Platelets of pseudohypoparathyroid patients: Evidence that distinct receptor-cyclase coupling proteins mediate stimulation and inhibition of adenylate cyclase

(α2-adrenergic receptor/prostaglandin I2/GTP binding protein)

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ABSTRACT We studied platelets of patients with the genetic disorder pseudohypoparathyroidism (PHP) to test whether the nucleotide-binding proteins mediating stimulation of adenylate cyclase (termed N₅) are identical to those mediating inhibition of cyclase (termed N₆). Functional responses to hormones that work through stimulation of adenylate cyclase are blunted in PHP patients. The erythrocytes of many of these patients (PHP-Ia) have been shown to have decreased N₂ activity whereas those of other PHP patients (PHP-Ib) have normal N₁ activity. We find that this decreased N₂ activity (measured by the ability to restore adenylate cyclase activity to membranes prepared from S49 cyc⁻/cell) also occurs in the platelets of PHP-Ia but not of PHP-Ib patients. Platelets from both groups of patients accumulate less cAMP in response to prostacyclin than do platelets from control subjects. In contrast to the decreased N₂ function in patients with PHP-Ia, we find that N₁ function in platelets is similar in these patients and control subjects in several types of experiments: (i) epinephrine-mediated inhibition of prostacyclin-stimulated cAMP production in intact platelets; (ii) the affinity of platelet α₂-adrenergic receptors for epinephrine, as determined by competition for [³H]yohimbine binding; (iii) the decrease in receptor affinity for epinephrine produced by Na⁺ and GTP; and (iv) the concentration dependence of GTP for decreasing the affinity of these receptors for epinephrine. Because N₁ is expressed normally in platelets from patients that are genetically deficient in N₂, we conclude that N₁ and N₂ are likely to be distinct gene products.

Hormonal stimulation of adenylate cyclase involves the interaction of several components: the hormone receptor, the adenylate cyclase catalytic unit, and one or more coupling proteins (herein termed N₅) that link the two and bind GTP (for review, see refs. 1 and 2). Hormonal inhibition of adenylate cyclase also requires GTP, and GTP alters the binding of agonists to the inhibitory receptors (1); thus, the existence of an inhibitory coupling protein (N₆) has been inferred. Whether N₁ and N₂ activities are simply two separate functions of one gene product is unclear. Both N₁ and N₂ interact with hormone receptors, adenylate cyclase, and GTP; thus, they are clearly similar. Yet receptor occupation is translated into stimulation of adenylate cyclase by N₁ and into inhibition of adenylate cyclase by N₂.

Our approach to determining whether N₁ and N₂ are the same was to examine their activity and properties in a single cell type, the human platelet. Platelets possess GTP-regulated adenylate cyclase that is both stimulated by prostaglandin I₂ (prostacyclin) (PGI₂) and certain other prostaglandins and inhibited by epinephrine and ADP (3–5). Thus, both N₁ and N₂ activities are present in human platelets.

We have compared N₁ and N₂ activities in platelets from control subjects and patients with pseudohypoparathyroidism (PHP), a genetic disorder associated with decreased responses to hormones that work through stimulation of adenylate cyclase (6). The activity of N₁, as assayed by its capacity to restore hormone-sensitive adenylate cyclase activity to the N₂-deficient membranes of S49 cyc⁻/cells or by its [³²P]ADP ribosylation in the presence of cholera toxin and NAD, is decreased in erythrocytes (and other tissues) in some patients with PHP (PHP-Ia) (7–10). N₁ is normal in erythrocytes of other patients (PHP-Ib) who respond defectively to hormones. We report here the results of our studies of the platelets of seven PHP patients. We show that (i) the N₁ activity of PHP platelets corresponds with the previously determined erythrocyte N₁ assay; (ii) platelets from all PHP patients accumulate decreased amounts of cAMP in response to maximal stimulation with PGI₂; and (iii) N₁ is expressed normally in platelets of PHP-Ia patients, as assessed by epinephrine-mediated inhibition of adenylate cyclase and by interaction of epinephrine with α₂-adrenergic receptors linked to N₁. The latter findings strongly suggest that N₁ and N₂ activities are likely to be mediated by different proteins.

