Activated Gαq Inhibits p110α Phosphatidylinositol 3-Kinase and Akt*

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Lisa M. Ballou‡, Hong-Ying Lin‡§, Gaofeng Fan‡, Ya-Ping Jiang‡, and Richard Z. Lin‡¶

From ‡Research Service, Department of Veterans Affairs Medical Center, Northport, New York 11768 and the ¶Department of Medicine, Stony Brook University, Stony Brook, New York 11794

Some Gαq-coupled receptors have been shown to antagonize growth factor activation of phosphatidylinositol 3-kinase (PI3K) and its downstream effector, Akt. We used a constitutively active Gαq(Q209L) mutant to explore the effects of Gαq activation on signaling through the PI3K/Akt pathway. Transient expression of Gαq(Q209L) in Rat-1 fibroblasts inhibited Akt activation induced by platelet-derived growth factor or insulin treatment. Expression of Gαq(Q209L) also attenuated Akt activation promoted by coexpression of constitutively active PI3K in human embryonic kidney 293 cells. Gαq(Q209L) had no effect on the activity of an Akt mutant in which the two regulatory phosphorylation sites were changed to acidic amino acids. Inducible expression of Gαq(Q209L) in a stably transfected 293 cell line failed to increase in PI3K activity in p110α (but not p110β) immunoprecipitates. Receptor activation of Gαq also selectively inhibited PI3K activity in p110α immunoprecipitates. Active Gαq still inhibited PI3K/Akt in cells pretreated with the phospholipase C inhibitor U73122. Finally, Gαq(Q209L) co-immunoprecipitated with the p110α-p85α PI3K heterodimer from lysates of COS-7 cells expressing these proteins, and incubation of immunoprecipitated Gαq(Q209L) with purified recombinant p110α-p85α in vitro led to a decrease in PI3K activity. These results suggest that agonist binding to Gαq-coupled receptors blocks Akt activation via the release of active Gαq subunits that inhibit PI3K. The inhibitory mechanism seems to be independent of phospholipase C activation and might involve an inhibitory interaction between Gαq and p110α PI3K.

Phosphatidylinositol 3-kinase (PI3K) mediates many of the cellular actions of receptor tyrosine kinases, including effects on glucose metabolism, cell survival, and cytoskeletal rearrangements (1). Of the three classes of PI3K, only the class I enzymes preferentially phosphorylate phosphatidylinositol (PI) 4,5-bisphosphate in vivo. Class I PI3Ks are divided into two groups: IA enzymes are heterodimers between a p110 catalytic subunit and a p85 or p55 (or their splice variants) regulatory subunit. Mammals have three class IA, p110 catalytic subunits (α, β, and δ) that can associate with at least seven regulatory subunits that are generated by alternative splicing of three different genes (p85α, p85β, and p55γ). p110α and p110β are ubiquitously expressed, whereas p110δ is present almost exclusively in leukocytes. Class IA PI3Ks can be activated by receptor tyrosine kinases, and the p110β isofrom is also activated by some G protein-coupled receptors (2). The p85 regulatory subunit contains two SH2 (Src homology 2) domains that bind to specific phosphotyrosine motifs on receptor tyrosine kinases or their substrates. This binding leads to translocation of p110 to the membrane and enhances its catalytic activity. The class IA PI3K consists of a p110γ catalytic subunit and a p101 regulatory protein and is activated by some receptors coupled to heterotrimeric G proteins.

An important downstream effector of PI3K is the serine/threonine protein kinase Akt. Akt is activated by phosphorylation of Thr<sup>308</sup> in the activation loop of the kinase domain and Ser<sup>473</sup> in the C-terminal tail (reviewed in Ref. 3). It is believed that phosphorylation of both sites requires an interaction between the N-terminal pleckstrin homology domain of Akt and membrane phosphoinositides generated by PI3K. Although a few cell treatments have been reported to activate Akt in a PI3K-independent manner (4, 5), receptor tyrosine kinases that activate Akt also in general activate PI3K.

Cell-surface receptors that transmit signals through heterotrimeric G proteins regulate the PI3K/Akt pathway in a variety of ways. Receptors that couple to proteins in the G<sub>i/o</sub> family can increase PI3K activity. This effect is mediated mainly by Gβγ heterodimers, which activate both the p110β and p110γ PI3Ks (6–8). Recent reports indicate that G<sub>αi</sub>-coupled receptors also activate Akt (9–11). Receptors that couple to G<sub>αq</sub> have also been shown to activate Akt. In some cases, this response is unusual in that it appears to be independent of PI3K (4, 12), whereas in other cases, it is thought to be due to activation of PI3K by Gβγ subunits (11).

