Deficient Guanine Nucleotide Regulatory Unit Activity in Cultured Fibroblast Membranes from Patients with Pseudohypoparathyroidism Type I

A CAUSE OF IMPAIRED SYNTHESIS OF 3',5'-CYCLIC AMP BY INTACT AND BROKEN CELLS

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ABSTRACT Deficient activity of the guanine nucleotide regulatory protein (G unit), an integral component of the membrane-bound adenylate cyclase complex, has been implicated as the biochemical lesion in many patients with pseudohypoparathyroidism (PHP) type I. In addition to renal resistance to parathyroid hormone in this disorder, there is decreased responsiveness of diverse tissues to hormones that act via 3',5'-cyclic AMP (cAMP). To assess whether a deficiency of G units could account for impaired adenylate cyclase activity, we studied cAMP production in intact cultured fibroblasts and fibroblast plasma membranes from five patients with PHP in response to several activators of adenylate cyclase.

The number of G units in PHP fibroblast membranes, measured by cholera toxin-dependent [^{32}P]ADP ribosylation of G-unit peptides, as well as the G-unit activity, determined by the ability of detergent extracts to reconstitute adenylate cyclase activity in G-unit-deficient S49 CYC$^-$ membranes, were found to be markedly reduced compared with control membranes (43 and 40%, respectively), The activation of fibroblast membrane adenylate cyclase by effectors that act directly through the G unit (guanosine triphosphate, guanosine 5'-*O*-[3-thiotriphosphate] [GTP-γ-S], NaF) was significantly greater in control membranes than in membranes from patients with PHP. Moreover, we found that hormone (prostaglandin E_1)-stimulated adenylate cyclase activity was also greater in control membranes than in PHP membranes. Neither the apparent affinity of membrane adenylate cyclase for GTP-γ-S (apparent $K_m = 5 \times 10^{-8}$ M) nor the rate of enzyme activation by GTP-γ-S was significantly different in fibroblast membranes from control subjects and patients with PHP. In contrast to the notable differences in hormone and G-unit-activated adenylate cyclase shown in fibroblast membranes from PHP patients and control subjects, the intrinsic catalytic activity of membranes, as determined by forskolin-stimulated adenylate cyclase, was not significantly different in the two groups.

Intact fibroblasts derived from patients with PHP accumulated significantly ($P < 0.001$) less cAMP (46±21 pmol cAMP/mcg DNA, $n = 5$) than cells from normal individuals (170±51 pmol cAMP/mcg DNA, $n = 11$) when stimulated with PGE_1. PGE_1-stimulated accumulation of cAMP by intact fibroblast monolayers correlated closely with PGE_1 plus GTP-activated membrane adenylate cyclase activity in both patients and controls ($r = 0.97$, $P < 0.001$).

Our data show that, in patients with PHP, (*a*) fibroblast membranes show a decreased complement of

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G units, (*b*) membrane catalytic activity is normal, but adenylate cyclase activity is reduced when stimulated by hormone or by effectors which activate the G unit, (*c*) the ability of cells to accumulate cAMP in response to hormone stimulation is reduced, and (*d*) reduced membrane adenylate cyclase activity correlates well with impaired cellular cAMP synthesis. These results, taken together, indicate that a deficiency of G-unit activity can impair synthesis of cAMP by both intact and broken cells, and may explain the resistance of multiple tissues to hormones that act via cAMP observed in PHP.

INTRODUCTION

Pseudohypoparathyroidism (PHP)[1] is an uncommon metabolic disorder characterized by the biochemical features of hypoparathyroidism (hypocalcemia and hyperphosphatemia), despite apparently normal parathyroid hormone (PTH) secretion, and resistance of the target organs, bone and kidney, to PTH (1–3). The biologic action of PTH is mediated by 3′,5′-cyclic AMP (cAMP), and patients with PHP Type I exhibit a markedly blunted nephrogenous cAMP response to intravenous infusion of PTH in comparison with normal individuals and patients with other forms of hypoparathyroidism (4). This observation suggested that PHP Type I (as distinguished from PHP Type II, in which patients are characterized by an absent phosphaturic response to PTH despite a normal urinary cAMP response) is caused by a biochemical defect proximal to cAMP generation, i.e., in the plasma-membrane bound hormone-receptor adenylate cyclase complex. An integral component of the adenylate cyclase complex is the guanine nucleotide regulatory unit (G unit), a protein that couples hormone receptors to the catalytic unit of the enzyme. Recently, it has been found in this and other laboratories that many patients with PHP Type I show a reduced complement of G units in membranes prepared from erythrocytes (5, 6). Deficient erythrocyte membrane G-unit activity in erythrocyte membranes is associated with decreased responsiveness of diverse tissues (e.g., kidney, thyroid, gonad, and liver) to hormones (e.g., PTH, TSH, gonadotropin, and glucagon) acting via cAMP; patients with normal erythrocyte membrane G-unit activity generally manifest hormone resistance limited to PTH target tissues (7).

