Agonist-specific alterations in receptor–phospholipase coupling following inactivation of $G_{12}\alpha$ gene¹

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Different forms of phospholipase A_2 , together with pertussis toxin-sensitive G-proteins, $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration), protein kinase C, calmodulin, protein tyrosine kinases, mitogen-activated protein kinases and Ca^{2+} /calmodulin-dependent protein kinase appear to play a role in agonist-mediated release of arachidonic acid. Here we report that fibroblasts from 14-day-old mouse embryos with inactivated $G_{12}\alpha$ (α -subunit of the heterotrimeric G-protein G_{12}) gene display a marked decrease in the ability of lysophosphatidic acid, thrombin and Ca^{2+} ionophores to release arachidonic acid compared with their normal counterparts. The requirement for $G_{12}\alpha$ in the release of

arachidonic acid following increased $[Ca^{2+}]_i$ may be explained by the incomplete translocation of cytosolic phospholipase A_2 observed in $G_{12}\alpha$ -deficient cells. Paradoxically, inactivation of the $G_{12}\alpha$ gene resulted in up-regulation of bradykinin receptors and their coupling to increased arachidonic acid release, phospholipase C activity and $[Ca^{2+}]_i$. A concomitant increase in basal phospholipase C activity was also observed in the $G_{12}\alpha$ -deficient cells. These observations establish a pleiotropic and essential role for $G_{12}\alpha$ in receptor-phospholipase coupling that contrasts with its less obligatory participation in agonist-mediated inhibition of adenylate cyclase.

INTRODUCTION

G-proteins are membrane-bound heterotrimeric GTPases that transduce and amplify the signal carried by hormones, agonists or sensory stimuli, eliciting changes in the activity of cellular enzymes and ion channels. Different forms of phospholipase A_2 [1–5], together with PTX-sensitive G-proteins [6–8], intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), protein kinase C (PKC), calmodulin [9], protein tyrosine kinases [10,11], mitogen-activated protein kinases [12–16] and Ca^{2+} /calmodulin-dependent protein kinase [17] appear to play a role in the release of arachidonic acid (AA) driven by agonists. The role of G-proteins in AA release may exclusively reflect their ability to activate PLC isoforms and increase $[Ca^{2+}]_i$, together with the activity of PKC and other kinase cascades, or may include additional events. In this context, expression of a negative dominant mutant form of $G_{12}\alpha$ blocked agonist-mediated release of AA in Chinese-hamster ovary cells without altering activation of PLC or Ca^{2+} mobilization [6,8]. Mice deficient in $G_{12}\alpha$ [18–22] constitute an interesting system to study the role of this pertussis toxin (PTX) substrate in receptor–effector coupling. The $G_{12}\alpha$ -deficient cells used in the present study originate from 129/Sv transgenic mice [18], which are homozygous for a natural inactivating mutation disrupting the group II phospholipase A_2 (PLA_2) gene [23,24]. This circumstance allows a more selective study of the interaction between G_{12} and other PLA_2 enzymes. We have studied the coupling of agonists to AA release and phospholipase C (PLC) in control and $G_{12}\alpha$ -deficient fibroblasts from 14-day-old 129/Sv mouse embryos and obtained evidence consistent with a pleiotropic and essential role for $G_{12}\alpha$ in receptor–phospholipase coupling.

A partial account of these results has been published in abstract form [7,24a].

EXPERIMENTAL

Materials

myo-[2-³H]Inositol (22 Ci/mmol) and [5,6,8,9,11,12,14,15-³H] AA (60–100 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, U.S.A.). 1-Stearoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine (55 mCi/mmol) and the cAMP radioimmunoassay kit were purchased from Amersham (Arlington Heights, IL, U.S.A.). DMEM culture media was from GIBCO-BRL. 1,2-Dioleoyl-*sn*-glycerol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). BSA, DMSO, lysophosphatidic acid (LPA), thrombin, unlabelled AA, D-Arg, [Hyp³,D-Phe⁷]bradykinin (Hyp is hydroxyproline) and des-Arg⁹-bradykinin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bradykinin was purchased from United States Biochemical Corp. (Cleveland, OH, U.S.A.).

Cell culture

T-antigen-immortalized fibroblasts derived from primary cultures of 14-day-old $G_{12}\alpha$ -deficient ($\alpha_{12}/-$) and control ($\alpha_{12}/+$) mouse embryos were grown under a air/CO₂ (37:3) atmosphere in DMEM high glucose, supplemented with 10 % fetal-calf serum (FCS), 100 μ M β -mercaptoethanol, 100 units/ml penicillin and 100 μ g/ml streptomycin (for up to 30–35 passages).

AA release

Cells grown in 12-well plates (up to 70–80 % confluence) were labeled for 15–18 h in 700 μ l/well of DMEM/0.5 % FCS containing 0.7 μ Ci of [³H]AA (approx. 90 % of precursor was

Abbreviations used: $G_{12}\alpha$ (or α_{12}), α -subunit of the heterotrimeric G-protein G_{12} ; cPLA₂, (cytosolic) phospholipase A_2 ; PLC, phospholipase C; AA, arachidonic acid; PKC, protein kinase C; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase (or microtubule-associated protein 2 kinase); ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle medium; HBSS, Hanks balanced salt solution; LPA, lysophosphatidic acid; DIFP, di-isopropyl fluorophosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PTX, pertussis toxin; DTT, dithiothreitol; FCS, fetal calf serum; IBMX, isobutyl-1-methylxanthine; IP, IP₂ and IP₃, *myo*-inositol mono-, bis- and tris-phosphate respectively; Bt₂cAMP, dibutyl cAMP; CaLB, Ca^{2+} -dependent lipid-binding domain.

