

# Mediation of Growth Factor Induced DNA Synthesis and Calcium Mobilization by $G_q$ and $G_{i2}$

V. J. LaMorte\*<sup>‡</sup>§, A. T. Harootunian<sup>‡</sup>||, A. M. Spiegel<sup>†</sup>, R. Y. Tsien<sup>‡</sup>||, and J. R. Feramisco\*<sup>‡</sup>§

University of California, San Diego, Departments of \*Medicine and ‡Pharmacology, §Cancer Center, ||Howard Hughes Institute, La Jolla, California 92093; and †Molecular Pathophysiology Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

**Abstract.** A newly identified subclass of the heterotrimeric GTP binding regulatory protein family,  $G_q$ , has been found to be expressed in a diverse range of cell types. We investigated the potential role of this protein in growth factor signal transduction pathways and its potential relationship to the function of other  $G\alpha$  subclasses. Recent biochemical studies have suggested that  $G_q$  regulates the  $\beta 1$  isozyme of phospholipase C ( $PLC\beta_1$ ), an effector for some growth factors. By microinjection of inhibitory antibodies specific to distinct  $G\alpha$  subunits into living cells, we have determined that  $G_{\alpha_q}$  transduces bradykinin- and thrombin-stimulated intracellular calcium transients which are likely to be mediated by  $PLC\beta_1$ . Moreover, we found that  $G_{\alpha_q}$  function is required for the mitogenic action of both of these growth factors. These results indicate that both thrombin and bradykinin utilize  $G_q$  to couple

to increases in intracellular calcium, and that  $G_q$  is a necessary component of the mitogenic action of these factors. While microinjection of antibodies against  $G_{\alpha_{i2}}$  did not abolish calcium transients stimulated by either of these factors, such microinjection prevented DNA synthesis in response to thrombin but not to bradykinin. These data suggest that thrombin-induced mitogenesis requires both  $G_q$  and  $G_{i2}$ , whereas bradykinin needs only the former. Thus, different growth factors operating upon the same cell type use overlapping yet distinct sets of  $G\alpha$  subtypes in mitogenic signal transduction pathways. The direct identification of the coupling of both a *pertussis* toxin sensitive and insensitive G protein subtype in the mitogenic pathways utilized by thrombin offers an *in vivo* biochemical clarification of previous results obtained by pharmacologic studies.

**S**IGNAL transduction mechanisms for growth factor receptors in the plasma membrane have conventionally been classified into two predominant types. One type involves a mechanism in which receptors that span the membrane a single time utilize an intrinsic protein tyrosine kinase activity (Hunter, 1991; Cantley et al., 1991) whereas the other type is for receptors which span the membrane seven times and couple to heterotrimeric G proteins (Dohlman et al., 1991; Gupta et al., 1992). For the latter case, while it is thought that each of these receptors couple to a single subtype of heterotrimeric G protein, numerous signaling pathways are activated by these receptors; and this suggests that multiple coupling mechanisms may be utilized. Presently, there are 15 genes encoding the  $\alpha$  subunits of heterotrimeric G proteins (Simon et al., 1991). The best studied subtype is  $G_{\alpha_s}$ , which activates adenylylcyclase, while, a second subclass,  $G_{\alpha_i}$ , is best known as an inhibitor of this effector (Gilman, 1987). This diversity is thought to underlie the numerous stimuli and effectors that utilize this general type of signal transduction mechanism.

A newly identified subclass of this family,  $G_q$ , has recently been suggested to couple to  $PLC\beta_1$  by biochemical studies (Smrcka et al., 1991; Taylor et al., 1991; Berstein et al., 1992). This new subclass possesses four members, two

of which have  $\alpha$  subunits that are 88% homologous,  $G_{\alpha_{i1}}$  and  $G_{\alpha_q}$ . The carboxy termini of  $G_{\alpha_q}$  and  $G_{\alpha_{i1}}$  are identical, and they lack sites for ADP-ribosylation by *pertussis* toxin (Strathmann and Simon, 1990). These biochemical findings make  $G_q$  a likely candidate for the activity called  $G_p$ , which is responsible for GTP-dependent PIP<sub>2</sub> hydrolysis by PLC in a *pertussis* toxin insensitive manner (Gutowksi et al., 1991). This activity leads to the subsequent rise in intracellular calcium (calcium transient). The role of this subclass of G proteins in living cell systems and its potential role in mitogenesis has not as yet been explored.

Identification of a specific subtype of heterotrimeric G protein that provides a functional coupling between a specific hormone receptor, its corresponding second messenger effectors, and downstream events has proven to be a complex task. While it is thought that G proteins transduce signals from numerous hormone receptors to a variety of cellular effectors, only in a few cases is it possible to make a clear assignment of a signal pathway. One approach to establishing functional relationships has been based upon reconstitution of the receptors, G proteins, and effectors. This method has been proven powerful but can be confounded by the ability of different subtypes to substitute for one another in reconstitution studies (Jones et al., 1990;

Kurose et al., 1991). A second approach that is, as yet, less well utilized but promising has been through the microinjection of antisense oligonucleotides into living cells to block expression of discrete G proteins (Kleuss et al., 1991; Wang et al., 1992).

As an alternative approach to establishing the functional role of particular G proteins in signaling pathways in living cells, we have combined the use of functionally inhibitory antibodies specific to the carboxy-terminal regions of G proteins and needle microinjection into living somatic cells. This approach has been shown to be an effective means to inhibit the function of single classes of G proteins in living cells. It results in a rapid onset of inhibition and is not dependent on the turnover rate of the G protein as is the case for antisense oligonucleotides. The antibodies used for microinjection have been raised to various members of the G protein family through the use of distinct carboxy-terminal peptide sequences as antigens. These sequences are found in the region of each G protein that is thought to interact with an appropriate receptor, and antibodies directed against these sequences have been shown to uncouple G proteins from their respective receptors *in vitro* (Simonds et al., 1989a,b; Shenker et al., 1991). By microinjection, these inhibitory antibodies can be introduced into living cells and the resultant phenotype of the cells may be determined. This approach, for example, has been used to identify the role of  $G_{i2}$  in serum-stimulated mitogenesis of fibroblasts (La Morte et al., 1992); and  $G_s$  in the mediation of TSH-induced DNA synthesis in thyroid cells (Meinkoth et al., 1992).

In the present experiments, we have examined the role of  $G_q$  in the mobilization of intracellular calcium in response to a host of growth factors including PDGF, bradykinin, thrombin, and vasopressin. All of the examined factors appear to stimulate a similar response in cells with respect to an increased formation of the second messenger inositol (1,4,5) trisphosphate and a subsequent increase in intracellular calcium upon interaction with their respective receptors (Berridge and Irvine, 1989). The mechanisms responsible for these responses, however, appear to be quite distinct. PDGF appears to increase phosphatidylinositol hydrolysis through its intrinsic tyrosine kinase activity and subsequent activation of the  $\gamma$  isozyme of phospholipase C (Cantley et al., 1991). Bradykinin induced phosphatidylinositol hydrolysis and subsequent mobilization of intracellular calcium is thought to be regulated by the putative G protein,  $G_p$ , which is *pertussis* toxin insensitive (Vicentini and Villereal, 1984); while for thrombin, it has been reported to be both *pertussis* toxin sensitive (Chambard et al., 1987) and insensitive (Hung et al., 1992). In support of this, recent biochemical experiments have suggested that the  $G_q$  subfamily may regulate bradykinin activation of PLC in membranes *in situ*. (Gutowski et al., 1991). The present studies offer further identification of the molecular connections between these receptors and the subsequent transduction reactions.