Because human erythrocyte membranes do not contain a hormone-sensitive adenylate cyclase, one cannot test for abnormalities in hormone response in this cell. Hormone receptors and adenylate cyclase have been found, however, in human diploid fibroblasts. We thus chose to study cultured fibroblasts from five patients with PHP Type I and from 11 normal subjects in order to examine the functional consequences of G-unit deficiency. We report here detailed biochemical studies of cultured fibroblasts, which include measurement of G-unit activity, membrane adenylate cyclase activity, and cellular cAMP accumulation. We found decreased G-unit activity in fibroblast membranes from patients manifesting PHP and reduced erythrocyte membrane G-unit activity. We further found reduced synthesis of cAMP with intact fibroblasts, as well as fibroblast plasma membranes. These observations provide strong support for the hypothesis that a deficiency of G units is the cause of impaired hormone-responsive adenylate cyclase activity in vivo.

METHODS

Patients. Five female patients were studied; four were siblings from a large kindred and the fifth was unrelated. The diagnosis of PHP Type I was confirmed by documenting a markedly blunted urinary cAMP response to a standard intravenous infusion of PTH (4). Characteristic features of Albright's hereditary osteodystrophy were present in all patients, including obesity, brachydactyly, subcutaneous ossifications, and short stature in each. These five patients had been previously shown to have deficient (~50% of control) G-unit activity in their erythrocytes and resistance to multiple hormones (i.e., PTH, TSH, and glucagon) with actions mediated by cAMP (5, 8). The control group comprised five males and six females. All fibroblast strains were not used in all experiments.

Cell culture. Skin specimens from each subject were obtained by 4-mm punch biopsy in the deltoid region, and fibroblast cultures were established by explanation of minced skin fragments. Informed consent was obtained from patients and volunteers for this procedure. At the time of skin biopsy, all PHP Type I patients were receiving vitamin D and thyroid hormone replacement. The ages of the patients (17–28 yr) were comparable to the control group (20–25 yr). Cells were grown in Improved Modified Eagles Medium (IMEM) supplemented with 10% fetal calf serum, 10^{-7} M crystalline zinc insulin, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Confluent monolayers of fibroblasts were harvested for passage by trypsinization and subcultured 1:3. Cells were periodically examined for the presence of mycoplasma contamination (9), and the cell lines used in all experiments were maintained for less than 20 passages in culture.

Intact cell studies. Cell cultures from PHP patients and normal subjects were used at passages 6 through 10. Confluent cell monolayers were dispersed with trypsin and seeded onto 24-well plates (16-mm diam, Costar, Cambridge, MA) at a density of 50,000 cells/well. Fibroblasts grown in these wells in complete growth medium under the usual incubation conditions reached confluency within 24 h.

Hormone response experiments were performed in the 16-mm wells with intact adherent cells. The growth medium

[1] *Abbreviations used in this paper:* G unit, guanine nucleotide regulatory unit; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); IMEM, improved modified Eagle's medium; PGE_1, prostaglandin E_1; PHP, pseudohypoparathyroidism; PTH, parathyroid hormone.

was first removed and the cells were washed twice with 1.0 ml of cold Hepes (25 mM) buffered IMEM (without bicarbonate; pH 7.4). This solution was replaced by 0.5 ml of Hepes-buffered IMEM containing 0.2% bovine serum albumin, 5×10^{-4} M 3-isobutyl-*l*-methylxanthine and the desired concentration of freshly dissolved agonist (standard medium) and the plates were placed in a 37°C water bath. At the end of incubation, 0.5 ml of 10% perchloric acid (final concentration of 5%) was added to each well. To determine cAMP in intracellular and extracellular pools, the standard medium was removed before adding 0.5 ml of 5% perchloric acid to each well.

The contents (cells and/or medium) of each well were transferred to a 12 × 75-mm glass test tube and precipitated protein was removed by centrifugation at 10,000 rpm for 15 min. A 500-μl aliquot of each supernate was neutralized with 250 μl of 2 N potassium bicarbonate, and after a second centrifugation this supernate was diluted with 50 mM sodium acetate buffer (pH 4.6), and acetylated according to the method of Harper and Brooker (10). Aliquots of each sample were then assayed for cAMP by automated radioimmunoassay using the Squibb gamma-flo (E. R. Squibb and Sons, Princeton, NJ) (11). Recovery of exogenous [^{3}H]cAMP added to the perchloric acid averaged 90%.

The contents of three 16-mm wells/cell line per experiment were reserved for determination of DNA. Cells were harvested with trypsin and disrupted by sonication. Cellular DNA content was measured by a highly sensitive fluorometric technique (12).

