Stimulatory guanine nucleotide binding protein activity in the erythrocyte membrane of patients with pseudohypoparathyroidism type I and related disorders

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Abstract. The activity of stimulatory guanine nucleotide regulatory protein (Ns) in the erythrocyte membrane was assayed by the reconstitution method using plasma membrane of cyc- S49 mouse lymphoma cells in 18 patients with type I pseudohypoparathyroidism (PHP-I), 2 with pseudopseudohypoparathyroidism (PPHP) and 30 normal subjects, in parallel with other clinical parameters. The Ns activity as expressed by per cent of pooled standard (mean ± se) was 78.9 ± 6.1 in PHP-I patients, which was significantly lower (P < 0.01) than the value in normal subjects, 99.5 ± 2.4. In PHP-I patients, the Ns activities (Y) were in significant correlation with three clinical parameters examined (X), i.e., with body height in standard deviation score from the mean of the normal population at the corresponding age, Y = 89.4 + 10.4X (r = 0.616, P < 0.01); with urinary cAMP excretion in relation to creatinine [cAMP(nmol)/Cr(mg)], Y = 56.3 + 7.2X (r = 0.501, P < 0.05); and with TSH levels in plasma (μU/ml), Y = 129 - 3X (r = 0.639, P < 0.01). The Ns activities of PPHP were as low as 53.8 and 60.0. The decrease of Ns activity in the cell membrane may be implicated in the development of the clinical symptoms such as short stature, decrease in urinary excretion of cAMP and latent or manifest primary hypothyroidism in PHP-I and possibly in skeletal abnormality in PPHP.

Patients with pseudohypoparathyroidism type I (PHP-I) have a variety of disturbances in hormonal actions, which are manifested through cAMP production by the activation of adenylate cyclase. They include primary hypothyroidism (Marx et al. 1971) and hypogonadism (Wolfsdorf et al. 1978). As the pathogenesis of disturbances due to...
failure to produce cAMP, a deficiency of stimulatory guanine nucleotide binding protein (Ns) in the cell membrane of the target organ may be involved. Two groups have reported that the Ns activity in the erythrocyte membrane is below normal in approximately half of the patients with PHP-I (Farfel et al. 1980; Levine et al. 1980). The deficiency of Ns was further reported in thrombocytes (Farfel & Bourne 1980), skin fibroblasts in culture (Bourne et al. 1981; Levine et al. 1982), transformed lymphocytes (Farfel et al. 1982) and renal tissue (Downs et al. 1982) taken from patients with PHP-I showing low Ns activity in the erythrocyte membrane. Recently, it has been reported that patients with PHP-I having a low Ns activity in the erythrocyte membrane have a high incidence of symptoms of Albright's osteodystrophy, exaggerated response of TSH to TRH infusion and impaired response of cAMP in plasma to glucagon administration (Levine et al. 1983). In order to further clarify the significance of Ns deficiency in the cell membrane in the development of PHP-I, the activity of Ns in the erythrocyte membrane was determined in the present study in patients with PHP-I or other related disorders and in normal subjects, and the correlation between the activities of Ns and several clinical parameters was examined in the patients and controls.

Materials and Methods

Subjects

The activity in the erythrocyte membrane was assayed in 30 normal subjects, 18 patients with PHP-I, 2 patients with pseudopseudohypoparathyroidism (PHP), 3 with pseudohypoparathyroidism type II (PHP-II), 4 with idiopathic hypoparathyroidism and 6 with primary hypothyroidism. The patients with PHP-I consisted of 3 males and 15 females ranging in age from 15 to 30 years. The diagnosis of PHP-I was established by hypocalcaemia and impaired renal response to PTH in the absence of renal failure. The renal response to PTH was examined by the urinary excretion of cAMP during a 1-h period and that of phosphate during a 2-h period before and after infusion of 100 U of synthetic 1-34 hPTH. The response was judged to be normal if the increase in the urinary excretion of cAMP was larger than 1 μmol/h and 10-fold and that of phosphate was higher than 35 mg/2 h in the presence of hypocalcaemia (Yamamoto et al. 1982). The criteria for the diagnosis of Albright's hereditary osteodystrophy (AHO) were the radiographic metacarpal sign (Archibald et al. 1959) and/or sc calcification in the cases of PHP-I. All patients with PHP-I were treated by the administration of 1α-hydroxycholecalciferol for hypocalcaemia and were normocalcaemic at the time of Ns assay. Only 1 patient with PHP-I was given thyroid hormone for the treatment of hypothyroidism. The diagnosis of PHP was established by sc calcification or the metacarpal sign, normocalcaemia, normal level of PTH concentration in plasma and normal response of cAMP to PTH infusion. The radiographic evaluation of the metacarpal sign was made by one of the authors (T.S.), who was given X-ray film of both hands without knowing the assay values of the patients.