¹ In memory of Dr. M. Leonardo Satz (deceased 10 October 1997).

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incorporated). Labelled cells were washed at 37 °C with DMEM plus 0.1 % BSA and assays started by removal of the wash media and addition of 1 ml of DMEM supplemented with 0.1 % BSA, 100 μ M unlabelled AA and the indicated agonists. Following incubation for 10 min at 37 °C (unless otherwise indicated), samples of media were collected, centrifuged at 14000 *g* for 2 min and counted. ^3H radioactivity (d.p.m.) in the extracellular media represented [^3H]AA release, as shown in parallel extractions followed by ascending chromatography in the presence of unlabelled AA as standard (performed as described in [25]). Results were normalized for incorporation measuring ^3H radioactivity in either cell lysates (1 M NaOH) or lipid extracts. Variations in incorporated [^3H]AA between wells was typically lower than 15–20 %; because of this, some results were directly expressed as d.p.m./well. Pretreatment with PTX (100 ng/ml) took place during labelling with [^3H]AA (similar labelling was observed with or without toxin). Differences in agonist-mediated release of AA in control and α_{i2} -deficient fibroblasts represented differences in the production of this metabolite, as intracellular concentrations of free [^3H]AA in cells incubated with or without agonists accounted for 3 and 2 % of incorporated radioactivity ($\alpha_{i2} + / +$ and $\alpha_{i2} - / -$ respectively). Distribution of incorporated [^3H]AA into individual phospholipid species was studied following separation of lower phases of cell extracts (see above) by ascending TLC in Whatman K6 silica-gel plates (20 cm $\times$ 20 cm; 60 Å average pore diameter) previously embedded in 4–5 cm of 0.4 M boric acid (running solvent was chloroform/methanol/ammonium hydroxide/water, 65:35:2:3 by vol.). Inositol-containing phospholipids contributed most of the [^3H]AA mobilized by agonists (results not shown).

cPLA₂ activity in cytosolic fractions prepared from $\alpha_{i2} + / +$ and $\alpha_{i2} - / -$ cells: assays using exogenous substrates

Cells were grown in 100 mm dishes (to 70–80 % confluence) in complete media (see under 'Cell culture' above) followed by overnight incubation in 0.5 % FCS-containing media. Cells were washed in Ca^{2+} - and Mg^{2+} -containing Hanks balanced salt solution (HBSS) supplemented with 1 mg/ml glucose, followed by resuspension in 100 mM KCl/5 mM NaCl/3 mM MgCl_2 /50 mM Hepes (pH 7.4)/200 μ M PMSF/2 μ M pepstatin A/2 μ g/ml leupeptin/1 mM EGTA (lysis buffer). Suspensions were subjected to nitrogen cavitation [3105 kPa (450 lbf/in²), 20 min, 4 °C] in lysis buffer, followed by centrifugation for 8 min at 800 *g* and 4 °C. Supernatants were further centrifuged for 30 min at 195000 *g*. Protein concentration was determined in aliquots of the final supernatants (cytosolic fractions) as described by Lowry et al. [26], using lysis buffer as blank. In experiments aimed at the fractionation of cytosols by FPLC, the cells were washed and resuspended in EPB buffer [20 mM Hepes (pH 7.4)/120 mM potassium glutamate/20 mM potassium acetate, 3 mM NaCl/1 mM EGTA/11 μ g/ml Phenol Red] supplemented with 3 mM MgCl_2 , 1 mg/ml glucose, 3 mg/ml BSA, 2 μ M pepstatin, 200 μ M PMSF and 2 μ g/ml leupeptin (EPB+protease inhibitors). The suspensions were further supplemented with 4 mM di-isopropyl fluorophosphate (DIFP) and incubated for 30 min at 4 °C. The cells were pelleted, resuspended in EPB+protease inhibitors and subjected to nitrogen cavitation and preparation of high-speed supernatants as described above. The cytosolic fractions (195000 *g* supernatants) were subjected to anion-exchange chromatography on an FPLC[®] (Pharmacia LKB Biotechnology) using a Mono Q column (HR 5/5, 0.5 cm $\times$ 5 cm) previously equilibrated in 20 mM Tris/HCl (pH 8.5)/3 mM MgCl_2 /1 mM EGTA/0.1 mM PMSF (start buffer). The column

was eluted with 25 ml of a 0–1 M gradient of NaCl in start buffer at a flow rate of 1 ml/min (1 ml fractions were collected).

