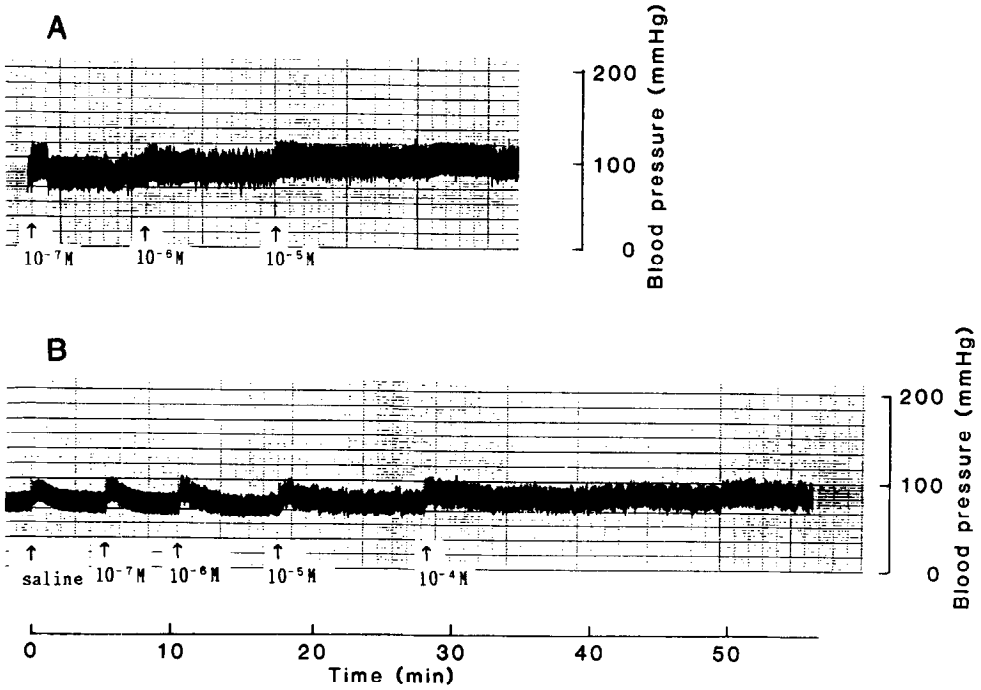

Fig. 7. Effect on rat blood pressure of ouabain (A) and hemin (B). Ouabain or hemin dissolved in 250 µl of physiological saline at various concentrations were injected to Wistar rats (250 g) at the times indicated by arrows. After the transient rise artificially caused by injection shock, the sustained increases in blood pressure evoked by ouabain ($\geq 10^{-6}$ M) or hemin ($\geq 10^{-5}$ M) were observed.

that the yield of activity should remarkably decrease as the purification advances if treated with the conventional isolation methods. Thus, some of the compounds which were purified as the digitalis activity but not finally identified might possibly be hemin. Furthermore, it is noteworthy that porphyrins, which are generated from hemin by elimination of an iron atom and acquire higher water solubility than hemin, also inhibited Na^+/K^+-ATPase activity.

The inhibitory activity of hemin to Na^+/K^+-ATPase was comparable to that of ouabain and the dose dependent curve of inhibition by hemin paralleled the curve of ouabain. Accordingly, it seemed that the inhibitory activity of hemin was not due to the irreversible break down of the enzyme but to the reversible inhibition of the enzyme in the same manner as ouabain. Though the displacement activity of the binding of [^{3}H]ouabain to the enzyme by hemin was 1/100 of ouabain, the dose dependent curve also paralleled that of ouabain and the IC_{50} (5×10^{-6} M) was low enough to consider the physiological significance of hemin. The difference of inhibitory binding activity between ouabain and hemin can be attributed to differences between the phytotoxin's and mammalian endogenous substance's mode of action or the affinity at the binding site. Ouabain has a strong toxicity besides its cardiotonic effect. In fact, ouabain showed cytotoxicity to cultured bovine aorta endothelial cells at a concentration as low as 1×10^{-9} M, whereas hemin did not affect the viability of the same cells even at a concentration of 1×10^{-5} M (data not shown). Daily production of heme by metabolic degradation is calculated at about 300 mg, ranging over every organ. This indicates that hemin is relevant to the regulation of Na^+/K^+-

ATPase activity in every organ through heme/hemin interchange caused by biological oxidation/reduction reaction, or as porphyrins and their related compounds.

