Impaired Formation of β-Adrenergic Receptor-Nucleotide Regulatory Protein Complexes in Pseudohypoparathyroidism

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Abstract. Decreased activity of the guanine nucleotide regulatory protein (N) of the adenylate cyclase system is present in cell membranes of some patients with pseudohypoparathyrodism (PHP-Ia) whereas others have normal activity of N (PHP-Ib). Low N activity in PHP-Ia results in a decrease in hormone (H)-stimulatable adenylate cyclase in various tissues, which might be due to decreased ability to form an agonist-specific high affinity complex composed of H, receptor (R), and N. To test this hypothesis, we compared β-adrenergic agonist-specific binding properties in erythrocyte membranes from five patients with PHP-Ia (N = 45% of control), five patients with PHP-Ib (N = 97%), and five control subjects. Competition curves that were generated by increasing concentrations of the β-agonist isoproterenol competing with [^{125}I]pindolol were shallow (slope factors < 1) and were computer fit to a two-state model with corresponding high and low affinity for the agonist. The agonist competition curves from the PHP-Ia patients were shifted significantly ($P < 0.02$) to the right as a result of a significant ($P < 0.01$) decrease in the percent of β-adrenergic receptors in the high affinity state from 64±22% in PHP-Ib and 56±5% in controls to 10±8% in PHP-Ia. The agonist competition curves were computer fit to a "ternary complex" model for the two-step reaction: H + R + N ⇌ HR + N ⇌ HRN. The modeling was consistent with a 60% decrease in the functional concentration of N, and was in good agreement with the biochemically determined decrease in erythrocyte N protein activity.

These in vitro findings in erythrocytes taken together with the recent observations that in vivo isoproterenol-stimulated adenylate cyclase activity is decreased in patients with PHP (Carlson, H. E., and A. S. Brickman, 1983, *J. Clin. Endocrinol. Metab.* 56:1323–1326) are consistent with the notion that N is a bifunctional protein interacting with both R and the adenylate cyclase. It may be that in patients with PHP-Ia a single molecular and genetic defect accounts for both decreased HRN formation and decreased adenylate cyclase activity, whereas in PHP-Ib the biochemical lesion(s) appear not to affect HRN complex formation.

Introduction

Pseudohypoparathyroidism (PHP)[1] is a rare genetic disorder characterized by end organ resistance to the effects of parathyroid hormone. Albright and colleagues (1) initially reported this disease and recognized that a particular phenotype, marked by short stature with shortening of the metacarpals and/or metatarsals, may accompany the biochemical disorder in PHP (1).

Recently, several investigators have recognized that end

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1. *Abbreviations used in this paper:* C, catalytic unit of adenylate cyclase; EC_{50}, concentration of isoproterenol inhibiting 50% of radioligand binding; Gpp(NH)p, guanylyl-imidodiphosphate; H, hormone; N, guanine nucleotide regulatory protein; PHP, pseudohypoparathyroidism.

organ resistance to a variety of hormones may occur as part of the PHP disorder. Thus, dysfunction of thyroid (2–4), gonadotropin (5), prolactin (6–8), and glucagon (4) action have been described as a consequence of hormone resistance. Very recently, a blunted plasma cyclic adenosine monophosphate (cAMP) response to the infusion of the β-adrenergic agonist isoproterenol has been described in patients with PHP (9).

Numerous investigations have been directed at determining whether a ubiquitous biochemical defect underlies the end organ resistance to PHP. These investigations have focused on the hormone (H) receptor-adenylate cyclase complex since it was first found that patients with PHP do not have a significant rise in urinary cyclic AMP excretion after the administration of PTH (10).

The adenylate cyclase-receptor complex consists of at least three separable plasma membrane components: the hormone receptor (R), the catalytic unit (C) of adenylate cyclase, and a guanine nucleotide regulatory protein (G/F or N protein) which serves to couple the receptor to the catalytic moiety (11). Several lines of evidence have suggested decreased activity of this N protein in activating adenylate cyclase in various tissues (including circulating cells) of some patients with PHP (PHP-Ia) most of whom had Albright's hereditary osteodystrophy (12–17). By contrast, phenotypically normal patients, who demonstrate abnormal urinary cAMP excretion in response to parathyroid (PTH) infusion, have normal assayable N protein activity in circulating cells (PHP-Ib).

The β-adrenergic receptor-N protein-adenylate cyclase complex is the most extensively studied and best understood hormonal-adenylate cyclase system (18–20). Previous work has demonstrated that hormonal agonists promote the coupling of β-adrenergic receptors with the N protein to form a high affinity complex (HRN), which is an intermediate in the pathway to adenylate cyclase activation (21). Guanine nucleotides, which are required for the hormonal activation of adenylate cyclase, revert the high affinity state of the receptor to its low affinity form (HR), concurrent with activation of the adenylate cyclase enzyme. Quantitation of the percentage of receptors in the high and low affinity complexes can be accomplished by computer modeling of agonist competition curves with β-adrenergic antagonist radioligands (18–19). Cells that either lack the N protein on a genetic basis (cyc^-) (22) or in which there is impaired RN coupling for any reason show absent or impaired formation of this high affinity agonist-receptor complex. As a result, such competition curves are shifted to the right (to higher concentration of isoproterenol inhibiting 50% of radioligand binding [EC_{50}]).