Ca^{2+} -dependent PLA₂ activity, using 1-stearoyl-2-[1- ^{14}C]-arachidonoyl phosphatidylcholine as exogenous substrate, was measured in the different column fractions and in the unfractionated cytosols as described previously [25]. Briefly, the assay mixtures contained 45 μ l of chromatographic samples or total cytosol (representing 10–30 μ g of protein) in a final volume of 60 μ l of EPB+protease inhibitors supplemented with varying concentrations of CaCl_2 and 50 nCi (0.7 nmol) of labelled substrate (previously evaporated and resuspended in 3 μ l of DMSO). Free Ca^{2+} concentrations resulting from the use of buffers containing 1 mM EGTA and increasing concentrations of CaCl_2 were calculated using the FREECA program [27]. Incubations were carried out for 30 min at 37 °C and stopped by addition of an equal volume of ethanol/acetic acid (49:1, v/v) supplemented with 200 μ g/ml of arachidonic acid. Samples were immediately vortex-mixed and left on ice for 10 min, followed by centrifugation for 4 min at 14000 *g* and 4 °C. [^{14}C]AA present in the supernatants was measured by ascending chromatography (silica-gel plates developed with the upper phase of ethyl acetate/iso-octane/water/acetic acid, 11:15:20:2 by vol.), followed by staining with I_2 vapour, scraping and liquid-scintillation counting.

Effects of agonists on activation of extracellular-signal-regulated protein kinase (ERK) and cPLA₂: mobility-shift assays

Cells were grown in 12-well plates, followed by overnight incubation in 0.5 % FCS-containing media and washed as described in the previous paragraph. Washed cells were incubated for the indicated times in DMEM supplemented with 0.1 % BSA in the presence or absence of 10 μ M LPA, 0.05 unit/ml thrombin or 10 % FCS. Reactions were stopped by removal of extracellular media and addition of 400 μ l of Laemmli sample buffer [28] and samples subjected to SDS/PAGE (8 or 12 % acrylamide, for immunoblotting with anti-cPLA₂ or anti-ERK2 antisera; see [25]). Two anti-cPLA₂ antisera (one kindly provided by Dr. G. Johnson and Dr. R. Nemenoff, National Jewish Medical and Research Center and University of Colorado Health Sciences Center, Denver, CO, U.S.A., the other from Santa Cruz Biotechnology) yielded identical results. The anti-ERK2 antiserum (#a1Cp42, described in [29]) was generously given by Dr. M. Dunn and Dr. M. Simonson, Department of Medicine, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

Ca^{2+} -dependent translocation of cPLA₂

Cells grown in two 100-mm-diameter culture dishes, and incubated for 15–18 h in 0.5 % FCS-containing medium as described above, were washed in Ca^{2+} - and Mg^{2+} -containing HBSS supplemented with 1 mg/ml glucose followed by resuspension in 100 mM KCl/5 mM NaCl/3 mM MgCl_2 /50 mM Hepes (pH 7.4)/200 μ M PMSF/2 μ M pepstatin A/2 μ g/ml leupeptin/1 mM EGTA (lysis buffer). Suspensions were subjected to nitrogen cavitation (3105 kPa, 20 min, 4 °C) in lysis buffer with or without 2 mM CaCl_2 , followed by centrifugation for 10 min at 900 *g* and 4 °C. Supernatants were centrifuged for 30 min at 195000 *g* and 4 °C; protein concentration was determined in aliquots of final pellets resuspended in cavitation buffer (crude membranes) and of supernatants (cytosol). Membrane and cytosolic fractions were diluted with 3 $\times$ Laemmli sample buffer and subjected to SDS/PAGE and cytosolic PLA₂ (cPLA₂) immunoblotting as described above. Differences in the translocation of cPLA₂ in $\alpha_{i2} + / +$ and $\alpha_{i2} - / -$ cells were also studied using a different

protocol in which membrane and cytosolic fractions (representing 70 and 250 μg of protein respectively and obtained after cell cavitation in the absence of added CaCl_2) were combined and incubated for 25 min at 20 °C in a final volume of 400 μl of lysis buffer containing increasing concentrations of free Ca^{2+} (calculated as described for PLA_2 assays). At the end of this period, samples were centrifuged at 16000 g and 4 °C for 40 min. The resulting supernatants were subjected to a 1.5-fold dilution with 3 $\times$ Laemmli sample buffer; pellets were resuspended in 100 μl of lysis buffer containing the corresponding concentrations of CaCl_2 used during the preceding incubation, followed by 1.5-fold dilution with 3 $\times$ Laemmli sample buffer. Samples representing approx. 25 μg of protein were subjected to SDS/PAGE and immunoblotting with anti-c PLA_2 antiserum as described above. Differences in translocation were quantified by densitometry using an Automated Sci Scan model 5000 densitometer programmed with an OS-Scan Image analysis system (USB, Cleveland, OH, U.S.A.). Scannings were repeated three times (in some cases on films reflecting different exposure times from a single immunoblot) with similar conclusions.

Experiments aimed at measuring thapsigargin-induced c PLA_2 translocation in intact cells were also performed using cells grown on 100-mm-diameter culture dishes and incubated overnight in 0.5 % FCS-containing medium. Cells were washed twice at 37 °C with DMEM, followed by incubation for 10 min with DMEM in the presence or absence of 1 μM thapsigargin (four 100-mm-diameter culture dishes per condition). At the end of this period cells were washed once with ice-cold DMEM and resuspended in 5 ml of lysis buffer without EGTA. Cells were subjected to nitrogen cavitation and the fractions collected and subjected to electrophoresis and immunoblotting as described in the previous paragraph.

