RESEARCH COMMUNICATION

Suppression of $G_{i\alpha 2}$ enhances phospholipase C signalling

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G-proteins mediate transmembrane signalling from a populous group of cell-surface receptors to a smaller group of effectors that includes adenylate cyclase, various ion channels and phospholipase C. Stem cells (F9 teratocarcinoma) or rat osteosarcoma 17/2.8 cells in which $G_{i\alpha 2}$ expression is abolished by antisense RNA display markedly elevated basal inositol 1,4,5-trisphosphate accumulation and a potentiated phospholipase C response to stimulatory hormones. Expression of the Q205L mutant of $G_{i\alpha 2}$, which is constitutively active, was found to block persistently hormonally stimulated phospholipase C activity, implicating $G_{i\alpha 2}$ as an inhibitory regulator of phospholipase C signalling. Analysis using $G_{i\alpha 2}$-deficient adipocytes of transgenic mice provided further evidence for a role for $G_{i\alpha 2}$ in phospholipase C regulation, demonstrating *in vivo* that loss of $G_{i\alpha 2}$ elevates basal, and markedly potentiates hormonally stimulated, phospholipase C activity. This report demonstrates for the first time that a single G-protein, G_{i2}, can regulate two distinct signalling pathways, i.e. adenylate cyclase and phospholipase C.

INTRODUCTION

Receptor-regulated hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) isoenzymes generates the intracellular second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol [1–3]. Three classes of mammalian PLC isoenzymes (β, γ, δ) have been described [4,5]. Stimulation of the PLC-β isoenzymes appears to be mediated by members of the G_q family of G-protein α-subunits [6–8]. Additionally, selective activation of PLC-β isoenzymes (especially PLC-β2) by G-protein $\beta\gamma$-subunits has been reported [9–11]. The role of $G_{i\alpha 2}$ in the regulation of PLC was explored utilizing antisense RNA strategies for cells in culture [12] as well as for transgenic mice *in vivo* [13].

MATERIALS AND METHODS

The pCW1 expression vectors harbouring wild-type and Q205L mutant $G_{i\alpha 2}$ were obtained from Dr. Gary L. Johnson (National Jewish Center for Immunology, Denver, CO, U.S.A.). ROS 17/2.8 rat osteosarcoma cells were provided by Dr. Gideon Rodan (Merck Research Laboratory, West Point, PA, U.S.A.). The antiserum specific for $G_{q\alpha}$ (E973) was a gift from Dr. John Exton (HHMI, Vanderbilt University, Nashville, TN, U.S.A.). The antiserum specific for $G_{i\alpha 3}$ (EC2) was purchased from NEN/DuPont. Antisera for $G_{i\alpha 2}$ (CM112) and $G_{\beta 1}$ (CM117) were prepared in the laboratory of C.C.M. Antibodies to PLC-β1 (1731) and PLC-β2 (195) were prepared to the C-terminal dodecapeptide sequences of each protein in the laboratory of A.J.M.

Cell culture

Wild-type and transfected F9 mouse teratocarcinoma stem cells, as well as the ROS 17/2.8 cells, were cultured on 100 mm Falcon Petri dishes in Dulbecco's modified Eagle's medium supplemented with 15% (F9 cells) or 10% (ROS cells) fetal calf serum.

Transfection protocol

Cells were either infected retrovirally using virus pLNCX-$ASG_{i\alpha 2}$ for the F9 cells lacking $G_{i\alpha 2}$ (F33) and ROS cells ($ASG_{i\alpha 2}$), or transfected via lipofectin with pCW1$G_{i\alpha 2}$Q205L to yield F9 cells in which the constitutively active mutant of $G_{i\alpha 2}$ is expressed under the cytomegalovirus promoter (FQ), as described previously [13]. Selection of neomycin-resistant colonies employed the analogue G418 (500 μg/ml; Gibco).

Cyclic AMP accumulation

Cells were released from culture plates with EDTA (or digested from tissue with collagenase; see below), washed and resuspended in Krebs–Ringer phosphate buffer (50000 cells/ml) with or without the indicated activators for 15 min at 37 °C. The reaction was terminated by addition of HCl (0.1 M) and heating to 100 °C. The samples were neutralized and the cyclic AMP accumulation was determined by a competitive protein binding assay [13]. Assays were performed in triplicate.

Immunoblotting

Crude membranes were prepared from cells and subjected to SDS/PAGE (20 μg/lane). The separated proteins were transferred electrophoretically to nitrocellulose and the blots were probed with primary antisera at 1:400 dilutions. Immune complexes were made visible by staining with a second antibody, goat anti-rabbit IgG conjugated with calf alkaline phosphatase [12]. For immunoblotting of PLC isoforms, the protein loaded on to large format gels was increased to 100–200 μg/lane.