Two or 3 h after breakfast, 10 ml of blood were withdrawn from the antecubital vein into a heparinized syringe. Urine was also collected at that time. The blood sample was transported on ice to the laboratory, and the erythrocyte membrane was separated within 24 h after sampling. The activity of Ns was assayed by the method of reconstitution of the plasma membrane of cyc' S49 mouse lymphoma cells as previously reported (Farfel et al. 1980; Ross & Gilman 1977; Kaslow et al. 1980) and partly by the method of ADP ribosylation (Kaslow et al. 1980) with modifications.

Erythrocyte membrane

The blood was centrifuged at 1000 × g for 10 min at 4°C, and the plasma was kept for the hormone assay. The buffy coat layer of the pellet was aspirated, and the packed red blood cell (one vol) was suspended in five vol of 0.15 M NaCl, 5 mM phosphate buffer, pH 8, and centrifuged for 10 min at 1000 × g. After two additional washings by this procedure, the erythrocytes were lysed in 40 vol of 5 mM phosphate buffer, pH 8, and centrifuged at 15 000 × g for 20 min. The pellet was then suspended in 40 vol of the same buffer and centrifuged. After an additional wash, the pellet was suspended in the buffer and stored at −70°C until assay.

Reconstitution method

For the extraction of Ns from the erythrocyte membrane, the membrane was suspended in 0.2% lubrol, which was deionized by AG501-X8 resin at the concentration of 20 mg/ml. The suspension was kept at 0°C for 16 h and centrifuged at 100 000 × g for 60 min. The supernatants in a vol of less than 10 μl containing the Ns extracted from 2.5, 5.0 and 7.5 μg of erythrocyte membrane were put into assay tubes. The total amount of protein and lubrol in the assay was kept constant at 10 μl by supplementing the reaction mixtures with appropriate vol of erythrocyte extract in which Ns had been inactivated by heating at 90°C for 10 min. The emergence of adenylate cyclase activity in the cyc' membrane by reconstitution with Ns of erythrocyte extract was completely eliminated in the Ns inactivated by this condition. The extract was mixed with 75 μl of
assembly mixture containing 10 mM MgCl₂, 0.1 mM RO 20-1724 (Roche), 10 μg/ml of pyruvate kinase, 0.1 mM Na l-asparate, 1 mM Na EDTA, 3 mM K-phosphoenol pyruvate, 0.1 mg/ml of bovine serum albumin, 100 μM Gpp(NH)p, 40 μg of plasma membranes of cyc S49 cells and 50 mM Hepes, pH 8.0. The mixture was incubated for 120 min at 30°C for the reconstitution of the membrane with Ns. One μCi [³²P]ATP (10 to 50 Ci/mmol) in 15 μl was then added and incubated for an additional 15 min for the assay of adenylate cyclase activity. The final concentration of ATP was 0.5 mM. The radioactivity of [³²P]cAMP was determined after purification with columns of Dowex AG50 and alumina by the method of Salomon (1979). In order to standardize the response of adenylate cyclase after reconstitution, the Ns activity of the standard erythrocyte membrane, which was prepared by pooling the membrane from 6 normal subjects and stored in aliquot at -70°C, was determined in every assay. The Ns activities of the samples were then expressed as the percentage of the standard membrane, which had the absolute activity of 654 ± 50 pmol cAMP/mg/min (mean ± sem, n = 12). The dose-response relationship was linear between the extract of the erythrocyte membrane and the adenylate cyclase activity after the reconstitution in the range of from 0 to 15 μg of protein.