METHODS

Patients. Seven patients with PHP were studied; these were the same patients reported previously (7). Previous assays demonstrated decreased activity of erythrocyte N₁ in three of the patients (PHP-Ia) but not in four others (PHP-Ib) (7). Control platelets were obtained from healthy men (ages 22–45) who had taken no medication for at least 1 wk.

Platelets. Blood was drawn into 60-mI plastic syringes containing 6 ml of 3.8% sodium citrate and centrifuged at 300 × g for 20 min, and the platelet-rich plasma was removed. The packed erythrocytes were mixed with an equal volume of 100 mM NaCl/5 mM EDTA/50 mM Tris·HCl, pH 7.5, and were centrifuged twice. The platelet-rich plasma and the two extracts were combined and centrifuged at 5,000 × g for 10 min and the pellet was washed twice by the same procedure. Determination of cAMP accumulation and some of the radioligand binding assays were carried out with intact platelets suspended in 50 mM Tris·HCl/100 mM NaCl/5 mM EDTA, pH 7.5. In other ra-

Abbreviations: PHP, pseudohypoparathyroidism; N₁, nucleotide binding unit coupled to inhibition of adenylate cyclase; N₂, nucleotide binding unit coupled to stimulation of adenylate cyclase; PGI₂, prostaglandin I₂ or prostacyclin.

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dioligand experiments, the platelets were suspended in 5 mM Tris, pH 7.5/0.5 mM EDTA at 4°C and homogenized with a “zero-clearance” motor-driven Teflon-tipped homogenizer (Kontes models K886031 and K885752). The homogenate was centrifuged at 30,000 × g for 10 min and the pellet was washed three times. The final particulate (or “membrane”) suspension (≈0.3 mg/mL) was kept ice cold until use.

N\textsubscript{2} Assay. The variant S49 cye- mouse lymphoma cells contain β adrenergic receptors and adenylate cyclase but no functional N\textsubscript{2}. One method to assay N\textsubscript{2} is to determine the degree to which adenylate cyclase activity can be restored to membranes prepared from these S49 cye- cells. We assayed N\textsubscript{2} activity in the platelets from the PHP patients by using this reconstitution assay as described (9). The platelet membranes were pelleted, resuspended to a concentration of 0.5 mg of protein per ml, and then treated with 0.2% Lubrol 12A9 for 60 min at 0°C. The suspension was centrifuged at 100,000 × g for 45 min and the supernatant fractions were incubated at 37°C for 15 min to inactivate the catalytic subunit of adenylate cyclase. Various amounts (0.5–5 μL) of the soluble platelet extract were then mixed with 15 μg of membranes prepared from cye- S49 cells, and cAMP synthesis was assayed after 40 min of incubation with 100 μM guanosine 5′-O-thio)triphosphate. The adenylate cyclase assay conditions were identical to those used previously (9, 10).

cAMP Assay. Washed platelets were suspended in Hanks' balanced salt solution lacking calcium and magnesium salts but including 5 mM NaHepes/0.5 mM 1-methyl-3-isobutylxanthine, pH 7.6, to a concentration of ≈0.25 mg/mL. Duplicate or triplicate aliquots of the platelet suspension (0.38 mL), dispensed into 12 × 75 mm polypropylene tubes, were incubated for 30 min at 37°C to allow basal cAMP levels to equilibrate. Drugs were added in a volume of 0.40 mL. Because PGI\textsubscript{2} is very unstable in solution at neutral pH, it was prepared at a concentration of 1 mg/mL in alkaline buffer and diluted immediately before use in the incubations with platelets. After 1 min of incubation of platelets with drugs, 0.1 mL of 40% (wt/vol) trichloroacetic acid, containing ≈15,000 cpm of [3H]cAMP, was added. The mixtures were incubated at room temperature for at least 30 min and then centrifuged at 900 × g for 5 min. The supernatants from the tubes were separately applied to columns containing 1 g of Dowex AG50-X8 resin, and the columns were washed twice with 1.2 mL of water to remove the trichloroacetic acid and with 4 mL of distilled water to elute the cAMP. This elute was lyophilized to dryness in a Savant-Vac centrifugal lyophiller and the lyophilate was stored at 0°C prior to assay for cAMP.