Accordingly, we assessed the ability of β-adrenergic receptors of human erythrocytes to form high affinity receptor complexes in erythrocyte membranes that were derived from five control subjects, five patients with PHP-Ia (low erythrocyte N protein), and five patients with PHP-Ib (normal erythrocyte N protein). This procedure required improvement of methods for detecting the small number of β-adrenergic receptors in these cells (23). The results shed further light both on the nature of the hormone-responsive adenylate cyclase system and on the pathophysiologic defects in patients with pseudohypoparathyroidism.

Methods

We studied five patients with pseudohypoparathyroidism type-Ia, five patients with pseudohypoparathyroidism type-Ib, and five healthy volunteers.

The clinical and laboratory diagnosis of PHP and assessment of the presence or absence of Albright's hereditary osteodystrophy was made by Dr. Drezner except for patient A.M. who was evaluated by Dr. Spiegel (see Table I). The diagnosis of PHP was further confirmed in the patients by measurement of the urinary cAMP response to PTH infusion (10), which in all cases was <20 μmol cAMP/g creatinine in the urine. In addition, all patients demonstrated a reduced phosphaturic response to PTH and elevated tubular maximum for reabsorption of phosphate. Each patient was initially evaluated while he was untreated and hypocalcemic. The present studies were performed after long-term treatment with vitamin D and/or calcium supplementation in each patient and with maintenance of normocalcemia.

At the time of the study, PTH hormone levels were determined by three different radioimmunoassays, which have been previously described and validated (24–26). "PTH assay 1" is a carboxy-terminal PTH assay performed at the Mayo Clinic (24). "PTH assay 2" is performed at Duke University and is also a carboxy-terminal PTH assay (25). "PTH assay 3" was performed on one patient (A.M.) and is a mid-region (amino acids 44–68) assay (26).

Human erythrocyte membrane preparation. After obtaining informed consent, we obtained 200 ml of whole blood via venipuncture. A 45-ml aliquot of this whole blood was collected into 5 ml of acid-citrate-dextrose anticoagulant and immediately placed on ice but not frozen. Erythrocyte membranes were prepared within 24 h of venipuncture for determination of N protein activity (13). N protein was assayed according to the reconstitution assay described by Kaslow et al. (27) but was modified by incubating the erythrocyte membrane extracts and cyc^- membranes for 20 min at 30°C in the presence of adenylate cyclase assay mixture before the addition of [^{32}P]ATP and the performance of the assay.

We anticoagulated the remaining 155 ml of whole blood by using U. S. Pharmacopoeia sodium beef lung heparin (The Upjohn Co., Kalamazoo, MI) (1,000 U of heparin per 49 ml of whole blood) and prepared erythrocyte β-adrenergic receptor-containing membranes. The erythrocytes were sedimented by the addition of 10 ml of 3% Dextran (Dextran T-500, Pharmacia Fine Chemicals, Piscataway, NJ) in normal saline to 25 ml of whole blood and left undisturbed for 20 min. The supernatant layer, which contained the plasma and most of the leukocytes and platelets, was discarded and 50 ml of erythrocytes was used for subsequent steps. These erythrocytes were washed three times with normal saline by centrifugation at 1,600 *g* and both the supernatant and small buffy coat layer on top of the erythrocyte layer were aspirated and discarded. We used 25 ml of the total volume of washed erythrocytes for the erythrocyte β-adrenergic receptor assays.

1 ml of the washed erythrocyte preparation was used for determination of the erythrocyte count and to assess the homogeneity of the erythrocyte preparation. Blood counts were made by use of an ELT-8/d.s. (Ortho Instruments, Westwood, MA) cell counter and by direct quantitative examination of a smear of the erythrocyte preparation for the presence of platelet and white cell contaminants.