IP_3 accumulation (PLC activity)

For determination of IP_3 accumulation, a procedure similar to that for cyclic AMP accumulation was followed. The assay was terminated, however, with perchloric acid and the mass of IP_3 was determined by direct assay using a competitive protein binding assay [14]. Assays were performed in triplicate.

Abbreviations used: PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate.

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Isolation of adipocytes from pPCK-ASG$_{i\alpha2}$-transgenic mice

Transgenic mice and their wild-type littermates were used at 16 weeks of age [13]. White adipocytes were isolated from parametrial adipose tissue using collagenase digestion, as previously described [13].

RESULTS AND DISCUSSION

Mouse F9 teratocarcinoma stem cells stably transfected with pLNC-ASG$_{i\alpha2}$ generate RNA antisense to G$_{i\alpha2}$ that suppresses the expression of G$_{i\alpha2}$ [12]. The expression of G$_{i\alpha3}$, G$_{q\alpha}$, G$_{\beta1}$ or G$_{s\alpha}$ was not altered in these clones (results not shown). Wild-type cells (F9), cells stably transfected with pLNC-ASG$_{i\alpha2}$ (F33) or cells stably transfected with pCW1G$_{i\alpha2}$Q205L (FQ) were prepared for study. Whereas F33 cells are rendered G$_{i\alpha2}$-deficient by antisense RNA, cells transfected with the pCW1G$_{i\alpha2}$Q205L express a G$_{i\alpha2}$ mutant with a Q205L substitution that reduces endogenous GTPase activity and constitutively activates the molecule. The inhibitory adenylate cyclase response was assayed first (Figure 1). Isoprenaline or forskolin was employed to activate the stimulatory adenylate cyclase pathway. Thrombin inhibited the stimulatory response in the wild-type cells, but not in the G$_{i\alpha2}$-deficient F33 clones. Stable expression of the constitutively active Q205L G$_{i\alpha2}$ mutant by cells resulted in the complete inhibition of isoprenaline- and forskolin-stimulated cyclic AMP accumulation by the clones (FQ). Thus, suppression of G$_{i\alpha2}$ attenuates, whereas expression of the Q205L mutant potentiates, the inhibitory adenylate cyclase response. In F9 cells, thrombin inhibited the isoprenaline response to a greater extent than the forskolin response. The basal cyclic AMP accumulation in F33 clones was higher than that of F9 cells stimulated with isoprenaline. These data suggest that some elements downstream from the G-protein may be altered as a result of the loss of G$_{i\alpha2}$. The existence of cross-regulation among G-protein pathways is now widely accepted.

PLC activity was measured by the accumulation of IP_3 in cells, using the mass assay [14]. Thrombin stimulated a modest accumulation of IP_3 in F9 cells (1.75 ± 0.3-fold stimulation; $n = 8$). For G$_{i\alpha2}$-deficient clones (F33), however, basal accumulation was found to be increased 3.3-fold, while thrombin-stimulated PLC activity increased an additional 2.2-fold (Figure 2). Expression of the constitutively active Q205L G$_{i\alpha2}$ (FQ) abolished the stimulatory PLC response to thrombin. It did not appear that alterations in the levels of precursor inositol lipids or changes in inositol phosphate metabolism accounted for the differences between the responses of the F9 cells and their derivatives to stimulation with thrombin. F9, F33 and FQ cells labelled with *myo*-[^{3}H]inositol for 24 h contained essentially equivalent amounts of phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate (results not shown). The patterns of inositol phosphates observed by anion-exchange h.p.l.c. analysis of extracts from resting and thrombin-stimulated F9 cells and their derivatives were also similar. In comparison with the F9 cells, the F33 line showed increased formation of inositol 1- and 4-monophosphates, inositol 1,4-bisphosphate and inositol 1,3,4- and 1,4,5-trisphosphates upon thrombin stimulation. Levels of each of these inositol phosphates were reduced in resting and thrombin-stimulated FQ cells (results not shown).

