Ouabain-Like Na\(^+\),K\(^+\)-ATPase Inhibitory Activity of a Plasma Extract in Normal Pregnancy and Pregnancy Induced Hypertension

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ABSTRACT—To estimate the participation of a Na\(^+\),K\(^+\)-ATPase-inhibiting plasma factor in pregnancy induced hypertension (PIH), the inhibitory activity and the characteristics of plasma extract eluted with ethanol through a C8 column were examined in normotensive non-pregnant women (N) and women with normal pregnancy (NP) and PIH. There were no differences among the 3 groups in the Na\(^+\),K\(^+\)-ATPase activity of erythrocyte ghosts. The heat- and acid-stable plasma extract dose-dependently inhibited Na\(^+\),K\(^+\)-ATPase activity with a pattern similar to that of ouabain, but different from that of vanadate. The inhibitory activity of plasma extract was not influenced by polyclonal digoxin antibody which almost completely prevented digoxin-induced inhibition and slightly but significantly reduced the ouabain-induced one. The results indicate that the plasma extract has ouabain-like inhibitory activity on Na\(^+\),K\(^+\)-ATPase and that it is not endogenously synthesized digoxin itself, but a substance differing in structure from digoxin. Furthermore, the ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in NP plasma was significantly lower than that in PIH plasma, which was similar to that in N plasma. There were significant relationships between the ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activities in plasma and the diastolic and systolic blood pressures in NP and PIH groups. The results suggest that the lower ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in plasma probably participates in maintaining the blood pressure within the normal range during pregnancy and its failure may be involved in the genesis of PIH.

A hypothesis that plasma-volume expansion associated with salt retention leads to the release of a circulating Na\(^+\),K\(^+\)-ATPase inhibitor and that the inhibitor causes not only natriuresis but also vascular contraction resulting in hypertension has been proposed (1, 2). A canine plasma extract that inhibits Na\(^+\),K\(^+\)-ATPase released by volume expansion has been observed to have digoxin-like immunoreactivity (3), and the endogenous digitalis-like substance increases sensitivity to the vasoconstrictive effects of norepinephrine and angiotensin (4). Hamlyn et al. (5) showed that the Na\(^+\),K\(^+\)-ATPase inhibitory activity of a plasma substance was related to the high blood pressure in subjects with essential hypertension.
hypertension and suggested the involvement of a circulating Na\(^+\) pump inhibitor in the genesis of essential hypertension.

In pregnancy-induced hypertension (PIH), decrease in circulating blood volume, increase in peripheral vascular resistance and increases in the pressor responses to both norepinephrine (6) and angiotensin (6, 7) are observed, while plasma volume expansion and decrease in peripheral vascular resistance are normal accompaniments of pregnancy. Therefore, the involvement of a Na\(^+,K\(^+\)\)-ATPase-inhibiting plasma substance in the genesis of PIH is suggested. In fact, several laboratories have assayed the plasma levels of a substance with digoxin-like immunoreactivity in women with normal pregnancy (NP) and PIH. However, different results have been reported: some reports indicated that the level of the substance in PIH was higher than that in NP (8–10), but others found that it was lower (11, 12) or comparable to the level in NP (13). Digoxin-like immunoreactive substances in unpurified material are not necessarily always the same as the substances with Na\(^+,K\(^+\)\)-ATPase inhibitory activity (14), but only one of the studies determined the Na\(^+,\)K\(^+\)-ATPase inhibitory activities of the substances (8). Unfortunately, even when the enzyme activity was assayed (4, 8), the assay mixtures employed contained a high K\(^+\) concentration; under such a condition, the observed inhibitory activity would include the non-specific (i.e., non ouabain-like) inhibitory activity (15).

Therefore, we performed the present study to investigate more precisely the characteristics of the Na\(^+,K\(^+\)\)-ATPase inhibitory substance extracted from plasma with a C8 column; we assayed the ouabain-like Na\(^+,\)K\(^+\)-ATPase inhibitory activity of plasma extracts obtained from normotensive non-pregnant women (N) and women with NP and PIH. From our data, we should be able to determine whether the Na\(^+\) pump inhibitory factor in plasma can be considered as a possible cause of PIH. Because a marked reduction in the Na\(^+,\)K\(^+\)-ATPase activity of the erythrocyte membrane has been observed in PIH (16), we also determined the Na\(^+,K\(^+\)\)-ATPase activity in PIH erythrocyte ghosts.