Immunoblotting

Expression of c PLA_2 in $\alpha_{12}/++$ and $\alpha_{12}/--$ cells was compared, subjecting cytosolic fractions prepared as described in the previous paragraph (no Ca^{2+} added to lysis buffer) to SDS/8 % PAGE and immunoblotting with the anti-c PLA_2 antisera described above. Expression of G-protein subunits was studied by subjecting the membrane fractions prepared under the same conditions to SDS/PAGE (12 % gels) and immunoblotting with specific antisera kindly provided by Dr. D. Manning, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A. (anti- $G_{12}\alpha$ #1521; anti- $G_s\alpha$ #1190; anti- $G_{q/11}\alpha$ #0946; anti- G_β #5357 and anti- $G_{13}\alpha$ #1518; described in [30,31]).

PLC activity

Cells grown in 12-well plates were labelled at 37 °C for 15–18 h with 1 ml/well of inositol-free DMEM (high glucose)/0.5 % FCS containing 1 μCi of *myo*-[^3H]inositol. Labelled cells were washed with 1 ml DMEM for 1 h at 37 °C, followed by pre-incubation for 15 min at 37 °C with 750 μl of HBSS containing 10 mM LiCl, 1.2 mM CaCl_2 , 1 mM MgCl_2 and 1.8 mg/ml glucose (supplemented HBSS). Assays were started by adding 250 μl of agonists in supplemented HBSS and further incubation for 30 min at 37 °C. Reactions were stopped by removal of medium and addition of 500 μl of methanol/HCl (100:1, v/v). Cells were scraped off and transferred into glass tubes containing 1 ml of chloroform followed by addition of 200 μl of 625 $\mu\text{g}/\text{ml}$ phytic acid hydrolysate and 200 μl of an aqueous wash of the well (the final chloroform/methanol/water ratio was 10:5:4, v/v). Inositol phosphates in the upper phases were separated on AG1-X8 (chloride form) columns by elution with 30, 90 and 500 mM HCl

[*myo*-inositol mono-, bis- and triphosphates (IP_1 , IP_2 and IP_3 , respectively)]. Lower phases were dried under nitrogen, dissolved in chloroform/methanol/water (75:25:3, by vol.) and counted for radioactivity. Release of [^3H] IP_n was expressed as a percentage of *myo*-[^3H]inositol incorporated into phosphoinositides [$100 \times \text{d.p.m. of } [^3\text{H}]\text{IP}_n \text{ released} / (\text{d.p.m. of } [^3\text{H}]\text{IP}_n \text{ released} + \text{d.p.m. } ^3\text{H in phosphoinositides})$].

Agonist-mediated changes in $[\text{Ca}^{2+}]_i$

Monolayers were detached using Ca^{2+} - and Mg^{2+} -free HBSS and centrifuged for 5 min at room temperature and 200 g . Cell pellets were resuspended in 130 mM NaCl/5 mM KCl/1.5 mM CaCl_2 /1 mM MgCl_2 /25 mM Hepes (pH 7.4)/1 mg/ml glucose/1 mg/ml BSA. Loading of cells with 1 μM fura-2 acetoxymethyl ester, and fluorimetry was conducted as described previously [32].

cAMP content in $\alpha_{12}/++$ and $\alpha_{12}/--$ fibroblasts

Cells were grown in six-well plates with or without overnight serum reduction (15–18 h in 0.5 % FCS-containing DMEM). Some of the cells grown overnight in low-serum-containing medium were washed twice with basal DMEM and incubated for 20 min at 37 °C with 2 ml of DMEM containing 500 μM isobutyl-1-methylxanthine (IBMX). Cells incubated with IBMX as well as resting cells, with or without overnight serum reduction, were washed twice with Ca^{2+} and Mg^{2+} -containing HBSS supplemented with 1.8 mg/ml glucose and resuspended in 0.05 M sodium acetate buffer, pH 5.8. Resuspended cells were boiled for 15 min, followed by centrifugation for 20 min at 4 °C and 16000 g . cAMP content in the supernatants was determined by radioimmunoassay using a commercial kit and the instructions provided by the supplier.

RESULTS

Effects on AA release

The release of AA in resting α_{12} -deficient ($\alpha_{12}/--$) 129/Sv mouse embryonic fibroblasts was $56 \pm 10\%$ (mean $\pm$ S.D.; $n = 12$) of that measured in their wild-type counterparts ($\alpha_{12}/++$). Moreover, as seen in concentration–response studies (Figures 1A and 1B) and time-course experiments (Figure 1C), stimulation of AA release by LPA or thrombin was markedly reduced in $\alpha_{12}/--$ cells. The markedly diminished response to agonists in the $\alpha_{12}/--$ cells was not an artifact of our standard assay conditions, which included 100 μM unlabelled AA in the incubation medium (to reduce the re-uptake of the released [^3H]AA): the same marked differences in the response of control and $\alpha_{12}/--$ cells to agonists were also observed in parallel experiments performed in the absence of unlabelled AA during incubation (results not shown).