## Materials and Methods

### Cell Culture

Mouse Balb/c3T3 fibroblasts (A31) were obtained from American Type Culture Collection (Rockville, MD) and propagated in DME (Fischer Scientific Co., Pittsburg, PA) containing 10% calf serum (Colorado Serum

Co., Denver, CO) at 37°C in an atmosphere containing 10% CO<sub>2</sub> as described by American Type Culture Collection. Rat embryo fibroblasts (REF52) were obtained from Cold Spring Harbor Laboratory (Cold Spring Harbor, NY) and propagated in DME containing 10% FBS (Gemini Bio-Products Inc., Calabasas, CA). For calcium ratio imaging experiments, both cell types were plated on 25-mm etched coverslips (Bellco Glass, Vineland, NJ); and for DNA synthesis experiments, cells were plated on 12-mm coverslips (Fisher Scientific Co.). Balb/c3T3 cells were rendered quiescent by starvation in DME containing 0.05% calf serum before injection whereas REF52 cells were starved in DME containing 0.05% FCS for 24 h.

### *Pertussis Toxin Treatment*

Quiescent cells were treated with *Bordetella pertussis* toxin (Sigma) at 25 and 50 ng/ml for 2 h and subsequently transferred to Hepes DME containing 0.05% calf serum for calcium ratio imaging experiments.

### Western Analysis

A 60-mm plate of logarithmic Balb/c3T3 and REF52 cells was solubilized in 200  $\mu$ l of 0.5% NP-40/PBS for 5 min on a rocker at 4°C. The supernatant was transferred to an Eppendorf tube and 200  $\mu$ l of 5 $\times$  Laemmli sample buffer was added. The tube was boiled for 15 min and 40  $\mu$ l of each sample was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was transferred to nitrocellulose and incubated with 5  $\mu$ g/ml of the affinity-purified QL antiserum. A secondary goat anti-rabbit biotinylated antibody (Vector Laboratories Inc., Burlingame, CA) and streptavidin-alkaline phosphatase colorimetric detection system (Bio Rad Laboratories, Hercules, CA) was used.

### Microinjection

Cells were microinjected with 10 mg/ml of affinity purified polyclonal antisera in microinjection buffer containing 100 mM KCl and 5 mM Na<sub>2</sub>PO<sub>4</sub>. In brief, QL antiserum was raised to a synthetic decapeptide (QLNLKEYNLV) which corresponds to the carboxy terminus of the  $\alpha$  subunit and is identical for both  $G_q$  and  $G_{11}$  (Shenker et al., 1991) AS antiserum was raised to a synthetic decapeptide (KENLKDCGLF) which is specific for  $G_{\alpha 2}$  in Balb/c3T3 fibroblasts (Simonds et al., 1989a). QQ, a control antiserum to GPA I, a yeast G protein, was generated to the carboxy terminus (QQNLKKIGI) and has no known homology with any mammalian G proteins (Shenker et al., 1991). Quiescent cells were microinjected with approximately  $5 \times 10^{-14}$  L/cell using an automated Eppendorf microinjection system and a Zeiss axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) which introduced  $>10^6$  molecules of IgG into each cell.

### Calcium Ratio Imaging

The methods and design for single cell calcium ratio imaging are described in detail in Tsien and Harootunian (1990). In brief, etched coverslips (Bellco Glass) with a defined injected area were loaded with fura-2/AM (Molecular Probes Inc., Eugene, OR) by incubating cells in a solution of DME containing 200 nM fura2/AM buffered with 20 mM Hepes at room temperature (23°C) for 1 h. Cells were imaged with a Zeiss im35 microscope and imaging system as described previously (Tsien and Harootunian, 1990). Growth factors were applied in a single bolus with a final concentration of 25 ng/ml PDGF (Upstate Biotechnology Inc., Lake Placid, NY), 5 U/ml thrombin (Sigma Chemical Co., St. Louis, MO), 1  $\mu$ M bradykinin (Calbiochem-Novabiochem Corp., La Jolla, CA), 50 ng/ml bombesin, 50 nM vasopressin, 10 nM and 20 nM insulinlike growth factor II (Upstate Biotechnology, Inc.), and 125 ng/ml insulinlike growth factor I (Upstate Biotechnology, Inc.). While 0.5 U/ml of thrombin elicited calcium transients equivalent to those seen for 5 U/ml, we routinely used the higher dose in these experiments to facilitate a rapid delivery of a single bolus of drug. Approximately 5 min elapsed between growth factor stimulation. After calcium imaging, the cells were fixed in 3.7% formaldehyde/PBS for 5 min and subsequently permeabilized with 0.3% Triton X-100 (Sigma Chemical Co.) for 5 min. The injected antibody was detected with a secondary goat anti-rabbit antibody conjugated to rhodamine (Cappell Laboratories, Malvern, PA) at 1:100 dilution of the stock concentration in 0.5% Nonidet P-40 (Sigma Chemical Co.). Its fluorescence intensity was calculated and assigned arbitrary units in order to quantitate the amount of injected antibody in each cell.

## DNA Synthesis

3 h postmicroinjection, Balb/c3T3 cells were stimulated with either 1  $\mu$ M bradykinin or 0.5 U/ml thrombin in DME containing 0.1% calf serum. For thrombin, 0.5 U/ml and 5 U/ml were found to both elicit maximal DNA synthesis. At this time bromodeoxyuridine (Amersham Corp., Arlington Heights, IL) was added to the media. Cells were fixed and stained 24 h after stimulation as previously described (La Morte et al., 1992) except that a secondary goat anti-mouse antibody conjugated to rhodamine (Cappell Laboratories) was used. The percentage of antibody-injected cells that were positive for BrdU incorporation was compared to the percentage of uninjected cells that were positive for BrdU incorporation. Background levels of DNA synthesis in DME containing only 0.1% calf serum were determined and subtracted from the percentage of injected and uninjected cells to calculate the percent reduction in DNA synthesis. A paired *t* test analysis  $\pm$  background was performed.