The contents of each tube were reconstituted in 50 mM NaOAc, pH 4.0/0.2 mM 1-methyl-3-isobutylxanthine, and an aliquot was assayed for cAMP by using a competitive protein binding method (11). The recovery of cAMP from the Dowex columns (routinely >90%) was calculated by comparing the amount of tritium in the reconstituted solution with the amount of [3H]cAMP added originally and was used to correct the determined amount of cAMP. Replicate determinations varied ±13%.

Protein Determination. Protein was determined by the method of Peterson (12), using log-log transformation to yield a linear standard curve. Albumin solutions containing NaN\textsubscript{3} at 1 mg/mL as a noninterfering bacteriocide were used as standards.

Radioligand Binding. Binding of [3H]yohimbine (an α\textsubscript{2}-adrenergic antagonist) to platelets or to platelet membranes was carried out in duplicate exactly as described (13, 14). Briefly, the platelets or membranes were incubated with [3H]yohimbine and various other drugs for 30 min at 25°C. In experiments with epinephrine, 0.8 mM ascorbic acid (freshly prepared) was included to prevent catecholamine oxidation. The platelets were then filtered over fiberglass filters (Whatman GF/C), which were washed with 20 mL of buffer. Specific binding was defined as that competed for by 10 μM phenolamine. Addition of Na\textsuperscript{+} to the membranes sometimes, but not always, increased specific [3H]yohimbine binding by <20%. Saturation binding isotherms were analyzed by Scatchard plots and competition curves were analyzed by a computer program that uses nonlinear regression to fit the data to the mass–action binding equation (15).

Statistics. Results are presented as mean ± SEM and statistical significance was calculated by two-tailed t tests.

RESULTS

Assessment of N\textsubscript{2} in Platelets. A decreased N\textsubscript{2} activity in platelets of two PHP-Ia patients was reported previously (9). We now confirm this finding with data from several additional patients. The platelets of PHP-Ia patients demonstrated 63% of the N\textsubscript{2} activity of simultaneously assayed control subjects (P = 0.002). The platelets of PHP-Ib patients, however, had nearly normal N\textsubscript{2} levels (84% of control, P > 0.05; Fig. 1).

N\textsubscript{2} couples hormone receptors to stimulation of adenylate cyclase. We measured PGI\textsubscript{2}-stimulated cAMP production in intact platelets of patients and control subjects. The shapes of the dose–response curves (Fig. 2) were similar but the maximal cAMP production of the platelets from the PHP-Ia patients (Fig. 3) was only 67 ± 2% of that simultaneously assayed controls (P = 0.001). The platelets of PHP-Ib patients (Fig. 3) also had lower maximal PGI\textsubscript{2}-stimulated cAMP accumulation than controls (80 ± 3% of control, P = 0.04). There was a great deal of variation between assays even for the same subject determined on separate occasions, making it necessary to assay cAMP accumulation in control subjects and PHP patients in parallel. In every experiment, PHP patients generated less cAMP than did the controls; PHP-Ia platelets produced a maximum of 407 ± 63 pmol of cAMP/mg of protein, whereas controls produced 605 ± 83 pmol/mg.

