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Na,K-ATPase from Placenta of Women with Pregnancy-Induced Hypertension Exhibits an Increased Affinity for Cardiac Glycosides

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Summary

Microsomes were prepared from placentas of normotensive women and of patients suffering from pregnancy-induced hypertension (PIH). Activity of Na,K-ATPase (estimated as ATP hydrolysis) from the hypertensive tissue was lower than from tissue of normotensive women, even if the number of Na,K-ATPase molecules (monitored by anthroyl ouabain binding) was actually greater in the hypertensive tissue. The affinity of Na,K-ATPase for anthroyl ouabain was about four times higher in plasma membranes of hypertensives, indicating some structural change in the Na,K-ATPase or in its vicinity. Assuming the presence of an endogenous digitalis-like factor, the results suggest a simple way of explaining not only the lower Na,K-ATPase activity in the placental membranes of hypertensives but also the different extent of enzyme inhibition in different tissues of PIH patients.

Key words

Pregnancy-induced hypertension – Cardiac glycosides – Human placenta – Na,K-ATPase – Endogenous digitalis-like factor

Introduction

Na,K-ATPase is an animal plasma membrane protein whose primary physiological function is to maintain the Na^+ and K^+ cation gradients across cell membranes. Despite years of intense study, the functional molecular mechanism and especially the enzyme regulation are still not fully understood. The enzyme is known to be composed of at least two subunits, the catalytic α-subunit and the smaller, glycoprotein, β-subunit. While the role of the β-subunit is not well defined, the α-subunit carries all the presently known functional properties of the enzyme, such as the ability to hydrolyze ATP, to transport Na^+ and K^+, and also to bind the specific inhibitor of Na,K-ATPase ouabain and related cardiac glycosides (Amler *et al.* 1992).

Conservation of a specific binding site for cardiac glycosides through evolution strongly argues for the presence of a natural ligand for the receptor – an endogenous digitalis-like factor (EDLF), proposed first by Gruber *et al.* (1980) and then detected with greater or lesser credibility in various tissues (Goto *et al.* 1988, Tamura *et al.* 1988). However, the simple presence of such a factor at an increased level would not explain the increased Na^+ pump activity (Parenti *et al.* 1991) and, in other cases, its inhibition (Pamnani *et al.* 1990). It would be even more difficult to explain the different extent of Na,K-ATPase inhibition in different tissues of the same hypertensive patient (Mazzanti *et al.* 1989). In the present study we used a fluorescent ouabain analog, anthroyl ouabain, to examine the possible reason for the lower Na,K-ATPase activity in human PIH placenta at the molecular level.

Materials and Methods

Full-term placentas (39–41 weeks of gestational age) were obtained within 15 min of delivery and placed on ice. We defined pregnancy-induced hypertension as the occurrence of diastolic blood pressure (DBP) equal or higher than 90 mm Hg (in at least two consecutive measurements, four or more hours apart) in previously normotensive non-proteinuric women. Mean DBP was 71.8 mm Hg in the normotensive group, and 99.8 mm Hg in the hypertensive group. Patients were not under pharmacological treatment before delivery. The microsomal membrane fraction was prepared according to Whitsett and Wallick (1980). Membrane protein concentration was determined by Lowry's method using bovine serum albumin as standard. The Na,K-ATPase activity was measured with the aid of the linked enzyme spectrophotometric assay as used by Ball and Friedman (1987). Anthroyl ouabain binding was determined with membranes incubated with anthroyl ouabain at 37 °C in 1.4 ml 50 mmol l^{-1} Pipes, 5 mmol l^{-1} Tris-phosphate, 5 mmol l^{-1} $MgCl_2$ at pH 7.4 (final concentration 1–2 mg protein per ml). The excitation wavelength used was 362 nm, the emission wavelength was 469 nm. The fluorescence intensity increase found upon anthroyl ouabain binding to the enzyme was used to determine both the number of binding sites per mg and their affinity as outlined by Fortes (1986). Anthroyl ouabain was purchased from Molecular Probes (USA), all other chemicals were from Sigma (FRG). The special high-yield fluorescence cuvette (Amler *et al.* 1992) was from Flura (Czech Republic).