The data regarding regulation of PI3K and/or Akt by G<sub>αq</sub>-coupled receptors are more controversial. One body of evidence suggests that some receptors that can couple to G<sub>αq</sub> might activate PI3K/Akt signaling in certain cellular settings (13–19). Indeed, we reported that norepinephrine stimulation of α<sub>1</sub>-adrenergic receptors in human aortic smooth muscle cells increases PI3K activity (20). However, this effect is completely blocked by pretreatment with pertussis toxin, indicating that it is not mediated by Gαq (20). A second body of evidence suggests that G<sub>αq</sub>-coupled receptors do not activate PI3K or Akt and in fact antagonize activation of these enzymes by growth factors that act through tyrosine kinase receptors (11, 21–25). In
agreement with these results, we found that stimulation of the α1A-adrenergic receptor in Rat-1 cells with the agonist phenylephrine (PE) does not increase PI(3,4,5)P₃ levels or P13K activity and does not activate Akt (26). Furthermore, the α1A-adrenergic receptor inhibits activation of P13K/Akt in response to platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor I (26, 27). To further explore the mechanism by which the α1A-adrenergic receptor and other Gₛ coupled receptors inhibit P13K signaling, we used Gₛα₂(Q209L) to test whether activated Gₛα₂ inhibits Akt activation by inhibiting P13K.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant PDGF-A/B, PE, doxycycline, insulin, PI, U73122, GTP-γ-S, and monoclonal antibody to FLAG were purchased from Sigma. [γ₃₂P]ATP (3000 Ci/mmol) and myo-[3H]inositol (10–25 Ci/mmol) were from PerkinElmer Life Sciences. Rabbit polyclonal antibodies against Gₛα2 and Akt (H-183) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p110α, anti-p110γ, and anti-p85α antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-Ser⁷²⁷ Akt antibody was from Cell Signaling Technology (Beverly, MA). Anti-HA and anti-Myc antibodies were from Covance (Richmond, CA).

Construction of an epitope-tagged Akt construct (Akt-HA) was obtained from Dr. Richard Roth (Stanford University, Stanford, CA). PCR with primers 5’-CGCTCTGAGGACAGTGGGCAAGC (forward) and 5’-GGACGATCTGAGGAGGTGCAGAGGGCTACCA (reverse) was used to amplify Akt from Akt-HA and the cdna fragment was subcloned into pBluescript II KS. Akt was excised using XhoI and XbaI and subcloned into pcDNA3.1/Myc-His (Invitrogen) to obtain Akt-Myc. The Akt (T308/S473D) double mutant (referred to as AktDD-Myc) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene) and forward primers 5’-GGTGCCACCATG-AAGGACATTGTCGGGACACAT (TCT07) and 5’-CATTCCCCTGGCATGACTGCGCAACG (S473D). Mouse wild-type p110α and constitutively active myristoylated (myr) p110α in pUSEamp were purchased from Upstate Biotechnology, Inc. FLAG-p110α was obtained by subcloning p110α from pUSEamp into the XbaI and BamHI sites of pcXFLAG-CMV-10 (Sigma). Mouse p85α was obtained by PCR from mouse liver cdna (Clontech) using primers 5’-CGGGATCTTGGGCAAGCAGGCTGAGCTACA (forward) and 5’-GCCGATCTCTACTGACGCACATCAGTGCTGGTCGGA (reverse). The p85α cdna fragment was subcloned into the BamHI and EcoRI sites of bluescript II KS and then subcloned into pcDNA3.1 using XbaI and EcoRV. PCR with primers 5’-CGGGATCTTGGGCAAGCAGGCTGAGCTACA (forward) and 5’-GCCGATCTCTACTGACGCACATCAGTGCTGGTCGGA (reverse) was used to obtain p110α from Swiss mouse 3T3 cell cDNA. The cdna fragment was subcloned into pcDNA3.1 using KpnI and EcoRV. HA-Gₛα₂ (Q209L) was constructed from HA-Gₛα2 using the QuikChange site-directed mutagenesis kit and the forward primer 5’-GTCAATGAGG-GGGCTTTAAGGCTGAGGAGAAG. PCR with primers 5’-CGGCGATGCTCCAGGACCTACCGGACTGCAG and 5’-CGGATCTTGGGCAAGCAGGCTGAGCTACA (reverse) was used to obtain Gₛα₂(Q209L) from HA-Gₛα2 (Q209L). Gₛα₂(Q209L) was subcloned into pcDNA5/FRT/TO (Invitrogen) using NosI and ApaI.

Cell Culture—Rat-1 fibroblasts stably expressing the human α1A-adrenergic receptor (28) or the human insulin receptor (29), human embryonic kidney (HEK) 293 cells, and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) with 10% fetal bovine serum (Sigma) in 5% CO₂ at 37 °C. Before agonist treatments, the cells were incubated overnight in serum-free medium. Twenty-four hours after transfection, cells were incubated in 1 ml of growth medium containing 3 μCi/ml myo-[3H]inositol for 1 h with or without 5 μM U73122 for 30 min. Cells were then treated with agonists for another 30 min. COS-7 cells were seeded at 2.5 × 10⁴ cells/well in 24-well plates at 2.5 × 10⁵ cells/well. The next day, cells were labeled for 16–24 h in 1 ml of serum-free medium containing 3 μCi/ml myo-[³H]inositol. After labeling, the cells were washed three times with lysis buffer and used for immunoblotting or washed three times with lysis buffer and once with the appropriate kinase assay buffer prior to performing kinase assays.

Cell Lysate Preparation—After treatment, cells were rinsed with ice-cold phosphate-buffered saline and scraped into lysis buffer (50 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM pyrophosphate, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μM/ml each aprotinin and leupeptin) with either 1% Triton X-100 or 0.5% Nonidet P-40 plus 0.25% sodium deoxycholate. Homogenates were centrifuged at 15,000 × g for 15 min at 4 °C, and protein concentrations were determined using the Bradford assay (Bio-Rad).