## Results

### Calcium Transients in Growth Factor Action

To establish assays for the diverse roles of G proteins in growth factor signaling suitable for the microinjection approach, several mitogens were tested for their ability to mobilize intracellular calcium in Balb/c3T3 fibroblasts. Mobilization of intracellular calcium in living cells was quantitated by single cell ratio imaging using the fluorescent calcium indicator dye, fura-2 (Tsien and Harootunian, 1990). This calcium transient results from the production of inositol (1,4,5)trisphosphate from the enzymatic hydrolysis of PIP2 from PLC (Berridge and Irvine, 1989). Bradykinin, thrombin, PDGF, and bombesin, but not vasopressin, IGF-I, or IGF-II elicited transient increases in intracellular calcium in quiescent Balb/c3T3 fibroblasts. To further clarify potential mediators of this response for these agonists, the sensitivity of the response to the ADP-ribosylating agent *Bordetella pertussis* toxin was determined. Calcium transients produced by bradykinin, thrombin, or PDGF appeared to be insensitive to *pertussis* toxin, whereas the transient produced by bombesin was reduced by the toxin (data not shown). These pharmacological data suggested that bradykinin, thrombin, and PDGF stimulate calcium transients in a *pertussis* toxin insensitive manner as expected. Additionally, REF 52 fibroblasts were tested and found to respond to vasopressin, bradykinin, and PDGF; and these cells were used in experiments as noted.

The newly identified subclass of G proteins,  $G_q$ , possesses a carboxy terminus lacking the cysteine residue that is the site of ADP-ribosylation and as a result is *pertussis* toxin insensitive. To investigate a potential role for  $G_q$  in the induction of calcium transients in these cells, we first determined that this protein was expressed in these cells. Western blot analysis showed that a 42-kD band corresponding to the mol wt of the  $\alpha$  subunit of the  $G_q$  family was identifiable (Fig. 1). Upon confirmation of the presence of this G protein  $\alpha$  subunit, we performed experiments to determine if a number of the  $G_q$  family is the G protein responsible for calcium transients in response to various growth factors. Microinjection of the affinity-purified antibodies specific for the carboxy-terminus decapeptide sequence of the  $\alpha$  subunit of  $G_{q,11}$ ,  $G_{12}$ , or a yeast G protein, GPA I into quiescent Balb/c3T3 fibroblasts was performed. Cells injected were readily identifiable because they were grown on numerically gridded coverslips and could be immunostained upon termination of the experiments for the presence of in-

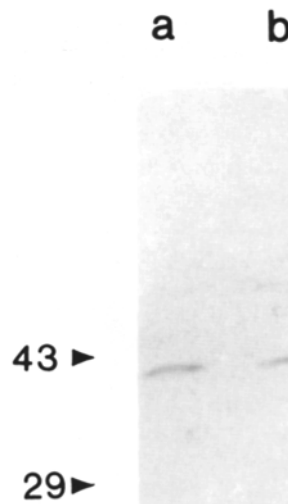
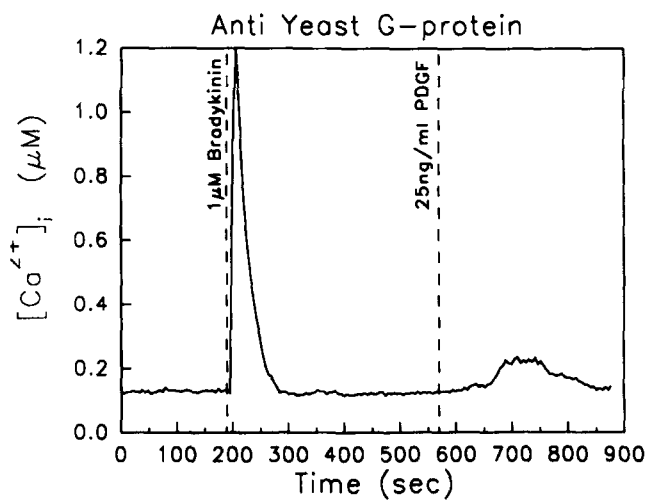
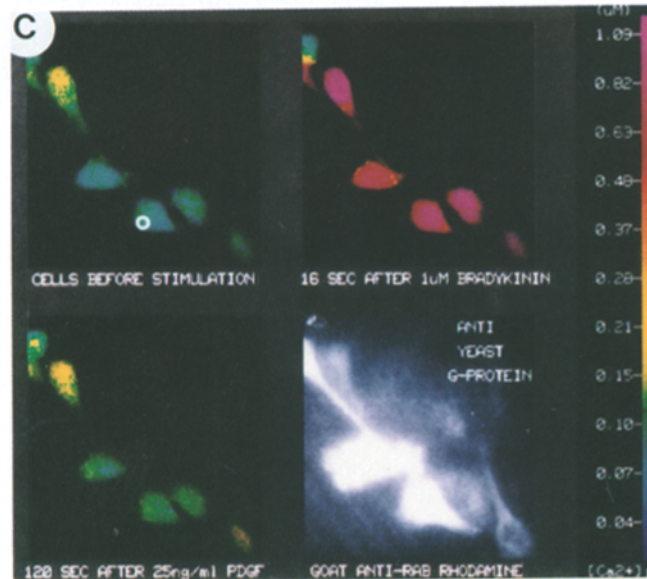
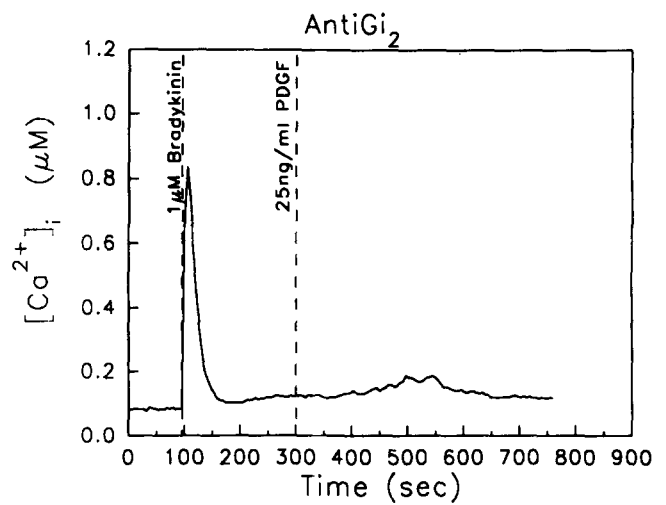
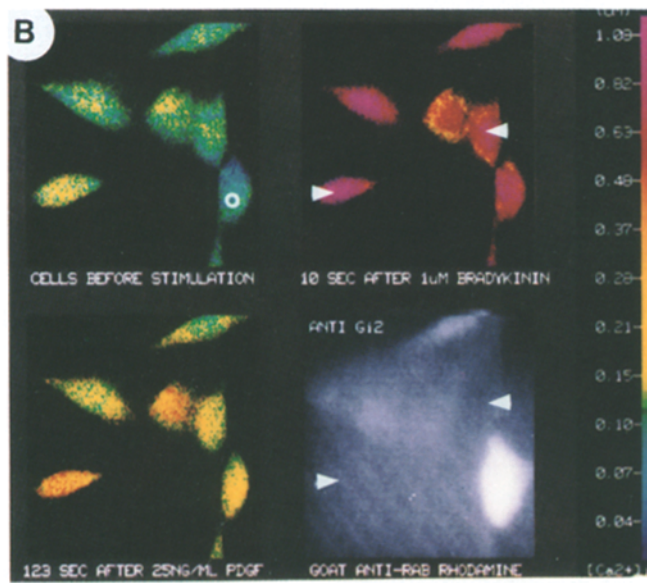
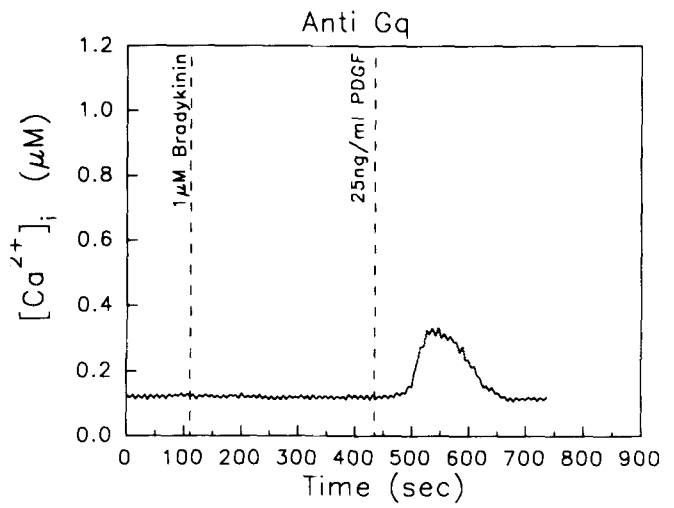
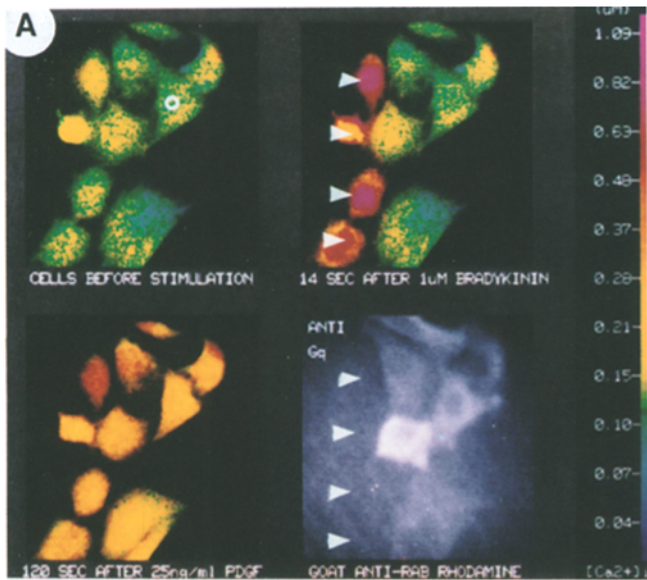


