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Constitutive Activation of a Phosphoinositidase C-Linked G Protein in Murine Fibroblasts Decreases Agonist-Stimulated Ca^{2+} Mobilization

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SUMMARY

We compared Ca^{2+} signaling and inositol polyphosphate metabolism in NIH-3T3 cells stably transfected with cDNA encoding either the wild-type G protein G_{16} α subunit or a GTPase-deficient α_{16} subunit (Q212L-α_{16}). Constitutive activation of phosphoinositidase C (PIC) in cells expressing Q212L-α_{16} was demonstrated by 1) an increased basal level of [^{3}H]inositol polyphosphates, 2) an enhanced rate of [^{3}H]inositol polyphosphate accumulation in cells treated with 10 mM LiCl, and 3) an increased rate of incorporation of [^{3}H]inositol into cell lipids. Q212L-α_{16} cells had a diminished cell growth rate. Basal intracellular Ca^{2+} concentration was equivalent in Fura-2 acetoxymethyl ester-loaded Q212L-α_{16} cells compared with controls; however, calcium release in Q212L-α_{16} cells exposed to ionomycin, ATP (a G protein-linked agonist), or platelet-derived growth factor (a tyrosine kinase-linked agonist) was decreased. Permeabilized, ^{45}Ca-loaded Q212L-α_{16} cells released less ^{45}Ca at each concentration of inositol-1,4,5-trisphosphate than did control cells. Accordingly, the total amount of inositol trisphosphate (IP_3) receptor protein was decreased in Q212L-α_{16} cells relative to controls. These data demonstrate that Q212L-α_{16} cells maintain physiological levels of cytoplasmic calcium and partially loaded Ca^{2+} stores in the face of constitutively active PIC. This is accomplished, at least in part, by down-regulation of IP_3 receptor number. Thus, diminution in cell growth rate in Q212L-α_{16} cells seems to be attributable to a combination of at least two effects: a direct effect of PIC activation leading to partial depletion of Ca^{2+} stores and an indirect, adaptive response resulting in a decreased IP_3 receptor number.

PIC catalyzes the hydrolysis of the plasmalemma-associated phospholipid phosphatidylinositol-4,5-bisphosphate to form the Ca^{2+} release signal (1,4,5)IP_3 and the PKC activator diacylglycerol. Numerous mitogenic hormones, neurotransmitters, and growth factors activate this signaling pathway, and intracellular Ca^{2+} concentration and PKC activity have been shown to regulate normal cell growth and proliferation (1, 2). Recent studies have demonstrated that constitutive activation of this signaling pathway, whether by overexpression of PIC-coupled receptors (3, 4) or by constitutive activation of transducing G proteins (5, 6), can cause cellular transformation and abnormal cell growth. Constitutive activation of PIC in cultured cells thus provides a model for studying the involvement of this signaling pathway in normal and abnormal cell proliferation.

Heterotrimeric G proteins are activated by the exchange of bound GDP for GTP and dissociation of the α from the $\beta\gamma$ subunits and are inactivated by the hydrolysis of GTP to GDP and subunit reassociation (7). Consequently, constitutively activated G proteins have been produced by introduction of point mutations into the GTP binding domains of the α subunits of G_s (8), G_i (9, 10), G_o (11), and G_q (5, 6, 12–14), resulting in impaired GTPase activity and constitutive activation of coupled effectors. The effects of constitutive activation of G_q on cell growth and transformation seem to be cell (5) and laboratory (5, 6, 13) specific: stable transfection of NIH-3T3 cells with GTPase-deficient G_q has been reported to lead to either cell transformation (5, 6) or cell death (13), and stable transfection of rat-1 cells was not transforming (5). The underlying effects of constitutive activation of PIC on cell Ca^{2+} homeostasis and inositol polyphosphate metabolism were not characterized in any of these studies.

ABBREVIATIONS: PIC, phosphoinositidase C; (1,4,5)IP_3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; IP_2, inositol bisphospate; IP_3, inositol trisphosphate; PMA, phorbol-12-myristate-13-acetate; $[Ca^{2+}]_i$, intracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PDGF, plate-derived growth factor; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; BAPTA; 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid.

In this study, we examined the effects of stable transfection of NIH-3T3 cells with a GTPase-deficient α subunit of G_{16} on cell growth, Ca^{2+} homeostasis, and inositol polyphosphate metabolism. G_{16} is a member of the G_q family of PIC-coupled G proteins that are specific for myelomonocytic and T cells (15). The data demonstrate that constitutive activation of PIC by G_{16} results in a decrease in cell growth rate and impaired Ca^{2+} signaling due, at least in part, to IP_3 receptor down-regulation.

Experimental Procedures

Materials. DMEM, fetal bovine serum, newborn calf serum, L-glutamine, penicillin/streptomycin, Geneticin, trypsin/EDTA, and Puck's Saline A were purchased from GIBCO BRL (Baltimore, MD). *myo*-[^{3}H]Inositol (10 mCi/ml) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Dowex AG 1 × 8 anion exchange resin (formate form) was purchased from BioRad (Hercules, CA). [^{3}H]Inositol polyphosphate standards and ^{45}Ca-$CaCl_2$ were purchased from DuPont-New England Nuclear (Boston, MA). Fura-2 acetoxymethyl ester and BAPTA were purchased from Molecular Probes (Eugene, OR). PDGF was purchased from R & D Systems (Minneapolis, MN). Disodium-ATP and BSA (Fraction V) were purchased from Sigma Chemical (St. Louis, MO). Thapsigargin was obtained from LC Services (Woburn, MA). Adsorbosphere SAX HPLC columns were purchased from Alltech Associates (Deerfield, IL). All other chemicals and solvents were of at least reagent grade.

Plasmid construction and transfection. cDNA coding for the G protein α subunit G_{16} (α_{16}) was cloned after amplification by PCR from reverse-transcribed HL-60 total RNA. Leucine was substituted for glutamine at amino acid 212 (Q212L-α_{16} mutation) by site-directed mutagenesis with a Stratagene kit (La Jolla, CA). The α_{16} construct cDNA was then packaged into a retroviral vector (LNCX) containing a cytomegalovirus promoter to drive constitutive expression of the α_{16} cDNA. A viral packaging cell line (GPE) was transfected with the α_{16} construct via electroporation. Positive clones were selected for Geneticin (GIBCO) resistance (*Neo* gene) and screened by Northern blot analysis. NIH-3T3 cells were infected with the supernatant from positive GPE clones, which contained shed virus. Cells expressing α_{16} were then selected in Geneticin-containing medium, and cell homogenates were screened by immunoblotting using a rabbit anti-α_{16} peptide-specific antibody (16).

Cell culture. Stably transfected NIH-3T3 cells were grown at 37° in a 5% CO_2 atmosphere in 175-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) in high glucose-DMEM containing 5% fetal bovine serum, 5% newborn calf serum, 2 mM glutamine, penicillin/streptomycin (500 units/ml and 50 μg/ml, respectively), and 375 μg/ml Geneticin. Cell monolayers were dispersed with trypsin/EDTA before confluence (approximately every 3 days) and split 1:20 with each successive passage. Cells that had attained 70–90% confluence were used in the experiments reported here. For inositol polyphosphate metabolism studies, cells were grown onto six-well tissue culture plates (Costar, Cambridge, MA). Cells used in Ca^{2+} experiments were resuspended from 175-cm^2 flasks using 5 mM EDTA in Puck's Saline A.

