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# Coupling of the PTH/PTHrP Receptor to Multiple G-Proteins

## *Direct Demonstration of Receptor Activation of $G_s$, $G_{q/11}$, and $G_{i(1)}$ by [α-$^{32}$P]GTP-γ-Azidoanilide Photoaffinity Labeling*

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**Parathyroid hormone (PTH) elicits many of its physiological effects by activating distinct G-protein-coupled signaling cascades that lead to synthesis of cyclic AMP and hydrolysis of phosphatidylinositol 4,5-bisphosphate. Using the nonhydrolyzable photoreactive GTP analog [α-$^{32}$P]GTP-γ-azidoanilide (GTP-AA) and peptide antisera raised against G-protein α-subunits, we studied coupling of the PTH receptor to G-proteins in rat osteoblast-like cells (ROS 17/2.8), and in human embryonal kidney cells expressing the cloned human PTH/parathyroid hormone-related peptide (PTHrP) receptor at 40,000 receptors/cell (C20) or 400,000 receptors/cell (C21). Incubation of C21 membranes (but not C20 membranes) with [Nle$^{8,18}$, Tyr$^{34}$]-bovine PTH(1-34) amide (bPTH[1-34]) led to concentration-dependent incorporation of GTP-AA into the two isoforms of $G\alpha_s$, into $G\alpha_{q/11}$, and to a much lesser extent into $G\alpha_{i(1)}$. In ROS 17/2.8 cells, bPTH(1-34) increased the incorporation of GTP-AA into $G\alpha_s$, but not into $G\alpha_{q/11}$ or $G\alpha_i$. The ability of bPTH(1-34) to increase labeling of $G\alpha_s$ and $G\alpha_{q/11}$ was correlated with the receptor-dependent sensitivity of the adenylyl cyclase and phospholipase C signaling pathways to the hormone.**

**Key Words:** Parathyroid hormone; parathyroid hormone-related peptide; signal transduction; G-protein; in vitro.

## Introduction

Parathyroid hormone (PTH) is the major calciotropic hormone, and regulates renal synthesis of calcitriol, resorption of calcium and phosphate from the glomerular filtrate, and release of calcium and phosphate from bone *(1)*. A second hormone, parathyroid hormone-related peptide (PTHrP), regulates maternal–fetal calcium transport *(2)*, and has paracrine actions in skeletal development *(3)* and hair growth *(4)*. Ectopic expression of PTHrP causes the syndrome of humoral hypercalcemia of malignancy *(5)*. PTH and PTHrP share 8 of the first 13 amino-terminal residues, and many of the physiological actions of these two hormones are mediated through the activation of a single receptor, the PTH/PTHrP receptor *(6)*. PTH, but not PTHrP, binds to and activates a second receptor that is encoded by a distinct gene, the PTH2 receptor *(7)*. The PTH/PTHrP receptor belongs to the family of seven transmembrane-spanning segment receptors that are coupled to intracellular signal effector molecules through heterotrimeric G-proteins. Binding of an agonist to its receptor activates the G-protein by stimulating the release of GDP. The subsequent binding of GTP to the α-subunit leads to dissociation of the G-protein into an activated α[GTP] and βγ dimer. Both the α[GTP] and βγ dimers regulate signal effector molecules, such as adenylyl cyclase and β-isoforms of phospholipase C.

PTH and PTHrP activate multiple second-messenger signaling pathways that are coupled by distinct G-proteins to the PTH/PTHrP receptor *(8)*. PTH increases intracellular cAMP, presumably via $G_s$. PTH also inhibits adenylyl cyclase via a pertussis toxin-sensitive G-protein (presumably $G_i$) *(9–11)*. Finally, PTH activates phospholipase C, and leads to the accumulation of $IP_3$ *(12)* and to increased intracellular $Ca^{2+}$ *(13)*, via a pertussis toxin-insensitive G-protein (presumably a member of the $G_q$ family). Direct evidence for involvement of α-subunits of the $G_q$ family in coupling of the PTH/PTHrP receptor to the phospholipase C pathway has come from experiments in which cells were cotransfected with both the PTH/PTHrP receptor and various α-subunits of the $G_q$ family *(14,15)*.

The ability of an agonist to promote binding of GTP to the active site of the α-subunit of specific G-proteins has

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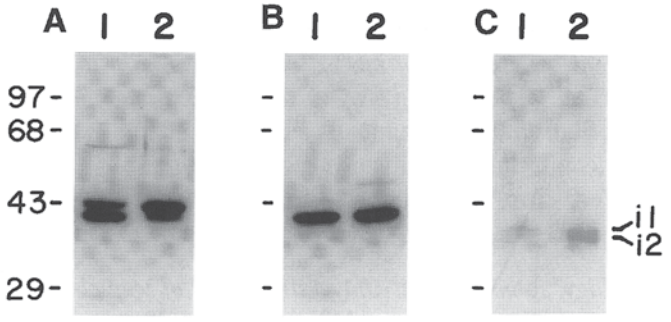


**Fig. 1.** Immunoblot: 50 μg of membrane protein prepared from HEK293 cells (lane 1) or ROS 17/2.8 cells (lane 2) were immunoblotted with polyclonal rabbit peptide antisera specific for $G\alpha_s$ (RM/1, NEI-805) **(A)**, $G\alpha_{q/11}$ (QL, NEI-809) **(B)**, and $G\alpha_{i1/i2}$ (AS/7, NEI-801) **(C)**. Both the 52- and 45-kDa form of $G\alpha_s$ are visible in both cell lines in (A). $G\alpha_q$ and $G\alpha_{11}$ were not resolved from each other (B). The 41-kDa $G\alpha_{i1}$ is present in both cells lines, but the 40-kDa $G\alpha_{i2}$ is seen predominantly in ROS 17/2.8 cells.

