# G$\alpha_{12}$/G$\alpha_{13}$ Subunits of Heterotrimeric G Proteins Mediate Parathyroid Hormone Activation of Phospholipase D in UMR-106 Osteoblastic Cells

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**PTH, a major regulator of bone remodeling and a therapeutically effective bone anabolic agent, stimulates several signaling pathways in osteoblastic cells. Our recent studies have revealed that PTH activates phospholipase D (PLD) -mediated phospholipid hydrolysis through a RhoA-dependent mechanism in osteoblastic cells, raising the question of the upstream link to the PTH receptor. In the current study, we investigated the role of heterotrimeric G proteins in mediating PTH-stimulated PLD activity in UMR-106 osteoblastic cells. Transfection with antagonist minigenes coding for small peptide antagonists to G$\alpha_{12}$ and G$\alpha_{13}$ subunits of heterotrimeric G proteins pre-**
**vented PTH-stimulated activation of PLD, whereas an antagonist minigene to G$\alpha$s failed to produce this effect. Effects of pharmacological inhibitors (protein kinase inhibitor, *Clostridium botulinum* exoenzyme C3) were consistent with a role of Rho small G proteins, but not of cAMP, in the effect of PTH on PLD. Expression of constitutively active G$\alpha_{12}$ and G$\alpha_{13}$ activated PLD, an effect that was inhibited by dominant-negative RhoA. The results identify G$\alpha_{12}$ and G$\alpha_{13}$ as upstream transducers of PTH effects on PLD, mediated through RhoA in osteoblastic cells. (*Endocrinology* 146: 2171–2175, 2005)**

P TH INTERACTION with its receptor on osteoblasts initiates events leading to both the production of anabolic mediators that stimulate bone formation, and to the production of cytokines that activate osteoclastogenesis, osteoclast activity, and osteoclast survival. The receptors for PTH are heptahedral transmembrane proteins that transduce their effects through heterotrimeric GTPase-activating proteins (G proteins). The activation of adenylyl cyclase through G$\alpha$-containing G proteins is a major downstream effect of PTH (1). However, there is also activation of the membrane phospholipases, phospholipase C (2) and phospholipase D (PLD) (3, 4). Our previous studies have shown that PTH activation of PLD and subsequent phosphatidic acid phosphatase action leads to translocation of protein kinase C$\alpha$ from the cytosol to plasma membranes (5). PLD-dependent signaling also contributes to PTH stimulation of IL-6 promoter activity in the osteoblastic cells (5). PTH activates the small G protein RhoA (6). The actions of PTH on PLD (4) and on protein kinase C$\alpha$ translocation (6) are dependent on RhoA, and small GTPases of the Rho/Ras family are also involved in PTH stimulation of IL-6 promoter activity (6).

Activation of small G proteins of the Rho family and subsequently PLD by membrane receptors can be mediated through the G$\alpha_{12}$/G$\alpha_{13}$ family of heterotrimeric GTPases (7–13), and these interactions are now recognized to play important roles in cell shape changes and cell survival (7,

14–17). Because our studies of PTH signaling in UMR-106 cells showed that PTH activates PLD in a RhoA-dependent manner (4), it was relevant to investigate the role of G$\alpha_{12}$ and G$\alpha_{13}$ subunits of heterotrimeric G proteins in PTH stimulation of PLD in the osteoblastic cells to more fully understand the process of PTH activation of the pathway.

## Materials and Methods

### Materials

Bovine PTH 1–34 was from Bachem (Torrance, CA). RhoA19N was generously provided by Dr. Said Sebti (University of South Florida, Tampa, FL). *Clostridium botulinum* exotoxin C3 was from Calbiochem (San Diego, CA). Constitutively active (GTPase-deficient) G$\alpha_{12}$ and G$\alpha_{13}$ were generated as described previously (18, 19). Minigenes that code for small peptides that act as antagonists of the endogenous G proteins, pcDNA-G$\alpha$s, pcDNA-G$\alpha_{12}$, and pcDNA-G$\alpha_{13}$, were obtained from Cue BIOtech (Evanston, IL) and have been described previously (20, 21).