Since we observed that the level of erythrocyte β-adrenergic receptor binding was very low, we sought to rigorously exclude the possibility

Table I. Patient Population Characteristics

Patient	Age	Sex	Serum calcium*	Serum phosphorus*	Serum PTH-assay 1*‡	Serum PTH-assay 2*§	N Protein‖*
			mg/dl	*mg/dl*	*μeq/ml*	*ng/ml*	*% of control*
PHP-Ia patients							
G.W.	33	F	8.7	4.4	85	1.7	56±9
L.H.	32	M	9.5	3.9	—	0.73	43±9
M.W.	7	M	8.9	4.9	—	3.14	62±6
J.P.	22	F	9.5	3.8	—	1.70	41±7
A.M.+	28	F	8.8+	5.0+	—	—	22±9
PHP-Ib patients							
K.R.	14	M	9.2	3.4	—	0.87	96±21
B.R.	13	F	9.1	5.4	110	2.34	84±13
S.R.	34	F	9.1	3.7	51	2.26	100±16
B.M.	49	F	9.6	3.7	—	1.1	97±11
R.J.	29	F	9.5	4.1	65	—	110±17
Normal values			8.8–10.2	2.5–4.5	<70	<1.5	100

* Measured while on vitamin D and/or calcium replacement. ‡ Mayo Clinic assay (see Methods and reference 24). § Duke University assay (see Methods and reference 25). ‖ N protein assay (see Methods and reference 27). ¶ All assays for patient A.M. were performed at the National Institutes of Health (NIH): PTH level for A.M. = 63 (nl = 2–50 fmol·eq/ml, see Methods and reference 26); normal NIH values (serum): calcium = 9.0–10.6; phosphorus = 2.4–4.4.

that the observed binding was associated with membranes derived from other cell types. We found a low level of leukocyte and platelet contamination in the erythrocyte layer before lysis of cells for membrane preparation. An average of 250±130 leukocytes and 5,500±3,000 platelets per cubic millimeter were present in control preparations; 100±50 leukocytes and 5,500±2,900 platelets, in PHP-Ia patients; and 100±170 leukocytes and 5,200±3,200 platelets, in PHP-Ib preparations. Thus, the level of leukocytes and platelets present in the erythrocyte preparation was consistently <5% of the level found in the whole blood of the donor.

25 ml of washed erythrocytes was lysed by the addition of 225 ml of ice-cold distilled water and allowed to sit undisturbed for 10 min on ice. Subsequently, the lysed erythrocytes were gently homogenized on ice in a 40-ml Dounce homogenizer. An equal volume of cold membrane buffer (75 mM Tris-HCl buffer, pH 7.5, containing 25 mM $MgCl_2$ and 1.5 mM EDTA) was added to the homogenate and centrifuged at 40,000 *g* for 10 min at 4°C. The hemoglobin-containing supernatant was discarded and the membrane pellet was then washed three times with membrane buffer by centrifugation at 40,000 *g* for 10 min at 4°C. The resulting white or light pink pellet was then resuspended to a volume of 50 ml with membrane buffer and used for the β-adrenergic receptor assay. This membrane preparation was also assayed for protein concentration according to the method of Lowry et al. (28).

Human erythrocyte membrane β-adrenergic receptor assay. We performed competition and saturation binding isotherms on membranes from all subjects by using standard radioligand binding techniques (29). [^{125}I]Iodopindolol was prepared from (−)pindolol by radioiodination with chloramine T and [^{125}I]NaI (30). The product was completely resolved from uniodinated pindolol and was therefore assumed to have theoretical specific activity of 2,200 Ci/mmol.

Saturation binding isotherms were constructed using 0.4 ml of the erythrocyte membrane preparation and [^{125}I]pindolol concentrations from 25 to 800 pM in a total assay volume of 0.6 ml. 10^{-4} M isoproterenol that was prepared in membrane buffer containing 10^{-3} M sodium ascorbate (Sigma Chemical Co., St. Louis, MO) was used to define nonspecific binding. Assays were incubated at 37°C for 30 min and were performed in duplicate. Bound [^{125}I]pindolol was separated from unbound [^{125}I]pindolol by filtering the membranes over Whatman GF/C glass fiber filters and then washing with 25 ml of cold membrane buffer. The filters were then counted in an Auto Gamma 800 gamma counter (Packard Instrument Co., Inc., Downers Grove, IL).

Competition binding isotherms were constructed by using isoproterenol concentrations in the assay from 10^{-9} to 10^{-3} M in competition with 200 pM [^{125}I]pindolol for binding to erythrocyte membrane β-adrenergic receptors. The incubation and washing conditions were as described for the saturation binding isotherms and the total volume of the assay was also 0.6 ml (100 μl of isoproterenol or buffer, 100 μl of [^{125}I]pindolol and 400 μl of membranes) and were performed in duplicate. Competition binding isotherms were performed in parallel in the presence and absence of 10^{-4} M guanylyl-imidodiphosphate (Gpp(NH)p) (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

We attempted to measure adenylate cyclase in these human erythrocyte membranes according to the method of Salomon et al. (31). Despite the addition of theophylline, forskolin, and the use of various incubation conditions and temperatures, no fluoride- or isoproterenol-stimulatable adenylate cyclase could be measured.