**MATERIALS AND METHODS**

**Experimental subjects**

The female subjects in the present experiment consisted of three groups: normotensive non-pregnant women [N: n = 11; age, 20–33 (29 ± 1) years old; systolic blood pressure (S.B.P.), 92–124 mmHg; and diastolic blood pressure (D.B.P.), 60–82 mmHg in the sitting position]; women with normal pregnancy [NP: n = 16, at the 19th–39th (33 ± 2) week of gestation; age, 25–38 (31 ± 1) years old; S.B.P., 90–122 mmHg; D.B.P., 52–80 mmHg]; and women with pregnancy-induced hypertension [PIH: n = 13, at 19th–41st (33 ± 2) week of gestation; age, 24–42 (34 ± 2) years old; S.B.P., 140–250 mmHg; D.B.P., 100–160 mmHg].

**Collection of blood**

Thirty milliliters of fresh venous blood from the subjects was collected into a chilled 50-ml polycarbonate tube containing 300 U/0.3 ml of heparin and centrifuged immediately at 1000 × g for 30 min at 4°C. The supernatant was stored at –80°C until the following extraction procedure was carried out. The Na\(^+,\)K\(^+\)-ATPase activity of the sedimented erythrocytes was assayed. After aspiration of the buffy coat, the erythrocytes were immediately washed by isotonic Tris buffer and used for the preparation of erythrocyte ghosts (17).

**Preparation of erythrocyte ghosts**

The erythrocytes were resuspended in isotonic Tris buffer (0.172 M, pH 7.6 at 4°C) and then centrifuged at 1000 × g for 30 min at 4°C; this washing procedure was repeated four times. After washing, an equal volume of isotonic Tris buffer was added to the washed erythrocytes to make an erythrocyte suspension with a 50% hematocrit. Thirty milliliters of hypotonic Tris buffer (11 mM, pH 7.6 at 4°C) was added into 5 ml of erythrocytes sus-
pension, and the tube was allowed to stand 5 min so that hemolysis would occur. The hemolytic suspensions were centrifuged at 20000 × g for 40 min at 4°C. The sediment was washed with 30 ml of hypotonic Tris buffer and centrifuged at 20000 × g for 40 min at 4°C; this washing procedure was repeated four times. The sediment was suspended in hypotonic Tris buffer, and 5 ml of the erythrocyte ghost suspension was prepared.

Assay of Na⁺,K⁺-ATPase activity of erythrocyte ghosts

The assay was performed by the method of Esmann (18). In the presence and absence of 1 mM ouabain (Merck), 100 µl of erythrocyte ghost was incubated in 1 ml of a reaction mixture containing 124 mM NaCl, 20 mM KCl, 4 mM MgCl₂ and 30 mM histidine buffer (pH 7.4) at 37°C. After a 3-min preincubation, the reaction was started by the addition of 3 mM 2Na-ATP (Sigma). After 30 min, the reaction was terminated by adding 100 µl of chilled 50% trichloroacetic acid and centrifuged at 11300 × g for 15 min at 4°C. Inorganic phosphate hydrolyzed in the supernatants was measured by the modified method of Fiske and Subbarow (19). Na⁺,K⁺-ATPase activity was calculated by subtracting Mg³⁺-ATPase activity in the presence of 1 mM ouabain from the total ATPase activity in the absence of ouabain.

Extraction of Na⁺,K⁺-ATPase inhibitory substance from plasma

Na⁺,K⁺-ATPase inhibitory substance was extracted from plasma according to the methods of Boschi et al. (20) and Masugi et al. (15). Two milliliters of plasma was mixed with 4 ml of ethanol, and the mixture was heated in a boiling water bath for 2 min. After cooling in ice, the precipitates were centrifuged at 10000 × g for 10 min at 4°C and removed. The supernatant was evaporated to dryness under aspiration at 50°C within 30 min, and the residue was reconstituted with 2 ml of distilled water. The solution was brought to pH 3 with 5% formic acid at room temperature and centrifuged at 11000 × g for 10 min at 25°C. The acidified supernatant was applied on a C8 disposable column (Analytichem International) which was previously equilibrated with methanol, distilled water and acidified water (pH 3). The column was then washed twice with 4 ml of the acidified water and eluted three times with 2 ml of ethanol (Nacalai), which had been brought to pH 1 with HCl. The eluate was evaporated to dryness under vacuum, and the residue was dissolved in 1 ml of TES-EGTA buffer (pH 7.4).