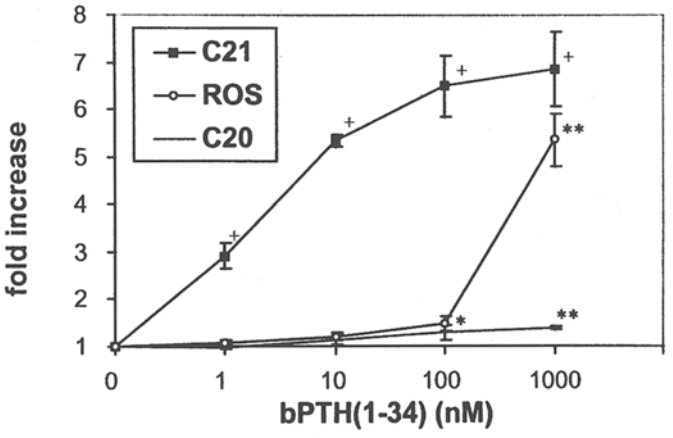


**Fig. 2.** Adenylyl cyclase activity of 20 μg of plasma membranes isolated from C21 (filled squares), ROS 17/2.8 (open circles), and C20 (no symbols) cells, during incubation for 20 min at 30°C in the presence of 10 μ*M* GTP and varying concentrations of bPTH(1-34). Results expressed as fold stimulation over basal activity represent the mean ± SE of 3 or 4 independent experiments. Repeated measure analysis of variance showed that when treated with bPTH(1-34), all three cell lines showed significant increases of adenylyl cyclase activity as illustrated ($+p < 0.001$, $**p < 0.01$, $*p < 0.05$).

facilitated the use of the nonhydrolyzable, photoaffinity label [α-$^{32}$P]GTP-γ-azidoanilide (GTP-AA) to identify the specific G-proteins that are coupled to cell-surface receptors *(16)*. In this study, we have developed a rapid technique to purify GTP-AA, and used the compound to examine the interaction of G-protein α-subunits with native and recombinant PTH/PTHrP receptors. With this technique, we extend the results of previous investigators and confirm that a single form of the PTH receptor is able to activate $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_i$.

## Results

### *Characteristics of Cell Lines*

We studied two cell lines that stably express the cloned human PTH/PTHrP receptor: C21 expresses approx 400,000 receptors/cell, whereas C20 express approx 40,000 receptors/cell *(17)*. These cell lines were compared to ROS 17/2.8 rat osteosarcoma cells, which express 72,000 endogenous receptors/cell *(18)*, and to untransfected HEK293 cells. As previously shown for C21 and C20 cells *(13)*, immunoblot analysis demonstrated that HEK293 and ROS 17/2.8 cells express $G\alpha_{q/11}$, and both the 52- and 45-kDa isoforms of $G\alpha_s$. However, although ROS 17/2.8 cells express both $G\alpha_{i1}$ and $G\alpha_{i2}$, HEK293 cells express predominantly $G\alpha_{i1}$ (Fig. 1). Low levels of $G\alpha_{i2}$ in HEK293 cells have been noted previously *(19)*.

### *Stimulation of Adenylyl Cyclase*

Adenylyl cyclase activity in response to bPTH(1-34) was dependent both on the concentration of bPTH(1-34) (1–1000 n*M*) and on the number of PTH/PTHrP receptors. C21 cells showed the greatest maximal response (210 ± 19 pmol cAMP/mg protein/min), followed by ROS 17/2.8 (131 ± 53 pmol cAMP/mg protein/min), and C20 cells (10 ± 3 pmol cAMP/mg protein/min) (Fig. 2). HEK293 cells, which do not bind PTH *(20)*, showed no increase in adenylyl cyclase activity in response to bPTH(1-34) (not shown).

### *Stimulation of Phospholipase C Signaling*

To examine PTH-dependent activation of the phospholipase C signaling cascade, we measured both IP accumulation and intracellular calcium transients in response to bPTH(1-34). In C21 cells, IP accumulation increased in response to carbachol (100 μ*M*, $p < 0.01$ compared to unstimulated), fluroaluminate (20 m*M* NaF, 30 μ*M* $AlCl_3$, $p < 0.01$ compared to unstimulated), and bPTH(1-34) (1–1000 n*M*, $p < 0.01$ for linear trend) (Fig. 3). The IP response to 1000 n*M* bPTH(1-34) was less than the response to either carbachol or fluroaluminate, suggesting that PTH-induced IP synthesis is not limited by the availability of G-protein α-subunits in HEK293 cells. Moreover, C21 cells demonstrated a rapid transient increase in intracellular $Ca^{2+}$ in response to 100 n*M* bPTH(1-34) (Fig. 4). By contrast, treatment of HEK293 or C20 cells with bPTH(1-34) did not elicit an increase in intracellular $Ca^{2+}$ (not shown).

### *bPTH(1-34)-Dependent Labeling with GTP-AA*

To determine which G-proteins are coupled to the PTH/PTHrP receptor in the adenylyl cyclase and phospholipase C signal transduction pathways, we used GTP-AA to label G-protein α-subunits activated by treatment of cell membranes with bPTH(1-34). Cell membranes were labeled with GTP-AA in the presence of varying concentrations of bPTH(1-34) and α-subunits were immunoprecipitated. Figure 5 demonstrates the agonist-dependent increase in labeling for both isoforms of $G\alpha_s$ and $G\alpha_{q/11}$ in C21 membranes.