### Cell culture

UMR-106 osteoblastic osteosarcoma cells were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured to confluence in DMEM supplemented with 15% heat-inactivated horse serum and 100 U/ml K-penicillin G at 37 C in a 5% $CO_2$ environment. Cells were passaged every 3–5 d in 75-cm² tissue culture flasks. Cells were then seeded on sterile culture dishes and used the following day. Cells from passages 12–18 were used for experiments.

### PLD activity/transphosphatidylation

Cells were seeded at 500,000 cells per well in six-well cell culture dishes, allowed to attach overnight, and then prelabeled with [$^{14}$C] palmitic acid (0.25 $\mu$Ci/ml) in DMEM containing 15% heat-inactivated horse serum and 100 U/ml K-penicillin G for 24 h at 37 C in a 5% $CO_2$ atmosphere. Cells were washed and then pretreated or treated with the indicated agonists or antagonists for the given times in DMEM containing 20 mM HEPES, 0.1% BSA, and 1% absolute ethanol. To terminate

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Abbreviations: PE, Phosphatidylethanol; PKI, protein kinase inhibitor; PLD, phospholipase D.

the reaction, media were quickly removed, and 1 ml ice-cold methanol was added to cells. Cells were scraped into chloroform, and lipids were extracted using the method of Folch *et al.* (22). The extract containing lipids was dried under nitrogen, lipids were re-equilibrated in 100 $\mu$l chloroform-methanol (9:1 vol/vol), of which 50 $\mu$l was spotted on a thin-layer chromatography plate, and a 10-$\mu$l aliquot was used to determine total lipid radioactivity. Phosphatidylethanol (PE) was separated from the total lipid fraction by thin-layer chromatography using chloroform-methanol-acetic acid (70:10:2 vol/vol/vol) as the running solvent. A 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol standard was run concurrently. Lipids were visualized by exposure to iodine vapor. For autoradiography, thin-layer chromatography plates were incubated at $-70$ C for 72 h. The PE bands were scraped and radioactivity determined by liquid scintillation counting. The $^{14}$C radioactivity recovered in PE at the end of the treatments was expressed as the percentage of total $^{14}$C lipid radioactivity and is presented as mean $\pm$ SE of three determinations.

*Constructs and transfections*

UMR-106 cells were seeded at 400,000 cells per well in six-well cell culture dishes, and were transfected 16 h later. For experiments involving G$\alpha$ C-terminal selective antagonist minigenes, 0.5 $\mu$g each of pcDNA$_3$ (parental vector), pcDNA-G$\alpha_{12}$, pcDNA-G$\alpha_{13}$, pcDNAG-$\alpha$s, or pcDNA-G$\alpha$q minigene DNAs were precomplexed with Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) in OPTI-MEM in the absence of antibiotics and serum. Cells were incubated with the constructs for 3 h at 37 C in a 5% CO$_2$ atmosphere, after which 1 ml OPTI-MEM medium containing 5% fetal bovine serum and 1% penicillin/streptomycin was added to each of the culture dishes. Medium was changed after 6 h, and incubation continued until 48 h. Cells were labeled with [$^{14}$C] palmitic acid for the final 24 h of the incubation. For experiments with constitutively active (GTPase-deficient) G$\alpha$ constructs, 0.4 $\mu$g each of pcDNA$_3$ (control vector), *G$\alpha_{12}$ (G$\alpha_{12}$Q226L), or *G$\alpha_{13}$ (G$\alpha_{13}$Q223L) were precomplexed with Lipofectamine Plus reagent in OPTI-MEM medium in the absence of antibiotics and serum. Cells were incubated with the constructs for 3 h at 37 C in a 5% CO$_2$ atmosphere, after which 1 ml OPTI-MEM medium containing 5% fetal bovine serum and 1% penicillin/streptomycin was added to each of the culture dishes, which were then incubated overnight. Cells were labeled with [$^{14}$C] palmitic acid and PLD activity measured as described. For experiments determining the effects of the dominant-negative mutant of RhoA (RhoA19N) on the actions of the constitutively active G$\alpha_{12}$ and G$\alpha_{13}$, 0.5 $\mu$g each of constitutively active mutants of G$\alpha_{12}$ and G$\alpha_{13}$ and dominant-negative mutant of RhoA (RhoA19N) were used, along with 0, 0.5, or 1 $\mu$g pcDNA3 (vector control), for a total of 1 $\mu$g DNA transfected.