The PLC response to α_1-adrenergic stimulation (phenylephrine; 100 μM) was similar to that of thrombin, i.e. amplified in G$_{i\alpha2}$-deficient cells and abolished in cells expressing the Q205L mutant. Phenylephrine-stimulated IP_3 accumulation for F9, F33 and FQ clones was 15.5, 47.2 and 12.8 pmol/10^6 cells respectively. These data suggest that the locus for the changes observed in

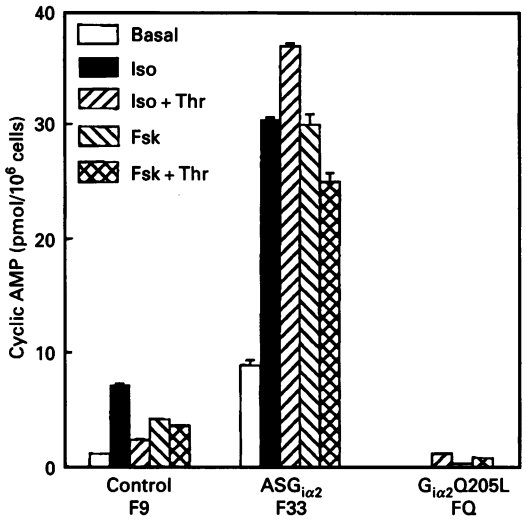

Figure 1 Suppression of G$_{i\alpha2}$ and expression of G$_{i\alpha2}$Q205L in F9 teratocarcinoma cells alters the inhibitory control of adenylate cyclase

F9 teratocarcinoma cells (F9) were retrovirally infected with pLNC-ASG$_{i\alpha2}$, which harbours an antisense sequence for G$_{i\alpha2}$ (F33), or transfected with an expression vector pCW1Q205L harbouring the cDNA encoding a mutant G$_{i\alpha2}$ which is constitutively active (FQ), as previously described [12]. Cells retrovirally infected with the vector alone (pLNCX) or vector harbouring a sequence antisense to G$_{i\alpha1}$ were examined as controls and found to display the wild-type phenotype [12]. Stimulatory adenylate cyclase was measured by cyclic AMP accumulation at 6 min following stimulation with isoprenaline (Iso; 10 μM) or forskolin (Fsk; 10 μM). The inhibitory adenylate cyclase was measured in response to the inhibitory ligand thrombin (Thr; 1 unit/ml) in the presence of either isoprenaline or forskolin. The data are means $\pm$ S.E.M. from at least four separate experiments.

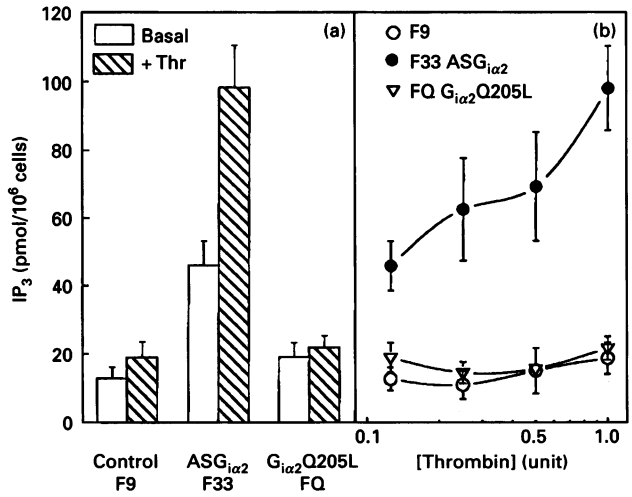

Figure 2 Suppression of G$_{i\alpha2}$ enhances and expression of G$_{i\alpha2}$ Q205L blunts the hormonal stimulation of PLC

F9 teratocarcinoma cells (F9) were retrovirally infected with pLNC-ASG$_{i\alpha2}$, which harbours an antisense sequence for G$_{i\alpha2}$ (F33), or transfected with an expression vector pCW1Q205L harbouring the cDNA encoding a mutant G$_{i\alpha2}$ which is constitutively active (FQ), as previously described [12]. PLC activity was measured by accumulation of IP_3 in cells at 15 s following stimulation with 1 unit/ml thrombin (**a**) or increasing concentrations (0.1–1.0 unit/ml) of thrombin [14]. The data are means $\pm$ S.E.M. from at least three separate experiments.

Table 1 Suppression of $G_{i\alpha 2}$ expression elevates basal and thrombin-stimulated IP_3 accumulation in rat osteosarcoma cells and mouse adipocytes

(**a**) Rat osteosarcoma 17/2.8 cells (generously provided by Dr. Gideon Rodan) were retrovirally infected with pLNC-$ASG_{i\alpha 2}$, which harbours a sequence producing RNA antisense to $G_{i\alpha 2}$, and the neomycin-resistant colonies were selected and propagated. PLC activity was measured by accumulation of IP_3 in cells at 15 s following stimulation with 1 unit/ml thrombin. The data are means ± S.E.M. from at least three separate experiments. (**b**) Adipocytes were acutely isolated from either control mice (BDF1) or transgenic mice carrying an inducible pPCK-$ASG_{i\alpha 2}$ gene that suppresses expression of $G_{i\alpha 2}$ in liver and adipose tissue [13]. Immunoblotting reveals that these cells are $G_{i\alpha 2}$-deficient. In this case, PLC activity was measured by the accumulation of IP_3 in cells at 30 s following stimulation with angiotensin II (100 μM).