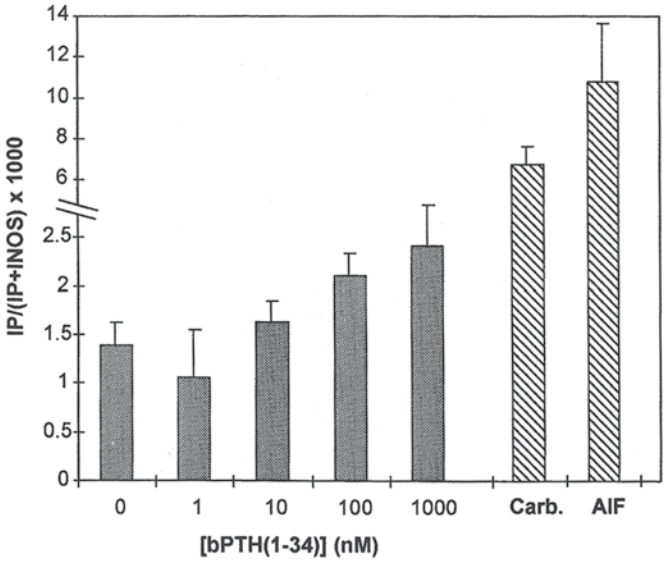


**Fig. 3.** IP accumulation in subconfluent cultures of C21 cells, grown in 24-well dishes, during a 30-min incubation at 37°C, with various concentrations of bPTH(1-34), 100 μ*M* carbachol (**Carb**), or 20 m*M* NaF and 30 μ*M* $AlCl_3$ (**AlF**). Results are expressed as IP divided by IP plus inositol, and represent the mean ± SE of 8 independent experiments.

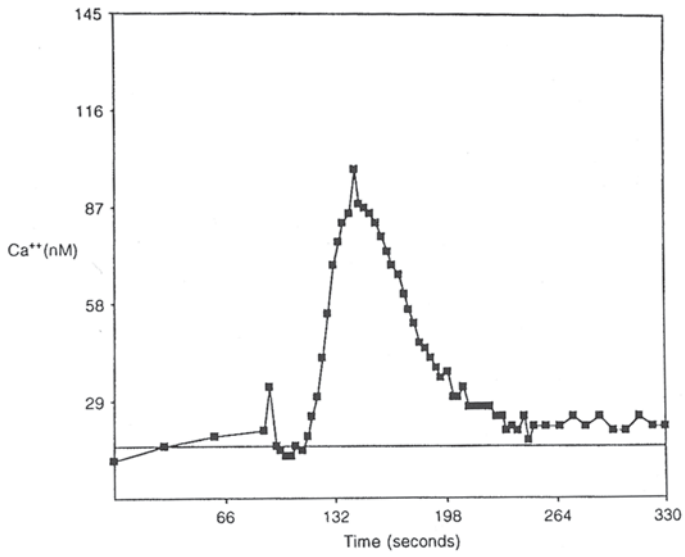


**Fig. 4.** Increased intracellular $Ca^{2+}$ in C21 cells in response to 100 n*M* bPTH(1-34) determined by digital video microscopy of a field of 25 cells. Results are expressed as concentration of intracellular $Ca^{2+}$ as determined by the formula of Grynkiewicz et al. *(40)*. In C21 cells $R_{min}$ was 0.72, $R_{max}$ was 2.46, and β was 2.92. A single representative experiment is shown.

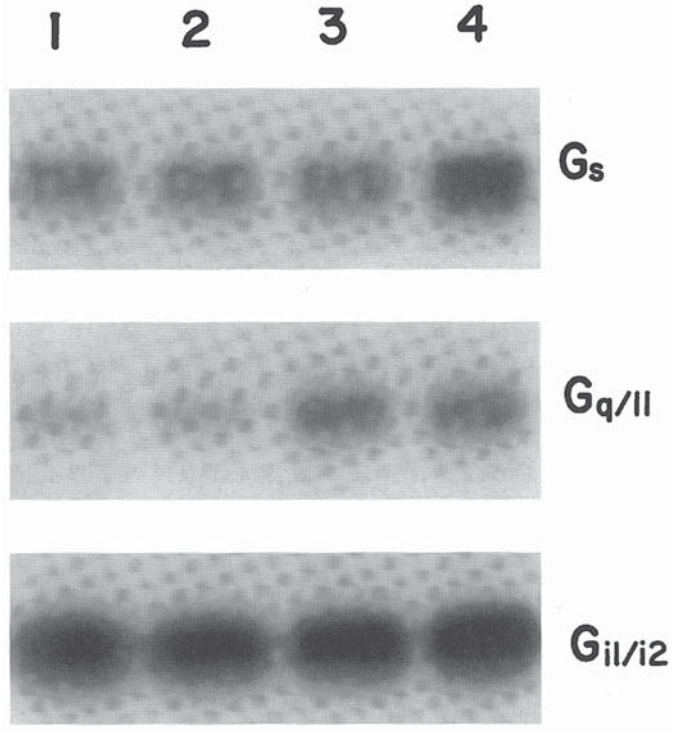


**Fig. 5.** Autoradiograph of agonist-induced photolabeling G-protein α-subunits with GTP-AA in C21 cells. C21 plasma membranes were incubated with GTP-AA and varying concentrations of bPTH(1-34), none (lane 1), 0.1 n*M* (lane 2), 10 n*M* (lane 3), 1 μ*M* (lane 4). Following UV crosslinking, G-protein α-subunits were immunoprecipitated with specific antisera, RM/1 for $G\alpha_s$, QL for $G\alpha_{q/11}$, and AS/7 for $G\alpha_{i1/i2}$, and resolved on SDS polyacrylamide gels.