Data analysis. Data from the saturation and competition binding assays were analyzed and modeled by computer techniques as previously described (18, 19, 32). Competition curves were fit with a four-parameter logistic equation, which provided the slope factor and EC_{50} for each curve (32). Next, the curves were modeled according to the law of mass action to provide the best fit to a model, assuming a single state of the receptor or to a model assuming two states (high and low affinity) of

the receptor. A two-affinity state model was accepted only if it was shown to significantly ($P < 0.05$) improve the fit over a single-affinity state model. This program provided the affinity constants and proportions of the receptors in the high affinity or low affinity state for isoproterenol (9). This program was also used to fit the saturation binding isotherms for [^{125}I]pindolol and invariably such data were adequately modeled to a one-site fit.

When modeling the competition curves according to the law of mass action, the following approach was taken. The data from each subject were first modeled individually and was then averaged with data from other subjects in three different ways. The first method was to plot every data point from each of the five people in each donor group (normal controls, PHP-Ia, PHP-Ib) and then have the computer fit the best curve to all the data points for each group of patients. The second method was to average the binding data from each group of patients at a given concentration of competing isoproterenol and to generate a single competition binding isotherm from the patient data within a donor group. The third technique was to average the parameter values obtained from each patient's data individually within each of the three donor groups. These three methods of averaging the data within a group of donors yielded results that were virtually identical. The data and curves obtained by the second method above are presented in this paper.

As a final approach, the isoproterenol competition curves from the three subject groups were analyzed by using the "ternary complex" model, which we have previously proposed and validated to account for ligand interactions with plasma membrane β-adrenergic receptors. The model is (19):

$$\begin{array}{ccc} H + R + N & \overset{K}{\rightleftharpoons} & HR + N \\ \downarrow\uparrow M & & \downarrow\uparrow L \\ H + RN & \overset{K'}{\rightleftharpoons} & HRN \end{array}$$

K, K', L, and M represent the equilibrium constants for the various reactions. The curve fitter based on this model provides estimates for the association constant (K) of isoproterenol with the free form of the receptor (R) and the equilibrium constant (L) for the isoproterenol-promoted coupling of the receptor with a guanine nucleotide binding protein (N) as well as estimates of the relative concentrations of R and N in the system.

The assumptions used for this modeling were the following. (*a*) There was negligible interaction or "precoupling" of R with N in the absence of any ligand. In essence this sets the parameter $M = 0$. This assumption was based on ititial results that indicated that including a precoupled form of the receptor (RN) in the modeling did not improve the accuracy with which the data could be fit. (*b*) The K_d for the radioligand [^{125}I]pindolol was the same for controls and PHP-Ia and PHP-Ib patients and was determined by the analysis of [^{125}I]pindolol saturation curves with the mass action model (see Fig. 2). (*c*) The radioligand had no effect on the equilibrium between the free and coupled forms of the receptor so that it had identical affinity for R and RN. This was based on the finding that [^{125}I]pindolol saturation curves were always adequately fit by a one-state model. (*d*) There was no difference in the properties of isoproterenol interactions with the receptors between the three subject groups, i.e., that the values of L and K were the same for all three groups. That the value of K is the same in the different groups is supported by the finding that the agonist competition curves in the presence of the guanine nucleotide analog Gpp(NH)p were all superimposable. It has previously been demonstrated that guanine nucleotides convert all high affinity state receptors to low affinity state receptors and hence the affinity constant measured under these conditions should represent K (18, 19). Although the parameter L might have varied between the groups, it was set constant since (*a*) excellent fits to the data could be obtained when L values were shared among the three groups and (*b*) the major hypothesis that we wished to test with the ternary complex modeling was whether the binding data were consistent with the biochemically established decrease in the concentration of N in the membranes.

Statistical comparisons. With each of the three types of curve-fitting programs used, parameter values derived from the different patient groups were compared by constraining the computer fit competition curves from the different groups to share common values (e.g., EC_{50}, slope factor, K_d, etc.) and testing for worsening of the fit while keeping other values constant. Parameters were judged as significantly different if sharing of these parameters led to a significant ($P < 0.05$) worsening of the fit compared with that obtained when parameters were determined independently for each group (19). In other cases, parameters from the differing groups were compared by a one-way analysis of variance.

Results

Characteristics of ligand binding to the human erythrocyte membrane beta-adrenergic receptor. As shown in Fig. 1, the binding of 200 pM [^{125}I]pindolol to the β-adrenergic receptor of human erythrocyte membranes reaches equilibrium by 30 min at 37°C (Fig. 1). This interaction appears to be stable for >60 min at this temperature without significant degradation of the assayable receptor. The dissociation of [^{125}I]pindolol from the receptor has a t½ of 12 min at 37°C.

Erythrocyte membrane β-adrenergic receptor binding of [^{125}I]pindolol has the typical specificity and stereospecificity of a β_2-adrenergic receptor (Fig. 2 *A–C*).