Preparation of fibroblast membranes. Plasma membrane-enriched fractions were prepared by a modification of the hypotonic lysis method of Kartner et al. (13). Flasks (Costar T150) containing confluent fibroblasts were washed three times with 20 ml of phosphate-buffered saline (without calcium or magnesium) at room temperature. The washed cells were then swollen by incubation in 15 ml of 1 mM sodium bicarbonate (pH 7.4) for 5 min with frequent mechanical agitation at 4°C. Cells that were still adherent to the plastic flask after 5 min were detached by manual scraping with a rubber policeman. Complete cell harvesting and lysis were confirmed by light microscopy and trypan blue exclusion. Sodium EDTA was added to the disrupted suspension to a final concentration of 0.5 mM, and the suspension was centrifuged at 27,000 *g* for 20 min at 4°C. The pellet (crude membrane preparation) was resuspended in a minimal volume of 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 10% (wt/vol) sucrose at 4°C, layered onto a cushion of 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 44.5% (wt/vol) sucrose and centrifuged at 70,000 *g* for 30 min in a SW 40 rotor. Material banding at the 10%:44.5% interface (plasma membrane-enriched fraction) was collected by Pasteur pipette, diluted 1:5 with 20 mM Tris-HCl (pH 7.4) with 0.25 M sucrose, and washed three times in this buffer. Membranes were collected by centrifugation and were resuspended in this buffer and stored in liquid nitrogen until used.

Adenylate cyclase assay. Adenylate cyclase activity of fibroblast membranes was assayed in a 100-μl final volume containing 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM cAMP, 5 mM creatine phosphate, 3.3 U of creatine phosphokinase, 100 μM [α-^{32}P]ATP (0.1 Ci/mmol), and 6–7 μg of membrane protein. Other modulators were added as shown in the table legends. Samples were incubated for 20 min at 30°C unless otherwise indicated. cAMP was isolated and measured by the method of Salomon et al. (14).

Measurement of G-unit activity. G units extracted from fibroblast membranes in detergent were assayed by addition to CYC$^-$ membranes and determining catecholamine- and sodium fluoride-sensitive adenylate cyclase activity. Membranes from the CYC$^-$ clone (94.15.1) of S49 mouse lymphoma cells, genetically deficient in G-unit activity (15), were prepared as previously described (16). Fibroblast membrane pellets were prepared by centrifugation and solubilized in buffer A (1.5 mg protein/ml) (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2% [wt/vol] Lubrol PX) and incubated overnight at 4°C. The membrane suspensions were then centrifuged for 30 min at 50,000 *g* and the supernatant solutions (membrane extract) collected and retained at 0–4°C for immediate assay of G-unit activity. CYC$^-$ membranes (200 μg), and the indicated amount of fibroblast membrane extract were incubated for 20 min at 30°C in a volume of 80 μl of buffer B containing 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM cAMP, 5 mM creatine phosphate, 3.3 U of creatine phosphokinase, 100 μM ATP, and the appropriate agonists. After the 20-min incubation, 20 μl of buffer B containing [α-^{32}P]ATP (0.1 Ci/mmol) was added and incubation continued for a further 20 min at 30°C. cAMP was isolated and determined by the method of Salomon et al. (14). Fibroblast extracts alone did not show measurable adenylate cyclase activity in response to stimulators, and the slight intrinsic activity of CYC$^-$ membranes in response to isoproterenol/guanosine 5′-*O*-(3-thiotriphosphate (GTP-γ-S) or NaF (~1.0–1.5 pmol cAMP/tube) was subtracted from the activity of combined fibroblast extract and CYC$^-$ membranes to obtain the increment due to addition of fibroblast G units (G-unit activity).

Cholera toxin-dependent [^{32}P]ADP-ribosylation. Fibroblast membranes (32 μg protein) were incubated for 20 min at 30°C in 100 mM potassium phosphate (pH 7.5), containing 0.1 mM guanosine triphosphate (GTP), 25 μM [^{32}P]NAD (5–10 Ci/mmol), 20 mM thymidine, 100 U/ml Trasylol, and either cholera toxin (100 μg/ml; preactivated during a 10-min 30°C incubation in 20 mM dithiothreitol) or an equivalent volume of 20 mM dithiothreitol without cholera toxin (final dithiothreitol concentration in cholera toxin labeling mix was 2 mM). The reaction was terminated by diluting the membrane suspension with 10 vol of ice-cold 100 mM potassium phosphate (pH 7.5), centrifuging (30,000 *g*, 15 min), and resuspending the treated membranes in 100 mM potassium phosphate buffer, pH 7.5. Samples were prepared for electrophoresis by the addition of gel sample buffer (final concentration 1% sodium dodecyl sulfate [SDS] [wt/vol], 10% sucrose [wt/vol] in 62.5 mM Tris-HCl [pH 6.8], 2.5% [vol/vol] β-mercaptoethanol, and 0.005% Bromphenol-Blue) and incubation for 5 min at 95°C.

SDS-polyacrylamide gel electrophoresis was performed in 10% acrylamide gels using the system of Laemmli (17). Standards for molecular-weight determination (phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydride) were obtained from Bio-Rad Laboratories, Richmond, CA. The areas corresponding to the 52,000- and 42,000-M_r [^{32}P]ADP-ribosylated subunits of the G unit were determined by weighing tracings cut from densitometric scans of the gel autoradiogram. The membrane G-unit value for each patient and control was the average value obtained from duplicate lanes. Protein was fixed and stained in 25% isopropyl alcohol, 10% acetic acid containing 0.05% (wt/vol) Coomassie Brilliant Blue, then destained in 10% acetic acid before drying and autoradiography.