[^{3}H]inositol polyphosphate metabolism. Cells were plated directly into culture medium containing 20–100 μCi/ml *myo*-[^{3}H]inositol. After 2–3 days, the medium was aspirated, and cells were grown overnight in serum-free culture medium containing 1% (w/v) BSA and equivalent *myo*-[^{3}H]inositol. On the next day, this medium was aspirated, and cells were equilibrated for 20 min in experimental medium (DMEM, 20 mM HEPES, pH 7.4, 2 mM glutamine, 1% (w/v) BSA). LiCl (10 mM) was then added directly to this medium as indicated. After the specified incubation period, experimental medium was aspirated, and inositol polyphosphates were extracted into 1 ml of 6% perchloric acid, 5 mM EDTA, and 250 μg/ml phytate on ice for 20 min. For HPLC analysis, acid extracts were neutralized by the freon/trioctylamine method (17) as modified by Shears *et al.* (18). [^{3}H]IP_3 and [^{3}H]inositol tetrakisphosphate isoforms were separated by HPLC (Adsorbosphere SAX column) with a linear ammonium phosphate gradient (0–1 M, pH 3.35) with radioactivity determined on-line (19). [^{3}H]IP_2 isoforms could not be separated using this protocol. For separation with the use of Dowex anion exchange columns, acid extracts were neutralized with 0.5 M KOH plus 9 mM Na-Borax, salt was precipitated overnight and pelleted, and the sample supernatant was loaded onto 0.8–1-ml Dowex anion exchange columns. The columns were first washed extensively with water to remove free [^{3}H]inositol; then, [^{3}H]inositol phosphates were eluted sequentially with ammonium formate/formic acid as described previously (20). Data were normalized to total cell [^{3}H]inositol lipids as determined by acidified chloroform/methanol extraction of the acid-precipitated cell monolayer as described below.

[^{45}Ca] release. Cell monolayers dispersed with trypsin/EDTA were pelleted, washed, and resuspended at 1 mg of total cell protein/ml in permeabilization buffer containing 20 mM NaCl, 100 mM KCl, 2 mM $MgSO_4$, 20 mM HEPES, pH 7.2, 1 mM EGTA, and 0.451 mM $CaCl_2$ (150 nM [Ca^{2+}]). Cells were permeabilized with 50 μg/ml saponin (10 min at 37°), pelleted, and resuspended in saponin-free permeabilization buffer. Triplicate aliquots were taken for protein normalization. Cells were then loaded with ^{45}Ca (1 μCi/ml) for 20 min at 37° in buffer with 3 mM Mg-ATP. ^{45}Ca efflux was initiated by the addition of 1–300 μM (1,4,5)IP_3 to duplicate 200-μl aliquots of cell suspension. Efflux was terminated by the addition of 5 ml of ice-cold stop solution (310 mM sucrose plus 4 mM EGTA, pH 7.2) followed by rapid filtration under vacuum through glass microfiber filters (Whatman G/C; Fisher Scientific, Pittsburgh, PA). Filters were rinsed three times with 5 ml of ice-cold wash buffer (310 mM sucrose), dried, and transferred to scintillation vials with 5 ml of scintillant (Hydrofluor; National Diagnostics, Atlanta, GA). Radioactive counts were corrected for filter background and normalized to total cell protein.

Immunoblot of IP_3 receptor protein. Cell monolayers were rinsed with ice-cold *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer containing 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, 20 mM sucrose, 100 mM NaF, 15 mM EDTA, and 2 mM EGTA and scraped into buffer containing protease inhibitors (2 μg/ml aprotinin, 0.6 μg/ml leupeptin, 0.9 μg/ml antipain, 0.9 μg/ml pepstatin A, 1 μg/ml E-64, 1 μg/ml calpain inhibitor I, 1 μg/ml calpain inhibitor II, 1 μg/ml phenylmethylsulfonyl fluoride). Cells were disrupted by sonication, and the resultant cell suspension was added to an equal volume of sample buffer [9% (w/v) SDS, 15% (v/v) glycerol, 6% (v/v) β-mercaptoethanol, 186 mM Tris·HCl, pH 6.7], boiled 10 min, and frozen at −20° until assayed. Thawed samples were electrophoresed on a 7% polyacrylamide gel (18 hr at 40 V) and transferred to nitrocellulose (4 hr at 200 mA). The blot was blocked overnight in 3% (w/v) BSA in Tris-buffered saline containing 10 mM Tris base, pH 8, 154 mM NaCl, and 0.05% Tween 20 and then incubated sequentially with affinity-purified goat anti-rat IP_3 receptor antibody (1:10,000 dilution for 1.5 hr at 37°; Ref. 21), Tris-buffered saline/Tween 20 rinse solution (5 × 10 min), rabbit anti-goat IgG (1:300 dilution for 30 min at room temperature), and goat peroxidase-anti-peroxidase (1:3000 dilution for 15 min at room temperature). After a final rinse (6 × 10 min with Tris-buffered saline/Tween 20), the banding pattern was revealed by enhanced chemiluminescence (DuPont-New England Nuclear).

Intracellular Ca^{2+} determination. Cell monolayers were disaggregated with 5 mM EDTA in Puck's Saline A; cells were then pelleted and resuspended (4 × 10^6 cells/ml) in modified Krebs-Ringer solution [115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 11 mM glucose, 25 mM HEPES, pH 7.4, 0.1% (w/v) BSA] containing 10 μM Fura-2 acetoxymethyl ester. Cells were incubated for 30 min at room temperature with shaking and then pelleted and resuspended (1 × 10^6 cells/ml) in Fura-2 acetoxymethyl ester-free solution for 20 min to cleave the dye. Two milliliters of cell suspension was placed in a stirred cuvette at 37° in a spectroflu-

orometer (Photon Technology International, Princeton, NJ). Fluorescence emission at 515 nm was monitored during alternating excitation at 340 and 380 nm. Autofluorescence was determined using unloaded cells. Calcium concentration was calculated from the fluorescence ratio (340/380 nm) as described previously assuming K_D = 224 nm (22).

Statistics and data analysis. Statistical comparisons of data were made using paired *t* tests and one and two-way analyses of variance (with appropriate *a posteriori* multiple-range comparison procedures). Data are expressed as mean ± standard error. Treatment groups were considered significantly different from control when $p < .05$.

Results

Expression of $G_{\alpha 16}$ in transfected cells. Nontransfected NIH-3T3 cells and several clones transfected with either the wild-type α subunit of G_{16} or the GTPase-deficient mutant α subunit (Q212L) were screened for expressed protein using polyclonal anti-α_{16} antisera (Fig. 1). As expected for a nonhematopoetic cell line, nontransfected NIH-3T3 cells did not express endogenous α_{16} (*lane 1*); however, all of the screened clones transfected with the wild-type α subunit and several of the clones transfected with Q212L-α_{16} expressed a 41-kDa protein. The clonal cell lines used in the current study were WT-3 (control cells) and Q212L-8 and Q212L-19 (GTPase-deficient mutants).

