Activation of Phospholipase C-β1 via Gαq/11 during Calcium Mobilization by Calcitonin Gene-related Peptide*

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Interaction of calcitonin gene-related peptide (CGRP) with its receptors leads to stimulation of adenyl cyclase and/or phospholipase C (PLC). While regulation of adenyl cyclase is thought to involve the G-protein Ga, it is not known whether activation of PLC results from coupling the receptor to Ga family proteins or whether βγ subunits released from receptor-activated Gs activate PLC. We used human bone cells OHS-4 bearing CGRP receptors in which CGRP activates only the PLC signaling pathway to determine how CGRP acts. CGRP increased the concentration of intracellular calcium ([Ca²⁺])₅₀ within 5 s via a Ca²⁺ influx through voltage-gated calcium channels and by mobilizing calcium from the endoplasmic reticulum. The activation of effectors, like PLC coupled to G-proteins, is the early event in the pathway leading to inositol 1,4,5-trisphosphate formation, which is responsible for Ca²⁺ mobilization. Western blotting demonstrated a range of PLC-β isoforms (β1, β3, β4, but not β2) and G-proteins (Gαq/11, and Gαi). Only phospholipase C-β1 is involved in the mobilization of Ca²⁺ from the endoplasmic reticulum of Fura-2-loaded confluent OHS-4 cells and the formation of inositol 1,4,5-trisphosphate by CGRP; PLC-γ have no effect. Activation of PLC-β1 by CGRP involves the Gαq/11 subunit, which is insensitive to pertussis toxin, but not βγ subunits. We therefore believe that CGRP causes the activation of two separate G-proteins.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide generated by alternative splicing of the calcitonin/CGRP gene product (1, 2), which effects numerous organs, including bone (3). The receptor for CGRP, which belongs to a subgroup of G-protein-coupled receptors (4–6), is a seven-transmembrane domain receptor protein, very like other receptors of the subfamily (7, 8). The binding of CGRP to these receptors activates multiple signaling pathways. The main signal pathway used by CGRP is the adenyl cyclase pathway (9–14), but CGRP has also been reported to activate guanylate cyclase (15, 16) and phospholipase C (17) and to increase the intracellular calcium concentration (18). However, in osteoblastic OHS-4 cells, which possess CGRP receptors (19), CGRP does not stimulate adenyl cyclase as do other cell types (20, 21), but it does increase intracellular calcium. This suggests that the CGRP receptor activates different G-proteins than do other calcitropic peptide hormones (22).

There are many similar heterotrimeric G-proteins that transduce a signal from a hormone-bound receptor to a variety of downstream effector molecules (23–25). Mammals have several types of G-proteins, which form the α subunit and the β and γ subunits. These include the Gαq, Gαi, Gαo, and Gαs subfamilies, which are classified on the basis of amino acid sequence of the α subunits. G-proteins are also divided traditionally into two types based on their sensitivity to Bordetella pertussis toxin (PTX) (26). The PTX-sensitive G-proteins are inactivated by ADP-ribosylation of the α subunit. This group includes members of the Gq and Gi subfamily. Gαi and Gαq proteins were originally purified as mediators of adenyl cyclase pathway (27). PTX-insensitive G-proteins are resistant to ADP-ribosylation and include members of the Gs subfamily (27). The α subunits of these proteins activate phospholipase C-β isoenzymes (28), but not phospholipases (PLCs) Cγ or Cδ (29–33). PLC-β isoenzymes may be stimulated by both G-protein α subunits of the Gq family and by free βγ subunits (34–36).

PLC can thus be stimulated via receptors with the dual signaling properties described above. Binding of the ligand to the receptor either stimulates adenyl cyclase via Gαo, and phospholipase C is activated by βγ subunits released from activated Gαo or the receptor couples to multiple G proteins leading to the activation of adenyl cyclase and PLC via Gαi and α subunits of the Gi family (37). The latter route appears to apply to the human TSH receptor (38), and the turkey erythrocyte β-adrenergic receptor (39). The present study identifies and characterizes the PLC isoform involved in the mobilization of Ca²⁺ from the endoplasmic reticulum and inositol 1,4,5 trisphosphate formation of bone OHS-4 cells in response to CGRP, and its regulation by G-protein α and βγ subunits.