A typical saturation binding isotherm of the erythrocyte β_2-adrenergic receptor of a normal subject for [^{125}I]pindolol is shown

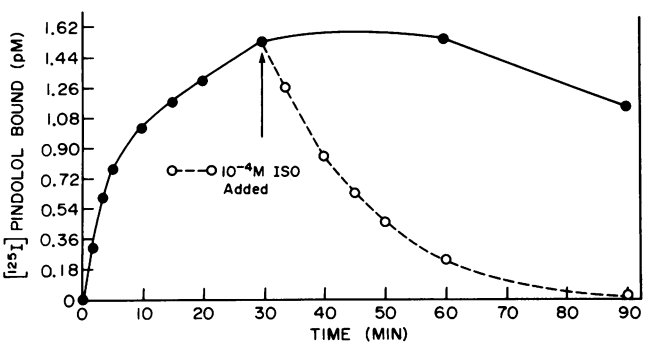

Figure 1. Kinetics of [^{125}I] pindolol binding to human erythrocyte β_2-adrenergic receptors. Human erythrocyte β_2-adrenergic receptors were prepared from healthy volunteer subjects as described in the text and were incubated with 200 pM [^{125}I]pindolol at 37°C for varying periods of time. At various times, as indicated by the abcissa, aliquots were taken and specific binding was assessed by using the filtration technique described in the text. In a parallel experiment, the off-kinetics were studied by the addition of 10^{-4} M isoproterenol to compete with the radiolabeled pindolol. Aliquots of the incubation mixture were then sampled at the times shown.

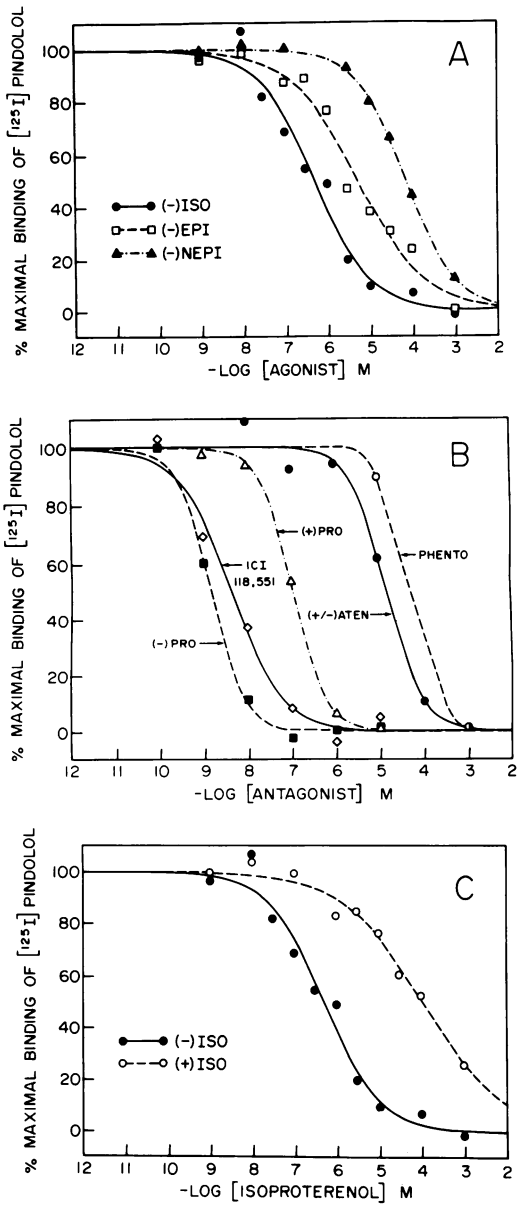

Figure 2. β_2-Adrenergic specificity and stereospecificity of adrenergic binding of human erythrocyte membranes. Competition binding isotherms were performed at 37°C for 30 min by using 400 μl of human erythrocyte membranes prepared as described in the text, 100 μl of [^{125}I]pindolol, and 100 μl of unlabeled agonist or antagonist. *A* shows the mean results of three competition experiments (determined in duplicate) in which increasing concentrations of (−)isoproterenol, (−)epinephrine, and (−)norepinephrine compete with [^{125}I]pindolol for binding to the human erythrocyte membranes. In *B*, increasing concentrations of the antagonists (−)propranolol, ICI-118, 551, (+)propranolol, (±)atenolol, and phentolamine are allowed to compete with [^{125}I]pindolol for binding to human erythrocyte membranes. In *C* (−)isoproterenol and (+)isoproterenol are shown in competition with [^{125}I]pindolol for binding to human erythrocyte membranes.