	PLC activity (pmol/mg of protein)	
	Control	$G_{i\alpha 2}$-deficient
(a) ROS 17/2.8 cells		
Basal	13.9 ± 0.7	71.0 ± 0.9
+ Thrombin (1 unit/ml)	17.1 ± 0.7	107.4 ± 1.7
(b) Mouse adipocytes		
Basal	8.2 ± 0.5	16.3 ± 2.4
+ Thrombin (1 unit/ml)	16.7 ± 0.9	83.8 ± 0.4

both basal and receptor-stimulated IP_3 accumulation is at the level of the G-protein and not the receptor. Unlike the adenylate cyclase response, the basal PLC response was not suppressed in the clones expressing Q205L $G_{i\alpha 2}$. Residual PLC activity probably reflects forms of PLC (γ and δ) that are not subject to G-protein-mediated regulation [4,5]. PLC-β1 levels, as measured by immunoblotting, were equivalent in all three clones, and PLC-β2 and PLC-β3 were not detected. Suppression of $G_{i\alpha 2}$ might yield a modest elevation of uncomplexed $G_{\beta\gamma}$ subunits, contributing to an activation of PLC [9–11]. The absence of PLC-β2 in the F9 stem cells (via immunoblotting; results not shown), the potentiation of hormonally stimulated PLC activity in $G_{i\alpha 2}$-deficient cells, and the ability of Q205L $G_{i\alpha 2}$ to block the PLC stimulatory responses, argue for a prominent role for the $G_{i\alpha 2}$-subunit. In addition, incubating F9 cells in dibutyryl cyclic AMP (5 mM) for up to 4 days did not lead to enhanced PLC signalling [15], eliminating elevated cyclic AMP as the basis for the potentiation of PLC signalling observed in $G_{i\alpha 2}$-deficient clones. These observations suggest that $G_{i\alpha 2}$ expresses an inhibitory control on PLC, similar to that on hormonally stimulated adenylate cyclase [12,13].

To further explore this new function of $G_{i\alpha 2}$ (i.e. regulation of PLC), we probed this linkage using two additional $G_{i\alpha 2}$-deficient cell types, one a cell line in culture, the other acutely prepared cells from transgenic mice. Rat osteosarcoma (ROS) 17/2.8 cells display thrombin-stimulated accumulation of IP_3, similar to that observed for the F9 cells (Table 1). ROS cell clones stably transfected with pLNC-$ASG_{i\alpha 2}$, like $G_{i\alpha 2}$-deficient F9 clones, showed a 5.0-fold elevation of basal PLC activity. Thrombin stimulated an additional 1.5-fold response in the ROS-$ASG_{i\alpha 2}$ clones, providing a 6.3-fold greater stimulation of PLC activity over that observed in cells expressing wild-type levels of $G_{i\alpha 2}$.

Recently we reported the development of transgenic mice in which $G_{i\alpha 2}$ is suppressed *in vivo* through the use of an inducible, promoter-driven expression of antisense RNA harboured within a hybrid mRNA molecule [13]. Adipocytes were isolated from control BDF1 mice and from transgenic (BDF1 pPCK-$ASG_{i\alpha 2}$) mice in which $G_{i\alpha 2}$ had been suppressed in adipose tissue [13]. Basal accumulation of IP_3 (pmol/mg of protein) by adipocytes was 8.1 ± 0.5 for cells from BDF-1 control mice and 16.3 ± 2.4 for those from littermates carrying the transgene suppressing $G_{i\alpha 2}$. Angiotensin II-stimulated PLC activity was elevated ~ 5.0-fold in the $G_{i\alpha 2}$-deficient cells, compared with 2-fold in the control mouse adipocytes, confirming *in vivo* the results obtained with $G_{i\alpha 2}$-deficient clones of F9 stem and rat osteosarcoma cells (Figure 2 and Table 1 respectively). Thus, in two different cell lines made deficient in $G_{i\alpha 2}$, as well as in adipocytes from transgenic mice made deficient in $G_{i\alpha 2}$ from birth, suppression of $G_{i\alpha 2}$ both elevates basal PLC activity and markedly potentiates the hormonal activation of PLC.