EXPERIMENTAL PROCEDURES

Materials—The ECL kit, and l-3-phosphatidyl [2-3H]inositol 4,5-bisphosphate and Fura-2/AM were purchased from Amersham, Life Technology (Les Ulis, France). Polyclonal rabbit anti-PLC antibodies to PLC-β1, PLC-β2, PLC-β3, and PLC-β4, polyclonal rabbit anti-G-protein antibodies to Gαq/11, Gαo, Gαs, Gαi, and Gαs, and their blocking peptides were obtained from Santa Cruz Biotechnology, Inc. and Tebu (Le Perray-en-Yvelines, France). Peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad (Ivry-sur-Seine, France). Human CGRP and all chemicals were purchased from Sigma (St. Quentin Fallavier, France). Dulbecco’s modified Eagle’s essential medium and fetal calf serum were supplied by Eurobio (Les Ulis, France).

Human Cell Culture—The human bone cell line OHS-4, which exhibits most of the osteoblast phenotype (40), was kindly provided by Drs. B. Fournier and P. Price. Cells were grown on rectangular glass coverslips or in Petri dishes (100 cm²) for 4 days in Dulbecco’s modified Eagle’s essential medium and fetal calf serum.
Eagle’s essential medium supplemented with 10% heat-inactivated fetal calf serum. Cells were then incubated for 72 h in Dulbecco’s modified Eagle’s essential medium containing 1% heat-inactivated fetal calf serum and transferred to serum-free medium 24 h before use.

**Calcium Measurement and Experimental Protocol**—The cells were washed with Hank’s HEPES, pH 7.4 (137 mM NaCl, 5.6 mM KCl, 0.441 mM KH$_2$PO$_4$, 0.442 mM Na$_2$HPO$_4$, 0.885 mM MgSO$_4$, 7H$_2$O, 27.7 mM glucose, 1.25 mM CaCl$_2$, and 25 mM HEPES), and loaded with 1 µM Fura-2/AM before adding 1 nM CGRP. Neomycin and U-73122 both abolished the transient peak and had no effect on the plateau phase. These results are representative of eight different coverslips.

![Fig. 1. Effect of CGRP on the intracellular calcium concentration in confluent OHS-4 cells. A, direct effect of 1 nM CGRP on intracellular calcium. These results are representative of eight different coverslips. B, dose-dependent effects of CGRP on intracellular calcium. Intracellular Ca$^{2+}$ concentrations were determined at 10 s. Values are means ± S.E., n = 6, and are significantly different from the basal level. *p < 0.01 and **p < 0.001.](image1)

![Fig. 2. Mechanisms of CGRP actions on intracellular calcium in confluent OHS-4 cells. A, cells were incubated for 30 s with 2 mM EGTA or for 60 s with 1 µM nifedipine before adding 1 nM CGRP. EGTA and nifedipine both reduced the transient peak and abolished the plateau phase. B, cells were incubated for 3 min with 1 mM neomycin or 60 s with 3 µM U-73122 before adding 1 nM CGRP. Neomycin and U-73122 both abolished the transient peak and had no effect on the plateau phase. These results are representative of eight different coverslips for each experiment.](image2)

The intracellular calcium mobilization from intracellular stores. Two types of blocks-
bovine serum albumin as a standard.

The homogenate was centrifuged for 10 min at 6000 × g to remove nuclei. They were sonicated on ice twice for 20 s each at 40 KHz, and aliquots of the total homogenate and membrane fractions were stored at −80 °C. Protein was determined by the method of Bradford (45) with bovine serum albumin as a standard.