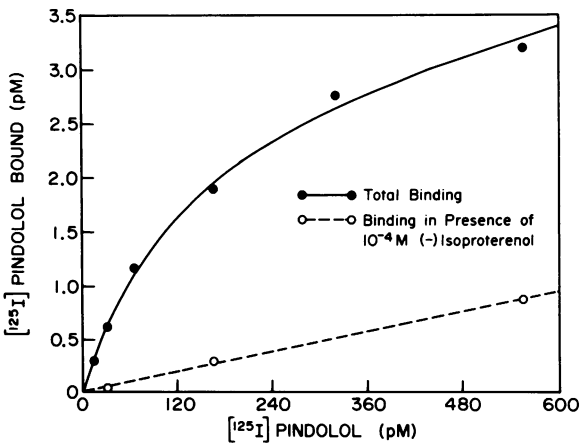

Figure 3. Saturation binding isotherm of the erythrocyte β_2-adrenergic receptor of a normal subject for [^{125}I]pindolol. Human erythrocyte membranes were prepared as described in the text and were assayed for β-adrenergic binding by using the radioligand [^{125}I]pindolol. Saturation binding isotherms were constructed with 400 μl of the erythrocyte membrane preparation and [^{125}I]pindolol concentrations from 25 to 800 pM in a total assay volume of 0.6 ml. Incubation was carried out at 37°C for 30 min. The nonspecific binding was defined as binding of [^{125}I]pindolol in the presence of 10^{-4} M isoproterenol. Specific β_2-adrenergic binding is the total binding as shown by the solid line minus the nonspecific binding as shown by the dashed line. The solid line represents the computer-generated best-fit curve for a single class of sites.

in Fig. 3. The specific binding was defined as the difference between [^{125}I]pindolol bound in the presence or absence of 10^{-4} M (−)isoproterenol. Such saturation curves were adequately modeled to a single site fit, which indicated interactions of the radioligand with an homogeneous population of receptor binding sites of uniform affinity.

The average (n = 5 subjects each) erythrocyte β-adrenergic receptor concentrations and dissociation constants (K_d) for [^{125}I]pindolol (Table II) did not differ between the groups. The density of sites was extremely low and presumably accounts for difficulties of others in demonstrating these binding sites in the past.

Since β-adrenergic receptors are known to exist on leukocytes (33) and platelets (34), we sought to exclude these cell types as the source of the β-adrenergic receptor activity in our preparations. We found that when the erythrocyte preparations were made entirely leukocyte- and platelet-free by cell elutriation that the erythrocyte β_2-adrenergic receptor binding was unchanged, i.e., K_d and receptor density were the same as in standard preparations containing a small amount (<5% of initial whole blood) of white cell or platelet contaminants (data not shown). Moreover, when platelets and leukocytes were deliberately left in the preparation and membranes were prepared, the β-adrenergic binding properties and total β_2-adrenergic receptor concentration were unchanged (data not shown).

Table II. Erythrocyte β_2-Adrenergic Receptor Binding Data

	Control	PHP-Ib*	PHP-Ia	*P* values for PHP-Ia patients vs. control
Saturation binding isotherm				
Receptor concentration (*fmol/mg protein*)	2.1±0.6	2.0±0.6	2.6±1.5	NS
K_d for [^{125}I]pindolol (*pM*)	141±69	153±44	177±119	NS
Competition curve data				
Slope factor				
Gpp(NH)p absent	0.6±0.1	0.8±0.1	0.9±0.1	0.05
Gpp(NH)p present	1.1±0.2	1.0±0.1	1.0±0.2	NS
EC_{50}				
Gpp(NH)p absent (*M*)	$4.7\pm1.5 \times 10^{-7}$	$4.9\pm2.0 \times 10^{-7}$	$1.2\pm0.2 \times 10^{-6}$	<0.001
Gpp(NH)p present (*M*)	$2.0\pm0.5 \times 10^{-6}$	$1.9\pm0.6 \times 10^{-6}$	$2.5\pm0.1 \times 10^{-6}$	NS
% Receptor in high affinity form				
Gpp(NH)p Absent	56.2±5.4	64.0±21.5	10.2±8.4‡	<0.001

* All values are not significantly different from control ($P > 0.05$). ‡ In this case a two-site fit was not significantly better than a one-site fit since so little of the high affinity state of the receptor was formed. However, for purposes of comparison this value was used rather than a value of 0% (the value implied by a one site fit).

All of these lines of evidence indicate that platelet or leukocyte binding did not account for the observed binding in these preparations but rather that a low level of receptors is, in fact, present on erythrocytes.

Comparison of isoproterenol competition curves between control subjects and PHP patients. Isoproterenol competition curves in the presence or absence of Gpp(NH)P, representing the averaged, normalized data for each of five control, PHP-Ia and PHP-Ib subjects, are shown in Fig. 4 and the various parameters derived from these curves are summarized in Table II. The competition curves for control subjects and patients with PHP-Ib are virtually identical. The average EC_{50} for controls was 4.7×10^{-7} M while that for patients with PHP-Ib was 4.9×10^{-7} M without Gpp(NH)p (P = NS). In the presence of Gpp(NH)p these values were 2.0×10^{-6} M and 1.9×10^{-6} M for controls and PHP-Ib patients, respectively (P = NS). In the absence of Gpp(NH)p the slope factor of the isoproterenol competition curve derived from subjects with PHP-Ib is not significantly steeper than for control subjects ($P = 0.48$); however both competition curves were steep with slope factors that approximated 1.0 in the presence of Gpp(NH)p. Similarly, both the PHP-Ib and control subjects have ~60% of their β_2-adrenergic receptor population in the high affinity form in the absence of Gpp(NH)p. The percentage of receptors in the high affinity state was 64% for the PHP-Ib patients and 56% for controls but this difference was not statistically different ($P > 0.5$).