The effects of thrombin and LPA on AA release in $\alpha_{12}/++$ cells were partially sensitive to pre-treatment with PTX (44 and 71 % inhibition respectively), whereas the response to the Ca^{2+} ionophore A23187 was (as expected) insensitive to treatment with this toxin (Table 1). Interestingly, and consistent with the effects of an inhibitory $G_{12}\alpha$ mutant [8], the release of AA driven by the Ca^{2+} ionophore was also markedly diminished in $\alpha_{12}/--$ cells (Table 1). Surprisingly, the pattern of decreased release of AA in cells lacking $\alpha_{12}/--$ was not invariable: although bradykinin was ineffective in control cells it triggered a relatively small, but significant, increase in AA release in $\alpha_{12}/--$ cells (Table 2). Mouse embryo fibroblasts exhibited an approx. 75 % decrease in both resting and agonist-mediated AA release when extracellular Ca^{2+} was lowered from 1.8 mM to 0.1 μM (results not shown).

Figure 1 Agonist-mediated release of AA in fibroblasts from α_{12} -deficient and control mouse embryos

(A) Concentration–response curves for LPA in $\alpha_{12}+/+$ (●) and $\alpha_{12}-/-$ (○) cells. (B) Concentration–response curves for thrombin in $\alpha_{12}+/+$ (●) and two different lines of $\alpha_{12}-/-$ cells (#5 and #11, symbolized by ○ and △ respectively). (C) Time courses corresponding to resting (▲, △) and LPA (20 μ M)-driven (●, ○) release of AA in $\alpha_{12}+/+$ (▲, ●) and $\alpha_{12}-/-$ (△, ○) cells. Results shown represent the average and range of duplicate determinations (bars not visible in close duplicates) and originate from a representative experiment (out of two independent ones). For details, see the Experimental section.

Table 1 Effects of agonists, A23187 and PTX on AA release in $\alpha_{12}+/+$ and two lines of α_{12} -deficient (#5 and #11) mouse embryonic fibroblast

	[3 H]AA release*			
	Control	A23187 (5 μ M)	Thrombin (0.02 unit/ml)	LPA (10 μ M)
$\alpha_{12}+/+$	64 $\pm$ 14	822 $\pm$ 164	457 $\pm$ 43	792 $\pm$ 72
$\alpha_{12}+/+$, PTX†	53 $\pm$ 9	717 $\pm$ 165	271 $\pm$ 75	264 $\pm$ 55
$\alpha_{12}-/-$ #5	30 $\pm$ 7	63 $\pm$ 7	40 $\pm$ 9	49 $\pm$ 9
$\alpha_{12}-/-$ #11	35 $\pm$ 1	188 $\pm$ 32	93 $\pm$ 13	112 $\pm$ 28

* Release of [3 H]AA was expressed as d.p.m./ μ g of protein; data shown represent the means $\pm$ S.D. for triplicate determinations from a single experiment that is representative of three independent ones.

† Conditions for treatment with PTX are described in the Experimental section.

Table 2 Effects of bradykinin on AA release in $\alpha_{12}+/+$ and $\alpha_{12}-/-$ mouse embryonic fibroblasts

Release of [3 H]AA was expressed as percentage of 3 H d.p.m. incorporated into cells; data shown represent the average and range of duplicate determinations from a single experiment that is representative of three independent ones.

	Release of [3 H]AA		
	Control	Bradykinin	
		10 nM	40 nM
$\alpha_{12}+/+$	1.17 $\pm$ 0.01	1.05 $\pm$ 0.10	0.91 $\pm$ 0.06
$\alpha_{12}-/-$	0.61 $\pm$ 0	0.99 $\pm$ 0.12	1.60 $\pm$ 0.08

Table 3 Release of [14 C]AA from 1-stearoyl-2-[14 C]arachidonoyl phosphatidylcholine in cytosolic fractions from $\alpha_{12}+/+$ and $\alpha_{12}-/-$ mouse embryonic fibroblasts

Specific activities shown represent the average $\pm$ S.D. of triplicate determinations from a single assay which is representative of three independent experiments (each conducted on different preparations of cytosolic fractions). The average activity (at 2 mM free Ca^{2+}) in the cytosolic fractions from the $\alpha_{12}-/-$ fibroblasts relative to that present in the $\alpha_{12}+/+$ cells was approx. 1.5 ($n = 3$). Free Ca^{2+} concentrations in the incubation media containing 1 mM EGTA and either 3 mM, 0.99 mM or 0 added CaCl_2 were calculated using the FREECA program [27]. The assays were performed using radioactive substrate resuspended in DMSO. For details, see the Experimental section.

Free Ca^{2+}	Specific activity (pmol/min per mg)	
	$\alpha_{12}+/+$	$\alpha_{12}-/-$
2 mM	47.6 $\pm$ 5.4	61.5 $\pm$ 5.3
1 μ M	51.0 $\pm$ 5.9	56.7 $\pm$ 2.9
$\approx$ 2 nM*	3.6 $\pm$ 0.5	4.0 $\pm$ 0.3

* The contribution of CaCl_2 from different reagents was estimated at 10 μ M, resulting in approx. 2 nM free Ca^{2+} in buffers containing 1 mM EGTA.