Constitutive activation of PIC. Three lines of evidence suggested that PIC was indeed constitutively activated in Q212L-α_{16} cells. First, 6–7-fold more [^{3}H]inositol had been incorporated into the lipids of Q212L-α_{16} mutants than into control lipids after a 2-hr labeling period (control = 35 ± 1 dpm/μg protein, Q212L-8 = 224 ± 6 dpm/μg protein, Q212L-19 = 257 ± 10 dpm/μg protein; five experiments; $p < .05$), despite the fact that labeling was equivalent after 72 hr (five experiments; $p > .05$). These data are consistent with an increased rate of turnover of inositol lipids in Q212L-α_{16} mutants. Second, in equivalently labeled cells (72 hr), basal levels of total inositol polyphosphate were significantly increased in Q212L-α_{16} cells relative to controls (Fig. 2). Finally, incubation of labeled cells with lithium to inhibit dephosphorylation of inositol phosphates had no effect on total inositol phosphate accumulation in unstimulated control cells but resulted in significant accumulation in Q212L-α_{16} mutants (Fig. 2).

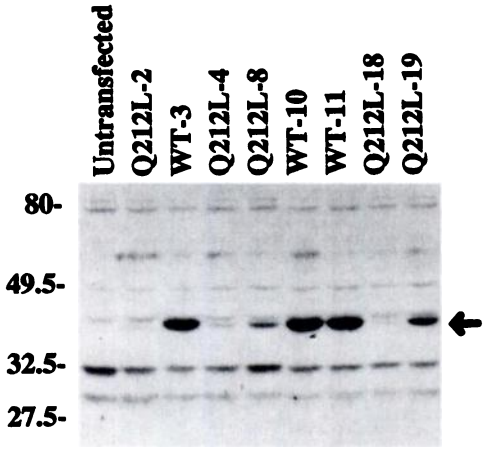

Fig. 1. GTPase-deficient and wild-type (*WT*) $G_{\alpha 16}$ protein expression in transfected NIH-3T3 cells. Cells were transfected with either wild-type α_{16} or GTPase-deficient Q212L-α_{16}. Protein expression was detected by immunoblotting with anti-α_{16} peptide-specific antibody. Clones WT-3 (Control) and Q212L-8 and Q212L-19 (GTPase-deficient) were used in this study. Nontransfected NIH-3T3 cells do not express $G_{\alpha 16}$.

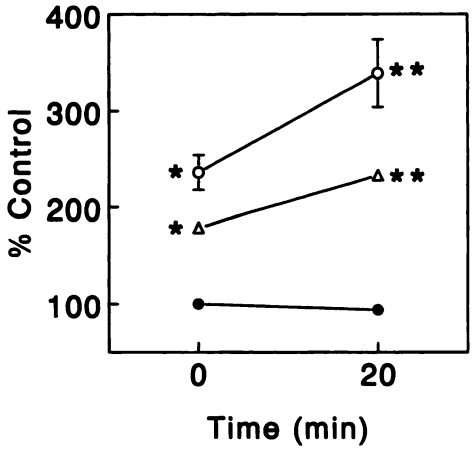

Fig. 2. The rate of total [^{3}H]inositol polyphosphate accumulation is increased in Li^+-treated Q212L-α_{16} cells. Cells were labeled for 72 hr with *myo*-[^{3}H]inositol and then treated with 10 mM LiCl for 0 or 20 min. Total [^{3}H]inositol polyphosphates were normalized to total cell lipids (eight or nine experiments). ●, Control cells; ○, Q212L-8 mutant cells; △, Q212L-19 mutant cells. *, Differs significantly from control; **, differs significantly from value at t = 0.

Cell growth rate. Fig. 3 shows that during their most rapid growth phase (days 2–4), Q212L-α_{16} cells have a slower growth rate than control cells. The Q212L-α_{16} cells also had a lower cell density at confluence that seemed to be related to an increased cell size. The Q212L-α_{16} cells did not have a transformed phenotype measured by their failure to form foci or grow in soft agar.[1]

Basal cytoplasmic Ca^{2+} and Ca^{2+} stores. There is considerable evidence that activation of PIC is associated with mitogenesis (2). Thus, the observation that Q212L-α_{16} cells actually grew more slowly than control cells was unexpected. In an attempt to resolve this apparent paradox, we carried out experiments designed to test the hypothesis that the observed decrease in cell growth rate in Q212L-α_{16} cells resulted from a secondary effect on cell Ca^{2+} homeostasis. Both basal $[Ca^{2+}]_i$ and the peak $[Ca^{2+}]_i$ after inhibition of endoplasmic reticular Ca^{2+}-ATPase with thapsigargin were equivalent in Q212L-α_{16} cells and controls (Figs. 4 and 5). In addition, $[Ca^{2+}]_i$ was not affected in either control or Q212L-α_{16} cells by the addition of the Ca^{2+} chelator BAPTA, indicating that despite PIC activation, Ca^{2+} entry across the plasma membrane was not activated. However, with the addition of thapsigargin, the rate of increase in $[Ca^{2+}]_i$ was greater in Q212L-α_{16} cells (increase in $[Ca^{2+}]_i$/time to peak): 7 ± 1, 11 ± 2,* and 14 ± 2* nM/sec for control, Q212L-8, and Q212L-19, respectively (four experiments; *differs significantly from control). These data implied that the rate of leak of Ca^{2+} from thapsigargin-sensitive intracellular stores was increased in the mutant cells, perhaps due to an increased basal level of (1,4,5)IP_3. In addition, because the peak $[Ca^{2+}]_i$ level in thapsigargin-activated cells depends in part on the rate of leak of Ca^{2+}, this result indicated that the peak $[Ca^{2+}]_i$ level in response to thapsigargin could not be taken as a reliable indicator of the size of intracellular Ca^{2+} stores.

[1] B. Eisfelder and G. L. Johnson, unpublished observations.

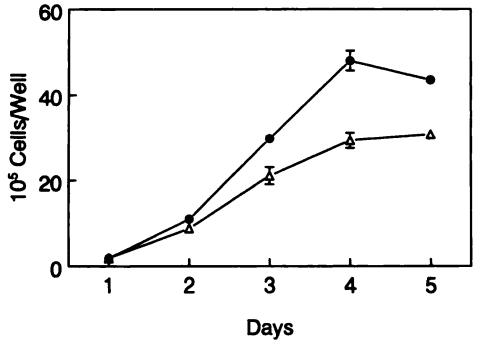

Fig. 3. The rate of cell growth is decreased in Q212L-α_{16} cells. Cells were plated onto six-well plates at 2 × 10^4 cells/well and counted at 24-hr intervals. A representative experiment is shown (three experiments). ●, Control cells; △, Q212L-19 mutant cells.

To avoid complications introduced by the increased basal permeability of Ca^{2+} stores in Q212L-α_{16} cells, we exposed cells to ionomycin, a Ca^{2+} ionophore that releases Ca^{2+} by increasing the permeability of intracellular Ca^{2+} stores to a high, uniform level (23). When control cells were exposed to ionomycin after thapsigargin (in the absence of extracellular Ca^{2+}), ionomycin was unable to release additional Ca^{2+} after thapsigargin exposure (data not shown), indicating that a single, homogeneous ionomycin- and thapsigargin-sensitive Ca^{2+} pool existed in NIH-3T3 cells. The data in Fig. 4 (*inset*) revealed that intracellular Ca^{2+} stores, determined by release by ionomycin, were in fact partially depleted in Q212L-α_{16} cells relative to controls.