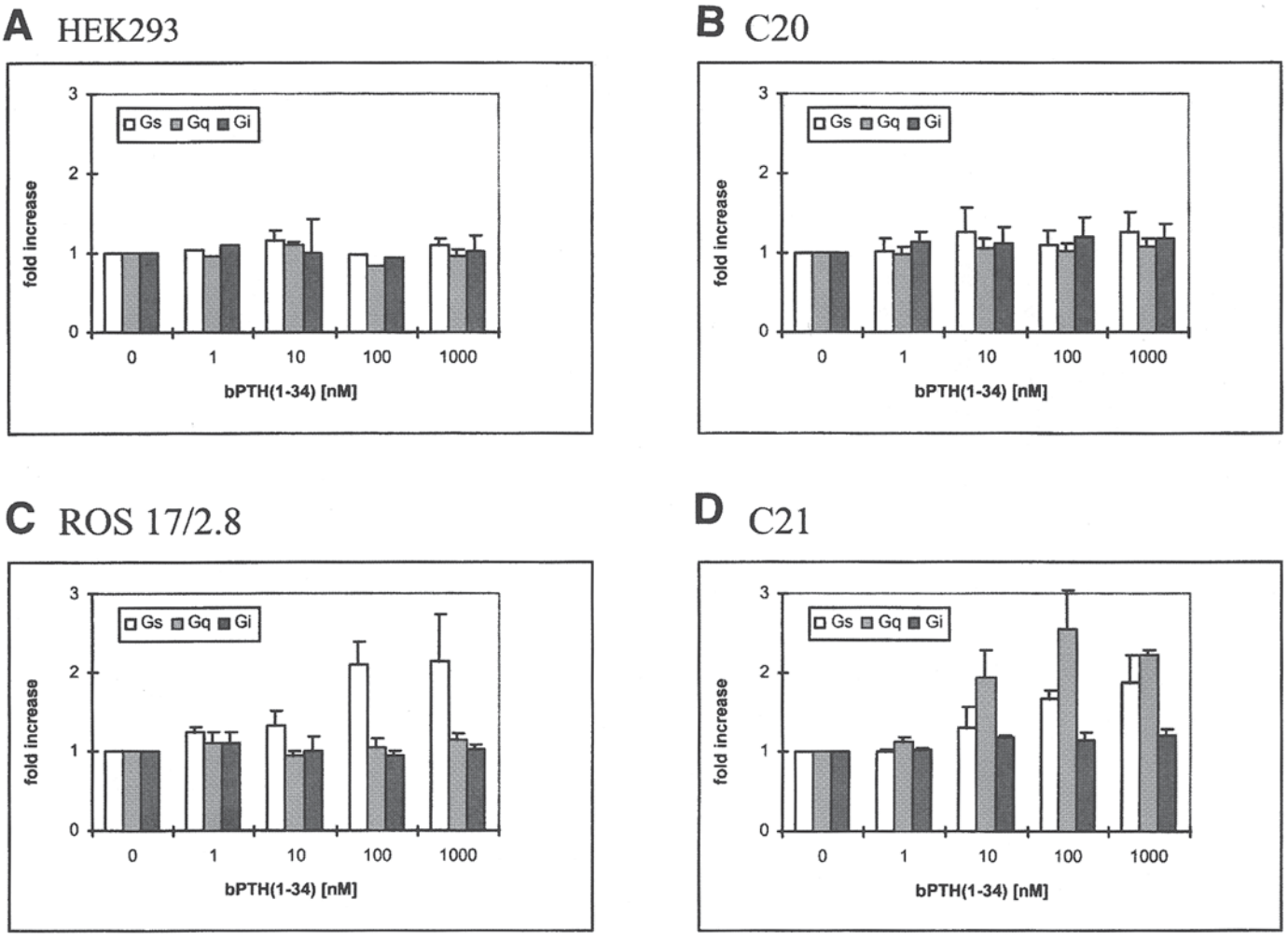


**Fig. 6.** Quantitation of agonist induced photolabeling. G-protein α-subunits photolabeled with GTP-AA in C21 cells were immunoprecipitated and resolved on SDS polyacrylamide gels. The dried gels were imaged with a Molecular Dynamics Phosphoimager and analyzed with ImageQuaNT software. Results are expressed as fold increase over basal labeling in response to various concentrations of bPTH(1-34), and represent the mean ± SE of 3 independent experiments. Repeated measures analysis of variance indicated that there was no significant increase in GTP-AA labeling of G-proteins in response to PTH in HEK293 cells **(A)**, or C20 cells **(B)**. Labeling of $G\alpha_s$ in ROS 17/2.8 cells **(C)** was significantly increased ($p = 0.037$). C21 cells **(D)** showed a significant increase in GTP-AA labeling of $G\alpha_s$ ($p = 0.025$), $G\alpha_{q/11}$ ($p = 0.017$), and $G\alpha_{i1}$ ($p = 0.036$).

A significant amount of GTP-AA is incorporated into α-subunits even in the absence of agonist. We therefore assessed a variety of incubation conditions in an attempt to decrease agonist-independent labeling. Increasing concentrations of GDP (0–30 μ*M*) or $K^+$ (10–100 m*M*), decreased concentrations of $Mg^{2+}$ (1–5 m*M*), shortened time (1–10 min), or lowered temperature (4–30°C) of incubation all reduced agonist-independent labeling, but also reduced agonist-dependent labeling, and did not improve the signal-to-noise ratio. Quantification of the amount of radioactivity bound by immunoprecipitated α-subunits is shown in Fig. 6. In membranes prepared from C21 cells bPTH(1-34) significantly increased the labeling of both $G\alpha_s$ and $G\alpha_{q/11}$. Nonlinear regression indicated that the maximal increase in labeling was 1.9- ± 0.3-fold for $G\alpha_s$ with an $EC_{50}$ of 24 n*M* bPTH(1-34) ($\log[EC_{50}] = -7.6 \pm 0.7$), values for $G\alpha_{q/11}$ were not significantly different with a maximal increase in labeling of 2.4- ± 0.2-fold and an $EC_{50}$ of 6 n*M* ($\log[EC_{50}] = -8.2 \pm 0.3$). Labeling of $G\alpha_{i(1)}$ was also slightly increased (1.18- ± 0.03-fold, $\log[EC_{50}] = -8.6 \pm 0.8$). Treatment of membranes prepared from ROS 17/2.8 cells with bPTH(1-34) also increased GTP-AA labeling of $G\alpha_s$ (2.2- ± 0.2-fold, $\log[EC_{50}] = -7.7 \pm 0.5$), but did not significantly increase labeling of $G\alpha_{q/11}$ or $G\alpha_{i1/i2}$. Treatment of membranes prepared from C20 or HEK293 cells with bPTH(1-34) did not increase GTP-AA labeling of G-protein α-subunits significantly.