Protein was measured by the method of Lowry et al. (18) with bovine serum albumin as standard. The *t* test was used to compare the means of PHP patients vs. control activities (19).

Materials. Prostaglandin E_1 (PGE_1) was purchased from the Upjohn Company, Kalamazoo, MI. PGE_1 was dissolved in absolute ethanol before dilution in standard medium or adenylate cyclase assay media. Ethanol content in the incubation did not exceed 0.5% (vol/vol) and this concentration did not significantly affect cAMP synthesis. [^{32}P]NAD was obtained from New England Nuclear, Boston, MA. GTP-γ-S was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Forskolin was obtained from Calbiochem-Behring Corp., San Diego, CA. Cholera toxin was purchased from Schwarz/Mann Div., Becton, Dickinson, and Co., Orangeburg, NY. Growth media (IMEM) and trypsin-EDTA were obtained from the NIH Media Unit, and fetal calf serum was purchased from Biofluids (Rockville, MD). Other chemicals were the best commercial grade available.

RESULTS

G-unit activity in fibroblast membranes. Fibroblast G-unit activity was measured by two independent methods: (*a*) the ability of detergent extracts from fibroblast membranes to reconstitute adenylate cyclase activity in membranes from S49 CYC$^-$ cells and (*b*) cholera toxin-dependent [^{32}P]ADP-ribosylation of the G unit in fibroblast membranes. Fibroblast G-unit activity was linear and proportional to the amount of detergent extract added to CYC$^-$ membranes with either of the activators, NaF (Fig. 1 *A*) or isoproterenol-GTP-γ-S (Fig. 1 *B*). The G-unit activity extracted from PHP samples was clearly reduced at any concentration tested and with either activator system (Fig. 1, *A* and *B*).

G-unit activity from control fibroblast membranes was 53.6±9.5 and 27.0±5.3 pmol cAMP/tube, whereas G-unit activity in the PHP group was 22.3±2.6 and 15.2±2.6 pmol cAMP/tube, for NaF- and isoproterenol-GTP-γ-S-stimulated activity with 10 μl of extract added per tube, respectively (mean±SD). These differences between control and PHP fibroblast G-unit activity were highly significant ($P < 0.001$).

The results of fibroblast G-unit measurement as determined by membrane [^{32}P]ADP-ribose incorporation are presented in Fig. 2. Cholera toxin catalyzes the transfer of [^{32}P]ADP-ribose from [^{32}P]NAD to 42,000- and 52,000-M_r membrane polypeptides, which are part of the G unit.

The electrophoretically separated 42,000- and 52,000-M_r bands were of greater density in the lanes with normal membranes than in the lanes corresponding to membranes from PHP subjects. The area of the peaks on densitometric tracings corresponding to the 42,000- and 52,000-M_r bands was significantly ($P < 0.005$) greater for control than for PHP membranes (58.2±13.3 vs. 24.7±6.6 arbitrary units, mean±SD). As the amount of [^{32}P]ADP-ribose incorporated into the two polypeptides is a function of their relative concentrations, this experiment confirms the reduced complement of G units in fibroblast membranes from PHP subjects.

Adenylate cyclase activity in fibroblast membranes. To test the hypothesis that a deficiency of G units

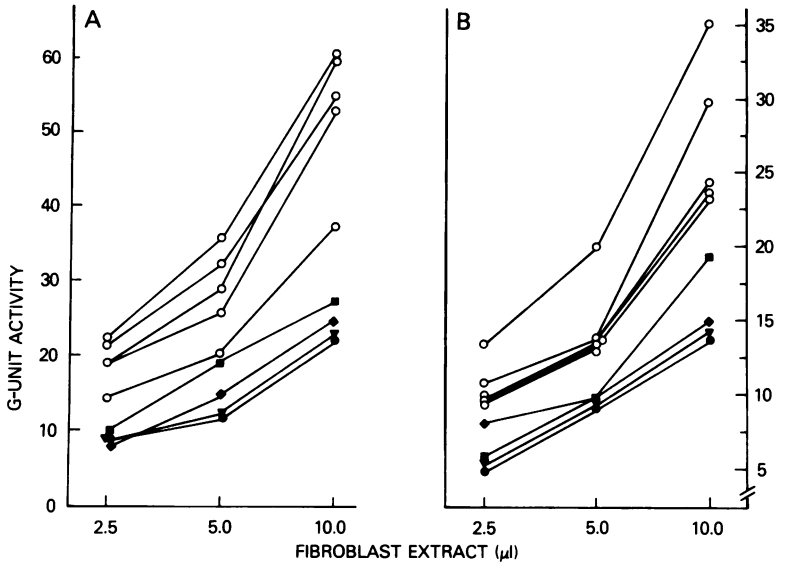

FIGURE 1 G-unit activity (pmol cAMP/tube) in detergent extracts of fibroblast membranes measured by reconstitution of adenylate cyclase in S49 CYC$^-$ membranes. Indicated amounts of extract from control (open symbols) or PHP (closed symbols: patient A, ●; B, ■; C, ◆; D, ▼) membranes were assayed in triplicate with either 10 mM sodium fluoride (*A*) or 100 μM *l*-isoproterenol plus 100 μM GTP-γ-S (*B*).