Protein Separation and Immunoblotting—Proteins were separated by SDS-PAGE (7.5% resolving gel for PLC and 13% for G-protein) in 25 mM Tris base, pH 8.3, 192 mM glycine, 0.1% SDS (46). They were electrophoretically transferred to nitrocellulose membranes (Immobilon P, Millipore, St. Quentin-en-Yvelines, France) in the same buffer with 20% ethanol for 2 h at 100 V (47). Nonspecific binding to nitrocellulose was prevented by incubating the membranes in 50 mM Tris-buffered saline (TBS) pH 7.5 containing 150 mM NaCl, 5% skimmed milk powder and 0.05% Tween-20 for 12 h at 4 °C. The membranes were washed in TBS containing 0.1% Tween-20 and incubated overnight at 4 °C with polyclonal rabbit antibodies against specific isoenzymes of PLC (PLC-β1, PLC-β2, PLC-β3, and PLC-δ1) and specific G-proteins (Gαq, Gαi, Gαs) (44). Cells were washed twice to remove saponin and incubated with the anti-PLC antibody, anti-G-protein antibody, or nonimmune rabbit serum (44). Cells were washed twice to remove saponin and incubated with the anti-PLC antibody, anti-G-protein antibody, or nonimmune rabbit serum for 1 h at 37 °C. 1 μM Fura-2/AM was added for the last 20 min of incubation. In some experiments, anti-PLC-β1 antibody and anti-Gαq/11 antibody were set up in competition with the antigens corresponding to the other anti-PLC antibodies for 2 h at room temperature (antibody:peptide 1:10 or 1:100, according to the specifications of the manufacturer), prior to use. GCRP was used at the concentration that gave the greatest increase in Ca2+ in confluent OHS-4 cells. The G-protein involved in the activity of GCRP was identified by incubating the membranes in 50 mM Tris-buffered saline (TBS) pH 7.5 containing 150 mM NaCl, 5% skimmed milk powder and 0.05% Tween-20 for 12 h at 4 °C. The membranes were washed in TBS containing 0.1% Tween-20 and incubated overnight at 4 °C with polyclonal rabbit antibodies against specific isoenzymes of PLC (PLC-β1, PLC-β2, PLC-β3, and PLC-δ1) and specific G-proteins (Gαq, Gαi, Gαs). The concentrations of PLC antibodies in TBS, 1.5% skimmed milk, 0.1% Tween 20 were as follows: 0.1 μg/ml for PLC-β1, 0.5 μg/ml for PLC-β2, 1 μg/ml for PLC-β3, and 0.5 μg/ml for PLC-δ1. The concentrations of Gαq, Gαi, and Gαs antibodies in the same buffer were 0.2 μg/ml and 0.5 μg/ml, respectively. The antibodies bound to the proteins on the nitrocellulose were detected using peroxidase-conjugated goat anti-rabbit IgG (1 mg/ml) (diluted 1/5000 in TBS, 1.5% skimmed milk, 0.1% Tween 20). The antigen was detected by enhanced chemiluminescence. Molecular size standards were used to estimate the apparent molecular mass of the PLC, myosin, 199 kDa; β-galactosidase, 120 kDa; bovine serum albumin, 87 kDa; and ovalbumin, 48 kDa. The molecular size standards for G-proteins were phosphorylase B, 105 kDa; bovine serum albumin, 82 kDa; ovalbumin, 49 kDa; carbonic anhydrase, 33.3 kDa; soybean trypsin inhibitor, 28.6 kDa; and lysozyme, 19.4 kDa.

Phosphatidylinositol 4,5-Bisphosphate Hydrolysis Assay—Phospholipid vesicles were prepared as described by Hofmann and Majerus (48), and assays were done essentially as described by Wu et al. (49). Diluted membranes (10 μg, 5–10 μg of protein) in 50 mM HEPES, pH 7.0, 0.5 mM EDTA, 2 mM EGTA, 0.6 mM pepstatin, 0.5 mM benzamidine, 0.1 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 0.125 mM aprotinin, and 1 mM diithiothreitol. They were sonicated on ice twice for 20 s each at 40 KHz, and the homogenate was centrifuged for 10 min at 8000 × g to remove nuclei. The supernatant was centrifuged at 100,000 × g for 1 h. The resulting membrane pellets were resuspended in homogenizing buffer, and aliquots of the total homogenate and membrane fractions were stored at −80 °C. Protein was determined by the method of Bradford (45) with bovine serum albumin as a standard.

Cell Homogenates and Membranes—Cells were washed three times with ice-cold phosphate-buffered saline, pH 7.4, and scraped off into ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM EGTA, 0.6 mM pepstatin, 0.5 mM benzamidine, 0.1 mM leupeptin, 2 mM phenylmethysulfonyl fluoride, 0.125 mM aprotinin, and 1 mM diithiothreitol). They were sonicated on ice twice for 20 s each at 40 KHz, and the homogenate was centrifuged for 10 min at 8000 × g to remove nuclei. The supernatant was centrifuged at 100,000 × g for 1 h. The resulting membrane pellets were resuspended in homogenizing buffer, and aliquots of the total homogenate and membrane fractions were stored at −80 °C. Protein was determined by the method of Bradford (45) with bovine serum albumin as a standard.
G-proteins and CGRP