*Statistics*

For each experiment, triplicate treatments were used, and the mean $\pm$ SE was calculated. Statistical significance was determined by one-way ANOVA and Tukey post test (GraphPad Prism Software; GraphPad Software, Inc., San Diego, CA). Treatment effects were confirmed in replicate experiments.

## Results

PLD activity was markedly increased when UMR-106 cells transfected with pcDNA3 were incubated with PTH 1–34 (10 nM) for 30 min (Fig. 1). This effect was prevented when the cells were transfected with minigene vectors that code for small peptide inhibitors of the G$\alpha_{12}$ or G$\alpha_{13}$ subunits of heterotrimeric G proteins, but was not prevented when the cells were transfected with minigene vectors that code for small peptide inhibitors of G$\alpha$s, G$\alpha$q, or with the pcDNA3 vector alone (Fig. 1). The lack of effect observed with transfection of the G$\alpha$s mutant vector is consistent with our findings using pharmacological agents. Specifically, a 30-min treatment with the protein kinase A antagonist protein kinase inhibitor (PKI) (Fig. 2A) failed to inhibit the PTH effect
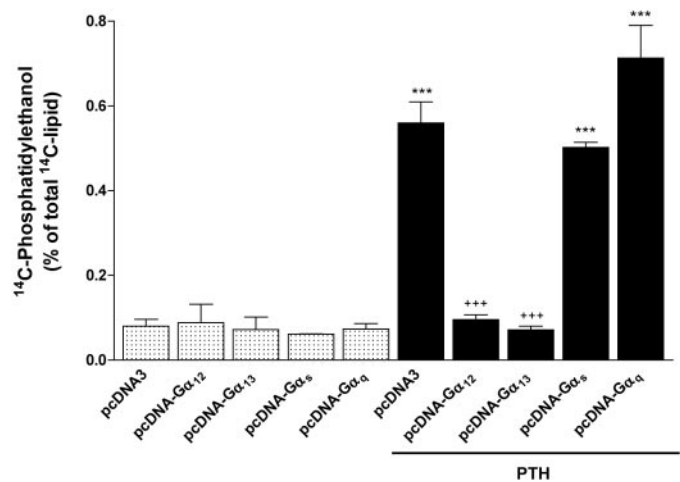


FIG. 1. PTH-stimulated PLD activity in UMR-106 cells is inhibited by antagonistic minigene vectors pcDNA-G$\alpha_{12}$ and pcDNA-G$\alpha_{13}$ (pcDNA-G$\alpha_{12}$, pcDNA-G$\alpha_{13}$) but not by antagonist minigene vectors pcDNA-G$\alpha$s (pcDNA-G$\alpha$s) or pcDNAG$\alpha$q (pcDNAG$\alpha$q). Cells were transfected with 0.5 $\mu$g of the minigene vector for 24 h and then treated with PTH (10 nM) for 30 min. PLD activity was assayed as described in *Materials and Methods*. n = 3; ***, $P < 0.001$ *vs.* control (pcDNA alone); +++, $P < 0.001$ *vs.* PTH in pcDNA-transfected cells.

on PLD, and the adenylyl cyclase activator forskolin failed to activate PLD in the osteoblastic cells (Fig. 2A). The PKI was able to antagonize effect of PTH on the activation of the MAPK ERK (Fig. 2B).