The ability of a C8 column to extract digitalis-like substance from plasma was examined. N plasma added with 1.5 × 10⁻⁷ M ouabain was treated in the same way as described above, and the Na⁺,K⁺-ATPase inhibitory activity of the extract was compared to that of the extract from the plasma without ouabain.

Assay of Na⁺,K⁺-ATPase inhibitory activity of vanadate, ouabain and plasma extract

The assay mixture contained 40 mM TES-NaOH buffer (pH 7.4), 100 mM NaCl, 4 mM MgCl₂, 5 mM EGTA, 0.02% 1-O-n-octyl-β-D-glucopyranoside, 0.6 mM 2Na-ATP and 0.1 mM or 10 mM KCl. Vanadate (Nacalai) at 10⁻⁹–10⁻³ M, as a non-digitalis-like Na⁺,K⁺-ATPase inhibitor; 10⁻⁹–10⁻³ M ouabain, as a specific Na⁺,K⁺-ATPase inhibitor; or 20, 100 and 200 µl of plasma extract was added to 1 ml of the reaction mixture. After a 3-min preincubation at 37°C, the reaction was started by adding approximately 4.6 µg protein as the Na⁺,K⁺-ATPase (Sigma, A-7510: the standard from porcine cerebral cortex) for the 10 mM K⁺ solution and approximately 23 µg protein as the Na⁺,K⁺-ATPase for the 0.1 mM K⁺ solution. After a 30-min incubation at 37°C, the reaction was terminated by adding 100 µl of chilled 50% trichloroacetic acid. The mixture was centrifuged at 25000 × g for 10 min at 4°C and inorganic phosphate hydrolyzed in the supernatant was measured according to the modified method of Fiske and Subbarow (19).
Digoxin-like immunoreactivity

Fab fragments (1.8 μg) from digoxin antibody (anti-digoxigenin) from sheep (Boehringer Mannheim) was added to the 0.1 mM K⁺ reaction mixture containing 100 μl of the extract from PIH plasma, 3 × 10⁻⁸ M ouabain or 3 × 10⁻⁸ M digoxin to test the effect of digoxin antibody on their Na⁺,K⁺-ATPase inhibitory activities. After a 15-min preincubation at 37°C, the reaction was started by adding approximately 23 μg protein as the Na⁺,K⁺-ATPase. The respective Na⁺,K⁺-ATPase activities were assayed in the presence and absence of digoxin antibody, and the % inhibitions were compared.

Statistics

Data were analyzed statistically with Student’s t-test and analysis of variance (ANOVA) for multiple comparison.

RESULTS

Na⁺,K⁺-ATPase activity of erythrocyte ghosts

Na⁺,K⁺-ATPase activities of erythrocyte ghosts in the N, NP and PIH groups are shown in Table 1. There were no significant differences among the groups.

Effects of ouabain and vanadate on Na⁺, K⁺-ATPase activity

The effects of ouabain and vanadate on commercially available standard Na⁺,K⁺-ATPase activity in the reaction mixture containing 10 mM K⁺ or 0.1 mM K⁺ are shown in Fig. 1. Ouabain, 10⁻⁹–10⁻³ M, dose-dependently inhibited Na⁺,K⁺-ATPase activity in both the 10 mM K⁺ and 0.1 mM K⁺ solutions. However, the % inhibitions were significantly greater in the 0.1 mM K⁺ than in the 10 mM K⁺ solution. The differences changed as shown in Fig. 1 (upper) and were significant at least in the range of 10⁻⁸–10⁻⁶ M ouabain. In contrast, vanadate, 10⁻⁹–10⁻⁵ M, did not inhibit Na⁺,K⁺-ATPase activity in the 0.1 mM K⁺ solution and vanadate above 10⁻⁴ M dose-dependently inhibited the enzyme activity. In the 10 mM K⁺ solution, vanadate below 10⁻⁸ M was also not inhibitory, but above 10⁻⁷ M, it dose-dependently inhibited the enzyme activity like ouabain.