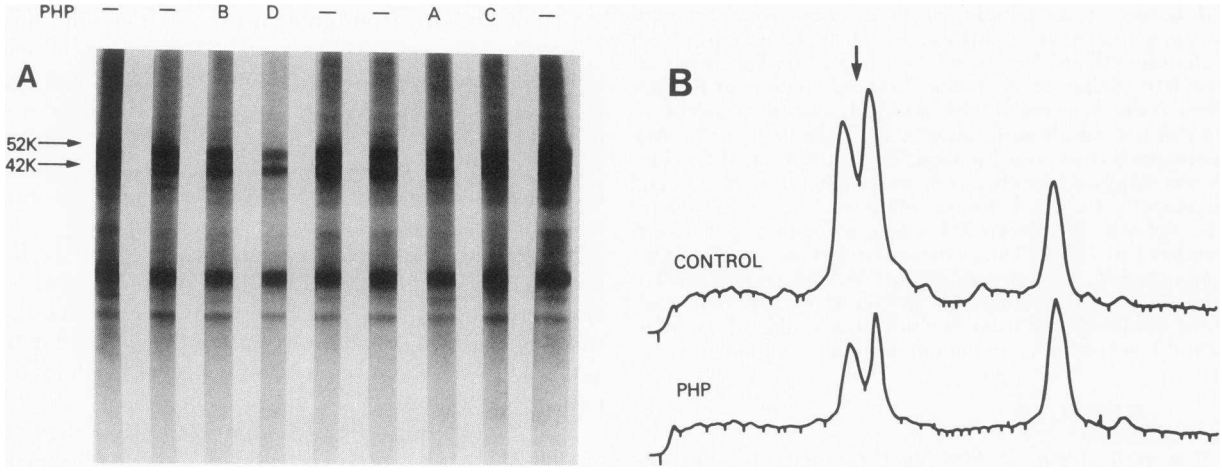

FIGURE 2 (*A*) Autoradiogram of SDS-polyacrylamide electrophoresis gel. Fibroblast membranes were treated with cholera toxin and [^{32}P]NAD as described in Methods. [^{32}P]ADP-ribosylation of the 42,000- and 52,000-dalton bands, corresponding to subunits of the G unit, was cholera toxin dependent. Labeling of other bands in each fibroblast membrane preparation was less uniform, and was not dependent upon the presence of cholera toxin. Coomassie Blue staining of the gel shown above gave patterns of equal intensity and distribution, consistent with the application of equivalent amounts of membrane protein to each lane. (*B*) Representative densitometric tracings from a control subject and a patient with PHP. The arrow indicates the peaks corresponding to the 52,000- and 42,000-M_r bands. Densitometric scans of the autoradiogram were done (as described in Methods) and the areas corresponding to the 52,000- and 42,000-M_r peaks were quantitated. The individual values (averaged from duplicate lanes) for patients with PHP were: patient *A* = 19.3, *B* = 29.3, *C* = 29.4, *D* = 17.9; for controls: 66.1, 38.5, 61.0, 49.0, 76.6.

should result in reduced synthesis of cAMP, we compared the effect of multiple stimulators of adenylate cyclase in cultured fibroblast membranes from four PHP patients and five control subjects (Table I). We found highly significant differences in the adenylate cyclase activity of membranes prepared from PHP fibroblasts as compared with normal.

Guanine nucleotides (GTP or its nonhydrolyzable analog GTP-γ-S) and NaF stimulate catalytic activity by interacting directly with the G unit, independent of any requirement for hormone receptor occupancy. The control membranes showed greater adenylate cyclase activation than PHP membranes when either GTP or GTP-γ-S was present in the assay mix (Table I). Moreover, the response to the nonphysiologic stimulator NaF was also significantly greater in control membranes than in PHP membranes, emphasizing further a G-unit deficiency as the cause of impaired membrane adenylate cyclase activity.

PGE_1 stimulates adenylate cyclase activity in fibroblast membranes by interacting with a membrane-bound receptor. Occupancy of this receptor by PGE_1 increases G-unit-mediated catalytic activity (7). PGE_1 stimulation of adenylate cyclase activity in cultured fibroblast membranes was almost totally dependent upon the addition of exogenous guanine nucleotides to the reaction media. We found that PGE_1-stimulated adenylate cyclase activity was significantly greater in control membranes than in PHP membranes, whether or not GTP or its nonhydrolyzable analog GTP-γ-S was added (Table I).