Basal [^{3}H]inositol polyphosphates. To characterize the mechanism(s) underlying the maintenance of normal basal $[Ca^{2+}]_i$ and partial Ca^{2+} pool depletion in Q212L-α_{16} mutant cells, we analyzed inositol polyphosphate levels by HPLC. The basal level of (1,4,5)IP_3 was not statistically elevated in Q212L-α_{16} cells (Fig. 6), despite the implication of the partial Ca^{2+} pool depletion and the increased Ca^{2+} permeability of intracellular stores in thapsigargin-treated cells noted above (Figs. 4 and 5). Our inability to detect an increase in (1,4,5)IP_3 in Q212L-α_{16} cells may be compromised by a high basal level of "silent" (1,4,5)IP_3, as previously described in AR4-2J cells (24). In fact, the mean of six measurements of the mass of (1,4,5)IP_3 by radioreceptor assay (25) was 9.1 ± 0.5 pmol/mg of protein, similar to the basal level in AR4-2J

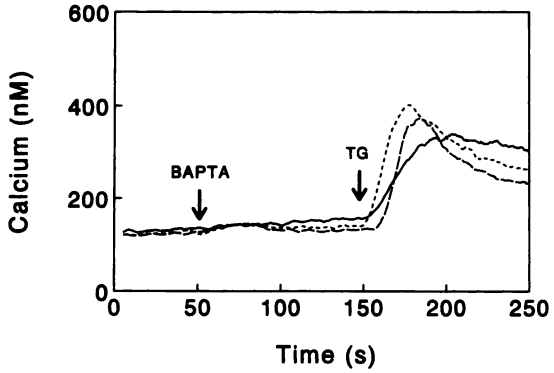

Fig. 4. Basal $[Ca^{2+}]_i$, basal Ca^{2+} influx, and the thapsigargin-induced $[Ca^{2+}]_i$ increase are equivalent in control and Q212L-α_{16} cells. Fura-2 loaded cells were suspended in 1.8 mM Ca^{2+} (37°). At the indicated times, cells were exposed to 3 mM BAPTA followed by 2 μM thapsigargin (*TG*). Representative traces are shown. *Solid line*, control cells; *dashed line*, Q212L-8 mutant cells; *dotted line*, Q212L-19 mutant cells.

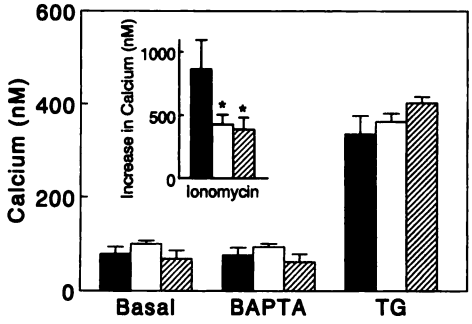

Fig. 5. Basal Ca^{2+} and the amount of thapsigargin (*TG*)-induced $[Ca^{2+}]_i$ increase are equivalent in control and Q212L-α_{16} cells. Compiled data were obtained as described in legend to Fig. 5 (five experiments). *Inset*, ionomycin-releasable Ca^{2+} is decreased in Q212L-α_{16} cells. Fura-2 loaded cells were suspended in Ca^{2+}-free buffer containing 0.3 mM EGTA. After 50 sec, cells were exposed to 10 μM ionomycin (three experiments). *Solid bars*, control cells; *open bars*, Q212L-8 mutant cells; *hatched bars*, Q212L-19 mutant cells. *, Differs significantly from control.

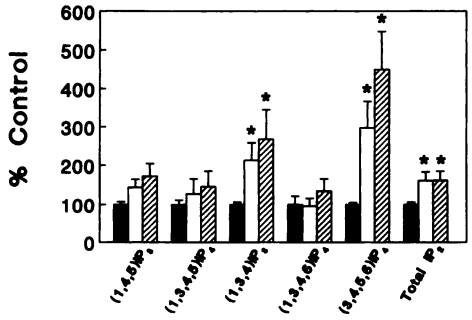

Fig. 6. Levels of [^{3}H]inositol polyphosphates in control and Q212L-α_{16} cells. Cells were labeled for 72 hr with *myo*-[^{3}H]inositol, and then [^{3}H]inositol polyphosphates were separated by HPLC and expressed as a ratio to total cell lipids. Data are presented relative to control values, set at 100% (10–12 experiments). *Solid bars*, control cells; *open bars*, Q212L-8 mutant cells; *hatched bars*, Q212L-19 mutant cells. Basal levels of [^{3}H](1,4,5)IP_3 in control cells represent an average of 1218 ± 199 dpm (eight experiments). *, Differs significantly from control.

cells (24). This would indicate an apparent basal concentration of ~2 μM, far in excess of the concentration of (1,4,5)IP_3 normally required for maximal Ca^{2+} mobilization. This likely is also the explanation, as discussed below, for the fact that activation of Ca^{2+} mobilization through PIC-linked receptors failed to significantly elevate (1,4,5)IP_3 levels. However, basal levels of inositol-1,3,4-trisphosphate and IP_2, metabolites produced by 5-phosphatase-mediated dephosphorylation of inositol-1,3,4,5-trisphosphate and (1,4,5)IP_3, respectively, were elevated in Q212L-α_{16} cells (Fig. 6). The basal elevation of these metabolites is most easily explained as a result of increased (1,4,5)IP_3 production. The increased level of inositol-3,4,5,6-tetrakisphosphate is believed to arise from a substrate cycle with inositol pentakisphospate (26) which generally occurs in parallel with PIC activation (27); its physiological significance in the present content is not known.

Agonist-stimulated Ca^{2+} release. Consistent with the decreased Ca^{2+} pool size noted above, the Ca^{2+} release in response to agonist stimulation was significantly decreased in Q212L-α_{16} cells relative to controls (Figs. 7 and 8). Ca^{2+} signals in response to both ATP, a purinergic agonist whose receptor is linked to activation of PIC-β via a transducing heterotrimeric G protein, and PDGF, a growth factor whose receptor is a tyrosine kinase that directly phosphorylates and

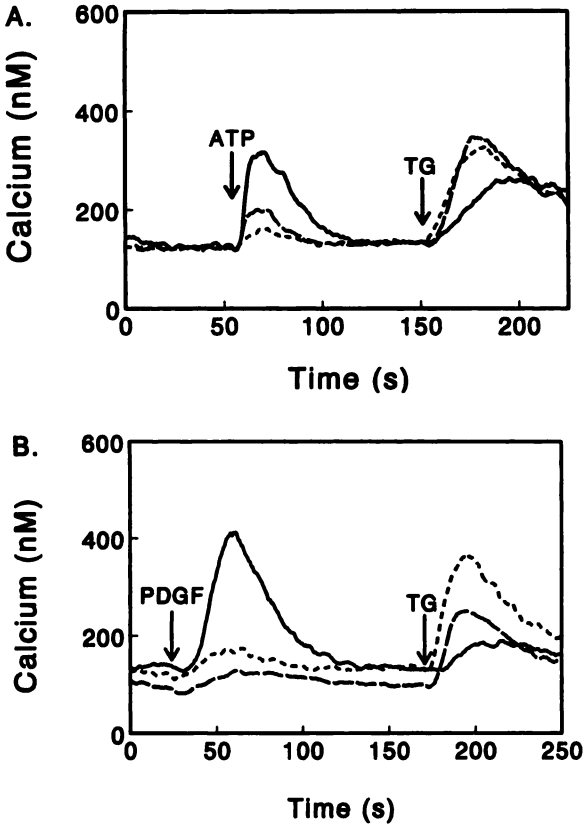

Fig. 7. The Ca^{2+} signal in response to agonist stimulation is decreased in Q212L-α_{16} cells. Fura-2 loaded cells were suspended in 1.8 mM Ca^{2+} plus 3 mM EGTA and stimulated with 1 mM ATP (A) or 50 ng/ml PDGF (B) followed by 2 μM thapsigargin (*TG*). Representative traces are shown. *Solid lines*, control cells; *dashed lines*, Q212L-8 mutant cells; *dotted lines*, Q212L-19 mutant cells.