Extraction of ouabain-like factor from plasma

The ability of the C8 column to extract ouabain and ouabain-like substance from plasma is shown in Table 2. The N plasma extract inhibited Na⁺,K⁺-ATPase activity in the 0.1 mM K⁺ reaction mixture to 69.8 ± 2.5% of the control level (mean ± S.E., n = 3), corresponding to the effect of approximately 2 × 10⁻⁸ M ouabain. In contrast, extract from the plasma added with 1.5 × 10⁻⁷ M ouabain (final concentration of 3 × 10⁻⁸ M) inhibited the enzyme activity to 45.8 ± 2.5% (n = 3, P < 0.01 vs. plasma extract), corresponding to the effect of approximately 5 × 10⁻⁸ M oua-

| Group | n  | total ATPase | Mg²⁺ ATPase | Na⁺,K⁺-ATPase |
|-------|----|--------------|-------------|---------------|
| N     | 9  | 230 ± 17     | 122 ± 14    | 108 ± 12      |
| NP    | 8  | 230 ± 12     | 110 ± 4     | 119 ± 9       |
| PIH   | 8  | 269 ± 17     | 140 ± 15    | 129 ± 14      |

Na⁺,K⁺-ATPase activity was calculated by subtracting Mg²⁺-ATPase activity in the presence of 1 mM ouabain from total ATPase activity in the absence of ouabain. Mean ± S.E., N: normotensive non-pregnant women, NP: normal pregnancy, PIH: pregnancy induced hypertension.
Fig. 1. Effects of $10^{-9} - 10^{-3}$ M ouabain (left, $n = 4$) and $10^{-9} - 10^{-3}$ M vanadate (right, $n = 5$) on Na$^+,K^+$-ATPase activity in the 10 mM K$^+$ (●) and 0.1 mM K$^+$ reaction mixtures (○). Specific activities of Na$^+,K^+$-ATPase in the 10 mM K$^+$ and the 0.1 mM K$^+$ solutions without ouabain and vanadate were, respectively, approximately 31.7 µmol Pi/mg protein/30 min and 2.94 µmol Pi/mg protein/30 min at 37°C, which are taken as 100% activity. Vertical lines are the S.E. *P < 0.05 vs. 100% Na$^+,K^+$-ATPase activity. Differences of the % inhibitions (upper) were calculated by subtracting the mean values in the 10 mM K$^+$ solution from the mean values in the 0.1 mM K$^+$ solution. *P < 0.05 and **P < 0.01: significant differences between the % inhibitions in the 0.1 mM K$^+$ and the 10 mM K$^+$ solutions.

Table 2. Na$^+,K^+$-ATPase inhibitory effects of extracts from N plasma with or without $1.5 \times 10^{-7}$ M ouabain

| Na$^+,K^+$-ATPase activity (%) | No. 1 | No. 2 | No. 3 |
|-------------------------------|------|------|------|
| TES-EGTA buffer               | 100 µl | 100  |
| Ouabain                       | $10^{-8}$ M | 83.5 |     |
|                               | $10^{-7}$ M | 40.0 |     |
| Plasma extract                | 100 µl | 65.5 | 74.4 | 69.4 |
| Plasma (added ouabain) extract* | 100 µl | 42.3 | 44.5 | 50.7 |
| (ouabain $3 \times 10^{-8}$ M) |      |      |      |

Specific activity of Na$^+,K^+$-ATPase in the 0.1 mM K$^+$ reaction mixture without ouabain and plasma extract was 2.85 µmol Pi/mg protein/30 min at 37°C, which is taken as 100% activity. *: N plasma added with $1.5 \times 10^{-7}$ M ouabain was treated in the same manner, and the final concentration of ouabain in the reaction mixture was $3 \times 10^{-8}$ M. N: normotensive non-pregnant women.
bain, that is, inhibition was equal to the sum of the effects of plasma extract and ouabain added to the plasma.

**Na\(^+\),K\(^+\)-ATPase inhibitory activity of plasma extract**

Na\(^+\),K\(^+\)-ATPase inhibitory activities of the plasma extracts in the N, NP and PIH groups are shown in Fig. 2. Plasma extracts in the 3 groups inhibited dose-dependently Na\(^+\),K\(^+\)-ATPase activity in both the 10 mM K\(^+\) and 0.1 mM K\(^+\) reaction mixtures. In the 10 mM K\(^+\) solution, the % inhibition by 100 \(\mu\)l of the plasma extracts were 14–20%, and there were no significant differences in the enzyme inhibitory effect by 20–200 \(\mu\)l of the plasma extracts among the 3 groups. In the 0.1 mM K\(^+\) solution, 21% inhibition by 100 \(\mu\)l of the NP plasma extract was significantly smaller than the 38% inhibition in the N and PIH groups. The % inhibitions produced by 20 and 200 \(\mu\)l of the plasma extract in the NP group were also significantly smaller than those in the other 2 groups, and there were no significant differences between the N and PIH groups. The differences between the % inhibitions in the 10 mM K\(^+\) and in the 0.1 mM K\(^+\) solutions are shown in Fig. 3. The differences in the NP group increased dose-dependently, but were significantly smaller than those in the other 2 groups, and there were no significant differences between the N and PIH groups. The differences in the PIH group were not statistically significant but tended to be higher than those in the N group.