Fig. 7. Responses of OHS-4 cells treated with antibodies against G-proteins to CGRP. Cells were cultured, loaded with Fura2/AM, and incubated with G-protein antibodies as described. The concentrations of anti-G-protein antibodies were 2 μg/ml for Goq/11, 5 μg/ml for Goq, 5 μg/ml for Goq, and 5 μg/ml for Gβ plus 5 μg/ml for Gγ. Only anti-Goq/11 antibody blocked Ca2+ mobilization induced by CGRP. These results are representative of at least six cover-slips for each experimental case.

with 2 μl of serially diluted peptide control before adding to the membranes and incubation for 2 h. The reaction was started by adding GTPγS with or without CGRP, followed by incubation at 37 °C for 15 min. The reaction was stopped by adding 0.5 ml of chloroform/methanol/ HCl (40:20:0.5), mixing, and chilling on ice. Soluble inositol phosphates (indicating PIP2 hydrolysis) were extracted by adding 150 μl of chloroform and 200 μl of 0.1 M HCl. Phases were separated by centrifugation, and 200 μl of the upper aqueous phase were taken for liquid scintillation counting.

Statistical Analysis—The data were analyzed by one-way analysis of variance. Treatment pairs were compared by Dunnett’s method. A value of n indicates the number of glass coverslips used for a specific experiment or the number of cultures.

RESULTS

Direct Effects of CGRP on Intracellular Calcium Concentration—The basal intracellular calcium concentration in confluent OHS-4 cells was 115 ± 5 nM (mean ± S.E., n = 18). The transient increase in [Ca2+]i, induced by 1 nM CGRP formed a sharp peak which fell rapidly after 15 s, but remained above the basal level (plateau phase) (25 ± 2%, mean ± S.E., n = 6, p < 0.001) (Fig. 1A). The concentration-dependent effects of CGRP were bell-shaped, with a maximum activity at 1 nM (Fig. 1B).

Mechanisms of CGRP-induced Changes in Intracellular Calcium Concentration—1 nM CGRP was added 30 s after 2 mM EGTA, or 60 s after 1 μM nifedipine or 1 μM verapamil. Verapamil itself caused a small (5%) decrease in [Ca2+]i, whereas nifedipine caused a small (5%) transient increase (43). EGTA, nifedipine, and verapamil reduced the magnitude (50 ± 10%, mean ± S.E., n = 8, p < 0.001) of the transient peak induced by CGRP, and the plateau phase was completely abolished (Fig. 2A; data shown only for EGTA and nifedipine).

Cells were incubated for 3 min with 1 mM neomycin, with 3 μM U-73122 or with 2 μM U-73343, a closed analog of U-73122 but inactive agent (43), before adding 1 nM CGRP. Neomycin itself caused a small (5%) decrease in [Ca2+]i, whereas U-73122 caused a small (5%) increase (43). Neomycin and U-73122 both abolished the transient peak, but not the plateau phase (Fig. 2B). U-73343 (0.3–5 μM) had no effect on the intracellular calcium response to CGRP (data not shown).

The cells incubated for 16 h with 100 ng/ml PTX showed no change in the basal level of [Ca2+]i, or in the [Ca2+]i response to 1 nM CGRP (Fig. 3).

Western Immunoblotting of the PLC and G-proteins—All the anti-PLC-β antibodies used in this study were raised against amino acid sequences in the carboxyl terminus of each PLC-β. Western immunoblotting showed a 150-kDa immunoreactive band using the anti-PLC-β1 antibody, a 158-kDa immunoreactive band using the anti-PLC-β2 antibody, and a 153-kDa immunoreactive band using the anti-PLC-β4 antibody (Fig. 4). No immunoreactive reactive band for PLC-β2 was found (Fig. 4). Immunoblots probed with the anti-Goq/11 antibody or the anti-Gαq antibody showed a 42-kDa immunoreactive band (Fig. 5). A competitive Western blot with polyclonal PLC-β1, PLC-β3, and PLC-β4 antibodies and the antigens against which they were raised showed that immunoreactivity was completely abolished when a 100-fold excess of antigen was included (data not shown). This also shows that each antibody reacted with the intended target isoform.