The effect of PTH on PLD was antagonized by a 30-min treatment with *C. botulinum* exoenzyme C3 (Fig. 3), an inhibitor of Rho family small G proteins, consistent with our previous studies showing that the effects of PTH on PLD are inhibited by the more general Ras family small G protein inhibitor *Clostridium difficile* toxin B, by the geranylgeranyl-transferase I inhibitor GGTI-2166 and by dominant-negative RhoA (4).

Consistent with the finding that the antagonistic pcDNA-G$\alpha_{12}$ and pcDNA-G$\alpha_{13}$ minigene vectors inhibited PTH-induced PLD activation, transfection of the UMR-106 cells with constitutively active G$\alpha_{12}$ and G$\alpha_{13}$, but not the control vector (pcDNA3), activated PLD independent of PTH stimulation (Fig. 4A). Dominant-negative RhoA, which inhibited the effect of PTH on PLD (Fig. 4B) prevented the observed stimulatory effects of constitutively active G$\alpha_{12}$ and G$\alpha_{13}$ constructs on PLD activity (Fig. 4C), suggesting that RhoA mediates the effects of G$\alpha_{12}$ and G$\alpha_{13}$ on PLD in the osteoblastic cells.

## Discussion

The present findings establish G$\alpha_{12}$ and G$\alpha_{13}$ subunits of heterotrimeric G proteins as upstream regulators of PLD in osteoblastic UMR-106 cells and as mediators of PTH stimulation of the phospholipase. Furthermore, the results show that the Rho small G proteins and specifically RhoA are intermediates in the G$\alpha_{12}$/G$\alpha_{13}$ regulation of the PTH-stimulated PLD response.

Studies in other tissues have shown that G$\alpha_{12}$ and G$\alpha_{13}$ activate Rho family small GTPases. In COS-7 cells, constitutively active G$\alpha_{12}$ and G$\alpha_{13}$ increase Rho-GTP. Constitu-
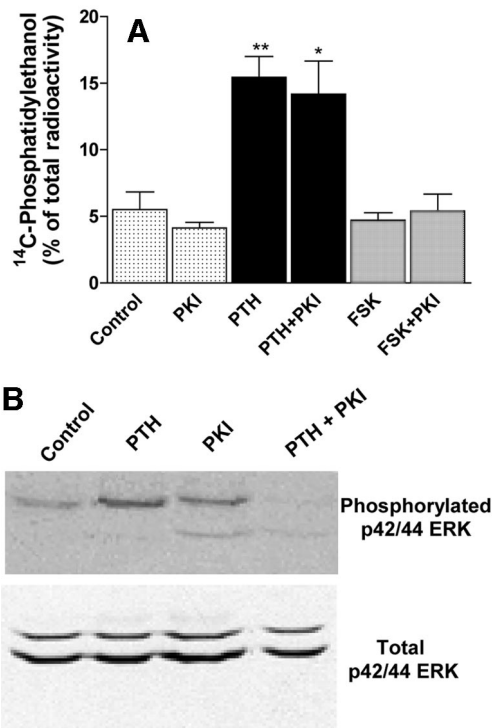
FIG. 2. A, PTH-stimulated PLD activity is not inhibited by the protein kinase A inhibitor PKI, and the adenylyl cyclase activator forskolin (FSK) does not stimulate PLD activity in UMR-106 cells. Cells were treated with PTH (10 nM) or forskolin (10 $\mu$M) for 30 min. PKI (10 $\mu$M) was added 60 min before PTH or forskolin and was present throughout the incubation. n = 3; *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control. B, Activity of PKI was demonstrated by its effectiveness to inhibit PTH activation of p42/44 ERK. Cells were incubated for 5 min with 10 nM PTH. PKI (10 $\mu$M) was added 60 min before treatment with PTH.