## Discussion

In the present study, we have used GTP-AA, a nonhydrolyzable, photoreactive GTP analog, to label G-protein α-subunits that are coupled to the PTH/PTHrP receptor.

Our modification of existing techniques for purification of GTP-AA simplifies production of this reagent and facilitates widespread application of this labeling approach to the study of receptor–G-protein interactions. Our studies indicate that the PTH/PTHrP receptor can couple to multiple G-protein α-subunits, but these interactions are dependent on receptor density. In C21 cells, which express 400,000 human PTH/PTHrP receptors/cell, we found direct coupling of the PTH/PTHrP receptor to $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i1}$. In C20 cells, which express only 40,000 human PTH/PTHrP receptors/cell, bPTH(1-34) did not significantly increase GTP-AA labeling of any of these G-protein α-subunits. In ROS 17/2.8, which express endogenous rat PTH/PTHrP receptors at 72,000 receptors/cell, bPTH(1-34) increased labeling of $G\alpha_s$, but it did not induce guanyl nucleotide exchange into $G\alpha_{q/11}$ or $G\alpha_{i1/i2}$.

GTP-AA labeling of G-proteins in the HEK293 cell lines corresponded to activation of the adenylyl cyclase and phospholipase C pathways in these cells. PTH-dependent activation of adenylyl cyclase activity was much greater in C21 membranes than in C20 membranes. cAMP accumulation in response to bPTH(1-34) was previously shown to be greater in intact C21 cells than intact C20 cells *(17)*. Similarly, GTP-AA labeling of $G\alpha_s$ in response to bPTH(1-34) was seen in C21 cells, but could not be demonstrated in C20 cells. Increased synthesis of IP and intracellular $Ca^{2+}$ transients in response to bPTH(1-34) were observed in C21 cells, but not in C20 cells. PTH-stimulated calcium transients occur in C20 cells, but only at concentrations of PTH approx 100 times greater than those required in C21 cells *(13)*. GTP-AA incorporation into $G\alpha_{q/11}$ in response to bPTH(1-34) was seen in C21 cells, but not in C20 cells. Finally, bPTH(1-34) failed to increase G-protein labeling or activate adenylyl cyclase or phospholipase C in wild-type HEK293 cells, which do not express PTH receptors.

Studies with ROS 17/2.8 have consistently demonstrated that PTH analogs increase intracellular cAMP *(9,10,21)*. Some studies of ROS 17/2.8 have also demonstrated that PTH analogs increase intracellular $Ca^{2+}$ *(22)* and IP accumulation *(23)*, and can activate the protein kinase C signaling pathway *(24)*. However, these effects have not been consistently found, and other studies failed to demonstrate increases in intracellular $Ca^{2+}$ *(25)* or IP accumulation *(22)* in response to PTH. Our studies with ROS 17/2.8 show that treatment with bPTH(1-34) increases GTP-AA labeling of $G\alpha_s$, but not $G\alpha_{q/11}$. One interpretation of our inability to demonstrate labeling of $G\alpha_{q/11}$ in ROS 17/2.8 cells is that photoaffinity labeling is too insensitive to detect activation of small numbers of $G_q$ or $G_{11}$ molecules. Alternatively, other α-subunits of the $G_q$ family (e.g., $G\alpha_{14}$ or $G\alpha_{15/16}$), or βγ-subunits released after activation of $G_s$, may mediate PTH stimulation of phospholipase C in ROS 17/2.8 cells.

A small, but significant increase in labeling of $G\alpha_{i1}$ was observed in C21 cells in response to bPTH(1-34). Moreover, PTH-stimulated cAMP accumulation in C21 cells is approx 30% greater following treatment with pertussis toxin *(13)*. These results are consistent with studies in transiently transfected COS-7 cells *(26)* and in ROS 17/2.8 cells *(10,21)* that showed that pertussis toxin treatment increased PTH-stimulated adenylyl cyclase activity. These studies suggest that pertussis toxin modification of $G_i$ can uncouple PTH receptors from inhibition of adenylyl cyclase, and provide additional support for the notion that the PTH/PTHrP receptor can interact with members of the $G_i$ family.