PLC Isoenzymes and G-proteins Involved in the Effects of CGRP on Intracellular Calcium—Treatment of the cells with saponin for 5 min followed by incubation for 60 min with the anti-PLC antibody or anti-G-protein antibody in the absence of...
saponin did not affect the basal $[Ca^{2+}]$. Nonimmune serum had no effect on basal $[Ca^{2+}]$, or on the $[Ca^{2+}]$, response to CGRP.

The CGRP-induced increase in $[Ca^{2+}]$, was reduced by anti-PLC-$\beta$1 antibody, whereas antibodies to PLC-$\beta$3 and PLC-$\beta$4 had no effect (Fig. 6). The residual increase was due to $Ca^{2+}$ influx because it was totally blocked by incubating the cells with 2 mM EGTA. Anti-PLC-$\gamma$1 and anti-PLC-$\gamma$2 antibodies had no effect on the $[Ca^{2+}]$, response to CGRP (Fig. 6), as did anti-PLC-$\beta$2 antibody (data not shown).

Polyclonal anti-PLC-$\beta$1 antibody was incubated for 2 h with its corresponding antigen or with the antigens used to produce the other anti-PLC antibodies (antibody:antigen 1:10 or 1:100) before use. The inhibition of the CGRP-induced increase in $[Ca^{2+}]$, due to anti-PLC-$\beta$1 antibody totally disappeared only when the anti-PLC-$\beta$1 antibody was incubated with its own antigen, but not with the antigens used to raise the other anti-PLC antibodies (Fig. 6; data shown only for the antigen corresponding to PLC-$\beta$1).

The CGRP-induced transient peak in $[Ca^{2+}]$, was blocked by anti-Go,q/11 antibody (Fig. 7). The residual increase was due to a $Ca^{2+}$ influx because this was totally blocked by incubating the cells with 2 mM EGTA (Fig. 7). The anti-G$\alpha$ q antibody (1 $\mu$g/ml), which cross-reacts with Go,q11, Go,q2, and Go,q3, and anti-G$\alpha$ i antibodies did not alter the $[Ca^{2+}]$, response to CGRP (Fig. 7). Adding the anti-G$\beta$ antibody, which reacts with G$\beta$1, G$\beta$2, G$\beta$3, and G$\beta$4, and the anti-G$\gamma$ antibody to the cell had no effect on the $[Ca^{2+}]$, response to CGRP (Fig. 7).

**PIP$_2$ Hydrolysis**—Since testing the effect of GTP$\gamma$S on intracellular calcium needed permeabilized cells, and this made it impossible to keep the antibodies inside the cell, we used a cell-free membrane system to test the activity of the antibodies. OHS-4 membranes containing endogenous PLC and G subunits were mixed with phospholipid vesicles containing radioactive substrate ($^3$H)PIP$_2$. Fig. 8A shows the effect of CGRP on the hydrolysis of PIP$_2$ in the absence of GTP$\gamma$S. The effect of GTP$\gamma$S in the absence and presence of 1 nM CGRP showed that GTP$\gamma$S was ineffective below 1 $\mu$M (Fig. 8B). GTP$\beta$S (100 $\mu$M) inhibited the increase induced by 10 $\mu$M GTP$\gamma$S, 10 $\mu$M GTP$\gamma$S plus 1 nM CGRP, or 1 nM CGRP alone (data not shown). OHS-4 membranes were incubated with antibodies prior to stimulation with 100 $\mu$M GTP$\gamma$S. All anti-PLC antibodies (G$\beta$, G$\beta$, and G$\beta$4), like anti-Go,q/11 and anti-G$\beta$ G$\beta$, antibodies, inhibited the PIP$_2$ hydrolysis induced by GTP$\gamma$S (Fig. 8C), whereas anti-G$\alpha$ q and anti-G$\alpha$, antibodies had no effect (data not shown). Membranes incubated with both the antibody and the blocking peptide, gave the same amount of $^3$H)inositol 1,4,5-trisphosphate as obtained with preimmune serum (Fig. 8C).

**DISCUSSION**

We have shown that Go,q/11-protein coupled to phospholipase C-$\beta$ is involved in the signaling of CGRP in human bone cells, and that G$\beta$ G$\beta$ subunits have no effect. The data also indicate that $Ca^{2+}$ mobilization from the endoplasmic reticulum following the increased formation of inositol 1,4,5-trisphosphate induced by CGRP is due to activation of a Go,q/11 that is insensitive to pertussis toxin.