Immunoprecipitation—Cells lysates containing equal amounts of protein were incubated with the appropriate antibody for 2 h and then with 25 μl of protein A- or protein G-agarose (Sigma) for 1 h. The beads were either washed three times with lysis buffer and used for immunoblotting or washed three times with lysis buffer and once with the appropriate kinase assay buffer prior to performing kinase assays.

Western Blotting—Immunoprecipitates or equal amounts of cell lysate protein were subjected to SDS-PAGE, followed by electrophoretic transfer onto nitrocellulose or polyvinylidene difluoride membranes. Signals were visualized using horseradish peroxidase-linked secondary antibodies (Amersham Biosciences) and a chemiluminescence kit (PerkinElmer Life Sciences). Blots were stripped as described (26). P110α and Akt Assays—P110α and Akt activities were assayed following methods described previously (26).

Phospholipid Analysis—Rat-1 cells were labeled with myo-[³H]inositol as described (26) and treated with agonists. Phospholipids were extracted and analyzed as described previously (26), except the HPLC gradient was modified to better separate the PI(3,4,5)P₃ derivative from other compounds (30). The solutions used to develop the gradient were ultrapure water (solution A) and 1.25 M (NH₄)₂HPO₄ (pH 3.8) (solution B). The gradient was developed as follows: 0% solution B from 0 to 10 min, increased to 15% from 10 to 48 min; 15% solution B from 48 to 60 min, increased to 25% from 60 to 75 min, and increased to 30% from 75 to 100 min. The flow rate was 1 ml/min, and 1-ml fractions were collected. The PI(3,4,5)P₃ derivative emerging after appropriate kinase treatments were confirmed using ³²P-labeled standards as described (26).

Purification of Recombinant p110α-p85α—Baculovirus expressing human p85α was purchased from Orbigen (San Diego, CA). To produce baculovirus expressing p110α, mouse p110α from pUSEamp was subcloned into pBlueBacHis2A (Invitrogen). SF9 cells were cotransfected with the plasmid and linear Autographa californiae nuclear polyhedrosis virus viral DNA using the Invitrogen BAC-N-BLUE transfection kit, and a baculovirus clone expressing recombinant p110α protein was isolated. To make the p110α-p85α complex, a 400-μl culture of SF9 cells was co-infected with the two baculoviruses. Two days later, the cells were pelleted and lysed, and the lysate was passed over a 5-ml HiTrap QFF column (Amersham Biosciences). The proteins were eluted with a gradient of NaCl; PI3K activity emerging at 180–270 mM NaCl was pooled. Material from three QFF columns was passed over a column of Ni²⁺-nitrilotriacetic acid-agarose (QIAGEN Inc.). After extensive washing with 50 mM imidazole, the PI3K complex was eluted with 200 mM imidazole. Fractions with the highest activity contained amounts of two major Coomassie Blue-stained proteins corresponding to recombinant p110α and p85α.

Quantitation of Inositol Phosphates—Rat-1 cells expressing the human α1A-adrenergic receptor were seeded in 24-well plates at 2.5 × 10⁴ cells/well. The next day, cells were labeled for 16–24 h in 1 ml of serum-free medium containing 3 μCi/ml myo-[³H]inositol. After labeling, the cells were washed three times with lysis buffer, harvested, and homogenized in 0.5 ml of buffer A (10 mM NaCl, 0.5 mM CaCl₂, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 20 mM LiCl, and 25 mM PIPES (pH 7.2)) with or without 5 μM U73122 for 30 min. Cells were then treated with agonists for another 30 min. COS-7 cells were seeded at 2.5 × 10⁴ cells/well in 24-well plates 1 day before transfection with FLAG-p110α using TransIT-LT1 (Mirus, Madison, WI) following a protocol supplied by the manufacturer. Twenty-four hours after transfection, cells were incubated in 1 ml of growth medium containing 3 μCi/ml myo-[³H]inositol for 16–24 h. Cells were then washed and incubated in buffer A with or without 5 μM U73122 for 1 h. After cell treatments, 1 ml of 16 mM HCl in methanol was added to each well, and the solutions were applied to 500-μl collector plates (Dowex X5 for Na⁺) or 500-μl collector plates (Dowex X8 for K⁺). The collectors were washed with 5 ml of sodium tetraborate and 60 mM sodium formate, and then total inositol phosphates were eluted with 2 ml of 1 mM ammonium formate and 100 mM formic acid. Radioactivity in the eluted material was determined by scintillation counting. Assays were performed in triplicate.
RESULTS

Galpha(q)(Q209L) Inhibits Growth Factor Activation of Akt—Stimulation of G protein-coupled receptors leads to GTP loading of the Go subunit and release of the Gbeta subunits. The Go and Gbeta subunits can then independently exert their effects on downstream effectors. Although the alpha2-adrenergic receptor couples mainly to alpha proteins in the Go11 family, the receptor can also activate alpha proteins in other families (31, 32). Inhibition of Akt upon PE stimulation of the alpha2-adrenergic receptor in Rat-1 cells is probably not due to coupling of the receptor to Go, or Galpha, because the effect was not blocked by pretreatment with pertussis toxin or 2',5'-dideoxyadenosine, an adenyl cyclase inhibitor, respectively (data not shown). Because Gbeta subunits have been shown to activate P13K, we decided to explore a possible role for Galpha in P13K/Akt inhibition. To do this, we used an HA-tagged version of the Q209L mutant of Galpha, which is GTPase-deficient and therefore constitutively activates downstream effectors such as phospholipase C beta without agonist stimulation of a receptor (33).

