Deficient Adenylate Cyclase Regulatory Protein in Renal Membranes from a Patient with Pseudohypoparathyroidism

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ABSTRACT Recent studies have established that some patients with pseudohypoparathyroidism have a deficiency of the adenylate cyclase regulatory protein (the G unit) in plasma membranes from erythrocytes, platelets, and fibroblasts. We have directly measured the activity of the G unit in renal membranes from a patient with pseudohypoparathyroidism who, in addition to parathyroid hormone resistance, has resistance to thyrotropin and gonadotropins. Erythrocyte membrane G unit activity was 57% that of control erythrocyte membranes. Lubrol PX extracts of renal membranes had only 30% of the G unit activity of control renal membrane extracts, whether assayed with sodium fluoride or guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S). In cholate extracts, the G unit activity was 37 and 48% of control with fluoride or GTP-γ-S, respectively. Cholera toxin-dependent incorporation of [32P]ADP-ribose into the 42,000-Mr subunit of the G unit was decreased in renal membranes from the patient compared with control renal membranes. The data demonstrate that the membrane G unit deficiency in pseudohypoparathyroidism extends to the cells of a clinically relevant parathyroid hormone target tissue.

INTRODUCTION

Pseudohypoparathyroidism (PHP)1 is a genetic disorder characterized by target organ insensitivity to parathyroid hormone (PTH). Patients with this condition display the features of hypoparathyroidism, hypocalcemia, and hyperphosphatemia, despite circulating levels of parathyroid hormone that are generally high. In addition, they do not respond to exogenous PTH with hypercalcemia or phosphaturia (1, 2). Chase and Aurbach (3, 4) showed that the action of PTH on target tissues, bone and kidney, is mediated by cyclic AMP, and found that patients with PHP lack the normal brisk rise in urinary cyclic AMP after infusion of PTH(5). Their studies indicated that in PHP there is a defect in the target organ adenylate cyclase system, proximal to the generation of cyclic AMP.

Recent studies (reviewed in ref. 6) have shown that the adenylate cyclase system is composed of at least three separable components: a hormone-specific receptor; a catalytic unit; and a guanine nucleotide-binding regulatory protein, the G unit (also termed G/F and N). The G unit interacts with hormone receptors and with the catalytic unit, and mediates the activation of adenylate cyclase by hormones, guanine nucleotides, cholera toxin, and sodium fluoride. Dreznner and Burch (7) studied renal membranes from a patient with PHP, obtained during a urologic surgical procedure. They found that PTH-stimulated adenylate cyclase activity was subnormal at low ATP concentrations, and was corrected with small concentrations of GTP, leading them to postulate a G unit abnormality in their patient.

Studies by Farfel et al. (8) and by our group (9) have now shown that some patients with PHP have a decrease in the number of functional G units present in erythrocyte plasma membranes. Our studies indicate

1 Abbreviations used in this paper: G units, the adenylate cyclase regulatory protein; GTP-γ-S, guanosine 5'-O-(3-thiotriphosphate); PHP, pseudohypoparathyroidism; PTH, parathyroid hormone.
that the G unit deficiency is restricted to those with PHP and the characteristic phenotype of short stature and brachydactyly (Albright’s hereditary osteodystrophy). Many of these patients are also resistant to other hormones whose effects are mediated by cyclic AMP (10). The G unit deficiency has been documented in membranes from erythrocytes (8, 9), platelets (11) and cultured fibroblasts (12) (Levine, M. A. Unpublished observations,) using several assay methods.

No defect in erythrocyte, platelet, or fibroblast function has been reported in patients with PHP. Available evidence suggests that the G unit is similar in all tissues (6), so it is assumed that the abnormality in these accessible cells reflects a comparable defect in tissues clearly dependent on hormone-stimulated cyclic AMP accumulation for normal function. Such a generalized deficiency of G units could be the basis for resistance to multiple hormones, including renal and skeletal resistance to PTH. However, a deficiency of G unit activity has yet to be demonstrated directly in membranes from a clinically affected target organ. We have used recently developed methods to directly examine G unit activity in renal membranes from the patient with PHP reported previously by Drezner and Burch (7). We report here data that show that this patient is resistant to multiple hormones and has a deficiency of functional G units in membranes from kidney, a target tissue for PTH. Although only a limited amount of renal tissue from a single patient with PHP was available for study, our observations significantly extend previous reports of G unit deficiency in other cell types to a clinically relevant tissue.

METHODS

The preparation of renal cortical membranes from the patient with PHP and from three control subjects undergoing urologic surgery was described previously (7). All membranes had been stored in liquid nitrogen for the same period of time. Frozen aliquots were removed, coded, and shipped to Bethesda, MD on dry ice.