Figure 1. Identification of  $G_{\alpha_q}$  in Balb/c3T3 and REF 52 fibroblasts by Western analysis. Experimental details are described under Materials and Methods. The QL antiserum specific for  $G_{\alpha_{q,11}}$  recognize a 42-kD band in lane a Balb/c3T3 fibroblasts and lane b REF 52 fibroblasts.

jected IgG. 4–6 h postinjection, the cells were loaded with the calcium indicator fura-2. 1 h later, the cells were stimulated with bradykinin or thrombin and examined for calcium transients. Shortly thereafter, the cells were stimulated with PDGF as a control for those cells that did not mobilize intracellular stores of calcium to bradykinin, i.e., to show that inhibited cells are capable of eliciting a calcium response to a tyrosine kinase mediated growth factor. Fig. 2 shows the results of such an experiment. Each panel of four images (i.e., A–C) shows a different field of cells injected with one of the antibody preparations, while the same field is shown within each panel (A–C). The top left of each matrix illustrates cells loaded with fura-2 that have not been stimulated with any of the factors. The top right depicts cells stimulated with 1  $\mu$ M bradykinin and the bottom left depicts the same cells stimulated with 25 ng/ml PDGF. After quantitation of the calcium response to bradykinin or thrombin followed by PDGF, the cells were fixed and stained for the injected antibody to allow for unambiguous identification of the injected cells. The bottom right of each panel shows the stained cells. As seen in Fig. 2 A, the cells injected with anti  $G_{\alpha_{q,11}}$  (QL) did not show a calcium transient in response to bradykinin but did to subsequent stimulation with PDGF as a control. Those cells that were uninjected as indicated by the arrows were able to produce calcium transients in response to both bradykinin and PDGF. To determine if the block of the calcium transient was a specific effect of anti  $G_{\alpha_{q,11}}$ , antibodies to a *pertussis* toxin-sensitive G protein,  $G_{12}$ , were injected. Previous studies have established the presence of this protein in these cells and an involvement in serum stimulated mitogenesis (La Morte et al., 1992). Cells injected with these antibodies (AS) elicited a normal response to bradykinin and subsequently to PDGF (Fig. 2 B). As an additional control, the cells were injected with a control antibody, QQ, raised to the carboxy terminus of the yeast G protein GPA I (Fig. 2 C). The QQ-injected cells responded normally to both bradykinin and PDGF stimulation. The calcium tracing to



the right of each matrix corresponds to an individual cell in the field and is indicated by the open white circle. These data show a virtual quantitative block of the calcium transient induced by bradykinin by anti-G $\alpha_{q,11}$ .

To further establish that the block of the calcium transient seen by anti-G $\alpha_{q,11}$  was specific, we preabsorbed the antibody with the antigen peptide to which it was raised. The remaining solution was then microinjected into cells. The preabsorbed antibodies did not inhibit the calcium transients induced by bradykinin (data not shown). The inhibition of bradykinin-induced calcium transients by anti-G $\alpha_{q,11}$  was also observed in REF52 fibroblasts. As a control, no inhibition was seen in response to PDGF. In response to vasopressin stimulation, anti-G $\alpha_{q,11}$ -injected cells exhibited a partial block (data not shown).

Fig. 3 (i.e., A-C) depicts three fields of cells that have been injected with the same series of affinity purified antibodies, but in this case were stimulated with 5 U/ml thrombin and subsequently with PDGF. Fig. 3 A demonstrates that antibodies specific for G $_q$  inhibited calcium mobilization in response to thrombin, whereas antibodies to G $_{12}$  (Fig. 3 B) and a yeast G protein (Fig. 3 C) had no effect on thrombin's ability to mobilize intracellular calcium, as was found for calcium transients induced by bradykinin.

To compensate for the inherent variability of microinjection, the amounts of injected antibody were quantified at the end of each experiment by fixing and staining with a secondary anti-rabbit antibody. The immunofluorescence intensity integrated over each cell was retrospectively compared with the inhibition of the agonist-induced [Ca $^{2+}$ ]<sub>i</sub> response in that cell (Fig. 4). Throughout this analysis, we found that cells that were either uninjected or injected with the lowest detectable amount of anti-G $\alpha_{q,11}$  behaved similarly; they produced transients in response to both bradykinin and thrombin. Injections of higher levels of anti-G $\alpha_{q,11}$ , but not anti-G $\alpha_{12}$  nor anti-GPA I, led to an abolition of the calcium transients and showed what appeared to be a threshold relationship for the inhibition.