Results and Discussion

The observed fluorescence F_{obs} is a function of the total ligand concentration $[L_T]$ and the concentration of bound ligand [EL]:

$$F_{obs} = \phi\delta[L_T] + \phi(\gamma-\delta)[EL] \qquad (1)$$

where ϕ is the fluorescence intensity per unit concentration, δ is the ratio of fluorescence quantum yield of bound fluorophore to fluorescence quantum yield of anthroyl ouabain in the buffer, γ is the ratio of fluorescence quantum yield of nonspecifically bound anthroyl ouabain to that in the buffer (Fortes 1980). For a single class of identical sites:

$$[EL] = S - (S^2 - 4[L_T][E_T])^{1/2} / 2 \qquad (2)$$

where

$$S = [E_T] + [L_T] + K_D \qquad (3)$$

The total number of binding sites $[E_T]$ was determined from the intersection of the extrapolated straight segments of the curve. At this point $[L_T]=[E_T]$ and hence K_D was given by:

$$K_D = ([E_T] - [E_L])^2 / [EL] \qquad (4)$$

Crude membranes prepared from placentas (three pieces for each preparation) of patients suffering from pregnancy-induced hypertension contained a lower activity of Na,K-ATPase than found in normotensive tissue (see Table 1). This is in accordance with Mazzanti *et al.* (1989) and Whitsett and Wallick (1980). Clearly, an inhibition of Na,K-ATPase in the tissue of hypertensives leads to an imbalance in cellular sodium homeostasis with a number of possible consequences for pathology. The enzyme might be, in principle, inhibited by a circulating EDLF or its lower activity might be due to a genetic disposition altering the enzyme in the cell membrane.

Table 1
Na,K-ATPase activity (A), number of binding sites for anthroyl ouabain (N) and the dissociation constant of anthroyl ouabain (K_D) in placental microsomal membranes. Means ± S.D. of three experiments are shown

Tissue	A mol P_i per mg protein per h	N pmol per mg protein	K_D nmol per l
Healthy	1.40±0.11	58±7	3.5±1.5
PIH	1.04±0.09*	100±9*	0.9±0.2*

*Means ± S.D., * $p < 0.01$.*

To resolve this question, membranes from normotensive and PIH tissues were examined for anthroyl ouabain binding which is known to occur at the active enzyme only. Thus, the binding capacity reflects the number of active Na,K-ATPase molecules in the plasma membrane. A typical experiment shown in Fig. 1 provides the following information. The straight line shows anthroyl ouabain fluorescence in the presence of excess nonfluorescent ouabain which occupies virtually all the specific binding sites. Hence, only nonspecific binding of the Na,K-ATPase is observed. This straight line was virtually identical in the concentration range used with that obtained with anthroyl ouabain in the buffer without enzyme. Lines 2 and 3 (in their left-hand segments) show that specific binding resulted in a higher quantum yield (higher initial slopes), so that the quantum yield of anthroyl ouabain in normal placenta was higher than in the PIH placenta. The binding sites of Na,K-ATPase from PIH

and normal tissues thus appear to differ in their microenvironment. Fluorescence intensities increased linearly up to a transition region which lay at a lower concentration of anthroyl ouabain in the normal tissue than in the PIH tissue.

Three such determinations were performed and the anthroyl ouabain binding capacity and dissociation constant were calculated (Table 1). The values for the tissue of normotensive women agree with a previous radioactive study (Whitsett and Wallick 1980). However, somewhat unexpectedly, the ouabain-binding capacity in the hypertensive tissue did not decrease but rather increased together with a decrease of the dissociation constant.