Rat-1 cells were cotransfected with Akt-Myc and with either HA-Galpha(Q209L) or empty vector as a control. The cells were treated with or without PDGF, and Akt activity was assayed in Myc immunoprecipitates. PDGF treatment stimulated Akt activity 3.6-fold in control cells, and this response was suppressed 58% by expression of HA-Galpha(Q209L) (Fig. 1A, graph). Western blotting showed that Akt-Myc and HA-Galpha(Q209L) were expressed appropriately (Fig. 1A, blots). An additional experiment using a different growth factor was done to test the generality of Akt inhibition by HA-Galpha(Q209L). Rat-1 cells stably expressing the human insulin receptor (Rat-1/HIR cells) were transfected with either HA-Galpha(Q209L) or empty vector as a control. The cells were treated with or without insulin, and the activity of endogenous Akt was assayed in Akt immunoprecipitates. Insulin activated Akt 3.4-fold in control cells, and this response was suppressed 38% by expression of HA-Galpha(Q209L) (Fig. 1B, graph). Western blot analysis showed that the reduction in Akt activity was paralleled by a reduction in Ser473 phosphorylation (Fig. 1B, upper blot). HA-Galpha(Q209L) did not affect the expression of the Akt protein (Fig. 1B, middle blot). The blot was also probed with an anti-HA antibody to demonstrate that HA-Galpha(Q209L) was appropriately expressed (Fig. 1B, lower blot). Thus, expression of HA-Galpha(Q209L) opposes the activation of Akt induced by both the PDGF and insulin receptors. The apparently stronger effect of HA-Galpha(Q209L) in Fig. 1A compared with Fig. 1B is due to the fact that Akt-Myc was cotransfected with HA-Galpha(Q209L) in the former experiment, whereas endogenous Akt was analyzed in the latter. Transfection of Rat-1/HIR cells with a vector expressing green fluorescent protein showed that the transfection efficiency under the conditions used in Fig. 1B was ~60% (data not shown).

Galpha(q)(Q209L) Inhibits Myristoylated p110alpha Activation of Akt—The inhibitory effect of Galpha(Q209L) on Akt activation induced by two distinct receptors raised the possibility that active Galpha might have a direct inhibitory effect on either Akt or P13K. To test whether Galpha(Q209L) targets Akt, HEK 293 cells were cotransfected with HA-Galpha(Q209L) or empty vector as a control and with an activated form of Akt in which the two phosphorylation sites were mutated to Asp (AktDD-Myc). Cells were serum-starved overnight prior to assaying Akt activity in Myc immunoprecipitates. Extracts from cells transfected with AktDD-Myc had four to six times more Akt kinase activity than extracts from cells transfected with Akt-Myc (Fig. 2A). The presence of HA-Galpha(Q209L) did not affect the activity of AktDD-Myc (Fig. 2A). These results suggest that activated Galpha does not have a direct inhibitory effect on Akt.

Expression of membrane-localized myr-p110alpha is sufficient to trigger downstream signaling events in the absence of growth factors. It is believed that when p110 is directed to the membrane, the increased availability of lipid substrates leads to increased production of PI(3,4,5)P3 and subsequent activation of Akt, even though the enzymatic activity of myr-p110alpha is no higher than that of wild-type p110alpha (34). We tested whether activated Galpha can inhibit myr-p110alpha signaling to Akt. HEK 293 cells were cotransfected with Akt-Myc in the presence or absence of myr-p110alpha or HA-Galpha(Q209L). Cells were serum-starved overnight, and extracts were analyzed on a Western blot to detect phospho-Ser473 Akt (P-S473,Akt), total Akt, and HA (blots). Western blotting was repeated with similar results. FIG. 1. Effect of Galpha(q)(Q209L) on Akt activation. A, Rat-1 cells were cotransfected with Akt-Myc and with either HA-Galpha(Q209L) or empty vector as a control. Serum-starved cells were treated for 5 min with or without 25 ng/ml PDGF. Akt activity was measured in Myc immunoprecipitates (graph). Data shown are means ± S.E. from three independent experiments. Expression of Akt-Myc and HA-Galpha(Q209L) was confirmed by Western blot analysis of cell lysate proteins using anti-epitope antibodies (blots). B, Rat-1/HIR cells were transfected with HA-Galpha(Q209L) or empty vector as a control. Serum-starved cells were treated for 5 min with or without 100 nM insulin, and endogenous Akt activity was measured in Akt immunoprecipitates (graph). Data shown are means ± S.E. from three independent experiments. A Western blot of cell lysate proteins was probed sequentially with antibodies to phospho-Ser473 Akt (P-S473,Akt), total Akt, and HA (blots). Western blotting was repeated with similar results.
Myc, HA, and p110 cell line that expresses untagged G but not on Akt.