tively active G$\alpha_{12}$ and G$\alpha_{13}$ stimulate formation of actin stress fibers and focal adhesions in Swiss 3T3 cells (7, 23). The effect is prevented by blocking Rho, indicating that Rho is a downstream effector of the actions of these heterotrimeric G protein subunits (7). Neither activated forms of other G$\alpha$ subunits, nor $\beta\gamma$ complexes have the cytoskeletal effects of the G$\alpha_{12}$ and G$\alpha_{13}$ subunits (7). In CCL39 fibroblasts, G$\alpha_{13}$ stimulates Na$^+$-H$^+$ exchange through both RhoA-dependent
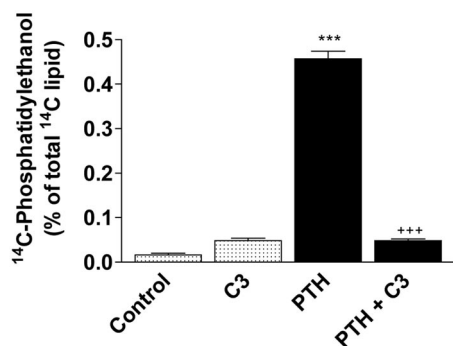


FIG. 3. The stimulatory effect of PTH 1–34 (10 nM) on PLD activity in UMR-106 cells is inhibited by *C. botulinum* C3 exotoxin, which inhibits the activity of Rho small G proteins by ADP ribosylation. PLD activity was measured after a 30-min incubation. n = 3; ***, $P < 0.001$ *vs.* control; +++, $P < 0.001$ *vs.* PTH alone.
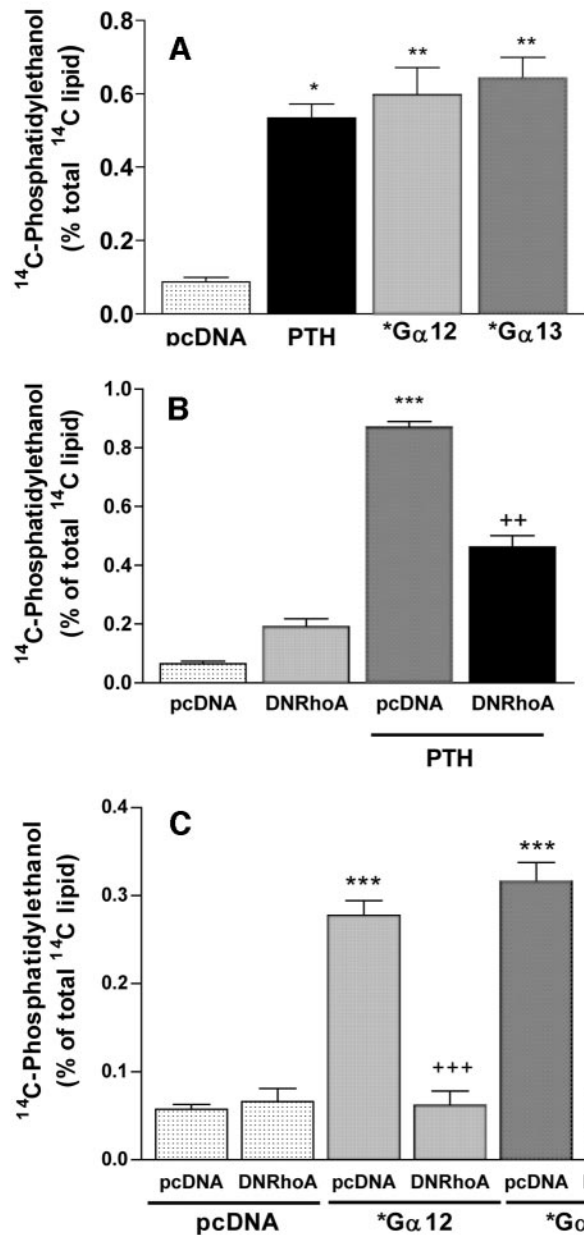