TABLE I
Adenylate Cyclase Activity in Cultured Fibroblast Membranes

Effector*	Controls (n = 5)	PHP Patients (n = 4)
Basal	196±44	61±8‡
GTP	230±60	62±11‡
GTP-γ-S	1,700±140	670±45§
PGE_1	580±140	225±60‡
PGE_1 + GTP	2,260±380	1,010±170‡
PGE_1 + GTP-γ-S	3,470±270	1,800±170‖
NaF	2,270±190	1,670±160‡
Forskolin	1,160±110	1,080±300

All results are expressed as mean±SE for the group. Units are picomoles of cAMP per milligram of membrane protein per 20 min.
* Concentrations: GTP, GTP-γ-S (100 μM), PGE_1 (10 μM), NaF (10 mM), forskolin (10 μM).
‡ $P < 0.05$, different from control.
§ $P < 0.001$, different from control.
‖ $P < 0.01$, different from control.

Decreased adenylate cyclase activity in cultured fibroblast membranes from PHP patients in response to PGE_1, guanine nucleotides, and NaF could be due to decreased membrane catalytic activity. We therefore examined the response of cultured fibroblast membranes to forskolin, a diterpene activator of adenylate cyclase whose effects are considered to be due to direct stimulation of the catalytic moiety (20). The response of adenylate cyclase to forskolin did not differ among control and PHP membranes (Table I).

We also examined the apparent affinity of the adenylate cyclase complex for GTP-γ-S and the activation time of the enzyme with GTP-γ-S in cultured fibroblast membranes from control subjects and patients with PHP. Fig. 3 shows the results of an experiment comparing the adenylate cyclase activity of membranes from four patients with PHP with membranes from five control subjects. Adenylate cyclase activity of the membranes from patients with PHP was lower than that of control membranes for all concentrations of GTP-γ-S tested, but there was no difference in affinity detected (apparent K_m for GTP-γ-S was 5×10^{-8} M for both groups).

We analyzed the rate of enzyme activation by GTP-γ-S in cultured fibroblast membranes from four patients with PHP and five control subjects (Fig. 4). Adenylate cyclase activity in membranes from both groups was linear with respect to time, but the activity of PHP membranes was significantly less than that of normal membranes at each time point.

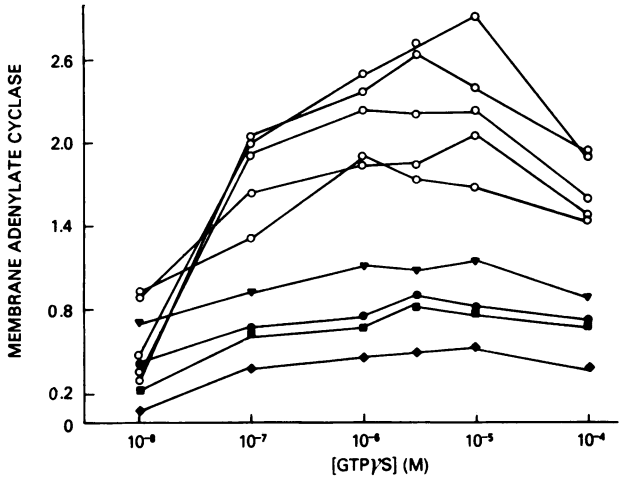

FIGURE 3 Adenylate cyclase activity (nmol cAMP/mg protein per 20 min) at differing concentrations of GTP-γ-S. Fibroblast membranes were assayed for adenylate cyclase for 20 min at 30°C. Open symbols (O) represent membranes prepared from control fibroblast lines, and closed symbols (patient A, ●; B, ■; C, ◆; D, ▼) represent membranes prepared from cell lines derived from patients with PHP. Each point is the mean of triplicate determinations.

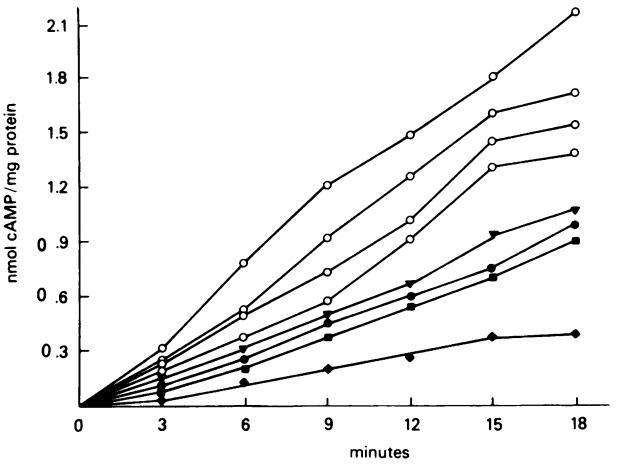

FIGURE 4 Membrane adenylate cyclase activity as a function of time. Fibroblast membrane adenylate cyclase activity was assayed in the presence of 100 μM GTP-γ-S for incubation periods indicated. Adenylate cyclase activity of membranes prepared from normal cell lines is represented by open symbols (O), and activity of membranes prepared from PHP cell lines is represented by closed symbols (patient A, ●; B, ■; C, ◆; D, ▼). Each point is the mean of triplicate determinations.