PLA₂ assays

The marked decrease in the responses to LPA, thrombin and A23187 following inactivation of the $\text{G}_{12}\alpha$ gene suggested changes in the expression and/or function of relevant PLA₂ enzyme(s) in the $\alpha_{12}-/-$ cells. PLA₂s include at least four different groups of secreted low-molecular-mass enzymes requiring millimolar Ca^{2+} (I, II, III and V), a cytosolic form (group IV or cPLA₂) that translocates to cell membranes in response to micromolar $[\text{Ca}^{2+}]_i$ [33–37] and a Ca^{2+} -independent form (for a review on different PLA₂ enzymes, see [1]). Both groups IV and VI, as well as secreted forms of PLA₂, may participate in agonist-mediated release of AA in different cell types [2,3,38]. The 129/Sv mouse strain (from which both control and α_{12} -deficient fibroblasts were derived [18]) is homozygous for a natural inactivating mutation disrupting the gene encoding group IIA secreted PLA₂ [23,24]; this rules out the participation of this enzyme and prompted us to investigate possible alterations in cytosolic PLA₂ activation in the $\alpha_{12}-/-$ cells.

High-speed supernatants prepared following nitrogen cavitation of $\alpha_{12}-/-$ cells displayed similar (or higher) PLA₂ activity than the corresponding material from control cells (as determined using 1-stearoyl-2-[14 C]arachidonoyl phosphatidylcholine in the presence of either 1 μ M or 2 mM free Ca^{2+}) (Table 3). The activity measured under these conditions truly represented cPLA₂, as indicated by the coincidence of the peak of activity

Figure 2 Fractionation of cytosolic extracts of $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ mouse embryonic fibroblasts by FPLC

Cytosolic fractions were prepared as described in the Experimental section and subjected to anion-exchange chromatography over a Mono Q column (0.5 cm $\times$ 5 cm) equilibrated in 20 mM Tris/HCl (pH 8.5)/3 mM $MgCl_2$ /1 mM EGTA/0.1 mM PMSF (start buffer) and eluted with 25 ml of a 0–1 M gradient of NaCl in start buffer at a flow rate of 1 ml/min. Fractions were assayed for both PLA_2 activity, using 1-stearoyl-2-[^{14}C]arachidonoyl phosphatidylcholine as substrate (upper graph) and anti-c PLA_2 immunoreactivity (autoradiogram). The peak of activity was eluted at approx. 0.3 M NaCl and coincided with that of anti-c PLA_2 immunoreactivity. Results shown are from one experiment which is representative of three independent ones, carried out with different preparations of cytosolic fractions.

Figure 3 Expression of c PLA_2 in $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ mouse embryonic fibroblasts

Cytosolic fractions, prepared following nitrogen cavitation in 1 mM EGTA-containing buffers and no added Ca^{2+} , were subjected to immunoblotting using an anti-c PLA_2 antiserum. The amount of cytosolic protein loaded into the different lanes was 22.5 μ g (lanes 1 and 4), 15 μ g (lanes 2 and 5) and 7.5 μ g (lanes 3 and 6). Similar results were obtained following immunoblotting of total cell lysates of $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ fibroblasts. Results shown are representative of three independent experiments.

with that of anti-c PLA_2 immunoreactivity following ion-exchange chromatography of cytosols through a MonoQ FPLC column (Figure 2). Consistent with this finding was the relative insensitivity of the activities measured in the PLA_2 assay using exogenous substrate to addition of 2 mM dithiothreitol (DTT). Activities measured in 2 mM free- Ca^{2+} -containing buffers and in the presence or absence respectively of 2 mM DTT were 90.3 ± 0.2 and 97 ± 3 pmol/min per mg ($\alpha_{i2}^{+/+}$ cytosol) and 90.9 ± 0.4 and 105 ± 2 pmol/min per mg ($\alpha_{i2}^{-/-}$ cytosol) (average and range of duplicate determinations from a single assay that is representative of three independent experiments, each performed on different cytosol preparations). Immunoblotting of cytosolic fractions prepared following cell cavitation in the absence of added Ca^{2+} showed similar levels of expression of c PLA_2 in $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ cells (Figure 3).

We subsequently studied whether the diminished release of AA in response to LPA and thrombin reflected the inability of c PLA_2 to undergo activation following receptor occupancy in intact $\alpha_{i2}^{-/-}$ cells, in spite of both normal levels of expression of this enzyme and similar specific activities as measured in the *in vitro* assays. Agonist-mediated activation of c PLA_2 occurs concomitantly with enzyme phosphorylation and can be revealed by a slight decrease in its electrophoretic mobility [12], similar to that which is observed during activation of MAP kinases

Figure 4 Activation of c PLA_2 and MAP kinase ERK2 (A and B respectively) by agonists in control and $\alpha_{i2}^{-/-}$ mouse embryonic fibroblasts

Washed cells were incubated for 6 min in the presence or absence of 10 μ M LPA, 0.05 unit/ml thrombin or 10% FCS, followed by SDS/PAGE and immunoblotting with anti-c PLA_2 or anti-ERK2 antisera (all zero times shown are equivalent and represent activity preceding addition of agonists). Results shown are representative of at least three independent experiments. Additional time courses for LPA-mediated activation of ERK2 showed that activation declined after 10 min and that only inactive form was detected following 20 min of stimulation for both control and $\alpha_{i2}^{-/-}$ cells (results not shown). For details see the Experimental section.

(MAPKs) [39,40]. Although an ‘activating’ phosphorylation of c PLA_2 that does not result in a mobility shift has been described in thrombin-stimulated platelets [14], the reverse situation (i.e., a shift that is not associated with enzyme activation) has not been observed. This indicates that the shift in electrophoretic mobility of c PLA_2 , caused by phosphorylation on residues Ser⁵⁰⁵ (catalysed by either ERK1,2 or p38 MAP kinases [12,14]) is a genuine indicator of enzyme activation.