FIG. 4. A, Constitutively active G$\alpha_{12}$ and G$\alpha_{13}$ (*G$\alpha_{12}$, *G$\alpha_{13}$) (0.5 $\mu$g) stimulate PLD activity similarly to PTH (10 nM) in UMR-106 cells. PLD activity was measured after a 30-min incubation. n = 3; *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control. B, The stimulatory effect of PTH (10 nM) on PLD activity is prevented by transfection with dominant-negative RhoA. Cells were transfected with 0.5 $\mu$g/ml of the dominant-negative RhoA as described in *Materials and Methods*, and then treated with PTH for 30 min. n = 3; ***, $P < 0.001$ *vs.* pcDNA control; ++, $P < 0.01$ *vs.* PTH. C, Stimulatory effects of constitutively active G$\alpha_{12}$ and G$\alpha_{13}$ on PLD activity in UMR-106 cells are prevented by transfection with dominant-negative RhoA. Cells were cotransfected with 0.5 $\mu$g/ml of the constructs as described in *Materials and Methods*. n = 3; ***, $P < 0.001$ *vs.* pcDNA control; +++, $P < 0.001$ *vs.* *G$\alpha_{12}$; \$\$\$, $P < 0.001$ *vs.* *G$\alpha_{13}$.

and independent pathways (24). The interaction of the G$\alpha$ subunits and RhoA appears to be through Rho-specific guanine nucleotide exchange factors, including p115 RhoGEF (25, 26), PDZ-RhoGEF (27) and Lbc RhoGEF (28).

Activation of several endogenous receptors leads to stim-

ulation of $G\alpha_{12}/G\alpha_{13}$-Rho signaling. In neuronal cells this leads to formation of a F-actin cortical structure that effects changes in cell shape (29, 30). $\alpha_1$ Adrenergic receptor-induced cardiac hypertrophy is mediated in part by a $G\alpha_{12}/G\alpha_{13}$-Rho-JNK pathway (31). The $G\alpha$ subunits can have independent effects. Serotonin 5-HT4(a) receptor activation of a serum response element, mediated through Rho activation, is potentiated by overexpression of $G\alpha_{13}$, but not $G\alpha_{12}$ (32). Lysophosphatidic acid activation of Rho in Swiss 3T3 cells involves $G\alpha_{13}$, but not $G\alpha_{12}$ (23).

$G\alpha_{12}$ and $G\alpha_{13}$ are involved in PLD activation in other tissues. $G\alpha_{12}/G\alpha_{13}$ transduces signaling from the calcium sensing receptor in Madin-Darby canine kidney cells (12). $G\alpha_{12}$ is involved in angiotensin II receptor coupling to PLD in vascular smooth muscle cells (11), whereas $G\alpha_{13}$ mediates PLD activation by 5-hydroxytryptamine 2C receptors in rat choroid plexus epithelial cells (9). Expression of a constitutively active $G\alpha_{13}$ subunit in COS-7 cells stimulated rat brain PLD (8).

Bone remodeling requires the action of both osteoblasts and osteoclasts. RhoA is already known to play an important role in osteoclast activity (33, 34). *C. botulinum* C3 exoenzyme disrupts the actin ring and inhibits bone resorption by isolated osteoclasts (33). Expression of a constitutively active RhoA in avian osteoclasts results in increased podosome assembly, formation of stress fibers, increased motility, and bone resorbing activity (34). Phosphatidylinositol-3-kinase is also increased. These effects are inhibited by *C. botulinum* C3 exoenzyme and by expression of dominant-negative RhoA.

PTH action on osteoblasts leads to significant changes in bone, including therapeutically significant anabolic effects that may be mediated through changes in cell survival (35), as well as activation of osteoclastogenesis through production of IL-6 (36) and the membrane cytokine, receptor activator of nuclear factor $\kappa$B ligand (37). The finding that the $G\alpha_{12}/G\alpha_{13}$-RhoA-PLD pathway, which is important in cell survival in other tissues, is activated by PTH in osteoblasts suggests that this PTH-stimulated, cAMP-independent pathway is likely to be important in bone biology.

## Acknowledgments

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