![Fig. 2](image_url)

**Fig. 2.** Effects of 20–200 \(\mu\)l plasma extract in the groups of normotensive non-pregnant women (Δ, n = 7) and women with normal pregnancy (○, n = 8) and pregnancy induced hypertension (△, n = 10) and the effect of \(10^{-8}–10^{-7}\) M ouabain (n = 19) on Na\(^+\),K\(^+\)-ATPase activity in the 10 mM K\(^+\) (upper) and in the 0.1 mM K\(^+\) solutions (lower). Na\(^+\),K\(^+\)-ATPase activities without the plasma extracts and ouabain are taken as 100% activity. Vertical lines are the S.E. *P < 0.05 and **P < 0.01 vs. N group. All other explanations are as in the legend to Fig. 1.

![Fig. 3](image_url)

**Fig. 3.** Differences between the % inhibitions in Na\(^+\),K\(^+\)-ATPase activity in the 10 mM K\(^+\) and the 0.1 mM K\(^+\) solutions by plasma extracts in the groups of normotensive non-pregnant women (II) and women with normal pregnancy (□) and pregnancy induced hypertension (■). The values are calculated from the results shown in Fig. 2, and vertical lines are the S.E. *P < 0.05 and **P < 0.01 vs. normotensive non-pregnant group. *P < 0.05 and **P < 0.01 vs. normal pregnancy group. All other explanations are as in the legends to Figs. 1 and 2.
Effect of digoxin antibody on Na⁺,K⁺-ATPase inhibitory activity of plasma extract

Na⁺,K⁺-ATPase inhibitory activities of PIH plasma extract, ouabain and digoxin in the presence and absence of polyclonal digoxin antibody in the 0.1 mM K⁺ reaction mixture are summarized in Table 3. The inhibitory activity of digoxin was significantly inhibited by digoxin antibody solution, and the inhibitory activity of ouabain was slightly but significantly inhibited. In contrast, the inhibitory activity of PIH plasma extract was not significantly changed by the antibody.

Relationships between blood pressure and Na⁺,K⁺-ATPase inhibitory activity of plasma extract

Relationships between the % inhibitions of Na⁺,K⁺-ATPase activity by 100 μl plasma extract in the 10 mM K⁺ and the 0.1 mM K⁺ solutions vs. diastolic and systolic blood pressures in the NP and PIH groups are shown in Fig. 4. The diastolic blood pressures (mean ± S.E.) were 71 ± 1, 58 ± 1 and 116 ± 6 mmHg, and the systolic blood pressures were 112 ± 2, 97 ± 2 and 186 ± 10 mmHg, respectively, in the N, NP and PIH groups. The Na⁺,K⁺-ATPase inhibitory activities of the plasma extracts in 0.1 mM K⁺ solution were significantly related to the diastolic blood pressure and also to the systolic blood pressure in the NP and PIH groups. In contrast, there were no significant relationships between the enzyme inhibitory activities in 10 mM K⁺ solution and the diastolic and systolic blood pressures.

DISCUSSION

The Na⁺,K⁺-ATPase activity of erythrocyte ghosts in the PIH group is similar to those in the N and NP groups. This suggests that the Na⁺,K⁺-ATPase activity of the erythrocyte membrane in the PIH group is probably intact, being coincident with the previous report (21), and that the existence of a Na⁺,K⁺-ATPase inhibitor is important for the regulation of the enzyme activity and cell functions. It is difficult to explain the difference from the result of Tranquilli et al. (16), but their experimental conditions are different from ours: all their hypertensive patients had received antihypertensive drugs for at least one week, the blood samples were obtained at 37–40 weeks of gestation, and their assay medium for Na⁺,K⁺-ATPase activity is a little different.