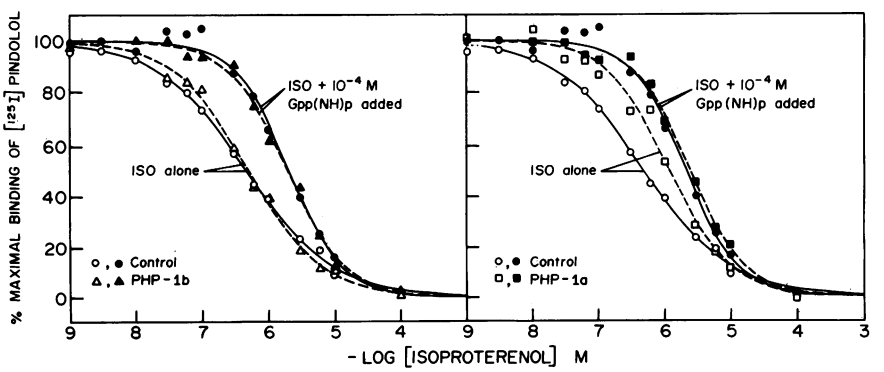

Figure 4. Isoproterenol competition curves in the presence and absence of 10^{-4} M Gpp(NH)p are compared for control subjects against PHP-Ib and PHP-Ia patients. Isoproterenol competition curves were performed as described in Methods by using membranes derived from human erythrocytes. In the left-hand panel, data for control subjects are shown by o and • in the absence and presence of Gpp(NH)p, respectively. The isoproterenol competition curves of the PHP-Ib patients are shown in the absence of Gpp(NH)p (Δ) or in the presence of 10^{-4} M Gpp(NH)p (▲). In the right-hand panel the same control data are shown again by the o and • in the absence or presence of 10^{-4} Gpp(NH)p, respectively. The competition data for the PHP-Ia patients are shown in the absence (□) or presence (■) of 10^{-4} M Gpp(NH)p. All data represent the mean of duplicate determinations from five patients or controls. The lines represent the computer-generated best fits according to the law of mass action (see Methods).

In contrast, visual inspection of the curves in Fig. 4 illustrates that the competition curve representing data from patients with PHP-Ia is different from that of subjects with PHP-Ib or controls in the absence of Gpp(NH)p. The curve for the PHP-Ia group is shifted to the right with an EC_{50} of $1.2\pm0.2 \times 10^{-6}$ M, which is significantly different than the curve for the PHP-Ib or control groups ($P < 0.001$).

The rightward shift and steepness of this curve are compatible with decreased formation of the high affinity receptor complex (19), which is presumably a reflection of decreased formation of HRN (18, 19). As shown in Table II, only 10% of the receptors are found to be in the high affinity form by computer modeling. Thus, a significantly lower percentage of the β_2-adrenergic receptors are able to form a high affinity complex in the erythrocytes of PHP-Ia patients than in the controls ($P < 0.01$) or the PHP-Ib patients ($P < 0.01$). Importantly, the dissociation constants for the isoproterenol competition curves in the absence of Gpp(NH)p were not statistically different between control and PHP-Ib and PHP-Ia patients.

To assess if a change in the stoichiometric ratio of N/R could explain the alterations in isoproterenol competition curves documented in Fig. 4 and Table II, we simultaneously analyzed the curves obtained for all three groups in the absence of Gpp(NH)p, together with a common competition curve in the presence of Gpp(NH)p as described in Methods using the "ternary complex" curve fitter. The results of the analysis indicated that the data from PHP-Ia subjects were best described as having a ratio of N to R that was ~40% of that determined for the other two subject groups (concentrations of N = 0.6±0.1 pM for PHP-Ia data vs. 1.5±0.1 pM for control and PHP-Ib data; see Table III). These results are consistent with a 60% loss in functional N from PHP-Ia subjects, as compared with the other groups.

N protein determinations. Erythrocyte N protein activity was 103±7% of normal (range = 92–109%) for controls, 97±9% (range = 84–110%) for the PHP-Ib patients, and 45±15% (range = 22–62%) for the PHP-Ia patients (Table III) as determined by the reconstitution assay discussed in Methods.