CGRP increases intracellular calcium concentration within 5 s via two mechanisms. It causes an influx of $Ca^{2+}$ from the extracellular milieu via voltage-gated calcium channels as indicated by the experiments with EGTA, nifedipine, and verapamil. This mechanism is different from that of rat osteoblastic UMR106 cells, in which depleting extracellular $Ca^{2+}$ induces an efflux of intracellular $Ca^{2+}$ involving K$_{ATP}$ channels (18). Activating the membrane K$_{ATP}$ channels with high concentrations of CGRP (100 nM) could inhibit transmembrane $Ca^{2+}$ uptake by hyperpolarizing the membrane potential, so reducing the voltage-dependent $Ca^{2+}$ channel activity (53). CGRP causes the release of $Ca^{2+}$ from the endoplasmic reticulum in human OHS-4 cells as it does in rat UMR106 cells (18). This seems to be a general mechanism of CGRP action. The activation of phosphoinositide-specific phospholipase C-$\beta$ upon stimulation of specific cell receptors results in the formation of second messengers, diacylglycerol, a direct activator of protein kinase C, and inositol 1,4,5-trisphosphate (50, 51). The latter binds to receptors on the endoplasmic reticulum, causing a transient release of calcium from the endoplasmic reticulum. As activation of PLC-$\beta$ is an early event in the signal transduction pathway resulting in a variety of cellular responses, we first identified the PLC-$\beta$ isoforms in bone OHS-4 cells. We find several isoforms of PLC-$\beta$, $\beta$, $\beta$, and $\beta$ but no PLC-$\beta$2, which is present in the rat brain (52) and in rat osteoblasts (44). As confluent rat osteoblasts do not possess PLC-$\beta$4 (44), whereas this isoform is present in the brain (52), these isoenzymes may well have a tissue- and/or species-specific distribution.

The next step was to identify the PLC-$\beta$ isoform involved in the effect of CGRP. This is the first report that PLC-$\beta$1 is involved in mobilization of $Ca^{2+}$ from the endoplasmic reticulum by CGRP. Anti-PLC-$\beta$1 antibody inhibits the CGRP-induced increase in $[Ca^{2+}]$, in much the same way as direct (U-73122) or indirect (neomycin) inhibitors of PLC. Anti-PLC
antibody, like PLC inhibitors, blocks only the part of the increase in [Ca$^{2+}$], that is due to Ca$^{2+}$ mobilization from the endoplasmic reticulum. The inhibition of the enzyme activity by anti-PLC-β1 antibody is totally abolished in competition experiments, in which polyclonal PLC-β1 antibody is incubated with the antigen against which it was raised, but not when using the antigen corresponding to other PLC-β. This type of enzyme inhibition by selective antibodies against phosphoinositide-specific PLC has also been demonstrated in fresh bovine erythrocytes (54) and rat osteoblasts (44), suggesting that the antibody directed against an amino acid sequence at the carboxyl terminus of the PLC-β binds to a part of the enzyme which is critical for the geometry of the active site. Anti-PLC-β1 antibody also inhibits the formation of inositol 1,4,5-trisphosphate in a OHS-4 cell-free membrane system. Neither PLC-β3 and PLC-β4 are involved in CGRP signal transduction, although both PLC stimulate PIP$_2$ hydrolysis in the presence of GTP$\gamma$S. Similarly, PLC-γ1 and -γ2 take no part in the effects of CGRP as expected, because PLC-γ are substrates for growth factor receptor protein-tyrosine kinases (55).

It is very likely that only PLC-β is involved in the action of CGRP, as only PLC-β types are regulated via heterotrimeric G-proteins in response to agonist binding to receptors (55, 56). Receptor activation of PLC via G-proteins occurs by pertussis toxin-sensitive and toxin-insensitive signaling pathways (26).

The α subunits of the G$_{q/11}$ family are presumed to mediate the toxin-insensitive pathway, but the nature of the G-proteins mediating the toxin-sensitive pathway is less well understood (55–58). This study shows that the PLC-β1 involved in the action of CGRP is linked to a pertussis toxin-insensitive G-protein, and this PTX-insensitive G-protein is a member of the G$_{q}$ family. These results are consistent with the fact that the G-proteins G$_{q/11}$ are the most prominent G-protein activators in receptor-mediated regulation of the PLC-β1 (29). Moreover, forskolin does not mimic the effects of CGRP on [Ca$^{2+}$], but increases cAMP formation in OHS-4 cells (19). Anti-G$_{α}$ and anti-G$_{α}$ antibodies do not block the [Ca$^{2+}$], response to CGRP or the stimulation of PIP2 hydrolysis induced by GTP$\gamma$S. G$βγ$ subunits, like PLC-β3, which is the preferred target effector for G$βγ$ subunits (36), are not involved in Ca$^{2+}$ mobilization induced by CGRP. These data indicate that the effects of CGRP on intracellular calcium in OHS-4 cells are not mediated via the G-protein linked to the cAMP pathway.