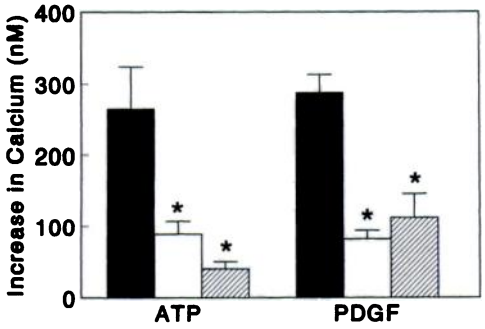

Fig. 8. ATP- and PDGF-stimulated Ca^{2+} release are decreased in Q212L-α_{16} cells. Compiled data were obtained as described in legend to Fig. 7 (four experiments). In some experiments, cells were resuspended in Ca^{2+}-free buffer plus 0.3 mM EGTA. *Solid bars*, control cells; *open bars*, Q212L-8 mutant cells; *hatched bars*, Q212L-19 mutant cells. *, Differs significantly from control.

activates PIC-γ (28), were decreased in Q212L-α_{16} cells. The diminished agonist responses probably resulted, in part, from the smaller Ca^{2+} pool size in Q212L-α_{16} cells. However, the agonists also seemed to release a smaller proportion of the intracellular Ca^{2+} stores; i.e., thapsigargin responses were not decreased by prior agonist treatment as much in Q212L-α_{16} cells as in control cells (Figs. 4 and 7 and data not shown). This result suggested that the decreased agonist response in Q212L-α_{16} cells is not totally accounted for by a decrease in Ca^{2+} pool size and thus may also involve either decreased agonist-stimulated production of (1,4,5)IP_3 or reduced Ca^{2+}-releasing efficacy of (1,4,5)IP_3.

Agonist-stimulated changes in [^{3}H]inositol polyphosphates. To evaluate the hypothesis that decreased agonist-stimulated Ca^{2+} signals in Q212L-α_{16} cells resulted from decreased agonist-stimulated production of (1,4,5)IP_3, we examined the effects of short term exposure of control and Q212L-α_{16} cells to ATP (30 sec) and PDGF (2 min) on levels of (1,4,5)IP_3 and its proximal metabolites. Agonist stimulation had no significant effect on (1,4,5)IP_3 levels in either control or mutant cells, whether measured by radioactivity or by radioreceptor assay (data not shown), perhaps because, as argued above for the effects of constitutive PIC activation, a relatively small increase in (1,4,5)IP_3 was obscured by a high level of "silent" (1,4,5)IP_3 (24). However, the agonist-stimulated increase in the proximal metabolites of (1,4,5)IP_3 (IP_2 plus inositol-1,3,4,5-tetrakisphosphate) in Q212L-α_{16} cells was the same or greater than that in control cells, suggesting that Q212L-α_{16} cells produced as much or more (1,4,5)IP_3 in response to agonist stimulation than control cells; the increase in control and Q212L-8 and Q212L-19 cells was 0.2 ± 0.1, 1.6 ± 0.4,* and 1.4 ± 0.7* dpm/dpm total lipid × 100 after 30 sec of ATP (1 mM) and 0.8 ± 0.2, 1.0 ± 0.2, and 1.8 ± 0.3* dpm/dpm total lipid × 100 after 2 min of PDGF (50 ng/ml) (*differs significantly from control). Consequently, we next examined the suggestion that the Ca^{2+}-releasing efficacy of (1,4,5)IP_3 was reduced in Q212L-α_{16} cells.

(1,4,5)IP_3-mediated ^{45}Ca release from intracellular stores. In agreement with the observed decrease in ionomycin-releasable Ca^{2+}, steady state ATP-dependent ^{45}Ca uptake was decreased in permeabilized Q212L-α_{16} cells relative to controls: steady state ^{45}Ca levels in control and Q212L-8 and Q212L-19 cells were 6.9 ± 0.4, 5.9 ± 0.6,* and 3.9 ± 0.4* nmol/mg protein, respectively (three or four experiments; *differs significantly from control). In addition, permeabilized Q212L-α_{16} cells released less of this steady state ^{45}Ca pool in response to (1,4,5)IP_3 (Fig. 9). This result confirmed that (1,4,5)IP_3 was less potent, and perhaps less efficacious, in releasing Ca^{2+} from intracellular Ca^{2+} stores in Q212L-α_{16} cells.

IP_3 receptor down-regulation in Q212L-α_{16} cells. One possible explanation for the decrease in Ins(1,4,5)P_3 respon-

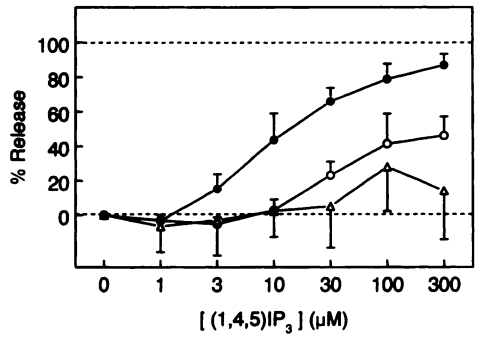

Fig. 9. (1,4,5)IP_3 released less ^{45}Ca in permeabilized Q212L-α_{16} cells. Cells were permeabilized with 50 μg/ml saponin and then loaded to steady state with ^{45}Ca in buffer containing 3 mM Mg-ATP (20–30 min). Loaded cells were exposed to the indicated concentration of (1,4,5)IP_3 for 1 min, and ^{45}Ca remaining in the cells was determined. Data are expressed as percentage of ATP-dependent ^{45}Ca uptake. Values are mean ± standard error of three experiments (●, ○, and △) performed in duplicate.

siveness observed in Q212L-α_{16} cells was a decrease in the number of IP_3 receptors. Therefore, we immunoblotted total cell proteins with a polyclonal antibody directed against rat cerebellar IP_3 receptor. Expression of wild-type α_{16} reduced the expression of IP_3 receptors. However, expression of GTPase mutant α_{16} (in Q212L-α_{16} cells) substantially suppressed the expression of IP_3 receptor protein below the level of either nontransfected NIH 3T3 cells or the wild-type transfected WT-3 cells (Fig. 10).