In this regard, Figure 4(A) shows that incubation of either control or $\alpha_{i2}^{-/-}$ fibroblasts with agonists led to accumulation of all the c PLA_2 in the activated (low-mobility) state, starting from an approx. 50–50% distribution in the resting cells (as shown in all zero times in this panel, which are equivalent and represent cells immediately preceding addition of the different agonists). On the other hand, activation of ERK2 by agonists, an event proposed to lay upstream of c PLA_2 , was decreased in the $\alpha_{i2}^{-/-}$ cells compared with their controls (Figure 4B). Analysis of agonist-induced mobility shifts in c PLA_2 by SDS/PAGE was performed immediately after preparation of lysates: storage at $-70^\circ C$ for 1–2 weeks decreased the magnitude of the mobility shifts upon electrophoresis of the lysates prepared from $\alpha_{i2}^{-/-}$, but not control, cells. This might result from differences in the concentration of phosphatases or in the c PLA_2 residues undergoing phosphorylation in both cell types (we have not investigated this in further detail).

Translocation of c PLA_2

Interestingly, $\alpha_{i2}^{-/-}$ cells displayed a 50–60% decrease in Ca^{2+} -dependent translocation of c PLA_2 compared with their controls (Figures 5A and 5B). This partial translocation was initially detected following nitrogen cavitation of cells in the presence or absence of 1 mM free Ca^{2+} (Figure 5A) and also following incubation of membrane and cytosolic fractions, originally prepared in the absence of added Ca^{2+} , with physiologically relevant (1–2 μ M) concentrations of this cation (Figure

Figure 5 Ca^{2+} -dependent translocation of cPLA₂ in control and α_{i2} -/- mouse embryonic fibroblasts

(A) Samples of cytosolic and membrane fractions (20 μg of protein), prepared following nitrogen cavitation of $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ cells in buffers containing 1 mM EGTA and the indicated concentrations of free Ca^{2+} , were subjected to immunoblotting using an anti-cPLA₂ antiserum (free Ca^{2+} contributed by water and other reagents in 1 mM EGTA-containing buffers was estimated at approx. 2 nM). Densitometry of the autoradiograms and calculation of the percentage of cPLA₂ associated with the membrane fractions under the different conditions showed the following results: $\alpha_{i2}^{+/+}$ cells, 6 and 95% (at ≈ 2 nM and 1 mM free Ca^{2+} respectively); $\alpha_{i2}^{-/-}$ cells, 2 and 54% (at ≈ 2 nM and 1 mM free Ca^{2+} respectively). Results are representative of six independent experiments. (B) Cytosolic fractions, prepared in the absence of added CaCl_2 as described above (≈ 2 nM free Ca^{2+}), were combined and incubated for 20 min at 20 °C in the presence of the indicated concentrations of free Ca^{2+} . Following incubation, the samples were centrifuged and the distribution of cPLA₂ in cytosolic and membrane fractions was analysed by immunoblotting as described in (A) (samples represented approx. 25 μg of protein). Densitometry of the autoradiograms and calculation of the percentage of cPLA₂ associated with membranes under the different experimental conditions showed the following results: 1, 85, 93 and 94% ($\alpha_{i2}^{+/+}$ cell fractions) incubated in the presence of ≈ 2 nM, 1.6 μM , 200 μM and 2 mM free Ca^{2+} respectively); 0, 48, 23 and 36% ($\alpha_{i2}^{-/-}$ cell fractions) incubated in the presence of ≈ 2 nM, 1.6 μM , 200 μM and 2 mM free Ca^{2+} respectively). Results shown are representative of four independent experiments. (C) Cells incubated for 10 min at 37 °C in the presence or absence of 1 μM thapsigargin were resuspended in lysis buffer without EGTA and subjected to nitrogen cavitation. Samples of cytosolic and membrane fractions were subsequently subjected to SDS/PAGE and immunoblotting using anti-cPLA₂ antiserum. Densitometric analysis showed a 91 and 48% distribution of immunoreactivity in membrane fractions from thapsigargin-treated $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ cells respectively. Results shown are representative of two independent experiments. For details see the Experimental section.

5B). The alterations in the translocation of cPLA₂ in $\alpha_{i2}^{-/-}$ cells were also observed following incubation of intact cells in the presence or absence of thapsigargin (Figure 5C), a specific inhibitor of intracellular Ca^{2+} pumps that increases cytosolic Ca^{2+} by depletion of intracellular stores and activation of Ca^{2+} influx [41].

Figure 6 Agonist-mediated accumulation of inositol phosphates (IP, IP₂ and IP₃) in α_{i2} -deficient and control fibroblasts

Results represent the means $\pm$ S.E.M. of triplicate determinations. Significant differences from the corresponding controls at $P < 0.05$ (*), $P < 0.02$ (**) and $P < 0.01$ (***) are shown (two-tailed Student's *t* test). Results originate from a single experiment that was repeated three times (IP and IP₃ were co-eluted in one of these experiments). An additional set of three experiments was carried out measuring only basal and LPA-dependent accumulation of IP and IP₃. The increase in basal release of IP in $\alpha_{i2}^{-/-}$ cells was detected in all six experiments (basal release of IP in the $\alpha_{i2}^{-/-}$ cells relative to that in control cells was 1.5 ± 0.2 , mean $\pm$ S.D.; $n = 6$). The decreased effects of LPA on accumulation of inositol phosphates in $\alpha_{i2}^{-/-}$ cells compared with controls were observed in five out of six experiments (small effects of LPA on both α_{i2} -deficient and control fibroblasts were seen in the remaining experiment).