signal comprising the wild-type and mutant proteins in assayed in Myc immunoprecipitates. Data shown are means control. Cells were serum-starved overnight, and Akt activity was as-

FIG. 2. Effect of Gq(Q209L) on myr-p110α activation of Akt. A, HEK 293 cells were cotransfected with Akt-Myc or AktDD-Myc and with combinations of myr-p110α, HA-Gq(Q209L), or empty vector as a control. Cells were serum-starved overnight, and Akt activity was assayed in Myc immunoprecipitates. Data shown are means ± S.E. from three independent experiments. B, cell extracts from A (representing the first, fourth, and fifth bars) were analyzed on a Western blot probed sequentially with antibodies to phospho-Ser 473 Akt (P-S473 Akt-myc), Myc, HA, and p110α. Western blotting was repeated with similar results.

Myc immunoprecipitates was 40% lower in cells expressing both myr-p110α and HA-Gq(Q209L) than in cells expressing only myr-p110α (Fig. 2A). The blot was reprobed with antibodies to Myc, HA, and p110α to demonstrate that Akt-Myc, HA-Gq(Q209L), and myr-p110α were appropriately expressed (Fig. 2B, lower three panels). Together, these results suggest that Gq(Q209L) might have a direct inhibitory effect on PI3K, but not on Akt.

Activated Gq Inhibits p110α PI3K Activity—To further examine the effect of constitutively active Gq on PI3K, a stable cell line that expresses untagged Gq(Q209L) in a doxycycline-responsive manner was constructed using a commercially available system (see “Experimental Procedures”). Flp-in T-REx/HEK 293 cells containing the empty vector (control) or Gq(Q209L) were treated overnight with 1 μM doxycycline. Western blotting of cell lysates using antibody to Gq detected the wild-type endogenous protein in control cells and a stronger signal comprising the wild-type and mutant proteins in Gq(Q209L) cells (Fig. 3A). Cell extracts were mixed with polyclonal antibody to either p110α or p110β, and the immunoprecipitates were assayed for PI3K activity. The activity in p110α immunoprecipitates from cells expressing Gq(Q209L) was only 39% of that in immunoprecipitates from control cells (Fig. 3B). In contrast, PI3K activities in p110β immunoprecipitates from control cells and cells expressing Gq(Q209L) were not significantly different (Fig. 3B). Western blotting showed that expression of the p110α and p110β proteins was the same in control and Gq(Q209L) cells (Fig. 3A).

Effect of Gq(Q209L) on PI3K activity. Flp-in T-REx/HEK 293 cells containing Gq(Q209L) or empty vector as a control were treated overnight with 1 μM doxycycline prior to making extracts. A, expression of Gq(Q209L) was confirmed by Western blotting with antibody to Gq (upper panel). The blot was reprobed with antibodies to p110α (middle panel) and p110β (lower panel). B, PI3K activity was assayed in p110α or p110β immunoprecipitates (IP). The autoradiogram shows a typical result from four independent experiments. Spots containing radioactive PI(3)P were scraped off the silica gel plates and quantitated by scintillation counting. Mean p110α activity in cells expressing Gq(Q209L) was 39% of the activity in control cells containing the empty vector (S.E. = 2.3%; p < 0.01 by t test). Mean p110β activity in cells expressing Gq(Q209L) was 99% of the activity in control cells (S.E. = 2.5%; not statistically significant).

We next investigated whether receptor activation of Gq also affects p110α activity. Rat-1 cells expressing the α1A-adrenergic receptor were treated with or without PE in the presence of PDGF, and PI3K activity was measured in p110α or p110β immunoprecipitates. PE treatment caused a 53% reduction in PI3K activity in p110α immunoprecipitates compared with cells not treated with PE (Fig. 4A). Similar to what was observed in Flp-in T-REx/HEK 293 cells expressing Gq(Q209L), PE activation of Gq did not have a significant effect on PI3K activity in p110β immunoprecipitates (Fig. 4A). We also assayed overall PI3K signaling by measuring levels of PI(3,4,5)P3 in Rat-1 cells following PE and PDGF treatment. The amount of PI(3,4,5)P3 in cells treated with PE alone was not significantly increased above control levels, whereas PDGF induced a 2.6-fold increase in PI(3,4,5)P3 (Fig. 4B). In cells co-treated with PE, the PDGF-induced increase in PI(3,4,5)P3 was reduced by 64% (Fig. 4B). Taken together, these results suggest that activated Gq inhibits p110α PI3K activity, resulting in decreased growth factor-mediated PI(3,4,5)P3 production and Akt activation.