Acute regulation by PKC. Low level, persistent activation of PKC is one likely consequence of constitutive activation of PIC in Q212L-α_{16} mutant cells, and data consistent with this interpretation were recently obtained in Q212L-α_{16}-transformed Swiss 3T3 cells (15). Activation of PKC with phorbol ester has been reported to decrease the potency of (1,4,5)IP_3 to release Ca^{2+} in human neuroblastoma cells (29); therefore, we examined the effects of acute activation and inhibition of PKC on Ca^{2+} signaling in control and Q212L-α_{16} mutant NIH-3T3 cells. Activation of PKC by preincubation of cells with the phorbol ester PMA inhibited the ATP-stimulated increase in $[Ca^{2+}]_i$ in both control and mutant cells; however, PMA had no effect on the PDGF-stimulated increase in $[Ca^{2+}]_i$ (Table 1). These results suggested that PKC was acting at a proximal step in the ATP signaling pathway, perhaps through inhibition of PIC-β (28). Inhibition of PKC by the kinase inhibitor staurosporine had no effect on the ATP-stimulated Ca^{2+} transient but completely blocked the PDGF-stimulated transient in both control and mutant cells (data not shown), presumably because of nonspecific inhibition of the tyrosine kinase activity of the PDGF receptor. Preincubation of cells with the more selective PKC inhibitor Ro 31–8220 (30) also had no effect on ATP-stimulated Ca^{2+} signaling in control or mutant cells. The ability of staurosporine and Ro 31–8220 to inhibit PKC in this system was confirmed by the fact that preincubation with either of these inhibitors lessened PMA-mediated inhibition of ATP-stimulated Ca^{2+} signaling in control cells (data not shown). We conclude that the decreased responsiveness to agonists and to (1,4,5)IP_3 in Q212L-α_{16} cells is unlikely to be a consequence of persistently activated PKC.

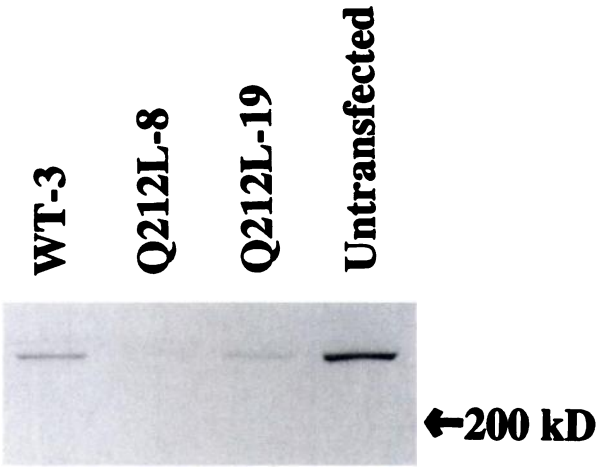

Fig. 10. Q212L-α_{16} cells contain less IP_3 receptor protein. Twenty micrograms of total cell homogenate was electrophoresed on a 7% polyacrylamide gel, transferred to nitrocellulose, and blotted with polyclonal antibody directed against rat cerebellar IP_3 receptor. The electrophoretic mobility of the protein band was identical to the mobility of IP_3 receptor in a control preparation of rat microsomal membranes (not shown). One of four similar experiments is shown. *WT*, wild-type.

TABLE 1

Effect of phorbol ester on agonist-stimulated increases in Ca^{2+} in control and Q212L-α_{16} mutant NIH-3T3 cells

Control and Q212L-α_{16} mutant cells were stimulated with either ATP (1 mM, 30 sec) or PDGF (50 ng/ml, 2 min). Ca^{2+} was determined in suspended cells preincubated for 15 min at room temperature with either PMA or the inactive PMA analog 4αPMA (1.6 μM), indicated with − below. Cells were then pelleted and resuspended in Ca^{2+}-free buffer plus 300 μM EGTA with or without PMA or 4αPMA at 37° immediately before agonist stimulation (three experiments unless otherwise noted).

Cell type	PMA	Increase in Ca^{2+}	
		ATP	PDGF
		nM	
Control	−	293 ± 72	257 + 37
	+	28 + 8[a]	221 + 45
Q212L-8	−	77 ± 28	110 + 16
	+	16 + 8	139 + 6
Q212L-19	−	51 ± 10	134 (n = 2)
	+	13 ± 5[a]	150 (n = 2)

NA = not assayed.
[a] Differs significantly from value obtained in the presence of 4αPMA.

Discussion

This is the first study to explore the effects of constitutive activation of PIC by stable transfection with GTPase-deficient G protein on cell Ca^{2+} homeostasis and inositol polyphosphate metabolism. The results suggest that growth inhibition in Q212L-α_{16} cells can be attributed, in part, to impaired Ca^{2+} signaling in response to mitogenic agonists due to down-regulation of IP_3 receptor number. Note that alterations in PIC activity and in cell growth do not necessarily indicate alterations in Ca^{2+} signaling. For example, Fee *et al.* (31) reported that down-regulation of PLC-β_1 and concomitant diminution in cell growth were not associated with substantial alterations in Ca^{2+} signaling. Presumably. in this system, other PIC forms are more important in generating Ca^{2+} signals.

Constitutive activation of G_{16} and cell proliferation. It is well established that activation of the PIC signaling pathway is associated with mitogenesis in several cell types and that overstimulation of this pathway can result in abnormal cell growth and oncogenesis (2). Constitutive activation of receptors coupled to PIC may result in cellular transformation (3, 4); similarly, a functional PIC signaling pathway has been found to be requisite for cellular transformation induced by growth factors and *ras* (32, 33). Whether constitutive activation of PIC by genetically altered GTPase-deficient G_q causes cellular transformation is more controversial. DeVivo *et al.* (5) reported that stable transfection with GTPase-deficient G_q resulted in transformation of NIH-3T3 cells but not of Rat-1 cells. The same researchers also demonstrated that the effects of the GTPase deficient G_q were dependent on the growth state of the cells (34). In confluent cells, PDGF caused little stimulation of thymidine incorporation in control cells but caused a substantial stimulation in cells expressing the mutant G_q; however, in subconfluent cells, PDGF markedly stimulated thymidine incorporation, and this was minimally augmented by the GTPase-deficient G_q (34). In contrast, a second group (13) found that transfection of NIH-3T3 cells with GTPase-deficient G_q was

cytotoxic. Kalinec *et al.* (6) reported that NIH-3T3 cells transfected with GTPase-deficient G_q displayed a transformed phenotype; however, the low colony-forming ability of these cells was indicative of a parallel growth-inhibitory effect. The mechanisms underlying the varied effects of constitutive activation of PIC on cell growth were not identified in these studies.

In our study, stable transfection of NIH-3T3 cells with GTPase-deficient G_{16} also caused inhibition of cell proliferation (Fig. 3) in the absence of cellular transformation. Our results suggest that the differences in cell growth inhibition and transformation with expression of $G_{\alpha q}$ family members may be related to secondary adaptive responses. For example, the secondary Ca^{2+} pool depletion observed in Q212L-α_{16} cells may be partially responsible for the decreased growth rate in these cells; previous reports have shown that prolonged Ca^{2+} pool depletion induced entry of smooth muscle cells into a quiescent G_0-like state (35–37). Thus, the biochemical responses downstream of constitutive PIC activity must be defined to understand the different cellular phenotypes in response to activated $G_{\alpha q}$ subunits.

Partial depletion of Ca^{2+} stores in Q212L-α_{16} cells. Despite our inability to directly document an increase in (1,4,5)IP_3, the biochemical evidence strongly suggests an increase in (1,4,5)IP_3 production in Q212L-α_{16} cells: three distinct markers of PIC activity were increased, and the levels of metabolites of (1,4,5)IP_3 were increased. In addition, Ca^{2+} stores in Q212L-α_{16} cells were partially depleted under basal conditions, and the Ca^{2+} permeability of thapsigargin-sensitive stores was increased (Figs. 4 and 5). These findings of decreased Ca^{2+} store size and increased Ca^{2+} store permeability are consistent with a basal elevation of (1,4,5)IP_3 in Q212L-α_{16} cells.