Our results indicate that the PTH/PTHrP receptor can interact with multiple G-proteins. Others' studies using GTP-AA have also demonstrated the interaction of other cell-surface receptors with multiple G-protein α-subunits. For example, the TSH receptor couples to $G\alpha_s$ and $G\alpha_{q/11}$ *(27)* and other G-protein α-subunits *(27,28)*, the vasopressin receptor to $G\alpha_q$ and $G\alpha_{11}$ *(29)*, the cholecystokinin receptor to $G\alpha_i$ and $G\alpha_s$ *(30)*, and the thrombin and thromboxane $A_2$ receptors to $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_{q/11}$ *(31)*. Although photoaffinity labeling with GTP-AA provides direct evidence of receptor–G-protein interaction, variability in the ability of GTP-AA to interact with different G-protein α-subunits limits comprehensive analysis. For example, whereas $G\alpha_{i2}$ and $G\alpha_o$ bind GTP-AA effectively at 100 m*M* free $Mg^{2+}$, $G\alpha_s$ requires 5 m*M* free $Mg^{2+}$ to bind GTP-AA, and $G\alpha_z$ does not bind GTP-AA appreciably at all *(31)*. Moreover, dissociation of GTP-AA from α-subunits is significant at 5 m*M* free $Mg^{2+}$ *(31)*. Thus, the GTP-AA system is best suited to analysis of receptor coupling to members of the $G\alpha_i$ family, must underestimate receptor coupling to $G\alpha_s$, and cannot measure coupling to $G\alpha_z$.

The results of the present study have relevance to understanding the relationship of PTH/PTHrP receptor number to PTH physiology. Based on the differential coupling efficacy of the PTH/PTHrP receptor to different G-protein α-subunits, it is tempting to speculate that changes in receptor number can have a profound effect on PTH action. Indeed, PTH stimulation of phospholipase C is highly dependent on receptor density, whereas stimulation of adenylyl cyclase occurs even at the lowest receptor densities *(32)*. Similar effects on PTH action are likely to result from changes in the number of G-proteins in the target cells, since transfection of HEK293 and COS-7 expressing the PTH receptor with α-subunits of the $G_q$ family enhanced the ability of PTH to simulate phospholipase C *(14)*. Finally, cell-specific expression of isoforms of adenylyl cyclase and phospholipase C that differ in their sensitivity to βγ-subunits and intracellular $Ca^{2+}$ can further modify hormone responses. Thus, the ultimate biological response to PTH can be influenced by the number of PTH/PTHrP receptors expressed as well as the number and type of G-proteins and effector molecules in the target cells.

## Materials and Methods

### *Materials*

[Nle$^{8,18}$,Tyr$^{34}$]-bovine PTH(1-34) amide [bPTH(1-34)] was from Bachem (Torrence, CA). Radionucleotides [$\alpha$-$^{32}$P]GTP (3000 Ci/mmol), [$\alpha$-$^{32}$P]ATP (800 Ci/mmol), [2,8-$^{3}$H]adenosine-3',5'-cyclic phosphate (cAMP) (28 Ci/mmol), and myo[2-$^{3}$H]inositol (22 Ci/mmol) were obtained from Dupont NEN (Boston, MA), as were polyclonal rabbit peptide antisera specific for $G\alpha_s$ (RM/1, NEI-805), $G\alpha_{q/11}$ (QL, NEI-809), and $G\alpha_{i1/i2}$ (AS/7, NEI-801). 4-Azidoaniline hydrochloride and 1-(3-dimethylaminopropyl)-3-ethylenecarbodiimide hydrochloride (NDEC) were from Aldrich Chemical Co. (Milwaukee, WI). 1,4-Dioxane and 2-[N-morpholino]ethanesulfonic acid (MES) were from Sigma Chemical Co. (St. Louis, MO). Triethylamine was from J.T. Baker (Phillipsburg, NJ). PEI-cellulose plates were obtained from EM Science (Gibbstown, NJ). Fetal bovine serum (Gibco cat. no. 26140-079) was from Life Technologies, Inc. (Gathersburg MD). Fura-2AM was from Molecular Probes, Inc. (Eugene, OR).

### *Membrane Preparation*

Human embryonal kidney cells (HEK293) and cell lines expressing the human PTH/PTHrP receptor (C20 and C21) *(17)* were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% fetal bovine serum. ROS 17/2.8 cells were grown in Ham's F-12 medium (Life Technologies) with 5% fetal bovine serum, 1 m*M* calcium chloride, and 28 m*M* HEPES, pH 7.4. Cells were grown at 37°C, 95% relative humidity, and 5% $CO_2$. To prepare membranes, confluent monolayers were first rinsed with phosphate-buffered saline (PBS), and then incubated for 30 min at 37°C in hypotonic buffer (5 m*M* HEPES, pH 8.0, 0.5 m*M* EDTA). Swollen cells were harvested by pipeting, collected by centrifugation, and resuspended in approx 9 vol of 10 m*M* Tris, pH 8.0, and 1 m*M* EDTA. Cells were disrupted with 20 stokes in a "loose" Dounce homogenizer (Wheaton, Millville, NJ) on ice. A crude membrane fraction was collected by centrifugation at 27,000*g* for 20 min at 4°C. The pellet was resuspended in 20 m*M* Tris, pH 7.4, 1 m*M* EDTA, 10% (w/v) sucrose, layered onto a cushion of 20 m*M* Tris, pH 7.4, 1 m*M* EDTA, 44.5% sucrose (w/v), and centrifuged at 100,000*g* at 4°C for 90 min. Plasma membranes were collected from the interface, washed in 5 vol of freezing buffer (250 m*M* sucrose, 20 m*M*, Tris pH 8.0, 1 m*M* $MgCl_2$, 1 m*M* DTT), and stored in small aliquots in at –70°C. Protein concentrations were determined by the BCA Protein Assay (Pierce, Rockford, IL).

### *Immunoblot Analysis*

Plasma membrane proteins were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and immunoblotted with specific G-protein $\alpha$-subunit antisera by published procedures *(33,34)*.