Cholera toxin-dependent G unit ADP-riboseylation. For each coded sample, two sets of duplicate tubes containing 200 µg of membrane protein were centrifuged for 10 min at 15,000 g. The membrane pellet was resuspended and incubated for 20 min at 30°C in 0.1 ml 100 mM potassium phosphate, pH 7.5, containing 100 U/ml Trasylol, 20 mM thymidine, 0.1 mM GTP, 25 µM [32P]NAD (sp act 10 Ci/mmol), and 2 mM dithiothreitol, with or without 100 µg/ml cholera toxin (activated for 10 min at 30°C in 20 mM dithiothreitol). The samples were then processed and 20 µg of protein applied to each lane of SDS slab gels according to the method of Laemmli (15). The gels were stained and dried for autoradiography as previously described (14). The pattern of stained protein bands was identical in all lanes.

G unit activity by adenylyl cyclase complementation assay. 300 µg of membrane protein from each coded sample were centrifuged for 10 min at 15,000 g and the membrane pellet resuspended in 0.1 ml 10 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 0.1 mM MgCl2, 1 mM dithiothreitol, and 0.2% (wt/vol) Lubrol PX. The suspension was incubated at 0°C overnight, then centrifuged for 60 min at 50,000 g and the supernatant Lubrol extract recovered for G unit assay.

500 µg of membrane protein from each coded sample were centrifuged 10 min at 15,000 g and the membrane pellet resuspended in 100 µl 30 mM Tris-HCl, pH 8.0, containing 0.25 M sucrose, 0.1 mM MgCl2, 1 mM dithiothreitol, and 1% (wt/vol) cholate. After an overnight incubation of 0°C, the suspension was centrifuged for 60 min at 50,000 g and the supernatant cholate extract recovered and diluted 10-fold in buffer without cholate before G unit assay.

G unit activity was determined in Lubrol PX and cholate extracts using a modification of the complementation assay described by Kaslow et al. (15), which depends on activation of adenylate cyclase by G units added to membranes from the S49 lymphoma clone cgc-, which lack functional G units. 5, 10, or 20 µl of detergent extract was incubated for 20 min at 30°C with 200 µg cgc- membranes in a final volume of 90 µl containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM cyclic AMP, 0.3 mM ATP, 5 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase, with 10 mM sodium fluoride or 100 µM guanosine-5’-O-(3-thiotriphosphate) (GTP-γ-S) as indicated. For volumes of extract <20 µl, an additional volume of the appropriate solubilizing buffer was included to keep the detergent concentration constant. At the end of this incubation, 10 µl containing 2 µCi [32P]ATP was added and the incubation continued at 30°C for an additional 20 min. The [32P]cyclic AMP accumulated during the second 20 min incubation was separated using the method of Salomon et al. (16). The assay of G unit activity is linear with respect to the amount of extract protein added (Fig. 1), and similar results are obtained when either fluoride or GTP-γ-S is used to activate the donor G units.

Other methods. Protein was measured using the method of Lowry et al. (17), with bovine serum albumin as standard. Sodium dodecyl sulfate (SDS) (0.5% wt/vol) was included in the alkaline copper solution to prevent the formation of a precipitate in samples containing Lubrol PX. Cells from the cgc- clone of S49 lymphoma cells (18) were grown and membranes prepared as previously described (19).

GTP-γ-S was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. [α-32P]NAD was from New England Nuclear, Boston, MA and [α-32P]ATP was from ICN Chemical and Radioisotope Division, Plainview, NY, and cholera toxin was purchased from Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, NY. Other reagents were of the best grade commercially available.

Case description

The patient was a 16-yr-old female. As reported previously (7), she had multiple subcutaneous calcifications and uniformly short metacarpals. Serum calcium was 7.8 mg/dl, and the serum phosphorus 5.1 mg/dl, with an elevated serum PTH. Intravenous infusion of parathyroid extract caused no increase in urinary cyclic AMP excretion. These findings are characteristic of PHP.

Additional data not previously reported include serum thyroxine, 2.6 µg/dl (normal, 3.0–7.0); resin uptake of triiodothyronine, 11.3% (normal, 10.0–14.6); and plasma thyrotropin, 20 µU/ml (normal, <10). Antithyroglobulin and antithyroid microsomal antibody titers were <16 and <4, respectively. The thyroid gland was not enlarged.

She had oligomenorrhea, and evaluation revealed a serum estrogen of 66 pg/ml (normal follicular phase, 82±58 pg/ml) and estradiol, 41.0 µ1/ml (normal follicular phase, 6.5–24.2) and follicle-
brol PX extracts of renal membranes from the patient with PHP had only 30% of the G unit activity of extracts from control and renal membranes, using either fluoride or GTP-γ-S as the G unit activator (Fig. 1). In similar studies with cholate extracts (Fig. 2), the G unit activity of PHP extracts was 37 and 48% of normal with fluoride and GTP-γ-S activation, respectively.

Cholera toxin-catalyzed mono-ADP-ribosylation of the G unit provides an independent means of measuring the number of G units in a membrane preparation (8). With [32P]NAD as substrate cholera toxin catalyzes [32P]ADP-ribosylation of the 42,000-M₃ subunit of the G unit, which can then be detected by polyacrylamide gel electrophoresis and autoradiography (8, 20, 21). Renal membranes from the patient with PHP have a clear deficit of G units by this method (Fig. 3, 42K arrow).

stimulating hormone 21.6 mIU/ml (normal follicular phase, 4.4–20.2). Vaginal cell maturation index was 0/90/10, indicative of a poor estrogen effect. Laparoscopy revealed ovaries that appeared poorly stimulated, and ovarian biopsy showed numerous primordial follicles and several larger atretic follicles, consistent with poor gonadotropin stimulation. Ovarian and peripheral leukocyte karyotypes were both 46XX.