Assessment of N\textsubscript{2} in Platelets. To assess whether N\textsubscript{2} activity was decreased in patients with deficient N\textsubscript{2}, we examined N\textsubscript{2}...
activity in platelets from three PHP-Ia patients by studying cAMP accumulation and binding to α2-adrenergic receptors. N1 mediates inhibition of platelet adenylate cyclase by several agents, including the adrenergic agonist epinephrine. We measured the inhibition of PGI2-stimulated cAMP production in intact platelets produced by epinephrine (Fig. 4). The dose-response curves were identical in patients and control subjects, and maximal concentrations of epinephrine inhibited PGI2-stimulated cAMP production similarly (by 86 ± 9% and 80 ± 3%, respectively) in patients and control subjects.

Inhibition of adenylate cyclase by epinephrine is mediated by an α2-adrenergic receptor linked to N1, α2-Adrenergic receptors can be directly probed with the selective antagonist [3H]yohimbine (13, 16, 17). Saturation binding isotherms of [3H]yohimbine to intact platelets of PHP-Ia patients were normal. There were 274 ± 66 (normal, 160–415; 5–95% of 35 subjects) α2-adrenergic receptors per platelet in the PHP-Ia patients and the Kd value for [3H]yohimbine binding was 2.7 ± 0.2 nM (normal, 2.0–4.0). The interaction of epinephrine with these receptors is complex. In washed platelet membranes (lacking GTP) epinephrine competes for two classes of α2-receptors, termed high- and low-affinity sites (18), that may correspond to receptors that are and are not attached to N1, respectively (17, 19). In the presence of GTP, epinephrine competes for only a single class of binding sites in equilibrium radioligand binding experiments; these binding sites correspond to the low-affinity sites. Epinephrine competed for [3H]yohimbine binding (tested in either the absence or presence of GTP) to membranes prepared from platelets of control and PHP-Ia patients (Fig. 5 and Table 1). We also found that the concentration of GTP required to decrease the ability of epinephrine to compete for [3H]yohimbine binding was identical in platelets from patients and from controls (Fig. 6). The binding of epinephrine to the α2-adrenergic receptors is also altered by Na+ (13, 18), although the molecular mechanism of this effect is obscure. Na+ (alone or with GTP) identically decreased the ability of epinephrine to compete for [3H]yohimbine binding to membranes from platelets of controls and PHP-Ia patients (Fig. 5 and Table 1).

**DISCUSSION**

The guanylyl nucleotide regulatory protein mediating hormonal stimulation of adenylate cyclase, N1, has been probed in biochemical, genetic, and functional studies (2, 20–23). Several peptides that comprise purified N1 have recently been identified (23). The existence of an inhibitory coupling protein, termed N1, has been surmised because some hormones act by inhibiting adenylate cyclase in a GTP-dependent manner (1, 24). No direct assay for N1 exists and its presence can only be assessed indirectly by the characteristics of hormone-mediated
three control nucleotides on reason, fore, are means himbine, epinephrine, and 100 mM NaCl (○), 100 mM GTP (△), both (○), or neither (□), and the specific binding was determined. Results are means of determinations with three PHP-Ia patients (Upper) and three control subjects (Lower). These data are analyzed in Table 1.

To determine whether one protein embodies both N_s and N_i activities, we studied the platelets of PHP-Ia patients. The tissue attenuation of adenylate cyclase or by the influence of guanine nucleotides on the binding of agonists to receptors. For this reason, comparatively little is known about N_i.

Table 1. Affinity of α2-adrenergic receptors in platelet membranes for epinephrine

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<th>Addition</th>
<th>K_d, μM</th>
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<td>NaCl (100 mM)</td>
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Data from experiments shown in Fig. 5 with platelets from either three control subjects or three PHP-Ia patients were simultaneously analyzed by a computer program that uses iterative nonlinear regression to fit data to equations based on the law of mass action (15). A model with two classes of binding sites fits the data for membranes lacking Na^+ and GTP much better than did a single-site model. Therefore, two K_d values are given for these experiments; numbers in parentheses are percentages of each class of sites. When either NaCl, GTP, or both were included, the two-site model did not fit the data statistically better than the one-site model. None of the differences between PHP and control platelets are significant.