### Mitogenic Regulation by G $\alpha$ Subunits

These data provided strong support for the notion that G $_q$  is the G protein that regulates bradykinin and thrombin receptor coupling to calcium transients in fibroblasts, which most likely represents molecular coupling of those receptors to the  $\beta 1$  isozyme of phospholipase C. This enzyme in turn catalyzes the hydrolysis of phosphatidylinositol to produce the second messenger IP $_3$  which leads to the mobilization of calcium from intracellular stores. While there is evidence that PLC activation is necessary for the mitogenic action of serum (Smith et al., 1990), it is not yet known if this is a requirement for these specific growth factors. Accordingly, to examine the role of G $_q$  in bradykinin and thrombin mito-

genesis in Balb/c3T3 cells, the effect of G $\alpha_q$  and G $\alpha_{12}$  antibodies on mitogenesis was assessed. Quiescent Balb/c3T3 fibroblasts were injected with antibodies specific for different G $\alpha$  subunits and 3 h later were stimulated with bradykinin, thrombin or PDGF. To monitor DNA replication, BrDU was added at the time of mitogen addition and the cells were incubated for a further 24 h. Injection of anti-G $\alpha_{q,11}$  resulted in a 95% reduction of bradykinin-stimulated DNA synthesis compared to unstimulated background levels of DNA synthesis (Table I). Thrombin-stimulated DNA synthesis was also assessed by these procedures. Quiescent cells were again injected with the antibodies specific for G $\alpha_{q,11}$ , G $\alpha_{12}$ , and GPA I; and subsequently stimulated with 0.5 U/ml thrombin. As observed for bradykinin, cells injected with anti-G $\alpha_{q,11}$  showed a significant reduction in DNA synthesis (96% reduction compared to background levels of DNA synthesis) (Table I). Anti-GPA I injected cells showed equal levels of DNA synthesis, as compared to uninjected cells. In contrast to bradykinin-stimulated mitogenesis, injected anti-G $\alpha_{12}$  completely blocked thrombin stimulated DNA synthesis. As a control, neither anti-G $\alpha_{q,11}$ , anti-GPA I (Table I), nor anti-G $\alpha_{12}$  as previously published (La Morte et al., 1992) had any effect on PDGF-induced DNA synthesis.

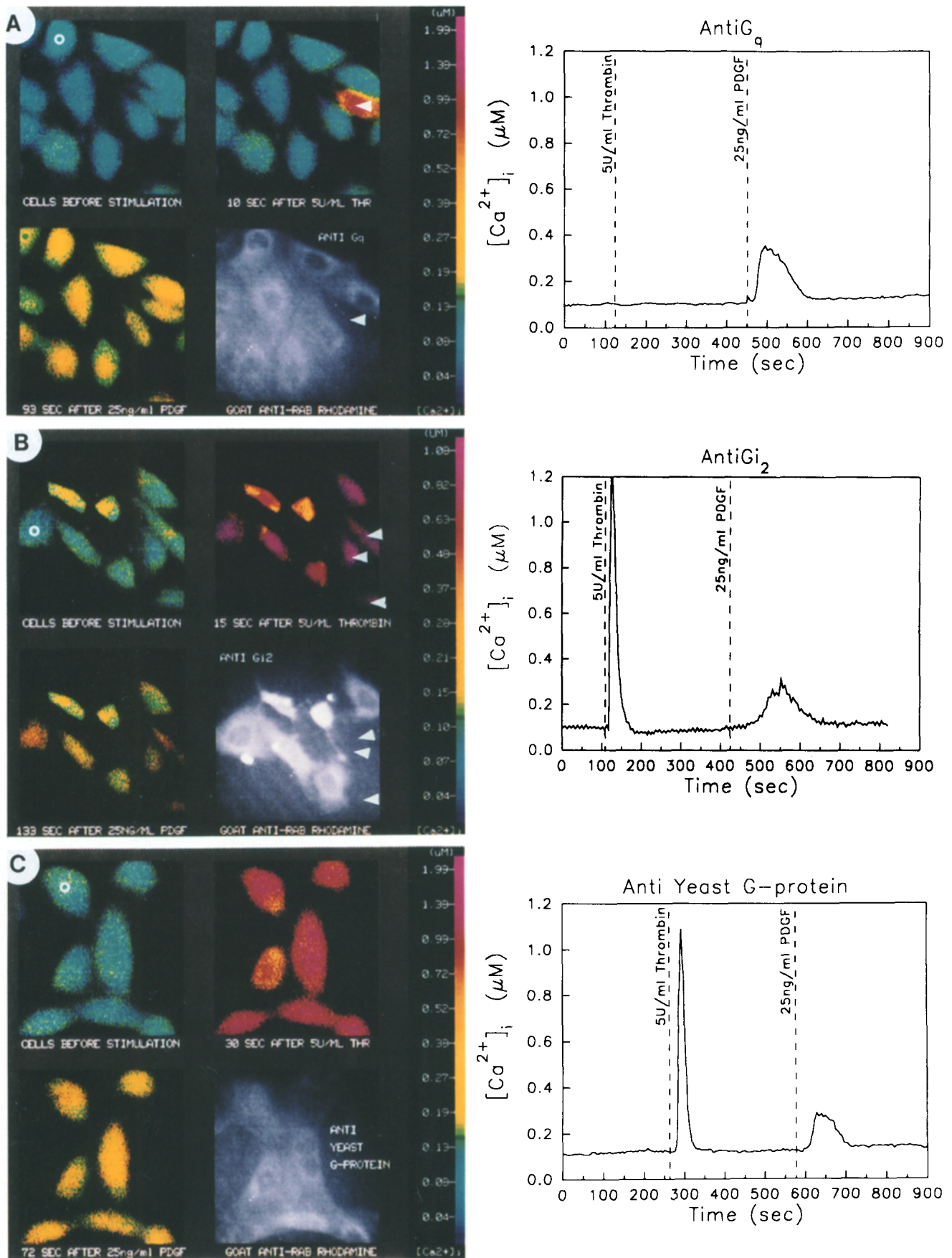
These experiments showed that while both thrombin and bradykinin required G $_q$  for coupling to increased intracellular calcium and for mitogenic responses, thrombin but not bradykinin appeared to require G $_{12}$  in addition to G $_q$  for the mitogenic response in cells. Moreover, PDGF required neither G $_q$  nor G $_{12}$  for these two cellular responses. Considering that G $_{12}$  but not G $_q$  is a pertussis toxin sensitive G protein, these data provide strong evidence for divergent signal transduction pathways emanating from the thrombin receptor in a manner dependent upon two distinct subtypes of G proteins. These experiments may also provide a molecular explanation for the observations that (a) calcium transients stimulated by thrombin or bradykinin are not sensitive to pertussis toxin (i.e., G $_q$ ) while (b) mitogenesis induced by thrombin but not bradykinin is sensitive to pertussis toxin (i.e., G $_{12}$ ) and that (c) neither response to PDGF appears to be mediated by G proteins.