Her adrenal responsiveness to endogenous ACTH was normal during a standard metyrapone test and after insulin-induced hypoglycemia.

RESULTS

G unit activity was deficient in both erythrocyte and renal membranes from the patient with PHP.

Erythrocyte membrane G unit activity determined as previously described (9) was 57% of the activity of a control pool of normal erythrocyte membranes. Lu-
FIGURE 3. Cholera toxin-dependent $^{32}$P-ribosylation of the 42,000-M$_s$, subunit of the G unit in control renal membranes and in renal membranes from a patient with PHP. Membranes were treated as described in Methods, and after electrophoresis an autoradiogram was prepared. The arrows indicate the position of the 42,000-M$_s$, subunit of the G unit (42K), and the position of the $\alpha_1$ subunit of cholera toxin (CT), which is auto-ADP-ribosylated (22). $^{32}$P incorporation into the other bands was not dependent on cholera toxin. The area of the 42,000-M$_s$, peak, calculated from densitometric tracings of the autoradiogram, was 513, 493, and 413 (arbitrary units) for the three controls, and 221 for the patient with PHP. Values are for the peak caused by toxin treatment minus the nontoxin lane background and represent the averages for duplicate determinations, which varied <10% from the mean.

There were also differences in the radiolabeling of other proteins of higher molecular weight. In control membranes, a complex of high molecular weight proteins is labeled (Fig. 3). This labeling occurred both with and without cholera toxin (not shown). In the PHP renal membranes the high molecular weight labeling is reduced. Since $^{32}$P incorporation into the high molecular weight band in control membranes is independent of cholera toxin, there is no evidence to indicate that this labeled band is related in any way to the adenylate cyclase system or to the hormone resistant state in patients with PHP. Instead, the high molecular weight band might reflect slight contamination of the membranes with nuclear proteins that are readily ADP-ribosylated even without cholera toxin (22). Others have observed that cholera toxin independent $^{32}$P incorporation into high molecular weight proteins is variable among membrane preparations, even from a single cultured cell line (23).

**DISCUSSION**

This patient showed, in addition to the typical features of PHP and Albright's hereditary osteodystrophy, evidence for resistance to thyrotropin and gonadotropins. Serum thyroxine was reduced, and thyrotropin elevated, compatible with primary hypothyroidism, but there was neither a goiter nor elevation of antithyroid antibodies. There was oligomenorrhea despite high serum gonadotropins and biopsy of the ovaries provided histologic evidence for poor stimulation by gonadotropins. Previous reports (24, 25) of resistance to multiple hormones in patients with PHP suggested that there might be a defect in a component of the adenylate cyclase system, distal to the hormone-specific receptor, and common to all tissues. Earlier studies of renal membranes from the patient described here (7), and a recent report (26) of abnormal adenylate cyclase activity in thyroid membranes from a patient with PHP and hypothyroidism, have indirectly implicated the G unit as the site of the molecular defect in PHP. We (9) and others (8) have shown that erythrocyte membranes from many patients with PHP (particularly those with the phenotypic features of Albright's hereditary osteodystrophy) have an ~50% reduction in G unit activity. The patient described here has a similar reduction in erythrocyte membrane G unit activity. Her characteristic habitus and resistance to multiple hormones make her typical of the patients with PHP who have G unit deficiency.

A generalized deficiency of G units could be the reason for resistance to multiple hormones in patients with PHP and Albright's hereditary osteodystrophy (10), but the biochemical results do not explain why hormone-dependent responses are more sensitive to the reduction in G units in some tissues than in others (10). Other factors, such as the amount of cyclic AMP required for protein kinase activation, the activity of phosphodiesterases, and even the membrane lipid composition, may influence the distal physiologic response to hormone stimulation.

This study indicates that the plasma membrane G unit deficiency extends to the cells of a classic PTH target tissue, the kidney. Renal membrane G unit deficiency was directly demonstrated by adenylate cyclase complementation assay, as well as by cholera toxin-catalyzed ADP-ribosylation. Earlier studies suggested that a subnormal response of the renal adenylate cyclase system to PTH in PHP could be corrected by small concentrations of GTP (7). Although insufficient renal tissue is available to test the sensitivity of G unit activity to guanine nucleotides, we have measured the sensitivity of G unit activity to GTP-$\gamma$-S in erythrocyte membranes from this patient. In agreement with other studies (9), the apparent affinity of the G unit for GTP-$\gamma$-S was similar in erythrocyte membranes from controls and the patient with PHP. Further studies of the kinetics and stoichiometry of the receptor-cyclase coupling process are necessary to learn whether GTP can recruit additional G units into the coupling reaction.
Our data suggest that studies with easily accessible cells, such as fibroblasts and circulating erythrocytes, should validly reflect the defect present in other less accessible tissues responsible for the clinical features of PHP.

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