### *Adenylyl Cyclase Assay*

Membranes (20 µg) were incubated in a final volume of 100 µL containing 0.4 m*M* [$\alpha$-$^{32}$P]ATP ($1–4 \times 10^6$ cpm) in 50 m*M* HEPES, pH 8.0, 0.2 m*M* EGTA, 1 m*M* DTT, 0.1 m*M* cAMP, 7.5 m*M* creatine phosphate, and 50 U/mL creatine phosphokinase, for 20 min at 30°C. Other modulators were added as indicated in the text. The reaction was terminated by adding an equal volume of stop solution (2% SDS, 50 m*M* HEPES, pH 8.0, 2 m*M* ATP, 0.5 m*M* [$^{3}$H] cAMP ($2–5 \times 10^4$ cpm), and heating at 100°C for 5 min. cAMP was isolated by Dowex and alumina chromatography *(35)*.

### *Phosphoinositide Assay*

C21 cells were seeded onto 24-well plates ($1 \times 10^5$ cells/well) and incubated with myo-[2-$^{3}$H]-inositol (2 µCi/mL) in inositol-free DMEM, supplemented with 10% fetal bovine serum, for 24 h. After 24 h of labeling, the medium was removed, and each well was washed twice with assay medium (DMEM without sodium bicarbonate, 20 m*M* HEPES, pH 7.4, 10 m*M* LiCl). Effectors were added in 250 µL of assay medium, and incubation was continued for 30 min at 37°C. The assay was terminated by the addition of 750 µL of cold 20 m*M* formic acid. Total phosphoinositides (IP) were separated from inositol by chromatography on Dowex AG1-X8 columns *(36)*.

### *Measurement of Intracellular Calcium Transients*

Subconfluent cells in 24-well plates were loaded with 4 µ*M* Fura-2AM for 60 min at 37°C in DMEM containing 5% FBS. Cells were then washed, covered with 0.5 mL of PAG (25 m*M* PIPES, pH 7.4, 110 m*M* NaCl, 5 m*M* KCl, 1 mg/mL BSA, 1 mg/mL glucose) with 2 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, and placed on the microscope stage where the temperature was maintained at 37°C throughout the experiment. Changes in intracellular $Ca^{2+}$ concentrations were determined by digital video microscopy *(37,38)*. Four frames (each frame is a single ratio measurement of a field of 40–50 cells), 30 s apart, were taken to establish a baseline calcium level. Modulators were added in a volume of 0.5 mL and a series of 60 frames of data were acquired every 2–8 s for a total of 3 min.

### *Synthesis of GTP-AA*

GTP-AA was synthesized from [$\alpha$-$^{32}$P]GTP according to the method of Schafer et al. *(39)*: [$\alpha$-$^{32}$P]GTP (1 mCi) was evaporated to dryness in a Speed Vac microconcentrator and 60 µL of a solution of NDEC (30 mg/mL) in 0.1 *M* MES, pH 5.6, plus 40 µL of a suspension of azidoaniline-HCl (40 mg/mL) in 1,4-dioxane were added. The mixture was incubated for 6 h at room temperature, in the dark, with constant agitation. The GTP-AA was purified by hydrophobic interaction chromatography *(16)* using a C-18 Sep Pak cartridge (Millipore Corp., Milford, MA). The cartridge was prewetted with methanol and equilibrated with 97.2% buffer A and 2.8% buffer B. (Buffer A was prepared