Involvement of Phospholipase C in PI3K/Akt Inhibition—Activated Gq directly stimulates phospholipase Cβ, leading to production of diacylglycerol and inositol 1,4,5-trisphosphate, release of Ca2+ from intracellular stores, and activation of protein kinase C (35). We noted earlier that PE still inhibits Akt activation by insulin-like growth factor I in cells depleted of intracellular Ca2+ (26). We therefore did additional experiments to assess the involvement of phospholipase C signaling in PI3K/Akt inhibition by activated Gq. First, we tested whether the phospholipase C inhibitor U73122 blocks the inhibitory effect of the α1A-adrenergic receptor on insulin activation of Akt. Insulin-induced phosphorylation of Akt was unaffected by pretreatment of Rat-1 cells with U73122 (Fig. 5A). More importantly, the inhibitory effect of PE on Akt phosho-
We wondered whether Gq/11 forms a complex with p110α. Equal amounts of cell lysate proteins of Rat-1 cells expressing the Gq(Q209L) mutant resulted in decreased activity of PI3K in p110α. In control COS-7 cells cotransfected with FLAG-p110α and p85α, expression of HA-Gq(Q209L) caused a 53% reduction in PI3K activity in FLAG immunoprecipitates (Fig. 5B). U73122 had a small inhibitory effect by itself on PI3K activity. However, expression of HA-Gq(Q209L) still caused a 59% decrease in PI3K activity in cells pretreated with U73122 (Fig. 5B). These data suggest that inhibition of PI3K by activated Gq occurs independently of the phospholipase C pathway.

Activated Gq Interacts with p110α in Vivo—It has been reported that endogenous Gqα11 forms a complex with p110α PI3K (17, 36). We wondered whether Gqα(Q209L) might directly bind to and inhibit PI3K. To test for binding, COS-7 cells were cotransfected with FLAG-p110α and p85α in the presence or absence of HA-Gqα(Q209L). Equal amounts of cell lysate proteins were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were examined on a Western blot probed with antibody to the HA epitope. HA-Gqα(Q209L) was found to coprecipitate with FLAG-p110α (Fig. 6B, upper panel). As expected, p85α was associated with FLAG-p110α in both HA and FLAG immunoprecipitates (Fig. 6, A and B, lower panels). In addition, the PI3K activity measured in FLAG immunoprecipitates from cells expressing HA-Gqα(Q209L) was only 44% of that recovered from control cells (Fig. 6C).

We next tested whether HA-Gqα(Q209L) inhibits PI3K in vitro. HA immunoprecipitates were prepared from COS-7 cells transfected with either HA-Gqα(Q209L) or empty vector as a control. The immunoprecipitates were incubated overnight with highly purified recombinant p110α-p85α complex prior to performing PI3K assays. The results show that PI3K activity in the presence of HA-Gqα(Q209L) was lower than in the control reaction (Fig. 6D). Together, these results suggest that activated Gqα might interact with p110α, either directly or indirectly through p85α, to inhibit its activity.

**DISCUSSION**

In this study, we investigated whether the inhibitory effect of Gqα-coupled receptors on the PI3K/Akt signaling pathway might be mediated by the activated Gqα subunit inhibiting PI3K. We found that expression of Gqα(Q209L) inhibited Akt activation induced by growth factors and constitutively active PI3K, but did not inhibit the activity of a constitutively active Akt mutant. We also demonstrated that expression of the activated Gqα mutant resulted in decreased activity of PI3K in p110α (but not p110β) immunoprecipitates. Furthermore, Gqα(Q209L) immunoprecipitates were examined on a Western blot probed with antibody to the HA epitope. HA-Gqα(Q209L) was found to coprecipitate with FLAG-p110α (Fig. 6B, upper panel). As expected, p85α was associated with FLAG-p110α in both HA and FLAG immunoprecipitates (Fig. 6, A and B, lower panels). In addition, the PI3K activity measured in FLAG immunoprecipitates from cells expressing HA-Gqα(Q209L) was only 44% of that recovered from control cells (Fig. 6C).

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In this study, we investigated whether the inhibitory effect of Gqα-coupled receptors on the PI3K/Akt signaling pathway might be mediated by the activated Gqα subunit inhibiting PI3K. We found that expression of Gqα(Q209L) inhibited Akt activation induced by growth factors and constitutively active PI3K, but did not inhibit the activity of a constitutively active Akt mutant. We also demonstrated that expression of the activated Gqα mutant resulted in decreased activity of PI3K in p110α (but not p110β) immunoprecipitates. Furthermore, Gqα(Q209L)

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**Fig. 4.** Effect of the α1α-adrenergic receptor on PI3K activity. A, Rat-1 cells expressing the α1α-adrenergic receptor were treated with or without 10 μM PE for 5 min in the presence of 50 ng/ml PDGF. PI3K activity was measured in p110α or p110β immunoprecipitates (IP). The autoradiogram shows a representative result from three independent experiments. Mean p110α activity in PE-treated cells was 47% of the activity in cells not treated with PE (S.E. = 3.2%; p < 0.01 by t test). Mean p110β activity in PE-treated cells was 98% of the activity in control cells (S.E. = 3.1%; not statistically significant). B, Rat-1 cells expressing the α1α-adrenergic receptor were labeled with myo-[3H]inositol and then treated for 5 min with 50 ng/ml PDGF in the presence or absence of 10 μM PE. Phospholipids were extracted, deacylated, and analyzed by HPLC (see “Experimental Procedures”). The amount of PI(3,4,5)P3 was normalized to the total amount of myo-[3H]inositol-labeled material recovered from the column. Data shown are means ± S.E. from three independent experiments. *, significant difference between PDGF and PE + PDGF (p < 0.01). Data were analyzed by one-way analysis of variance, and pairwise comparisons were made using Fisher’s post-hoc tests.