**ADP ribosylation method**

One mg/ml of the erythrocyte membrane was suspended in 100 μl of reaction mixture containing 50 μg/ml of cholera toxin activated by diithiothreitol, 100 μM [³²P]NAD with a specific activity of 1 to 6 Ci/mmol; 10 mM thymidine, 1 mM ATP, 4 mM GTP, 1 mM MgCl₂, 0.5 mM EDTA, 5 mM Hepes, 10 mM phosphoenol pyruvate and 50 μg/ml of pyruvate kinase. The mixture was then incubated for 10 min at 30°C. The reaction was stopped by adding 1 ml of cold 5 mM Tris hydrochloride, pH 7.5, and the solution was centrifuged at 27 000 x g for 10 min. The pellet was washed two more times with Tris buffer and applied to 10% acrylamide slab gel for SDS polyacrylamide electrophoresis by the method of Laemmli (1970). The gel was then exposed to Kodak XAR film using two intensifying screens for 3 h at -70°C. The band that corresponded to Ns, the protein with a molecular weight of 42 000, was identified on the film, and the gel strip corresponding to this protein was cut off and counted in a liquid scintillation counter.

**Hormone assay**

The levels of T₄, free T₄, TSH and cortisol in plasma and the urinary concentration of cAMP were determined by radioimmunoassay using commercial kits.

**Statistical method**

The statistical significance of the data was evaluated by Student's t-test.

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**Cells and chemicals**

cyc S49 mouse lymphoma cells were generously supplied by Dr. H. R. Bourne, University of California, San Francisco. [³²P]NAD was purchased from New England Nuclear Corp. [³²P]ATP and a kit for free T₄ were obtained from Amersham Medical Ltd. Co., Tokyo. Kits for radioimmunoassay of TSH, cortisol and thyroxine were from Daiichi Radioisotope, Tokyo. The kit for cAMP was from Yamasa, Chiba, Japan. The synthetic 1-34 hPTH was supplied by Toyojozo Ltd. Co., Tokyo, and RO 20-1724 was a gift from Roche Co., Tokyo.

**Results**

**Ns activity in the erythrocyte membrane**

The activity of Ns in the erythrocyte membrane assayed by the reconstitution method was 99.5 ± 2.4% (mean ± se) in normal subjects and 78.9 ± 6.1% in patients with PHP-I, which was significantly lower (P < 0.01) (Fig. 1). The lower limit of

![Fig. 1](image_url)
the normal range as defined by the mean-2 SD of the value of normal subjects was 73.2%. Out of 18 patients with PHP-I, 9 had the AHO sign, and their levels of Ns activity were 66.5 ± 7.3%, which was significantly lower than the activity of patients without the AHO sign, i.e., 94.5 ± 6.1% (P < 0.01). The activities assayed by ADP ribosylation in 9 of the patients with PHP-I correlated significantly with those assayed by the reconstitution method (Fig. 2). Accordingly, the activities were assayed by the reconstitution method thereafter.

Correlation of Ns activity with physical findings
There was no significant difference between the Ns activities of the males and females among the PHP patients or normal subjects. Furthermore, the Ns activities of the normal subjects and the patients were not in significant correlation with their ages. There was a significant positive correlation between the levels of Ns activity and the body height expressed as a score of the standard deviation from the mean of the normal Japanese population at identical ages. There was a tendency for the patients with AHO to be associated with a short stature and reduced Ns activity (Fig. 3).

There was also a significant positive correlation between Ns activity and metacarpal index, the mean of the ratios of the length to the width of the fourth metacarpus in right and left hand (Parish 1966) (Fig. 4).