Accumulation of cAMP in intact cultured fibroblasts in response to PGE_1. We measured the accumulation and release of cAMP by confluent cells in response to a submaximal stimulating concentration of PGE_1. The doubling time of cells used in these experiments was 24–36 h for both PHP- and control-derived cell lines. Response to PGE_1 was tested with nondividing cells, obtained from steady state monolayers and plated at cell numbers near confluence. This method of cell cycle synchronization was routinely used; stable content of DNA from 24 to 96 h after plating confirmed the establishment of the steady state. Hormone responsiveness was quantitatively similar when cells were challenged with PGE_1 at 24, 48, or 72 h after plating (not shown).

cAMP accumulation in response to PGE_1 was rapid and, in the presence of the phosphodiesterase inhibitor isobutyl methyl xanthine, reached a plateau at 15 min and then remained stable beyond 60 min (data not shown). We therefore elected to study confluent monolayers 48 h after plating and to incubate cells for 10 min. Two submaximal concentrations of PGE_1 (0.5×10^{-6} M and 0.5×10^{-5} M) were selected to enhance potential differences in cellular cAMP synthesis and to avoid hormone refractoriness or desensitization.

Cells derived from PHP patients accumulated significantly less cAMP than cells from normal individuals when challenged with PGE_1 (Fig. 5, Table II). The total cAMP accumulated was determined by measur-

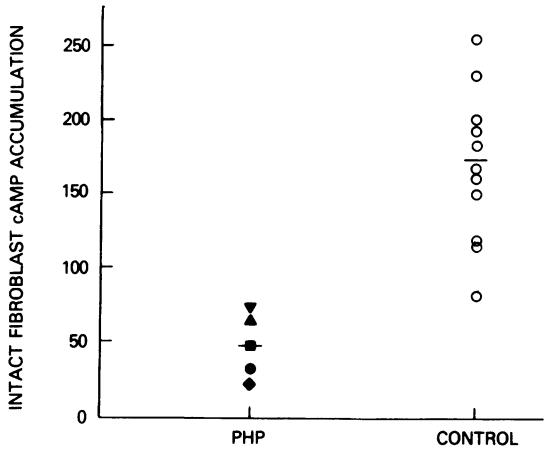

FIGURE 5 PGE_1-mediated accumulation (pmol cAMP/mcg DNA) (cells plus medium) by cultured fibroblasts from patients with PHP (patient A, ●; B, ■; C, ◆; D, ▼; E, ▲) vs. control (○). Each point represents the mean of three wells on individual subjects.

ing both the extracellular fraction and the intracellular fraction. In these experiments, cAMP in the extracellular compartment represented 8.5±1.5 and 14.4±3.3% of the total cAMP accumulated by PHP cells and normal cells, respectively. When only the intracellular cAMP was considered, PHP cells were still found to accumulate significantly less cAMP than normal cells.

Intact cell cAMP accumulation in response to PGE_1 correlates ($r = 0.97$, $P < 0.001$) with adenylate cyclase activity in membranes in individual patients with PHP (Fig. 6).

DISCUSSION

We found a reduced complement of functional G units in cultured fibroblast membranes from patients with PHP. These findings are in agreement with observations of deficient G-unit activity in membranes prepared from erythrocytes (5, 6) of many patients with PHP Type I. Furthermore, similar findings of deficient G-unit activity have been previously reported in membranes prepared from platelets of two patients (21) and renal cortex of one patient (22). These results thus support the hypothesis that an alteration of G-unit activity is the fundamental molecular lesion in many patients with PHP Type I.

Our studies document the consequences of deficient G-unit activity on adenylate cyclase activity in both membranes and intact cells. We found that accumulation of cAMP in response to PGE_1 is reduced in fibroblasts derived from patients with PHP, and that membranes prepared from these cells contain impaired adenylate cyclase activity. These results support the hypothesis that hormone resistance in PHP Type I is a consequence of impaired adenylate cyclase activation resulting from deficient G-unit activity.

Bourne et al. (23) have recently published similar findings of reduced G-unit content in PHP-cultured fibroblast membranes but, in contradistinction to our results, found no alteration in either membrane adenylate cyclase activity or cAMP response to PGE_1 or cholera toxin with intact cells. There are a number of possible explanations for the differences between our findings and those of Bourne et al. We harvested fibroblast monolayers by manual scraping, disrupted cells by hypotonic lysis, and prepared a purified membrane fraction by discontinuous sucrose gradient cen-

TABLE II
PGE_1-stimulated cAMP Accumulation in Intact Cultured Fibroblasts

PGE_1 concentration	Controls (n = 11)		PHP Patients (n = 5)	
	Total	Intracellular	Total	Intracellular
μM				
0.5	170±51	153±48	46±21*	42±19*
5.0	268±83	228±78	110±49*	100±22*

Results are expressed as mean±SD for the group. Units are picomoles cAMP/mcg DNA.
* $P < 0.001$, different from control.