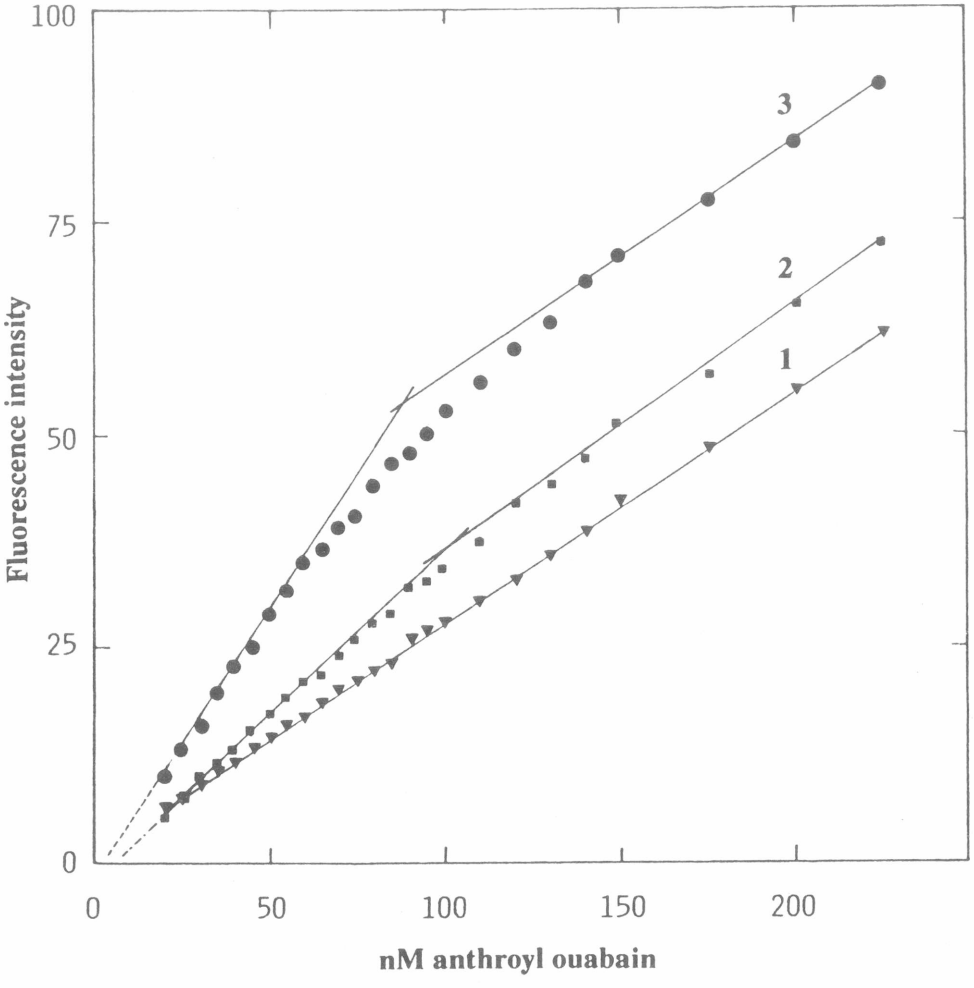

Fig. 1
Binding of anthroyl ouabain to placental microsomal membranes. 0.4 ml microsomal suspension from normal placenta (4.8 mg protein/ml) and 0.4 ml from placenta of PIH women (3.5 mg protein/ml) were placed in a buffer with anthroyl ouabain, either in the presence of 1 mM ouabain (1 – the same trace for both tissues) or alone (2 – PIH tissue, 3 – normotensive tissue). Total volume was 1.4 ml. After equilibrium had been reached, the sample was excited at 362 nm and fluorescence read at 469 nm.

The higher number of active Na,K-ATPase sites in PIH membranes implied that the Na,K-ATPase inhibition in hypertensive tissue was not due to a lower amount of the enzyme in the plasma membrane but was probably due to some structural changes of the enzyme. This resulted in a decreased efficacy of the individual enzyme molecules as was also indicated by a different initial slope of fluorescence of bound anthroyl ouabain. In fact, the higher number of the active Na,K-ATPase sites may reflect a cellular response to compensate for the insufficiency by a higher insertion of Na,K-ATPase into the plasma membrane. The putative structural changes of the enzyme in the hypertensive tissue caused a four-fold higher affinity of the enzyme for anthroyl ouabain. A hypothetical endogenous digitalis-like factor can thus easily be responsible for the Na,K-ATPase inhibition even if its level remains unchanged. This is also a way of explaining the different extent of Na,K-ATPase inhibition in different tissues of the same patient.