Activation of PLC via G-protein-coupled receptors requires higher ligand concentrations than for receptor-mediated adenylyl cyclase activation (22). But, we find that PLC is activated in OHS-4 cells at concentrations similar to those that increase CAMP in other systems.

Although the main second messenger produced in response to CGRP remains CAMP in various cell types, probably via G$_{α}$-protein, CGRP acts via the phospholipase C pathway and G$_{αq/11}$ protein in human bone cells. The present data therefore strongly suggest that the dual signaling of CGRP is due to the activation of more than one G-protein, G$_{α}$ for the CAMP pathway and G$_{αq/11}$ for the PLC pathway (Fig. 9).

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REFERENCES
1. Amara, S. G., Jones, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M. (1982) Nature 299, 240–249
2. Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., and Sawchenko, P. E. (1985) Nature 310, 130–135
3. Deftos, L. J., and Ross, B. A. (1989) in Bone and Mineral Research, Annual 6 (Peck W. A., ed.), pp 267–316, Elsevier, New York
4. Aiyar, N., Band, K., Elshourbagy, N. A., Zeng, Z., Adamou, J. E., Bergsma, D. J., and Li, Y. (1996) J. Biol. Chem. 271, 11253–11259
5. Han, Z.-Q., Coppage, H. A., Smith, D. M., Van Noorden, S., Makgoba, M. W., Nicholl, C. G., and Legon J. (1997) J. Mol. Endocrinol. 18, 267–272
6. Ueda, S., and Clark, A. J. (1995) Biochem. Biophys. Res. Commun. 217, 832–838
7. Letkowitz, R. J., Cotechia, S., Samana, P., and Costa T. (1993) Trends Pharmacol. Sci. 14, 305–304
8. Oliveira, L., Paiva, A. C. M., Sanders, C., and Friend, G. (1994) Trends Pharmacol. Sci. 15, 170–172
9. Pouyner, D. E. (1992) Pharmacol. Ther. 56, 32–51
10. Farley, J. R., Hall, S. L., and Herring, S. (1993) Trends Pharmacol. Sci. 14, 303–304
11. Deftos, L. J., and Ross, B. A. (1989) in Metabolism 39, 379S–384S
12. Stangl, D, Muff, R., Schmolck, C., and Fischer, J. A. (1993) J. Bone Miner. Res. 8, 1011–1019
13. Michelangeli, V. P., Findlay, D. M., Fletcher, A., and Martin, T. J. (1997) J. Cell Physiol. 2125–2131
14. Bajorek, B. M., and Legon S. (1997) J. Bone Miner. Res. 12, Suppl. i, F391
15. Drissi, H., Lasmoles, V., Le Mellay, P. J., Marie, P. J., and Lieberherr, M. (1986) Metabolism 35, 1325–1332
16. Parsons, A. M., and Seybold V. S. (1997) Endocrinology 138, 264–267
17. Kawase, T., Howard, G. A., Roos, B. A., and Burns, D. M. (1995) J. Biol. Chem. 270, 1137–1142
18. Laufer, R., and Changeux, J.-P. (1989) in Molecular Pharmacology, Annual 6 (Peck W. A., ed.), pp 267–316, Elsevier, New York
19. Drissi, H., Lasmoles, V., Le Mellay, P. J., Marie, P. J., and Lieberherr, M. (1986) Metabolism 35, 1325–1332
20. Neer, E. J. (1995) Trends Pharmacol. Sci. 16, 235–242
21. Lefkowitz, R. J., Cotechia, S., Samana, P., and Costa T. (1993) Trends Pharmacol. Sci. 14, 305–304
22. Neer, E. J. (1995) Trends Pharmacol. Sci. 16, 235–242
23. Vignery, A., and MacCarthy, T. L. (1996) Bone 18, 331–335
24. Gray, D., and Marshall, I. (1996) J. Biol. Chem. 271, 691–696
25. Parsons, A. M., and Seybold V. S. (1997) J. Bone Miner. Res. 8, 1011–1019
26. Goltzman, D., and Mitchell, J. (1985) Science 227, 1343–1345
27. Stangl, D, Muff, R., Schmolck, C., and Fischer, J. A. (1993) Endocrinology 132, 744–750
28. Oppermanns, S., ida-Klein, A., Segre, G. V., and Simon M. I. (1996) Mol. Endocrinol. 10, 566–574
29. Freissmuth, M., Casey, P. J., and Gilman, A. G. (1989) FASEBJ. 3, 2125–2131
30. Neer, E. J. (1995) Cell 80, 249–257
31. H. Drissi, F. Lasmoles, V. Le Mellay, P. J. Marie, and M. Lieberherr, unpublished data.
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G-proteins and CGRP