### Discussion

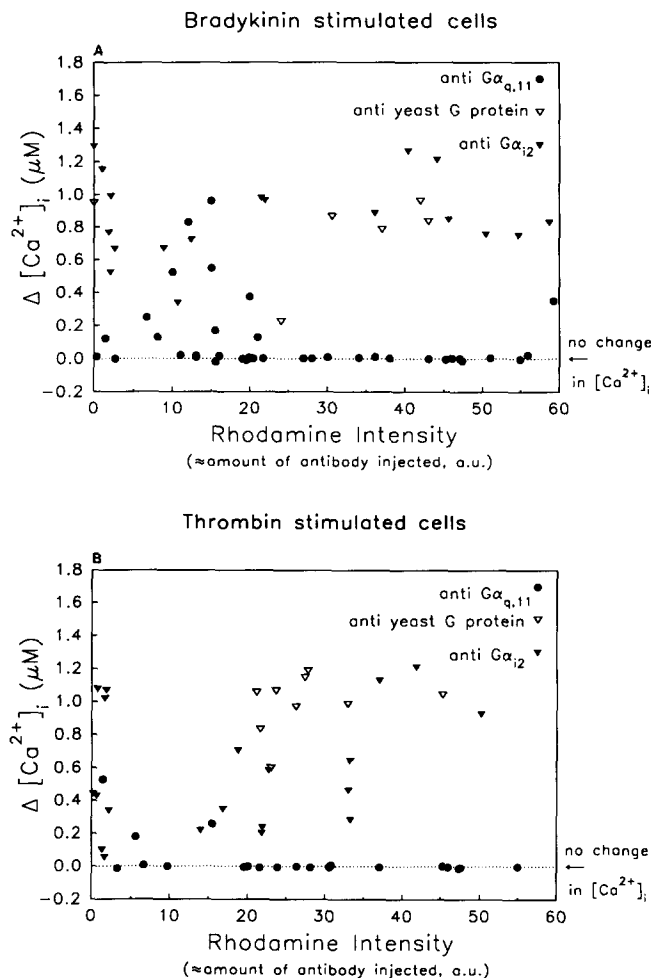
It appears that the function of G $_q$  in the case of both thrombin and bradykinin is, at the least, to provide transduction of the receptors to an increase in phosphoinositide breakdown and intracellular calcium, presumably via the action of PLC $\beta_1$ , and that this transduction is most likely an essential pathway for the mitogenic stimulation by these factors. It is unclear, however, if G $_q$  couples to other pathways or what role G $_{12}$  plays in the mitogenic action of thrombin, and why this is not also necessary for mitogenesis stimulated by

*Figure 2.* Single cell calcium ratio imaging of bradykinin-stimulated Balb/c3T3 fibroblasts. A-C each depict four different images of three different fields of cells loaded with fura-2/AM that have been injected with anti-G $\alpha_q$  (QL) (A); anti-G $\alpha_{12}$  (AS) (B); and anti-GPA I (C). The top left image of each panel illustrates the cells before stimulation. The top right corresponds to the image after bradykinin stimulation. The bottom left image depicts the same cells stimulated with PDGF; and, last, the bottom right image identifies the injected cells after fixation and indirect immunofluorescence. The white arrows indicate the cells in the field that have not been injected. The single cell tracing to the right of each panel (A-C) corresponds to the cell with the white circle.





**Figure 3.** Single cell calcium ratio imaging of thrombin-stimulated Balb/c3T3 fibroblasts. *A-C* each depict four different fields of cells loaded with fura-2/AM that have been injected with anti- $G_{\alpha_q}$  (QL) (*A*); anti- $G_{\alpha_{i2}}$  (AS) (*B*); and anti-GPA I (*C*). The top left image of each panel illustrates the cells before stimulation. The top right corresponds to the image after thrombin stimulation. The bottom left image depicts the same cells stimulated with PDGF; and, last, the bottom right image identifies the injected cells after fixation and indirect immunofluorescence. The white arrows indicate the cells in the field that have not been injected. The single cell tracing to the right of each panel (*A-C*) corresponds to the cell with the white circle.



**Figure 4.** Quantitation of injected antisera versus calcium ratio intracellular response. Immunofluorescence intensity (rhodamine) was integrated over each cell and assigned arbitrary units. Each cellular injection intensity was plotted against the cell's corresponding maximal change in intracellular calcium concentration ( $\mu\text{M}$ ). (A) Bradykinin-stimulated Balb/c3T3 fibroblasts. (B) Thrombin-stimulated Balb/c3T3 fibroblasts. The closed circle represents anti- $G_{\alpha_{q,11}}$  (QL) injected cells; the open triangle represents anti-GPA-I (QQ) injected cells; and the closed triangle represents the anti- $G_{\alpha_{12}}$  injected cells. Basal calcium levels ( $n = 101$ ) averaged  $122 \text{ nM} \pm 30 \text{ nM}$ . The mean increase in intracellular calcium for bradykinin-stimulated cells was as follows: QL ( $27.5 \text{ nM} \pm 80.6 \text{ nM}$ ); AS/QQ ( $718.5 \text{ nM} \pm 335.9 \text{ nM}$ ). The mean increase in intracellular calcium for thrombin-stimulated cells was as follows: QL ( $0 \text{ nM} \pm 4.4 \text{ nM}$ ); AS/QQ ( $846.0 \text{ nM} \pm 348.4 \text{ nM}$ ). The average increase in calcium was derived from injected cells with an injection fluorescence intensity  $>20$  (arbitrary units). *t*-test analysis of QL- compared to AS- and QQ-injected cells was significantly different with a  $p < 0.0001$  for both bradykinin- and thrombin-stimulated cells. QQ- and AS-injected cells were not significantly different for both bradykinin- and thrombin-stimulated cells.

bradykinin. It is conceivable that both growth factors stimulate more than one signaling pathway as a consequence of receptor occupancy. In the case of bradykinin, this could be accomplished by receptor coupling to  $G_q$  which would alter more than one biochemical effector, or by receptor coupling to both  $G_q$  and to some other signaling agent (other than  $G_{12}$ ). For thrombin, this could occur by receptor coupling to both  $G_q$  and  $G_{12}$ . A possible function for  $G_{12}$  in mitogene-