**Fig. 5.** U73122 resistance of PI3K/Akt inhibition by PE and Gqα(Q209L). A, serum-starved Rat-1 cells expressing the α1α-adrenergic receptor were incubated with 5 μM U73122 or vehicle for 30 min prior to stimulation with 1 μM insulin in the presence or absence of 10 μM PE for 5 min. Phospho-Ser108 Akt (P-S473 Akt; upper panel) and total Akt (lower panel) were detected on a Western blot of cell lysate proteins. Control experiments (see “Experimental Procedures”) showed that PE caused 6.8- and 2.9-fold increases in inositol phosphate levels in control and U73122-treated cells, respectively. B, COS-7 cells were cotransfected with FLAG-p110α and p85α in the presence of HA-Gqα(Q209L) or empty vector. Cells were treated 48 h later with 5 μM U73122 or vehicle for 1 h. PI3K activity was then assayed in FLAG immunoprecipitates. Control experiments (see “Experimental Procedures”) showed that expression of HA-Gqα(Q209L) caused 7.7- and 4.7-fold increases in inositol phosphate levels in control and U73122-treated cells, respectively. The experiments were repeated with similar results.
Fig. 6. $G_{\alpha_q}$ (Q209L) co-immunoprecipitates with p110α. A–C, COS-7 cells were transfected with FLAG-p110α and p85α in the presence or absence of HA-G$_{\alpha_q}$ (Q209L) or empty vector as a control. A, HA immunoprecipitates (IP) and total cell lysates were examined on a Western blot probed with antibodies to FLAG (upper panel) and p85α (lower panel). B, FLAG immunoprecipitates and total cell lysates were analyzed on a Western blot probed with antibodies to HA (upper panel) and p85α (lower panel). The experiment was repeated three times with similar results. C, shown is an autoradiogram illustrating PI3K activity measured in FLAG immunoprecipitates (left panel). The activity in cells expressing HA-G$_{\alpha_q}$ (Q209L) was 44% of the activity in control cells (mean activities from duplicate assays were 48,953 and 111,194 cpm, respectively). FLAG immunoprecipitates were probed with anti-FLAG antibody to show that the two assays contained similar amounts of p110α protein (right panel). D, highly purified recombinant p110α-p85α complex (see "Experimental Procedures") was mixed with HA immunoprecipitates from COS-7 cells transfected with HA-G$_{\alpha_q}$ (Q209L) or empty vector. Mixtures were then assayed for PI3K activity. To make the HA immunoprecipitates, COS-7 cells were seeded at 7.5×10⁵ cells/10-cm plate and transfected with 8 μg of HA-G$_{\alpha_q}$ (Q209L) or empty vector the next day. Two days later, cell lysate proteins were immunoprecipitated with anti-HA antibody as described under "Experimental Procedures." The immunoprecipitates were washed twice with lysis buffer and twice with PI3K assay buffer (26). Washed immunoprecipitates were suspended in 40 μl of PI3K assay buffer containing 1 mM GTPγS, 10 mM MgCl₂ and ~6 ng of purified PI3K. The mixtures were rotated at 4 °C overnight. The next day, PI3K assays were started by adding 5 μl of 10 mg/ml PI and 5 μl of 400 μM ATP, 2.5 μCi of [γ-32P]ATP, and 100 mM MgCl₂ in PI3K assay buffer. Decreased PI3K activity in the presence of immunoprecipitated HA-G$_{\alpha_q}$ (Q209L) was seen in three experiments. An autoradiogram of a representative result is shown (left panel). In a parallel experiment, mixtures that had been rotated at 4 °C overnight were probed with anti-p110α antibody to show that the two assays contained similar amounts of p110α protein (right panel).

bound to the p110α-p85α PI3K heterodimer and appeared to inhibit its activity in vitro, suggesting that there might be an inhibitory interaction between p110α and activated G$_{\alpha_q}$.

As mentioned in the Introduction, several reports indicate that some G$_{\alpha}$-coupled receptors can antagonize PI3K/Akt signaling. Because G protein-coupled receptors such as the α₁A adrenergic receptor can couple to more than one type of G$_{\alpha}$ subunit, assessing the involvement of a particular G$_{\alpha}$ protein can be difficult. Therefore, transfection studies using active G$_{\alpha}$ subunit mutants have been done to examine what effect the individual proteins have on Akt. In one report using COS-7 cells, transfection of activated mutants of G$_{\alpha_q}$ or G$_{\alpha_12}$ caused an increase in Akt activity, whereas G$_{\alpha_6}$ and G$_{\alpha_13}$ had no effect (37). However, in a separate study, expression of activated mutants of G$_{\alpha_q}$, G$_{\alpha_s}$, G$_{\alpha_12}$, or G$_{\alpha_13}$ in COS-7 or HEK 293 cells did not activate Akt (11). Similarly, G$_{\alpha_q}$, G$_{\alpha_s}$, G$_{\alpha_12}$, or G$_{\alpha_13}$ mutants did not activate Akt in NIH 3T3 cells (38). In agreement with the latter two reports, we found that G$_{\alpha_q}$ (Q209L) did not promote an increase in Akt activity in two different Rat-1 cell lines (Fig. 1).