25. Exton, J. H. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 481–509
26. Ui, M. (1990) in ADP-Ribosylating Toxins and G-Proteins (Moss, J., and Vaughan, M., eds) pp. 45–77, American Society for Microbiology, Washington, D. C.
27. Martin, T. F. J. (1991) Pharmacol. Ther. 49, 329–345
28. Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991) Science 251, 804–807
29. Noh, D.-Y., Shin, S. H., and Ree, S. G. (1995) Biochim. Biophys. Acta 1242, 99–114
30. Lee, C. W., Lee, K. H., Lee, S. B., Park, D., and Rhee, S. G. (1994) J. Biol. Chem. 269, 25335–25338
31. Kozasa, T., Hepler, J. R., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., and Gilman, A. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9176–9180
32. Rhee, S. G., and Choi, K. D. (1992) J. Biol. Chem. 267, 12393–12396
33. Conklin, B. R., and Bourne, H. R. (1993) Cell 73, 631–641
34. Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton J. H. (1991) Nature 350, 516–517
35. Camps, M., Carozzi, A., Schabel, P., Scherr, A., Parker, P. J., and Gierschick P. (1992) Nature 360, 684–686
36. Carozzi, A., Camps, M., Gierschick, P., and Parker, P. J. (1993) FEBS Lett. 315, 340–342
37. Birnbaumer, L., and Birbaumer M. (1995) J. Recept. Signal Transduct. Res. 15, 213–253
38. Allgeier, A., Offermanns, Van Sande, J. Spicher, K., Schultz G., and Dumont, J. E. (1994) J. Biol. Chem. 269, 13733–13735
39. James, S. R., Varizi, C., Walker, T. E., Milligan, G., and Downes, C. P. (1994) Biochem. J. 304, 359–364
40. Fournier, B., and Price, P. A. (1991) J. Cell Biol. 114, 577–583
41. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
42. Prenzel, M., Deeney, J. T., Matschinsky, F. M., and Joseph, S. K. (1986) FEBS Lett. 197, 285–288
43. Blesdale, J. E., Bundy, G. L., Bunting, S., and Fitzpatrick, F. A. (1989) Adv. Prostaglandin Thromboxane Leukotriene Res. 18, 590–593
44. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997) J. Biol. Chem. 272, 11902–11907
45. Bradford, M. (1976) Anal. Biochem. 72, 248–254
46. Laemmli, U. K. (1970) Nature 227, 680–685
47. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
48. Hofmann, S. L., and Majerus, P. W. (1992) J. Biol. Chem. 257, 14359–15364
49. Wu, D., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) J. Biol. Chem. 267, 1811–1817
50. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
51. Joseph, S. K., and Williamson, J. R. (1989) Arch. Biochem. Biophys. 273, 1–15
52. Tanaka, O., and Kondo, H. (1994) Neurosci. Lett. 82, 17–20
53. Nelson, M. T., Huang, Y., Brayden, J. E., Hescheler, J., and Standen, N. B. (1990) Nature 344, 770–773
54. Kuppe, A., Hedberg, K. K., Volwerk, J. J., and Griffith O. H. (1990) Biochim. Biophys. Acta 1047, 41–48
55. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) Annu. Rev. Biochem. 66, 473–509
56. Strathmann, M. P., and Simon, M. I. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9113–9117
57. Simon, M. I., Strathmann, M. P., and Gautum, N. (1991) Science 252, 802–808
58. Gudermann, T., Kalkbrenner, F., and Schultz, G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 429–459