sues of these patients show decreases in N_s activity, and this deficiency in N_i appears to be the molecular basis of hormonal resistance in this disease. We confirmed that the platelets of PHP-Ia patients had decreased N_s activity by a direct complementation assay and by showing that the maximal cAMP production of their platelets was abnormally low. Because the 30% loss of N_s causes clinically apparent abnormalities in PHP patients as well as biochemical changes in their platelets, it seems likely that a corresponding loss of N_i would lead to detectable changes. Thus, if N_s and N_i activities are expressed by the same moiety, one would expect a complementary decrease in N_i activity. We found, however, that epinephrine interacted with platelet α2-adrenergic receptors normally, with normal modulation by Na^+ and GTP, and that the dose–response curve for modulation by GTP was normal. Furthermore, the dose–response curves and maximal responses for epinephrine-mediated inhibition of PGI_2-stimulated cAMP production were normal. These observations indicate that N_i is expressed normally in the PHP-Ia platelets that are genetically deficient in N_s, therefore suggesting that N_i and N_s are distinct.

Our genetic evidence that N_i and N_s are distinct entities agrees with several other recent observations on platelets: that the concentrations of GTP required to enhance or inhibit platelet adenylate cyclase are quite different (4, 5); that the concentrations of Mn^2+ required to block N_i-mediated inhibition of adenylate cyclase by epinephrine are much lower than the concentrations required to block N_s-mediated stimulation of adenylate cyclase (1, 25); that cholera toxin appears to label proteins associated with N_s (26, 27) but does not appear to label solubilized components included in a high molecular weight complex of α_2-receptors, epinephrine, and (presumably) N_i (27); and that the maximal extent to which adenylate cyclase can be stimulated through N_s exceeds that to which it can be inhibited through N_i (28). Confirmation of the conclusion that N_i and N_s are distinct will require a more direct means of studying N_i. To date, however, we have been unsuccessful in our attempts to use platelets as donors of N_i in reconstitution experiments.

It is possible that there is indeed a deficiency of N_i in PHP-Ia platelets but this does not lead to an alteration in the ability of epinephrine to inhibit adenylate cyclase or to compete for ^3H)yohimbine binding. As noted above, if N_s and N_i were the
same and were depleted in PHP-Ia patients, it is unlikely that the N-mediated stimulation of cyclase would be impaired while the N-mediated inhibition of cyclase remained unaltered. This could, however, be possible if the concentration of N exceeded that of α2-adrenergic receptors but was smaller than the number of PGI2 receptors; then the concentration of N would be limiting for stimulation of cyclase but the concentration of receptors would be limiting for inhibition. However, the reported number of platelet prostaglandin receptors linked to stimulation of cyclase is not greater than the number of platelet α2-adrenergic receptors, making this argument untenable (29, 30).

Decreased N activity is not found in all PHP patients—thus, the distinction between type Ia (deficient N) and type Ib (normal N) PHP patients who are otherwise clinically indistinguishable. The lack of N presumably explains the hormonal resistance found in PHP-Ia patients. The hormonal resistance of PHP-Ib patients, however, currently defies explanation. The platelets of these patients have an impaired ability to accumulate cAMP, and further studies of these readily available cells may help untangle the molecular etiology of PHP-Ib.

Note Added in Proof. Our conclusion that N2 and N3 are distinct in platelets has been supported by new evidence that appeared while this article was in press: (i) additivity of prostaglandin E1- and epinephrine-promoted [3H]guanlylimidodiphosphate release (31), (ii) differential trypsin (32) and N-ethylmaleimide (33) sensitivity of prostaglandin E1 stimulation and epinephrine inhibition of adenylyl cyclase, and (iii) differences in sensitivity of prostaglandin E1 and epinephrine stimulation of GTPase to N-ethylmaleimide (33).

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