In the 10 mM K⁺ solution, both ouabain and vanadate similarly inhibited Na⁺,K⁺-ATPase activity even at the low concentrations; therefore, it is very difficult to differentiate between the ouabain-like Na⁺,K⁺-ATPase inhibitory effect and the non-ouabain-like inhibitory effect in the solution. In the 0.1

| Table 3. Effect of digoxin antibody on Na⁺,K⁺-ATPase inhibitory activity of PIH plasma extract |
|-----------------|-----------------|-----------------|-----------------|
| % Inhibition of Na⁺,K⁺-ATPase activity | antibody(−) | antibody(+) | antibody(−) − (+) × 100(%) |
| TES-EGTA buffer | 100 μl | 5 | 0 | antibody(−) | 34.0 ± 6.5 | − 3.4 ± 9.8 |
| PIH plasma extract | 100 μl | 6 | 32.5 ± 5.4 | 37.1 ± 2.8* | 23.9 ± 5.2† |
| Ouabain | 3 × 10⁻⁸ M | 9 | 48.8 ± 1.2 | 6.8 ± 2.6** | 87.0 ± 5.3†† |
| Digoxin | 3 × 10⁻⁸ M | 8 | 49.1 ± 2.7 |

Specific activity of Na⁺,K⁺-ATPase in the 0.1 mM K⁺ reaction mixture without digitalis and plasma extract was 2.93 ± 0.27 μmol Pi/mg protein/30 min at 37°C, which was taken as 100% activity and 0% inhibition. Mean ± S.E., PIH: pregnancy induced hypertension, *P < 0.05 and **P < 0.01 vs. digoxin antibody(−). †P < 0.05 and ††P < 0.01 vs. PIH plasma extract.
mM K\(^+\) solution, ouabain inhibited the enzyme activity dose-dependently at the lower concentration, but vanadate below 10\(^{-5}\) M did not inhibit the activity. Therefore, the % inhibition by plasma extract in the 0.1 mM K\(^+\) solution seems to represent the ouabain-like action. However, vanadate at higher concentration also inhibited the enzyme activity, so that it is still not appropriate to estimate the ouabain-like effect from the % inhibition in the 0.1 mM K\(^+\) solution alone. The Na\(^+,K\(^+\)-ATPase inhibitory activity of ouabain was always greater in the 0.1 mM K\(^+\) solution than in the 10 mM K\(^+\) solution, but that of vanadate was the opposite. The differences obtained by subtracting the enzyme inhibitory activity in the 10 mM K\(^+\) solution from that in the 0.1 mM K\(^+\) solution give a positive value for ouabain and a negative value for vanadate. Therefore, on the basis of the difference, we can decide whether the enzyme inhibitory activity is caused by a ouabain-like action or a vanadate-like (non ouabain-like) action. The conclusion supports the assay method of Masugi et al. (15) using 0.1 mM K\(^+\) solution for detecting the ouabain-like action. In the present experiment, the plasma extracts in the N, NP and PIH groups dose-dependently inhibited Na\(^+,K\(^+\)-ATPase activity in both the 0.1 mM K\(^+\) and 10 mM K\(^+\) solutions, but the inhibitory activities at the same concentrations were always greater in the 0.1 mM K\(^+\) solution. The results indicate that the plasma extracts have ouabain-like Na\(^+,K\(^+\)-ATPase inhibitory activity.

The possible existence of endogenously synthesized ouabain (22, 23) or digoxin (24) as one of several different types of endogenous digitalis-like substances has been shown (25). In addition, the present method which was useful for extracting the heat- and acid-stable plasma substance inhibiting Na\(^+,K\(^+\)-ATPase could also extract ouabain added in plasma. However, polyclonal digoxin antibody in the 0.1 mM K\(^+\) reaction mixture significantly inhibited the Na\(^+,K\(^+\)-ATPase inhibitory activ-