Discussion

The ability to assess β-adrenergic receptors and β-adrenergic receptor-N protein interactions in erythrocyte membranes from PHP patients and controls has afforded us a unique opportunity to probe the status of these components of the adenylate cyclase system in a human tissue under normal and pathophysiologic circumstances. In the present study we have shown that in patients in whom erythrocyte N protein is diminished (PHP-Ia), the interaction of the agonist isoproterenol with the β-adrenergic receptors is altered. We demonstrated an impaired ability of the receptors to form an agonist-promoted high affinity state as assessed by nonlinear least-squares curve fitting procedures. Such a reduction in high affinity agonist binding is highly suggestive of a reduction in the amount of the ternary complex HRN formed. This was directly demonstrated by computer modeling of isoproterenol competition curves with the ternary complex model (18). This modeling suggested a ~60% reduction in the functional N pool in the PHP-Ia patients. Although the results of the computer modeling do not categorically exclude a reduction in the L value for HRN complex formation, the calculated reduction in N protein concentration is consistent with the reduction in N protein activity observed in the biochemical assays of N.

In other situations where N is genetically deficient, such as the cyc^- variant of S49 lymphoma cells (22), or in situations where there is uncoupling of R from N such as desensitization (35), hypothyroidism (36), or hypoadrenalism (37), there is a reduction in the agonist-specific high affinity binding component that is demonstrated by a right-shifted and steepened agonist competition curve. This was precisely the situation documented in this study for the competition curves of the PHP-Ia patients. It is reasonably well established that formation of the HRN complex is a necessary prerequisite for cAMP formation (20). Thus, assuming that the results in erythrocytes are relevant to the situation in other tissues, it is possible that the decreased ability to form the HRN complex contributes to the decreased PTH, thyroid-stimulating hormone and other N protein-me-

Table III. Parameter Estimates from Data Analysis by using the Ternary Complex Model and Percentage Estimates of N-Protein Activity

Group	K_{ISO}	L_{ISO}	R_t	N_t	% N	% N protein activity
	pM	*pM*	*pM*	*pM*		
Control	$1.2\pm0.07 \times 10^6$	0.14±0.02	2.4	1.5±0.1	100	103±7
PHP-Ib	$1.2\pm0.07 \times 10^6$	0.14±0.02	2.4	1.5±0.1	100	97±9*
PHP-Ia	$1.2\pm0.07 \times 10^6$	0.14±0.02	2.4	0.6±0.1§	40	45±15‡

K_{ISO}, dissociation constant for isoproterenol with free form of receptor (R); L_{ISO}, equilibrium dissociation constant for isoproterenol-promoted coupling of R with N; R_t, estimate of total receptor concentration; N_t, total N concentration; % N, percentage of estimated N with respect to control values; % N protein activity, percentage of NIH laboratory controls (see Methods). * Not significantly different from control values. ‡ Significantly different from control values ($P < 0.001$). § $P < 0.001$ compared with fit where N for PHP-Ia is shared with that for other groups.

diated hormonal responses observed in patients with PHP-Ia. However, the present data do not permit us to assess the extent to which such a lesion, as opposed to decreased ability of N to activate C, determines the ultimately reduced generation of cyclic AMP in response to hormones in these patients. Again, as in other conditions of altered receptor coupling, computer modeling of agonist competition curves from PHP-Ia patients provided a direct quantitative estimation of the characteristics of the pathological changes in RN coupling.

Since patients with PHP-Ib have the same characteristics of agonist receptor interaction as controls, it is likely that the defect in this entity does not involve the coupling of H, R, and N. Thus, the site of the biochemical lesion in patients with PHP-Ib remains to be determined.

Although we were unable to measure adenylate cyclase responsiveness to β-agonist stimulation in these human erythrocyte membranes, a recent study (9) sheds important light on this question. Seven patients with PHP (three with PHP-Ia and four with PHP-Ib) were found to have a significantly blunted plasma cAMP response to isoproterenol infusion as compared with normal subjects. However, the PHP-Ib patients' cAMP response to isoproterenol infusion was blunted to an even greater degree than the PHP-Ia patients. Thus, in vivo, it appears that some component(s) of the adenylate cyclase-coupled β-adrenergic receptor system is abnormal in both PHP-Ia and PHP-Ib patients. However, the results of our work demonstrate that in vitro, agonist-induced receptor-N protein coupling is normal in erythrocytes from PHP-Ib patients. Thus, it is likely that a molecular understanding of the decreased cAMP formation in PHP-Ib patients will have to await development of further techniques for the investigation of this problem.

A major hypothesis that has been tested by the present work is whether the defect in the functional pool of N molecules present in the cell membranes of patients with PHP-Ia would be discerned in the patterns of agonist binding to β-adrenergic receptors. As noted, this hypothesis was confirmed by our observations. These data are consistent with the notion that in PHP-Ia patients a quantitative defect in a single gene product (i.e., N or some component thereof) might be responsible both for the ligand binding abnormalities demonstrated in this work and for decreased adenylate cyclase activation demonstrated in other work (12, 17). Our findings further support the idea of a single bifunctional N protein that interacts with both R and C.

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