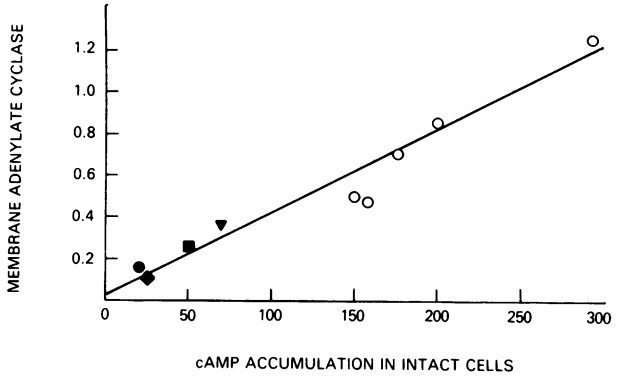

FIGURE 6 Relationship between cAMP accumulation by intact cells (pmol cAMP/mcg DNA) in response to PGE_1 (0.5×10^{-6} M) and membrane adenylate cyclase activity (nmol cAMP/mg protein per 20 min) stimulated by PGE_1 (0.5×10^{-6} M) plus GTP (10 μM). Membrane adenylate cyclase activity and cellular cAMP accumulation of fibroblast cell lines from PHP patients are represented by closed symbols (patient A, ●; B, ■; C, ◆; D, ▼) and from control subjects by open symbols (○). Each point represents the mean of three wells (cAMP accumulation) and the mean of triplicate determinations (adenylate cyclase activity). All correlations were significant ($P < 0.001$): r(PHP) = 0.93, r(control) = 0.94, r(PHP + control) = 0.97.

trifugation. Bourne et al. freed cultured cells with trypsin, used a Dounce homogenizer to disrupt cells, and studied particulate extracts. A receptor-enzyme system located in the plasma membrane might be perturbed by treatments, chemical or mechanical, that could interrupt the integrity of the membrane. Of these procedures, the exposure of fibroblasts to trypsin before preparing particulate extracts most deserves specific comment. Treatment of intact mammalian cells with trypsin under conditions similar to those used by Bourne et al. to harvest monolayers causes increases in basal and GTP-, Gpp(NH)p-, PGE_1-, and NaF-stimulated adenylate cyclase (24). These effects could obscure potential differences in membrane adenylate cyclase between PHP and control groups and may account for the large interstrain variability noted by Bourne et al. (23).

Since many factors can modify cyclic nucleotide metabolism in cultured cells, it is difficult to compare directly our study with that of Bourne et al. (23). Culture conditions such as passage number (25), cell density (26), time after subculture (27), serum concentration (28), and agonist used have been shown to influence significantly the response of the cAMP system.

With human fibroblasts in particular, response to β-adrenergic agonists is nonuniform, and cell content of cAMP varies with the cell cycle, the exponential growth phase showing higher cAMP content than cells at confluence (25). Moreover, the hormone responsiveness of cAMP accumulation by human diploid fibroblasts has been shown to vary with time after division (25, 27). We therefore carefully standardized our incubation conditions, synchronized cell growth to minimize artifactual influences of the cell cycle, and used PGE_1, which has given more uniform responses (29–31) to stimulate cAMP production in order to avoid variations that might preclude detection of statistically significant differences between cell lines.

The observation that cultured fibroblast membranes and erythrocyte membranes from patients with PHP Type I show a comparable reduction in G-unit complement further supports the genetic basis of this lesion. The growth of diploid fibroblasts from these patients in long-term culture did not interfere with the ultimate expression of the altered adenylate cyclase genotype. These patients represent a syndrome of hormone resistance generalized to diverse tissues (i.e., kidney, thyroid, gonad, liver) that can be explained by a defective adenylate cyclase component common to all these tissues. This component is the G unit, and it is apparent from our current results that a deficiency of G units can impair the ability of an intact cell to accumulate cAMP in response to hormone stimulation and can impair adenylate cyclase activation of membranes by agents acting via the G unit. A similar conclusion can be drawn from data recently reported by Motulsky et al. (32). Although Bourne et al. (23) were unable to see a significant difference in cAMP response to PGE_1 in intact fibroblasts derived from control subjects vs. patients with PHP, the same investigators (32) found deficient G-unit activity in platelet membranes as well as reduced cAMP response to prostacyclin in intact platelets from patients with PHP.

We know of no evidence to suggest that fibroblasts of PHP type I patients function abnormally in vivo. It is therefore not readily apparent what the functional consequence of an impaired cAMP system in these cells may be. However, the cultured fibroblast can provide the model system in which the specific molecular defect responsible for deficient G-unit activity may be elucidated. Furthermore, studies in cultured fibroblasts may lead to further appreciation of the stoichiometry and functional interactions of the multiple adenylate cyclase components, and may enable greater understanding of adenylate cyclase per se. It is entirely conceivable that other PHP genotypes with normal G-unit activity but resistance to multiple hormones will be identified with defects in other components of the cAMP system, e.g., the catalytic unit or phosphodiesterase. As yet, we have not had the opportunity to study cultured fibroblasts from patients with normal G-unit activity.

Finally, our studies with cultured fibroblasts suggest that expression of the genetic defect in PHP Type I may be anticipated in cells from amniotic fluid. If this can be demonstrated, prenatal diagnosis of low G-unit PHP can be achieved, and genetic counseling will become possible.

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