The absence of persistent activation of Ca^{2+} influx despite partial Ca^{2+} pool depletion in Q212L-α_{16} cells presents something of a paradox because depletion of intracellular stores is thought to act as a signal for calcium entry in nonexcitable cells, including 3T3 cells (38). The lack of Ca^{2+} entry in the Q212L-α_{16} cells may result from either desensitization of the influx pathway or a nonlinear relationship between pool depletion and activation of store-operated Ca^{2+} influx. In one study, partial desensitization of store-operated influx has been demonstrated in thapsigargin-treated HeLa cells (39). In another study, desensitization of influx was postulated based on the absence of store-operated Ca^{2+} influx in smooth muscle cells after overnight depletion of Ca^{2+} stores by thapsigargin (35). A nonlinear relationship between Ca^{2+} store depletion and activation of influx has been reported in human neutrophils (40). In the latter study, a 50% decrease in stored Ca^{2+} caused very little activation of influx, whereas further store depletion was proportional to activation of influx. If a similar relationship exists in NIH-3T3 cells, the 50% depletion of Ca^{2+} stores found in Q212L-α_{16} cells (Fig. 5) may be below threshold for significant activation of influx. Further experiments will be required to quantify the relationship between intracellular Ca^{2+} pool depletion and activation of Ca^{2+} influx and to explore the nature of desensitization of store-operated Ca^{2+} entry in NIH-3T3 cells.

Decreased responsiveness to agonists and to (1,4,5)IP_3 in Q212L-α_{16} cells. The observed decrease in agonist-induced Ca^{2+} release in Q212L-α_{16} cells in response to both a PIC-β and a PIC-γ coupled agonist suggested that the mechanism responsible was not directly related to G protein activation but rather occurred downstream from PIC activation. In a related study, Kalinec *et al.* (6) postulated that NIH-3T3 cells transfected with GTPase-deficient G_q accumulated less IP_3 in response to serum stimulation because these cells were selected for their ability to metabolize IP_3 rapidly. Such adaptive changes in signaling pathways in response to aberrant expression of one or more steps are not uncommon. Fee *et al.* (31) found that in Chinese hamster fibroblasts selected for diminished responsiveness to thrombin, a substantial diminution in the expression of PIC-β_1 was accompanied by increases in other PIC forms. In the current study, decreased agonist responses in Q212L-α_{16} cells can be accounted for by the combined effects of partial Ca^{2+} pool depletion, a direct effect of stimulated PIC activity, and down-regulation of IP_3 receptor number, likely an adaptive response to prolonged (1,4,5)IP_3 elevation. Also, a contribution from altered (1,4,5)IP_3 metabolism cannot be ruled out.

The decrease in IP_3 receptor number in Q212L-α_{16} cells exhibiting constitutively activated PIC hydrolysis is similar to the reported down-regulation of IP_3 receptor number in cultured human neuroblastoma cells occurring after prolonged exposure to the PIC-linked agonist carbachol (29, 41, 42). In one study (41), exposure of cells to carbachol for 6 hr caused a 90% decrease in IP_3 receptor number. This was shown to be accompanied by a 3-fold increase in the EC_{50} for (1,4,5)IP_3-mediated ^{45}Ca release from permeabilized cells and a decrease in the maximal extent of ^{45}Ca release to 34% (29). Down-regulation resulted from an increased rate of receptor degradation and required the presence of functional Ca^{2+} stores and persistent elevation of (1,4,5)IP_3 (41). The authors' conclusion that the signal for increased IP_3 receptor degradation was the increased efflux of Ca^{2+} from intracellular stores via the IP_3 receptor is consistent with the data obtained in the current study, although the mechanism(s) responsible for IP_3 receptor down-regulation in Q212L-α_{16} cells have not been explored.

In a second study, Honda *et al.* (43) demonstrated that microinjection of mRNAs for the platelet-activating factor receptor and constitutively activated G_q into *Xenopus* oocytes caused transient inhibition of platelet-activating factor-activated Cl^- current and down-regulation of IP_3 receptors. These authors invoked IP_3 receptor down-regulation as a potential mechanism for heterologous desensitization in inflammatory cells. Taken together, these results (29, 41–43) and our results suggest that IP_3 receptor regulation may be a general mechanism for termination or attenuation of prolonged activation of PIC-linked receptor signaling. In Q212L-α_{16} cells, down-regulation of IP_3 receptor number allowed these cells to maintain a physiological basal Ca^{2+} concentration and partially filled Ca^{2+} stores, at the expense of decreased agonist responsiveness and diminished cell growth rate.