Ns activity and hormonal levels
There was a significant correlation between the Ns activities, Y (%), and the ratio of cAMP to creatinine in the urine X [cAMP(nmol)/Cr(mg)]; Y = 56.3 + 7.2X (r = 0.501, P < 0.05). The patients with AHO showed a tendency to excrete less cAMP into the urine.

Among the 18 patients with PHP-I, 2 were positive for microsome antibody and another was receiving thyroxine replacement. These 3 were omitted from the analysis for the thyroid hormone and TSH. There was no significant correlation between the activities of Ns and plasma levels.
H.O. and Y.N., had sc calcification, normocalcaemia and normal response of urinary excretion of cAMP and phosphate in response to PTH infusion (Table I). Patient Y.N. had a daughter with PHP-I associated with a decrease in Ns activity. Patient H.O. had metacarpal sign and primary hypogonadism with an exaggerated response of LH to GnRH. The patients with PHP-II, idiopathic hypogonadism or primary hypothyroidism showed normal activity of Ns in the erythrocyte membrane.

Discussion

The average activity of Ns in the erythrocyte membrane in patients with PHP-I was lower than that of normal subjects. Half of the patients showed normal levels of Ns and the other half

of T4 (r = 0.052, P > 0.05) or cortisol (r = 0.070, P > 0.05). However, there was a significant negative correlation between TSH concentrations in plasma and Ns activity (Fig. 5). The body height in standard deviation score was also in significant negative correlation with TSH levels in plasma (Fig. 6). As shown in Fig. 6, the PHP-I patients with short stature showed a tendency to have an elevated level of TSH, high incidence of AHO and low activity of Ns (less than 73.2%) in erythrocyte membrane.

Ns activities in the disorder other than PHP-I

The Ns activities in the erythrocyte membrane in patients with PHP-II, PPHP, idiopathic hypoparathyroidism and primary hypothyroidism are shown in Fig. 7. It is noteworthy that 2 patients with PPHP showed a decrease in the activity of Ns in their erythrocyte membranes. Both patients,

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Correlation between TSH concentration in plasma and body height in PHP-I patients with or without AHO and with low (L) or without (N) decreased Ns activity in the erythrocyte membrane. The Ns activity below 73.2% was judged to be low. The body height represents the SD score from the mean of the normal Japanese population at corresponding ages.

showed levels that were below normal. Seven out of 9 PHP-I patients having low levels of Ns had bone abnormalities compatible with AHO, while 2 out of the other 9 patients having normal levels of Ns had signs of AHO. These incidences of low Ns levels in PHP-I patients and in the patients with or without AHO seem to be compatible with those previously reported in the Unites States (Levine et al. 1980, 1983; Farfel & Bourne 1980).

The negative correlation observed between the Ns activities and the serum levels of TSH may indicate the presence of latent hypothyroidism stemming from the impairment of the thyroid response to TSH and is apparently compensated for by the elevation of TSH. The hypothyroid state itself may not affect the Ns activity since the 6 patients with primary hypothyroidism showed normal levels of Ns. Mallet et al. (1982) have reported that the adenylate cyclase of the thyroid tissue in patients with PHP-I failed to be activated by TSH. The present result with TSH seems to be compatible with their in vitro finding as well as with the recent report about the exaggerated response of TSH release to TRH administration in patients with PHP-I having low Ns activity in the erythrocyte membrane (Levine et al. 1983).

It is noteworthy that Ns activities of erythrocyte membrane were in significant correlation with body height expressed in SD score as well as metacarpal index. As PTH may stimulate both bone formation and resorption, the ultimate effect of the hormone on the bone mass may be determined by the balance between these two processes (Parsons 1976). The impaired response to PTH in the bone tissue due to Ns deficiency may result in short stature. The possibility that the latent hypothyroidism may partly contribute to the reduced height as in the case of congenital hypothyroidism is also indicated. The measure-
ment of Ns activity in the erythrocyte membrane, thus, may permit prediction of body height in paediatric patients with this disorder. A careful follow-up of the thyroid function is required, especially in cases with low Ns activity.