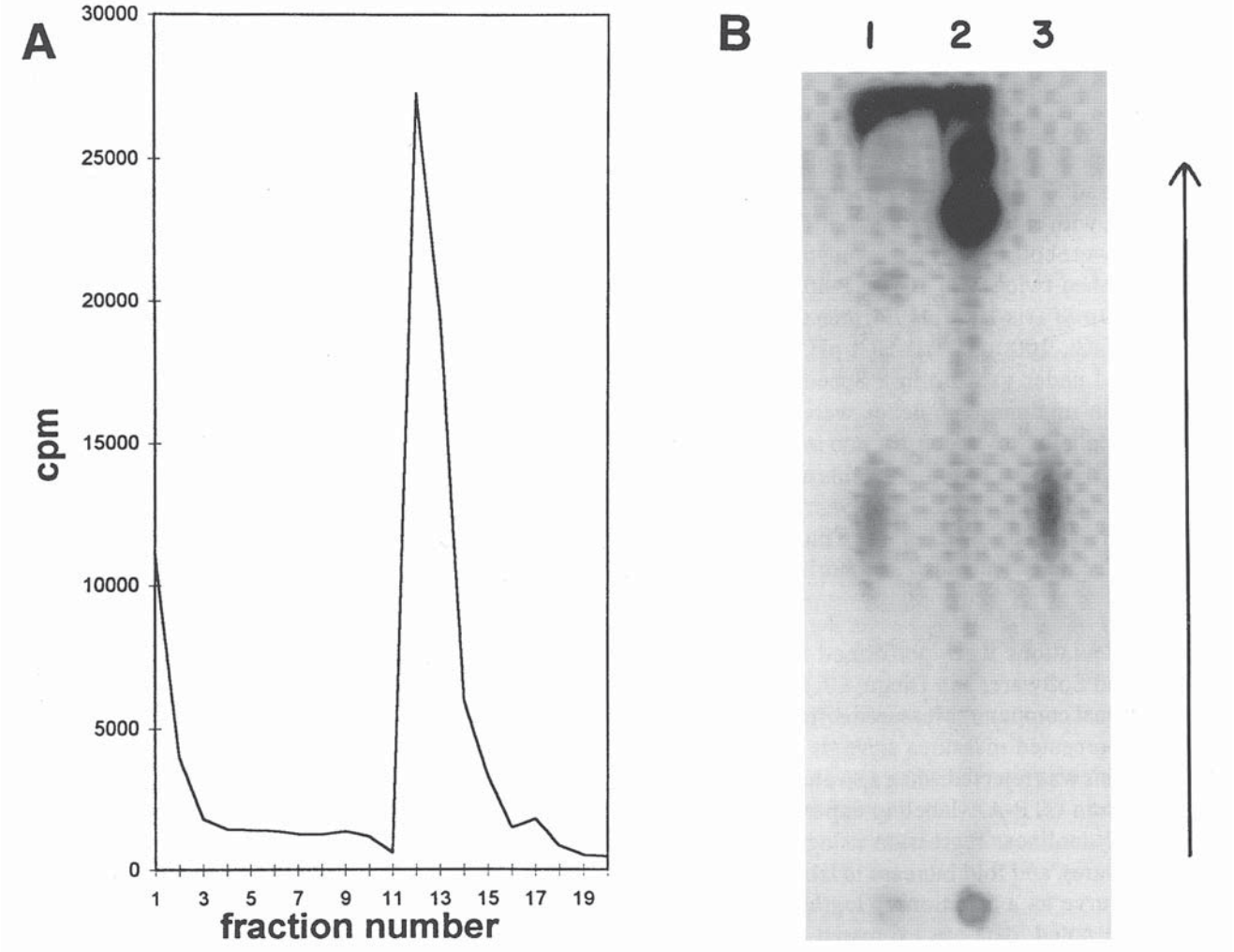


**Fig. 7.** Synthesis of GTP-AA. **(A)** Elution of GTP-AA from Sep-Pak cartridge. Fractions 1–10 are 1 mL of 97.2% buffer A (100 m*M* triethylammonium bicarbonate, pH 7), 2.8% buffer B (100 m*M* ethanolic triethylammonium bicarbonate). Fractions 11–20 are 0.5 mL of 10% buffer A, 90% buffer B. **(B)** PEI cellulose chromatography of fractions eluted from Sep-Pak indicating that the first peak (fraction 1) of radioactivity (lane 1) co-migrates with GTP (lane 3), whereas the second peak (fraction 13) (lane 2) demonstrates the expected increase in mobility of the GTP-AA derivative.

by bubbling $CO_2$ through 100 m*M* triethylamine until the pH reached 7.0; buffer B was prepared likewise with a 100-m*M* solution of triethylamine in ethanol.) Working behind a 5/8-in. Plexiglas shield, the sample was dissolved in 1 mL of equilibrating buffer and applied to the cartridge. The cartridge was washed with 10 mL of equilibrating buffer, and the wash was collected in 1-mL aliquots. The GTP-AA was eluted from the cartridge with 5 mL of 10% buffer A and 90% buffer B, and collected in 0.5-mL fractions. Aliquots (1/10,000) of each fraction were added to vials containing scintillation liquid (BioSafe II), and $^{32}P$ was quantified by scintillation spectrophotometry (Fig. 7A). Fractions containing GTP-AA were combined, evaporated to dryness, and stored at –20°C for up to 1 mo. Synthesis of GTP-AA was analyzed by thin-layer chromatography on PEI-cellulose plates with a solvent phase of buffer A (Fig. 7B). The overall yield of GTP-AA varied from 20 to 60%. All procedures were performed in a darkened room.

### *Photolabeling of α-Subunits*

Plasma membranes (150 µg) were incubated with 6 µCi of GTP-AA in 100 µL of buffer C (50 m*M* HEPES, pH 7.4, 30 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* benzamidine, 0.1 m*M* EDTA) in a darkened room. After incubation for 3 min at 30°C, membranes were placed on ice and collected by centrifugation (12,000*g*, 15 min, 4°C). The membranes were resuspended in 100 µL of buffer C containing 2 m*M* glutathione and exposed at 4°C to UV light (254 n*M*, 0.16 A, 115 V) at a distance of 5 cm for 10 min.

### *Immunoprecipitation*

GTP-AA-labeled membranes were collected by centrifugation (12,000*g*, 15 min, 4°C) and solubilized by repeated pipeting in 120 µL 2% SDS. Three hundred sixty microliters of buffer D (1% [v/v] Triton X-100, 1% [w/v] deoxycholate, 0.5% [w/v] SDS, 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* DTT, 1 m*M* EDTA, 0.2 m*M* PMSF,

10 mg/mL aprotinin) were added, and insoluble material was removed by centrifugation (12,000*g*, 4°C, 10 min). Solubilized membranes were divided into 150-µL aliquots. Each aliquot was incubated with 10 µL of an undiluted G-protein antisera at 4°C, with continuous agitation. After 2 h, 40 µL of a suspension of protein A sepharose (10% [w/v] in buffer D) were added, and the incubation was continued overnight at 4°C, with continuous agitation. The protein A sepharose beads were collected by centrifugation (12,000*g*, 4°C, 5 min), washed twice with 10% NP-40, 0.5% SDS, 600 m*M* NaCl, 50 m*M* Tris-HCl, pH 7.4, then washed twice with 300 m*M* NaCl, 100 m*M* Tris-HCl pH 7.4, 10 m*M* EDTA, and dried under vacuum in a Speed Vac microconcentrator. The immune complexes were dissociated from protein A sepharose by reconstitution in Laemmeli's buffer (80 µL) and boiling for 5 min. Proteins were resolved by electrophoresis through 10% SDS-polyacrylamide gels *(34)*. The gels were stained with Coomassie blue, dried, and imaged on a Molecular Dynamics PhosphorImager.

### *Statistics*

Statistical calculations were performed using InStat v2.04a (GraphPad Software, San Diego, CA) on an IBM-compatible personal computer. Measured differences were compared using repeated measures analysis of variance. The null hypothesis was rejected when a $p$ value $< 0.05$ was obtained. Data from GTP-AA-labeling experiments were also analyzed by nonlinear regression using InPlot v3.1 (GraphPad Software), and fold increase in labeling was fit to a sigmoidal curve as a function of log(bPTH[1-34]). Unless otherwise noted, data are expressed as the mean plus or minus standard error of the mean.

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