**Table I. Percent DNA Synthesis**

Background	Antibody	Injected	Uninjected	Percent reduction
<b>Bradykinin</b>				
18%	QL	7% ( $n = 69$ )	33% ( $n = 225$ )	100%
	QL	21% ( $n = 70$ )	37% ( $n = 251$ )	84%
	QL	24% ( $n = 50$ )	52% ( $n = 208$ )	82%
15%	QQ	32% ( $n = 53$ )	31% ( $n = 222$ )	0%
	QL	8% ( $n = 102$ )	31% ( $n = 247$ )	100%
	QL	12% ( $n = 56$ )	33% ( $n = 225$ )	100%
7%	QL	6% ( $n = 69$ )	15% ( $n = 203$ )	100%
	QL	5% ( $n = 92$ )	18% ( $n = 248$ )	100%
	QQ	21% ( $n = 47$ )	18% ( $n = 206$ )	0%
18%	QL	20% ( $n = 30$ )	31% ( $n = 206$ )	85%
	QL	17% ( $n = 88$ )	31% ( $n = 237$ )	100%
20%	AS	34% ( $n = 181$ )	37% ( $n = 323$ )	18%
	QQ	45% ( $n = 94$ )	46% ( $n = 219$ )	4%
<b>Thrombin</b>				
25%	QL	24% ( $n = 105$ )	38% ( $n = 216$ )	100%
14%	QL	14% ( $n = 78$ )	20% ( $n = 252$ )	100%
	QL	9% ( $n = 76$ )	35% ( $n = 206$ )	100%
	QQ	21% ( $n = 63$ )	19% ( $n = 273$ )	0%
15%	QQ	26% ( $n = 98$ )	25% ( $n = 204$ )	0%
	QL	18% ( $n = 101$ )	32% ( $n = 208$ )	82%
	QL	8% ( $n = 167$ )	20% ( $n = 225$ )	100%
23%	AS	18% ( $n = 66$ )	54% ( $n = 228$ )	100%
	AS	7% ( $n = 56$ )	44% ( $n = 248$ )	100%
	AS	23% ( $n = 74$ )	43% ( $n = 235$ )	100%
20%	QQ	43% ( $n = 116$ )	44% ( $n = 223$ )	5%
	AS	20% ( $n = 196$ )	59% ( $n = 200$ )	100%
	QQ	39% ( $n = 28$ )	42% ( $n = 235$ )	14%
<b>PDGF</b>				
20%	QL	84% ( $n = 89$ )	87% ( $n = 219$ )	4%
	QQ	84% ( $n = 116$ )	89% ( $n = 248$ )	7%

Percent reduction of DNA synthesis in microinjected fibroblasts. Briefly, as described in Materials and Methods, random areas of Balb/c3T3 fibroblasts were microinjected with the appropriate G protein antiserum and stimulated with either bradykinin, thrombin, or PDGF. Cells were analyzed using a Zeiss axiophot fluorescent microscope with a x63 oil immersion Apochromat objective. The background percentage of DNA synthesis for quiescent cells for a given experiment is reported in the first column, followed by the antisera that was microinjected. The percentage of injected cells that were positive for DNA synthesis are reported in addition to the number of cells that were injected ( $n$ ). Also, the percentage of DNA synthesis for a given number ( $n$ ) of uninjected cells on the same coverslip is reported. Percent reduction corresponds to percent of DNA synthesis (uninjected) minus percent of DNA synthesis (injected) divided by the percent of DNA synthesis (uninjected) after background has been subtracted. A paired *t*-test analysis was performed. Microinjection of QL resulted in a  $95 \pm 3\%$  reduction in bradykinin-stimulated DNA synthesis ( $p < 0.001$ ); a  $96 \pm 4\%$  reduction in thrombin-stimulated DNA synthesis ( $p < 0.006$ ), and, no significant difference in PDGF-stimulated DNA synthesis. Microinjection of AS resulted in a 100% reduction in thrombin-stimulated DNA synthesis ( $p < 0.0086$ ) and no significant reduction in bradykinin-stimulated DNA synthesis. As previously reported, microinjection of AS has no effect on PDGF-stimulated DNA synthesis (La Morte et al., 1992). Microinjection of QQ resulted in no significant reduction in DNA synthesis in response to bradykinin, thrombin, or PDGF.

sis may lie in its potential regulation of adenylylcyclase where it usually acts as an inhibitory agent, or in its activation of phospholipase  $A_2$  (Gupta et al., 1990) or in the activation of ERKs (Gupta et al., 1992). If these are necessary components of the mitogenic pathways for both of these growth factors, then we might suspect that bradykinin receptors may couple to these pathways as well, either through  $G_q$  or some other factor.

As transfection of activated *ras* (Robbins et al., 1992; Wood et al., 1992; Thomas et al., 1992) or  $G_{\alpha_{12}}$  leads to the rapid activation of extracellular signal regulated kinases,

as does stimulation by thrombin and bradykinin (Meloche et al., 1992; Kahan et al., 1992), it is conceivable that these growth factors stimulate extracellular signal regulated kinases through pathways shared by *ras* stimulated events. In preliminary experiments, we have found that the stimulation of mitogenesis by thrombin is blocked by prior microinjection of a mutant form of the *ras* protein which interferes with endogenous *ras* function (see Feig and Cooper, 1988). Together with the present results, this suggests that in addition to  $G_{12}$  and  $G_q$ , this mitogen also requires *ras* function. This points to a new potential linkage between the important protooncogene *ras* and heterotrimeric G proteins in cell signaling. The potential requirement for these GTP binding proteins in the mitogenic signaling suggests a complex interdependence of pathways. We are currently exploring this possibility. Mutations of  $G_{12}$  which cause an activation of the GTPase activity of the protein are mitogenic when transfected in fibroblasts (Hermouet et al., 1991). Antibodies that apparently inhibit the function of the endogenous  $G_{12}$  in fibroblasts cause a diminution of serum-stimulated DNA synthesis (La Morte et al., 1992) and a block of IGF-II-dependent mitogenesis (LaMorte, V. J., unpublished results). These observations provide strong evidence that  $G_{12}$  is a necessary cellular transducer of certain types of mitogens. As known functions of  $G_i$  subunits include the inhibition of adenylyl cyclase and activation of extracellular signal regulated kinases, this suggests important roles for these pathways in G protein dependent mitogenesis.

The identification of both a *pertussis* toxin sensitive and insensitive  $G_\alpha$  subtype in thrombin mitogenesis as shown here clarifies certain aspects of the complex pharmacologic data concerning this mitogen. The present data would suggest that the target of the toxin used in the pharmacologic studies was  $G_{\alpha_{12}}$ , which we show is involved in the mitogenic signaling of thrombin. The insensitivity of thrombin induced phosphoinositol hydrolysis to *pertussis* toxin (Hung et al., 1992) would, on the other hand, suggest a role for some other  $G_\alpha$  subtype in this transduction mechanism. The present results identify this as  $G_q$ . These results demonstrate that a single mitogen type can require functional coupling to two distinct subtypes of G proteins, and offer further support for the idea that growth factor receptors alter diverse biochemical pathways within the cell by such multiple linkages. The possible extent of the multitude of these linkages may be vast and will be determined by further analyses.

We thank C. Unson (Rockefeller University, New York) and P. Goldsmith (Molecular Pathology Branch [MPB]/National Institutes of Health) for peptides and antisera; J. Meinkoth for critical comments; and A. Majors and K. Martin for secretarial assistance. This work was funded by a grant from National Cancer Institute 39811 (J. R. Feramisco). V. J. LaMorte is a graduate student in the Biomedical Sciences Program and is supported by National Institutes of Health GM07752 training grant.

Received for publication 9 October 1992 and in revised form 9 December 1992.