Even if the binding site for the cardiac glycosides is known to be extracellularly exposed, one can hardly conclude that this part is responsible for the observed higher affinity of Na,K-ATPase for cardiac glycosides. The reason is that ouabain apparently promotes long-range changes in the enzyme structure (Fortes and Aquilar 1988). One might speculate about a reverse situation – a change in the intracellular segment may bring about a long-range disturbance of the ouabain binding site.

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References

AMLER E., ABBOTT A., BALL W.J.: Structural dynamics and oligomeric interactions of Na,K-ATPase as monitored using fluorescence energy transfer. *Biophys. J.* **61:** 553–568, 1991.

AMLER E., MAZZANTI L., BERTOLI E., KOTYK A.: Lifetime determination of low sample concentrations: A new cuvette for highly accurate and sensitive fluorescence measurements. *Biochem. Int.* **27:** 771–776, 1992.

BALL W.J., FRIEDMAN M.L.: Immunochemical evidence that the FITC labeling site on Na,K-ATPase is not the ATP binding site. *Biochem. Biophys. Res. Commun.* **148:** 246–253, 1987.

FORTES P.A.G.: A fluorometric method for the determination of functional Na,K-ATPase and cardiac glycoside receptors. *Anal. Biochem* **158:** 454–462, 1986.

FORTES P.A.G., AQUILAR R.: Distances between 5'-iodoacetamido-fluorescein and the ATP and ouabain binding sites of Na,K-ATPase determined by fluorescence energy transfer. In: *The Na,K-Pump.* Part A, J.C. SKOU, J.G. NORBY, A.B. MAUNSBACH, M. ESMANN (eds), A.R. Liss, New York, 1988, pp. 197–204.

GOTO A., YAMADA K., ISHII M., YOSHIOKA M., ISHIGURO T., EGUCHIO C., SUGIMOTO T.: Purification and characterization of human urine-derived digitalis-like factor. *Biochem. Biophys. Res. Commun.* **154:** 847–853, 1988.

GRUBER K.A., WHITAKER J.M., BUCKALEW V.M.: Endogenous digitalis-like substance in plasma of volume-expanded dogs. *Nature* **287:** 743–745, 1980.

MAZZANTI L., RABINI R.A., CESTER N., ROMANINI C., BERTOLI E.: Transmembrane cation transport: An approach to the study of the molecular basis of hypertension. In: *Molecular Basis of Membrane-Associated Diseases.* A. AZZI, Z. DRAHOTA, S. PAPA (eds), Springer-Verlag, Berlin, 1989, pp. 130–133.

MAZZANTI L., RABINI R.A., STAFFOLANI R., BENEDETTI G., CESTER N., LENAZ G.: Modifications induced by general anesthetics on Na,K-ATPase obtained from human placenta. *Biochem. Biophys. Res. Commun.* **173:** 1248–1251, 1990.

PAMNANI M.B., WHITEHORN W.V., CLOUGH D.L., HADDY F.J.: Effects of canrenone on blood pressure in rats with reduced renal mass. *Am. J. Hypertens.* **3:** 188–195, 1990.

PARENTI P., VILLA M., HANOZET G.M., FERRANDI M., FERRARI P.: Increased Na pump activity in the kidney cortex of the Milan hypertensive rat strain. *FEBS Lett.* **290:** 200–204, 1991.

TAMURA M., LAM T.-T., INAGAMI T.: Isolation and characterization of a specific endogenous Na,K-ATPase inhibitor from bovine adrenal. *Biochemistry* **27:** 4244–4253, 1988.

WHITSETT J.A., WALLICK E.T.: [^{3}H]-ouabain binding and Na,K-ATPase activity in human placenta. *Am. J. Physiol.* **238:** E38–E45, 1980.

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