\[\text{Fig. 4. Relationships between the % inhibitions in Na}\(^+,K\(^+\)-ATPase activity in the 10 mM K\(^+\) (upper) and the 0.1 mM K\(^+\) solutions (lower) by 100 \mu l\) plasma extracts in normal pregnancy (○) and pregnancy induced hypertension groups (●) vs. the diastolic (left) and the systolic blood pressure (right). Data are from individual subjects. D.B.P.: diastolic blood pressure, S.B.P.: systolic blood pressure.\]
ities of both ouabain and digoxin, but did not inhibit that of the PIH plasma extract. Polyclonal digoxin antibody probably recognizes some portions of the digitalis molecule but not the binding site to Na\(^+\),K\(^+\)-ATPase. Digoxin antibody binding to digitalis probably induces a conformational change in the digitalis molecule, which decreases the Na\(^+\),K\(^+\)-ATPase inhibitory activity. Therefore, the present results imply that the plasma extract probably does not have digoxin-like immunoreactivity if it is a small molecule like digitalis, although the binding to the antibody was not measured. It is obvious that the plasma extract is not endogenously synthesized digoxin itself but a substance with a structure different from that of digoxin, and yet it has ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity. The digitals of compounds usually has digoxin-like immunoreactivity (26), but in the present experiment, the immunoreactivity to digoxin antibody of ouabain was significantly lower than that of digoxin, although those Na\(^+\),K\(^+\)-ATPase inhibitory activities were not different. These results may support the observation of Yamada et al. (14) in which the Na\(^+\),K\(^+\)-ATPase inhibitory activity dissociates from the digoxin-like immunoreactivity. Therefore, the possibility that endogenously synthesized ouabain is contained in the plasma extract still remains.

The lower ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in the NP plasma than in the N plasma does not coincide with data obtained by the digoxin antibody binding method (9, 10, 27). One of the possible causes of the difference may be that the present plasma extract does not contain endogenously synthesized digoxin even if the substance is present in the plasma; that is, the substances determined are different. In addition, it has been reported that there are some digoxin-like immunoreactive factors without Na\(^+\),K\(^+\)-ATPase inhibitory activity (14, 28). Another cause may be the dilution of the ouabain-like substance inhibiting Na\(^+\),K\(^+\)-ATPase by the larger circulating blood volume in the NP group than in the N group. However, the present result does not contradict the physiological symptoms in NP (i.e., the larger circulating blood volume and the lower blood pressure), because it is probable that inhibitions of Na\(^+\),K\(^+\)-ATPase in the plasma membranes of urinary tubular cells and vascular smooth muscle cells may induce, respectively, natriuresis by decreasing Na\(^+\)-reabsorption and vasoconstriction by intracellular Na\(^+\) increase, resulting in an intracellular Ca\(^2+\) increase through the Na\(^+\)-Ca\(^2+\) exchange system. A ouabain-displacing factor has been observed in the urines of the N, NP and PIH groups by the receptor-binding assay using renal Na\(^+\),K\(^+\)-ATPase (29). Thus, the lower ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in the NP plasma may play a major role in the physiological conditions of NP. In contrast, the ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in the PIH plasma was not significantly higher than that in the N plasma in spite of the higher blood pressure. The results do not necessarily coincide with other reports (9, 10) in which significantly greater digoxin antibody binding substance was observed in the PIH plasma. The higher blood pressure in the PIH group than in the N group cannot be explained only by the ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in plasma in the present experiment. Endogenous digoxin-like immunoreactive factor, which we did not detect, may also be involved in the elevation of the blood pressure, because the factor increases markedly in both the NP and PIH groups after the 30th week of gestation (8, 10, 12, 13) where the blood pressure increases within the normal level in the NP group and to an even higher level in the PIH group. However, the ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activity in the PIH plasma was significantly greater than that in the NP plasma, and there were significant relationships between the ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory activities and the diastolic and systolic blood pressures in the NP and PIH groups. The ouabain-like Na\(^+\),K\(^+\)-ATPase inhibitory substance may also work together with norepinephrine, angiotensin or some other agents specific to pregnancy. This
is suggested by observations that digoxin (30) and the endogenous digitalis-like substance (4) increase sensitivity to the vasoconstrictive effects of both norepinephrine and angiotensin, and the pressor responses to the both agents increase in the PIH group (6, 7) and that to angiotensin decreases in the NP group (6, 7). In fact, the norepinephrine contraction of canine mesenteric artery in 4 mM K+ Krebs-Henseleit solution in in vitro experiments was greater in the presence of the present PIH plasma extract than the NP plasma extract (H. Moromizato et al., unpublished data). Thus, the lower ouabain-like Na+,K+-ATPase inhibitory activity in plasma probably participates in maintaining the blood pressure at a lower level or within the normal range in pregnancy, and the failure to maintain the lower inhibitory activity in pregnancy may be involved in the genesis of PIH.

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