## References

- Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature (Lond.)* 341:197-205.
- Berstein, G., J. L. Blank, A. V. Smrcka, T. Higashijima, P. C. Sternweis, J. H. Exton, and E. M. Ross. 1992. Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor,  $G_{q11}$ , and phospholipase C-beta 1. *J. Biol. Chem.* 267: 8081-8088.
- Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* 64:281-302.
- Chambard, J. C., S. Paris, G. L'Allemain, and J. Pouyssegur. 1987. Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. *Nature (Lond.)* 326:800-803.
- Dohlman, H. G., J. Thorner, M. G. Caron, and R. J. Lefkowitz. 1991. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* 60:653-688.
- Feig, L. A., and G. M. Cooper. 1988. Inhibition of NIH3T3 cell proliferation by mutated *ras* proteins with preferential affinity for GDP. *Mol. Cell. Biol.* 8:3235-3243.
- Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615-649.
- Gupta, S., C. Gallego, and G. Johnson. 1992. Mitogenic pathways regulated by G proteins. *Mol. Biol. Cell.* 3:123-128.
- Gupta, S. K., E. Dietz, L. E. Heasley, S. Osawa, and G. L. Johnson. 1990. A G protein mutant that inhibits thrombin and purinergic receptor activation of phospholipase  $A_2$ . *Science (Wash. DC)* 249:662-665.
- Gutowski, S., A. Smrcka, L. Nowak, D. G. Wu, M. Simon, and P. C. Sternweis. 1991. Antibodies to the alpha q subfamily of guanine nucleotide-binding regulatory protein alpha subunits attenuate activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by hormones. *J. Biol. Chem.* 266: 20519-20524.
- Hermouet, S., J. J. Merendino, Jr., J. S. Gutkind, and A. M. Spiegel. 1991. Activating and inactivating mutations of the alpha subunit of  $G_{i2}$  protein have opposite effects on proliferation of NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* 88:10455-10459.
- Hung, D. T., T.-K. H. Vu, N. A. Nelken, and S. R. Coughlin. 1992. Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. *J. Cell Biol.* 116:827-832.
- Hunter, T. 1991. Cooperation between oncogenes. *Cell* 64:249-270.
- Jones, D. T., S. B. Masters, H. R. Bourne, and R. R. Reed. 1990. Biochemical characterization of three stimulatory GTP-binding proteins. The large and small forms of  $G_s$  and the olfactory-specific G-protein,  $G_{olf}$ . *J. Biol. Chem.* 265:2671-2676.
- Kahan, C., K. Seuwen, S. Meloche, and J. Pouyssegur. 1992. Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts. Evidence for thrombin-induced signals different from phosphoinositide turnover and adenylyl cyclase inhibition. *J. Biol. Chem.* 267:13369-13375.
- Kleuss, C., J. Hescheler, C. Ewel, W. Rosenthal, G. Schultz, and B. Wittig. 1991. Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature (Lond.)* 353:43-48.
- Kurose, H., J. W. Regan, M. G. Caron, and R. J. Lefkowitz. 1991. Functional interactions of recombinant alpha 2 adrenergic receptor subtypes and G proteins in reconstituted phospholipid vesicles. *Biochemistry* 30:3335-3341.
- La Morte, V. J., P. K. Goldsmith, A. M. Spiegel, J. L. Meinkoth, and J. R. Feramisco. 1992. Inhibition of DNA synthesis in living cells by microinjection of  $G_{12}$  antibodies. *J. Biol. Chem.* 267:691-694.
- Meinkoth, J., P. Goldsmith, A. Spiegel, J. R. Feramisco, and G. N. Burrow. 1992. Inhibition of thyrotropin-induced DNA synthesis in rat thyroid follicular cells by microinjection of an antibody to the stimulatory G protein of adenylyl cyclase,  $G_s$ . *J. Biol. Chem.* 267:13239-13245.
- Meloche, S., K. Seuwen, G. Pages, and J. Pouyssegur. 1992. Biphasic and synergistic activation of p44<sup>mapk</sup> (ERK 1) by growth factors: correlation between late phase activation and mitogenicity. *Mol. Endocrinol.* 6:845-854.
- Robbins, D. J., M. Cheng, E. Zhen, C. A. Vanderbilt, L. A. Feig, and M. H. Cobb. 1992. Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. *Proc. Natl. Acad. Sci. USA*. In press.
- Shenker, A., P. Goldsmith, C. G. Unson, and A. M. Spiegel. 1991. The G protein coupled to the thromboxane A2 receptor in human platelets is a member of the novel  $G_q$  family. *J. Biol. Chem.* 266:9309-9313.
- Simon, M. I., M. P. Strathmann, and N. Gautam. 1991. Diversity of G proteins in signal transduction. *Science (Wash. DC)* 252:802-808.
- Simonds, W. F., P. K. Goldsmith, J. Codina, C. G. Unson, and A. M. Spiegel. 1989a.  $G_{12}$  mediates  $\alpha_2$ -adrenergic inhibition of adenylyl cyclase in platelet membranes: *In situ* identification with  $G_{12}$  C-terminal antibodies. *Proc. Natl. Acad. Sci. USA* 86:7809-7813.
- Simonds, W. F., P. K. Goldsmith, C. J. Woodard, C. G. Unson, and A. M. Spiegel. 1989b. Receptor and effector interactions of  $G_s$ . *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 249:189-194.
- Smith, M. R., Y. L. Liu, H. Kim, S. G. Rhee, and H. F. Kung. 1990. Inhibition of serum- and ras-stimulated DNA synthesis by antibodies to phospholipase C. *Science (Wash. DC)* 247:1074-1077.
- Smrcka, A. V., J. R. Hepler, K. O. Brown, and P. C. Sternweis. 1991. Regulation of phosphoinositide-specific phospholipase C activity by purified  $G_q$ . *Science (Wash. DC)* 25:804-807.
- Strathmann, M., and M. I. Simon. 1990. G protein diversity: a distinct class of  $\alpha$  subunits is present in vertebrates and invertebrates. *Proc. Natl. Acad. Sci. USA* 87:9113-9117.



- Taylor, S. J., H. Z. Chae, S. G. Rhee, and T. H. Exton. 1991. Activation of the  $\beta 1$  isozyme of phospholipase C by  $\alpha$  subunits of the  $G_q$  class of G proteins. *Nature (Lond.)*. 350:516-518.
- Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge. 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell*. 68:1031-1040.
- Tsien, R. Y., and A. T. Harootunian. 1990. Practical design criteria for a dynamic ratio imaging system. *Cell Calcium*. 11:93-109.
- Vicentini, L. M., and M. L. Villereal. 1984. Serum, bradykinin, and vasopressin stimulate release of inositol phosphates from human fibroblasts. *Biochem. Biophys. Res. Commun.* 123:663-670.
- Wang, H., D. C. Watkins, and C. C. Malbon. 1992. Antisense oligodeoxynucleotides to Gs protein  $\alpha$ -subunit sequence accelerate differentiation of fibroblasts to adipocytes. *Nature (Lond.)*. 358:334-337.
- Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis. 1992. ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell*. 68:1041-1050.