The existence of both tonically and acutely expressed inhibitory regulation of cellular PLC has been postulated [15–19]. In intact cells, hormonal inhibition of PLC has been observed in response to activation of PLC-inhibitory receptors, notably those for dopamine and adenosine, that appear to be G-protein-linked [15,16]. Evidence for a tonic inhibition of PLC activity in intact cells is suggested by the long-standing observation that, when assayed under optimal conditions, both crude and purified preparations of PLC isoenzymes exhibit catalytic activities that, if expressed *in vivo*, would be incompatible with the levels of their polyphosphoinositide substrates measured in cells and tissues [4]. Pertussis toxin-sensitive G-proteins have been implicated as mediators of inhibitory PLC responses [20–22]. Our results establishing PLC as an effector for $G_{i\alpha 2}$ are as follows: (i) suppression of $G_{i\alpha 2}$ increases basal PLC activity in several cell lines as well as in $G_{i\alpha 2}$-deficient adipocytes from transgenic mice; (ii) hormonal stimulation of PLC is potentiated in $G_{i\alpha 2}$-deficient cells; and (iii) expression of the constitutively active Q205L mutant $G_{i\alpha 2}$ persistently inhibits hormonal activation of PLC.

Our observations are consistent with a novel model in which a single G-protein, $G_{i\alpha 2}$, can regulate two parallel signalling pathways. Whereas the stimulatory pathways controlling adenylate cyclase and PLC-β are mediated by distinct G-proteins (G_s and G_q respectively), the inhibitory responses for adenylate cyclase and PLC reflect a common, prominent role for at least one pertussis toxin-sensitive G-protein, $G_{i\alpha 2}$, in transmembrane signalling. $G_{i\alpha 2}$ would appear to be an ideal locus for cross-talk, integrating the PLC and adenylate cyclase signalling pathways.

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REFERENCES

1 Berridge, M. J. (1992) Nature (London) **361**, 315–325
2 Nishizuka, Y. (1992) Science **258**, 607–614
3 Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
4 Rhee, S. G., Suh, P.-G., Ryu, S.-H. and Lee, S. Y. (1989) Science **244**, 546–550
5 Rhee, S. G. and Choi, K. D. (1992) J. Biol. Chem. **267**, 12393–12396
6 Smrcka, A. V., Hepler, J. R., Brown, K. O. and Sternwels, P. C. (1991) Science **251**, 804–807
7 Taylor, S. J., Smith, J. A. and Exton, J. H. (1990) J. Biol. Chem. **265**, 17150–17157
8 Waldo, G. L., Boyer, J. L., Morris, A. J. and Harden, T. K. (1991) J. Biol. Chem. **261**, 14217–14225
9 Camps, M., Hou, C. F., Sidiropoulos, D., Stock, J. B., Jakobs, K. H. and Gierschik, P. (1992) Eur. J. Biochem. **206**, 821–831
10 Katz, A., Wu, D. and Simon, M. I. (1992) Nature (London) **360**, 686–689
11 Park, D., John, D.-Y., Lee, C.-W., Lee, K.-H. and Rhee, S. G. (1993) J. Biol. Chem. **268**, 4573–4576
12 Watkins, D. C., Johnson, G. L. and Malbon, C. C. (1992) Science **258**, 1373–1375
13 Moxham, C. M., Hod, Y. H. and Malbon, C. C. (1993) Science **260**, 991–995
14 Palmer, S. and Wakelam, M. J. O. (1990) in Methods in Inositide Research (Irvine, R. F., ed.), pp. 127–133, Raven Press, New York
15 Deckmyn, H., Van Geet, C. and Vermylen, J. (1993) News Physiol. Sci. **8**, 61–63
16 Linden, J. and Delahunty, T. M. (1989) Trends Pharmacol. Sci. **10**, 114–120

17 Irvine, R. F., Hemmington, N. and Dawson, R. M. C. (1979) Eur. J. Biochem. **99**, 525–530
18 Hoffman, S. L. and Majerus, P. W. (1979) J. Biol. Chem. **257**, 14359–14364
19 Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G. and Pollard, T. D. (1991) Science **251**, 1231–1233
20 Simmons, S. H. and Strange, P. G. (1985) Neurosci. Lett. **60**, 267–272
21 Journot, L., Homburger, V., Pantaloni, C., Priam, M., Bockaert, J. and Enjalbert, A. (1987) J. Biol. Chem. **262**, 15106–15110
22 Bizzarri, C., DiGirolamo, M., D'Orazin, M. C. and Corda, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4889–4893

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