A limited number of similar experiments have been performed to investigate the inhibitory effects of G$_{\alpha_q}$ proteins on Akt activation. Bommakanti et al. (11) found that expression of G$_{\alpha_q}$ (Q209L) inhibited Akt activation induced by coexpression of Gβγ subunits or activated Ras in COS-7 cells. In addition, the activated mutant of G$_{\alpha_q}$ (but not of G$_{\alpha_s}$, G$_{\alpha_6}$, or G$_{\alpha_13}$) inhibited Akt activation induced by insulin-like growth factor I in HEK 293 cells. Here, we found that G$_{\alpha_q}$ (Q209L) inhibited the activation of Akt promoted by PDGF or insulin treatment of Rat-1 cells as well as by expression of myr-p110α in HEK 293
results could be that Gq immunoprecipitates, an alternative explanation for our ability of its upstream regulator, PI3K. However, because PI3K was accompanied by a 64% decrease in PI(3,4,5)P3 production in H9252 cells inhibited Akt activation induced by expression of Gq. We were surprised that activated Gq exerts a stable inhibitory effect on p110α that can be measured even after immunoprecipitation of the enzyme. We were surprised that activated Gq did not appear to have such an effect on p110β since Bommakanti et al. (11) found that Gq(Q209L) inhibited Akt activation induced by expression of Gβγ subunits in COS-7 cells. Activation of Akt by Gβγ in these cells is presumably mediated by the p110α isoform of PI3K, as COS-7 cells express p110β, but not p110γ (38). Thus, if activated Gq does inhibit p110α, it may either bind weakly to this PI3K or use a distinct mechanism that does not allow us to measure a stable change in activity using our in vitro assay.

Our data suggest that inhibition of PI3K by activated Gq is not mediated by the phospholipase Cβ signaling pathway. Activated Gq still exerted an inhibitory effect on Akt and p110α-p85α in the presence of a phospholipase C inhibitor (Fig. 5) and in cells depleted of protein kinase C (data not shown) or intracellular Ca2+ (26). Gq(Q209L) stimulation of GLUT4 translocation in 3T3-L1 adipocytes is also thought to be independent of phospholipase C signaling (39). We therefore postulated that activated Gq might bind to PI3K and directly inhibit its catalytic activity. In support of this hypothesis, we found that the p110α-p85α heterodimer coprecipitated with Gq(Q209L) (Fig. 6, A and B). In addition, the PI3K activity measured in these immunoprecipitates was lower than in control reactions that did not contain Gq(Q209L) (Fig. 6C). Finally, incubation of immunoprecipitated Gq(Q209L) with p110α-p85α in vitro also led to a decrease in PI3K activity (Fig. 6D). More studies are needed to determine the nature and relevance of the physical interaction between Gq and p110α.

In direct contrast to our hypothesis, Olefsky and co-workers (17) have proposed that stimulation of the Gq-coupled ETα receptor in 3T3-L1 adipocytes causes Gq to activate p110α PI3K. Acute treatment of these cells with endothelin-1 increased p110α activity almost as much as insulin did, but there was no corresponding increase in Akt phosphorylation. Stimulation with endothelin-1 also increased the amount of endogenous Gq(11) that coprecipitated with p110α (17). Similarly, adenosine expression of Gq(Q209L) increased the PI3K activity in p110α and p110γ immunoprecipitates, and yet Akt phosphorylation did not increase. Unlike our findings, Gq(Q209L) modestly inhibited insulin activation of p110α and Akt, whereas PDGF-induced activation of the two enzymes was not affected (36). The differences between our results and those cited above could be due to cell type variation. For example, 3T3-L1 adipocytes might contain unique proteins that join the Gq-p110α complex to cause a stimulatory effect, or a post-translational modification of Gq might cause the interaction with p110α to be stimulatory rather than inhibitory. Indeed, it was reported that tyrosine phosphorylation of Gq(11) increases following treatment of 3T3-L1 adipocytes with endothelin-1 or insulin (17, 36). On the other hand, some of the data in Refs. 17 and 36 are controversial, as Pessin and co-workers (39) believe that activation of Gq in 3T3-L1 adipocytes does not lead to an increase in PI3K activity.

Activation of Gq can have profound effects on cell survival. Simon and co-workers (40) reported that expression of the constitutively active Gq(R183C) mutant in COS-7 or Chinese hamster ovary cells promotes apoptosis. We used the inducible Flp-In T-REx system to study Gq(Q209L) (Fig. 3) because we were unable to isolate cell clones stably expressing the active mutant in a constitutive manner. In addition, we found that α1A-adrenergic receptor activation of Gq promotes apoptosis in Rat-1 cells (26). Finally, transgenic mice overexpressing Gqα were found to die of heart failure, and cardiomyocytes from these animals showed an increased rate of apoptosis (41). We speculate that direct inhibition of p110α by active Gq could be a common mechanism used by the α1A-adrenergic receptor and other Gq-coupled receptors to inhibit Akt activity and to promote apoptosis. This prediction could be tested by attempting to reverse the apoptotic effect of Gq(Q209L) by expression of constitutively active Akt or a p110α mutant that no longer binds to Gq.

In conclusion, our results demonstrate that activated Gqα modulates Akt activity by inhibiting PI3K. Further investigation into how Gq-coupled receptors inhibit this signaling pathway might reveal important physiological insights into diseases such as diabetes mellitus, heart failure, and cancer.

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