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References

1. Berridge, M. J. Inositol trisphosphate and calcium signalling. *Nature (Lond.)* **361:**315–325 (1993).
2. Rozengurt, E. Early signals in the mitogenic response. *Science (Washington D. C.)* **234:**161–166 (1986).
3. Julius, D., T. J. Livelli, T. M. Jessell, and R. Axel. Ectopic expression of the serotonin 1C receptor and the triggering of malignant transformation. *Science (Washington D. C.)* **244:**1057–1062 (1989).
4. Gutkind, J. S., E. A. Novotny, M. R. Brann, and K. C. Robbins. Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. *Proc. Nat. Acad. Sci. USA* **88:**4703–4708 (1991).
5. De Vivo, M., J. Chen, J. Codina, and R. Iyengar. Enhanced phospholipase C stimulation and transformation in NIH-3T3 cells expressing Q209LG_q-α-subunits. *J. Biol. Chem.* **267:**18263–18266 (1992).
6. Kalinec, G., A. J. Nazarili, S. Hermouet, N. Xu, and J. S. Gutkind. Mutated α subunit of the G_q protein induces malignant transformation in NIH-3T3 cells. *Mol. Cell. Biol.* **12:**4687–4693 (1992).
7. Bourne, H. R., D. A. Sanders, and F. McCormick. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.)* **349:**117–127 (1991).
8. Freissmuth, M., and A. G. Gilman. Mutations of $G_{s\alpha}$ designed to alter the reactivity of the protein with bacterial toxins. *J. Biol. Chem.* **264:**21907–21914 (1990).
9. Hermouet, S., J. Merendino, Jr., S. Gutkind, and A. M. Spiegel. Activating and inactivating mutations of the α subunit of G_{i2} protein have opposite effects on proliferation of NIH-3T3 cells. *Proc. Nat. Acad. Sci. USA* **88:**10455–10459 (1991).
10. Wong, Y. H., A. Federman, A. M. Pace, I. Zachary, T. Evans, J. Pouyssegur, and H. R. Bourne. Mutant α subunits of G_{i2} inhibit cyclic AMP accumulation. *Nature (Lond.)* **351:**63–65 (1991).
11. Kroll, S. D., J. Chen, M. De Vivo, D. J. Carty, A. Buku, R. T. Premont, and R. Iyengar. The Q205LG_o-α subunit expressed in NIH-3T3 cells induces transformation. *J. Biol. Chem.* **267:**23183–23188 (1992).
12. Conklin, B. R., O. Chabre, Y. H. Wong, A. D. Federman, and H. R. Bourne. Recombinant $G_q\alpha$: mutational activation and coupling to receptors and phospholipase C. *J. Biol. Chem.* **267:**31–34 (1992).
13. Wu, D., C. H. Lee, S. G. Rhee, and M. Simon. Activation of phospholipase C by the α subunits of the G_q and G_{11} proteins in transfected Cos-7 cells. *J. Biol. Chem.* **267:**1811–1817 (1992).
14. Schnabel, P., R. Schreck, D. L. Schiller, M. Camps, and P. Gierschik. Stimulation of phospholipase C by a mutationally activated G protein α_{16} subunit. *Biochem. Biophys. Res. Commun.* **188:**1018–1023 (1992).
15. Amatruda, D., III, A. Steele, V. Z. Slepak, and M. I. Simon. G alpha 16, a G protein alpha subunit specifically expressed hematopoietic cells. *Proc. Nat. Acad. Sci. USA* **88:**5587–5591 (1991).
16. Qian, N.-X., M. Russel, A. Mette Buhl, and G. L. Johnson. Expression of GTPase-deficient $G\alpha_{16}$ inhibits Swiss 3T3 cell growth. *J. Biol. Chem.* **269:**17417–17423 (1994).
17. Downes, C. P., P. T. Hawkins, and R. F. Irvine. Inositol 1:3,4,5-tetrakisphosphate and not phosphatidylinositol 3,4-bisphosphate is the probable precursor of inositol 1,3,4-trisphosphate in agonist-stimulated parotid gland. *Biochem. J.* **238:**501–506 (1986).
18. Shears, S. B., D. J. Storey, A. J. Morris, A. B. Cubitt, J. B. Parry, R. H. Michell, and C. J. Kirk. Dephosphorylation of *myo*-inositol 1:4,5-trisphosphate and *myo*-inositol 1,3,4-trisphosphate. *Biochem. J.* **242:**393–402 (1987).
19. Menniti, F. S., K. G. Oliver, K. Nogimori, J. F. Obie, S. B. Shears, and J. W. Putney, Jr. Origins of myoinositol tetrakisphosphates in agonist-stimulated rat pancreatoma cells: stimulation by bombesin of myoinositol (1,3,4,5,6)pentakisphosphate breakdown to myoinositol (3,4,5,6)tetrakisphosphate. *J. Biol. Chem.* **265:**11167–11176 (1990).
20. Sugiya, H., K. A. Tennes, and J. W. Putney, Jr. Homologous desensitization of substance-P-induced inositol polyphosphate formation in rat parotid acinar cells. *Biochem. J.* **244:**647–653 (1987).
21. Sharp, A. H., S. H. Snyder, and S. K. Nigam. Inositol 1:4,5-trisphosphate receptors: localization in epithelial tissue. *J. Biol. Chem.* **267:**7444–7449 (1992).
22. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450 (1986).
23. Morgan, A. J., and R. Jacob. Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. *Biochem. J.* **300:**665–672 (1994).
24. Bird, G. S. J., K. G. Oliver, D. A. Horstman, J. F. Obie, and J. W. Putney, Jr. Relationship between the calcium-mobilizing action of inositol 1:4,5-trisphosphate in permeable AR4–2J cells and the estimated levels of inositol 1,4,5-trisphosphate in intact AR4–2J cells. *Biochem. J.* **273:**541–546 (1991).
25. Challiss, R. A., I. H. Batty, and S. R. Nahorski. Mass measurements of inositol(1,4,5)trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem. Biophys. Res. Commun.* **157:**684–691 (1988).
26. Shears, S. B. Metabolism of inositol phosphates. *Adv. Second Messenger Phosphoprotein Res.* **26:**63–92 (1992).
27. Menniti, F. S., K. G. Oliver, J. W. Putney, Jr., and S. B. Shears. Inositol phosphates and cell signalling: new views of $InsP_5$ and $InsP_6$. *Trends Biochem. Sci.* **18:**53–56 (1993).
28. Rhee, S. G., and K. D. Choi. Regulation of inositol phospholipid-specific phospholipase C isozymes. *J. Biol. Chem.* **267:**12393–12396 (1992).
29. Wojcikiewicz, R. J., and S. R. Nahorski. Chronic muscarinic stimulation of SH-SY5Y neuroblastoma cells suppresses inositol 1,4,5-trisphosphate action. *J. Biol. Chem.* **266:**22234–22241 (1991).
30. Dieter, P., and E. Fitzke. Ro 31–8220 and Ro 31–7549 show improved selectivity for protein kinase C over staurosporine in macrophages. *Biochem. Biophys. Res. Commun.* **181:**396–401 (1991).
31. Fee, J. A., J. D. Monsey, R. J. Handler, M. A. Leonis, S. R. Mullaney, H. M. Hope, and D. F. Silbert. A Chinese hamster fibroblast mutant defective in thrombin-induced signaling has a low level of phospholipase C-β_1. *J. Biol. Chem.* **269:**21699–21708 (1994).
32. Matuoka, K., K. Fukami, O. Nakanishi, S. Kawai, and T. Takenawa. Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. *Science (Washington D. C.)* **247:**1074–1077 (1990).
33. Smith, M. R., Y. -L. Liu, H. Kim, S. G. Rhee, and H.-F. Kung. Inhibition of serum- and ras-stimulated DNA synthesis by antibodies to phospholipase C. *Science (Washington D. C.)* **247:**1074–1077 (1990).
34. De Vivo, M. and R. Iyengar. Activated Gq-α potentiates platelet-derived growth factor-stimulated mitogenesis in confluent cell cultures. *J. Biol. Chem.* **269:**19671–19674 (1994).
35. Ghosh, T. K., J. Bian, A. D. Short, S. L. Rybak, and D. L. Gill. Persistent intracellular calcium pool depletion by thapsigargin and its influence on cell growth. *J. Biol. Chem.* **266:**24690–24697 (1991).
36. Short, A. D., J. Bian, T. K. Ghosh, R. T. Waldron, S. L. Rybak, and D. L. Gill. Intracellular Ca^{2+} pool content is linked to control of cell growth. *Proc. Nat. Acad. Sci. USA* **90:**4986–4990 (1993).
37. Waldron, R. T., A. D. Short, J. J. Meadows, T. K. Ghosh, and D. L. Gill. Endoplasmic reticulum calcium pump expression and control of cell growth. *J. Biol. Chem.* **269:**11927–11933 (1994).
38. Villereal, M. L., and K. L. Byron. Calcium signals in growth factor signal transduction. *Rev. Physiol. Biochem. Pharmacol.* **119:**67–121 (1992).
39. Missiaen, L., J. B. Parys, H. DeSmedt, M. Oike, and R. Casteels. Partial calcium release in response to submaximal inositol 1:4,5-trisphosphate receptor activation. *Mol. Cell. Endocrinol.* **98:**147–156 (1994).
40. Montero, M., J. Alvarez, and J. Garcia-Sancho. Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular calcium stores. *Biochem. J.* **277:**73–79 (1991).
41. Wojcikiewicz, R. J. H., T. Furuichi, S. Nakade, K. Mikoshiba, and S. Nahorski. Muscarinic receptor activation down-regulates the type I inositol 1:4,5-trisphosphate receptor by accelerating its degradation. *J. Biol. Chem.* **269:**7963–7969 (1994).
42. Wojcikiewicz, R. J. H. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.* **270:**11678–11683 (1995).
43. Honda, Z., T. Takano, N. Hirose, T. Suzuki, A. Muto, S. Kume, K. Mikoshiba, K. Itoh, and T. Shimizu. G_q pathway desensitizes chemotactic receptor-induced calcium signaling via inositol trisphosphate receptor down-regulation. *J. Biol. Chem.* **270:**4840–4844 (1995).

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