Ferrous ion or ferrous compounds may contribute to central nervous system damage after brain hemorrhage as a consequence of their catalytic effects on hydroxyl radical production and lipid peroxidation. Levere *et al.* (17) reported that, though heme did not inhibit Na^+/K^+-ATPase at a concentration of 3×10^{-5} M, it showed 75 % inhibition when treated with heme oxygenase and suggested that this inhibitory effect could be ascribed to the ferrous ion liberated from heme. In our Na^+/K^+-ATPase inhibition assay system, the amount of hemin before and after the reaction was quantified and no decrease of hemin was observed (data not shown). Subsequently, the inhibitory activity of hemin observed in our experiments could not be due to the iron (ferrous or ferric) ion generated on degradation of hemin. Now, it seems very likely that the inhibitory effect caused by heme with oxygenase was derived from porphyrin-related compounds which were produced during the heme degradation cascade or hemin (oxidized heme). Similarly, the Na^+/K^+-ATPase inhibition activity of hemoglobin reported by Sadrzadeh *et al.* (18) also seemed to be explainable to the inhibitory activity of hemin. Taken together, it is conceivable that hemin is involved in the central nervous system damage evoked by brain hemorrhage through its inhibitory effect on Na^+/K^+-ATPase.

Hemin is not necessarily considered as the sole endogenous compound which is responsible for regulating Na^+/K^+-ATPase activity. There might still be another more suitable candidate for the endogenous digitalis substance than hemin. However, because of its distribution, ubiquity and adequate activity, in the course of investigation for digitalis-like substance one must examine whether that activity is derived from hemin or its related porphyrin compounds.

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REFERENCES

1. Haber, E. and Hauper, G.T. Jr (1987) *Hypertension* **9**, 315-324.
2. Haddy, F.J. (1987) *ISI Atlas Sci. Pharmacol.* **1**, 119-123.
3. Hamlyn, J.M., Harris, D.W. and Ludens, J. H. (1989) *J. Biol. Chem.* **264**, 7395-7404.
4. Boschi, S., Borghi, C., Munarini, A., Costa, F.V., Lu, Z., deVries, D. and Ambrosioni, E. (1990) *Biochem. Biophys. Res. Commun.* **169**, 360-368.
5. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T., Eguchi, C. and Sugimoto, T. (1988) *Bichem. Biophys. Res. Commun.* **154**, 847-853.
6. Tamura, M., Lam, T.T. and Inagami, T. (1988) *Biochemistry* **27**, 4244-4253.
7. Shimoni, Y., Gotsman, M., Deutsch, J., Kachalsky, S. and Lichtstein, D. (1984) *Nature* **307**, 369-371.
8. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T., Eguchi, C. and Sugimoto, T. (1989) *Biochem. Biophys. Res. Commun.* **161**, 953-958.
9. Graves, S.W. (1987) *Hypertension* **19 (Part 2)**, I84-I86.
10. Fagoo, M. and Godfraind, T. (1985) *FEBS Lett.* **184**, 150-154.
11. Lichtstein, D., Minc, D., Bouritt, A., Deutsch, J., Karlish, S.J.D., Belmaker, H., Rimon, R and Palo, J. (1985) *Brain Res.* **235**, 13-19.

12. Halperin, J.A. (1989) *J. Neurol. Sci.* **90**, 217–230.
13. Araki, K., Kuroki, J., Ito, O., Kuwada, M. and Tachibana, S. (1989) *Biochem. Biophys. Res. Commun.* **164**, 496–502.
14. Goto, A., Ishiguro, T., Yamada, K., Ishii, M., Yoshioka, M., Eguchi, C., Shimora, M. and Sugimoto, T. (1990) *Bichem. Biophys. Res. Commun.* **173**, 1093–1101.
15. Hamlyn, J.M., Harris, D.W., Clark, M. A., Rogowski, A.C. White, R.J. and Ludens, J.H. (1989) *Hypertension* **13**, 681–689.
16. Watford, W.H., Walsh, R.A., and O'Rourke, R.A. (1989) *Am. Heart J.* **118**, 738–747.
17. Levere, R.D., Escalante, B., Schwartzman, M.L. and Abraham, N.G. (1989) *Neurochem. Res.* **14**, 861–864.
18. Sadrzadeh, S.M., Anderson, D.K., Panter, S.S., Hallaway, P.E. and Eaton, J.W. (1987) *J. Clin. Invest.* **79**, 662–664.