The loose but significant correlation observed between the urinary excretory ratio of cAMP to creatinine and the Ns activity may indicate that the cAMP production in the body as a whole is impaired in patients with PHP-I having a low Ns activity in the erythrocyte membrane. The impaired responses may include the cAMP production in the kidney by PTH and in the liver by glucagon as major sources of the urinary cAMP (Hardman 1971).

The findings seem to confirm the implication of Ns deficiency in the cellular membrane in the pathogenesis of PHP-I, at least in some cases. In particular, the clinical symptoms of PHP-I, which are related with the hormonal responses mediated via cAMP, including the skeletal development and thyroid responsiveness to TSH, may be dependent on the activity of Ns in the cell membrane.

In our two cases of PPHP, the Ns activities were decreased, as reported in two other cases of this disorder (Farfel et al. 1981; Fischer et al. 1983). As to the relationship between PHP-I and PPHP, it has been suggested that PPHP is a variant of PHP-I. This was based on two reasons. The first is that these two disorders are often found in the same family (Arnstein et al. 1966; Boscerini et al. 1980) and a common genetic process seems to be involved in the pathogenesis. The second is that some patients with PHP-I having hypocalcaemia at a certain stage become normocalcaemic without treatment, compatible with PPHP, afterwards (Monn et al. 1967; Mautalen et al. 1967). The decrease in the Ns activity observed in the PPHP patients seems to provide the third basis for this hypothesis. The fact that case Y.N.’s daughter had PHP-I and decreased activity of Ns in the erythrocyte membrane as well seems to support this concept. The genetically induced deficiency of Ns activity in the cell membrane, thus, may be a common pathogenesis of short stature and/or AHO in some cases of PHP-I and PPHP.

Further studies are required as to the pathogenesis of PHP-I in which no decrease in Ns activity was observed. In the present study, however, two cases of PPHP with decreased Ns activity

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**Table 1.**

Characteristics of the 2 patients with PPHP.

<table>
<thead>
<tr>
<th>Case</th>
<th>H. O.</th>
<th>Y. N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Body height (cm, SD)</td>
<td>143.5 ± 3.0</td>
<td>151.0 ± 0.7</td>
</tr>
<tr>
<td>Sc calcification</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Brachydactyly</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Metacarpal sign</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Others</td>
<td>Primary hypogonadism</td>
<td>PHP-I in daughter (Ns1: 67.5%)</td>
</tr>
<tr>
<td>Serum Ca (mg/dl)</td>
<td>9.2</td>
<td>8.7</td>
</tr>
<tr>
<td>Serum PTH (ng/ml)</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>PTH (100 U)iv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UcAMP (before/after)</td>
<td>110.0</td>
<td>92.3</td>
</tr>
<tr>
<td>U phosphate (mg/2 h)</td>
<td>72.1</td>
<td>26.3</td>
</tr>
<tr>
<td>Ns activity in erythrocyte membrane (%)</td>
<td>53.8</td>
<td>60.0</td>
</tr>
</tbody>
</table>

1. Ns activity in the erythrocyte membrane in the daughter with PHP-I.
2. C-terminal immunoreactivity.
3. Ratio of urinary excretion of cAMP during the 2 h before and after infusion of PTH.
4. Difference of urinary excretion of phosphate during the 2 h before and after infusion of PTH.
in the erythrocyte membrane showed normal levels of serum calcium and obvious response of the kidney to the infusion of PTH, as evaluated by urinary excretion of cAMP and phosphate. It seems possible that distribution of Ns deficiency among organs in some patients is uneven, and Ns activity in the erythrocyte membrane may not closely represent the final responses of a hormone in other target tissues, such as the kidney or skeletal system, which are mediated through cAMP.

In conclusion, the significant correlation between Ns activity in the erythrocyte membrane and some clinical findings in patients with PHP-I or PPHP in the present study is consistent with the hypothesis that the deficiency of Ns in the cell membrane plays an important role in the pathogenesis of these disorders, at least in some cases, although it still remains to be elucidated to what extent the Ns activity assayed in the solubilized membrane from a tissue may reflect the Ns-mediated responses in other tissues.

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References


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