PLC activity and $[\text{Ca}^{2+}]_i$ levels

Regulation of PLC was examined in [³H]inositol-labelled $\alpha_{i2}^{+/+}$ and $\alpha_{i2}^{-/-}$ fibroblasts. Under our assay conditions (30 min at 37 °C in the presence of 10 mM LiCl), the $\alpha_{i2}^{-/-}$ cells consistently showed an increased resting accumulation of IP compared with controls (Figure 6). Similarly to observations on [³H]AA release, bradykinin was ineffective in activating PLC in the $\alpha_{i2}^{+/+}$ cells, while triggering small increases in accumulation of inositol phosphates over the resting state in $\alpha_{i2}^{-/-}$ fibroblasts (Figure 6). Interestingly, PTX treatment of the $\alpha_{i2}^{+/+}$ cells also resulted in an approx. 3-fold increase in basal accumulation of IP, together with an approximately 40%

Figure 7 Concentration–response curves for the effects of bradykinin (A) or LPA (B) on $[Ca^{2+}]_i$ in control and $\alpha_{i2}/-$ mouse embryonic fibroblasts (two clones of $\alpha_{i2}/-$ cells, #5 and #11, were assayed in B)

Results are representative of four independent concentration–response curves determined for bradykinin and LPA. The responses of $\alpha_{i2}/+$ and $\alpha_{i2}/- \#5$ cells to 150 nM and 1.5 μ M LPA respectively were not determined in the experiment shown in (B). Pretreatment with PTX resulted in $65 \pm 10\%$ (mean $\pm$ S.D., $n = 3$) inhibition of the response of $\alpha_{i2}/-$ cells to 0.01–1 μ M bradykinin (A). In other experiments, PTX inhibited the increase in $[Ca^{2+}]_i$ triggered by 5 μ M LPA in $\alpha_{i2}/+$ and $\alpha_{i2}/-$ by 70 ± 18 (average and range, $n = 2$) and $83 \pm 18\%$ (mean $\pm$ S.D., $n = 3$) respectively (not shown).

reduction in the effect of LPA on PLC activation, but did not elicit a response to bradykinin (results not shown). Although LPA significantly increased production of inositol phosphates in both $\alpha_{i2}/+$ and $\alpha_{i2}/-$ cells, the response was lower in the $\alpha_{i2}/-$ fibroblasts (Figure 6). The effects of agonists on accumulation of inositol phosphates correlated with those on $[Ca^{2+}]_i$: while LPA triggered similar concentration-dependent and PTX-sensitive increases in $[Ca^{2+}]_i$ regardless of the expression of α_{i2} (Figure 7B), we detected very significant responses to bradykinin only in the $\alpha_{i2}/-$ cells (Figure 7A). The similar increase in Ca^{2+} triggered by LPA in the $\alpha_{i2}/-$ fibroblasts, in spite of the partial decrease in the ability of this agonist to trigger production of IPs in these cells, is probably due to the downstream

Table 4 cAMP levels in $\alpha_{i2}/+$ and $\alpha_{i2}/-$ mouse embryonic fibroblasts

Results shown represent the average $\pm$ S.D. of sextuplicate ($\dagger$) or quadruplicate ($\ddagger$) determinations (three or two wells per condition respectively, assayed at two different dilutions each by radioimmunoassay).

Condition	cAMP (pmol/mg of protein)	
	$\alpha_{i2}/+$	$\alpha_{i2}/-$
10% FCS	34.8 ± 4.1	72.5 ± 14
0.5% FCS, 15 h $\dagger$	24.8 ± 2.4	56.5 ± 9.9
0.5% FCS, 15 h 0.5 mM IBMX, 20 min $\ddagger$	36.2 ± 4.3	73.7 ± 5.8

Figure 8 Western blotting of G-protein subunits expressed in $\alpha_{i2}/+$ and $\alpha_{i2}/-$ mouse embryonic fibroblasts

The amount of membrane protein loaded on to gels is shown under each lane. Results are representative of three independent immunoblots.

amplification in this signalling cascade which has been observed in other cell types [42].

Binding of [3 H]bradykinin and cAMP levels

The $\alpha_{i2}/-$ fibroblasts displayed a 3–16 fold increase in [3 H]bradykinin receptors, mainly B_2 (as detected with selective antagonists and agonists), compared with the very low levels measured in the $\alpha_{i2}/+$ fibroblasts (range of eight independent experiments with four different membrane preparations; results not shown). Thus the enhanced effects of bradykinin in $\alpha_{i2}/-$ cells may represent receptor up-regulation. Assays performed in cells grown in medium containing either high (10%) or low (0.5%) concentrations of FCS, with or without incubation in the presence of IBMX, showed that $\alpha_{i2}/-$ cells display a 2-fold increase in resting cAMP levels when compared with their corresponding controls (Table 4). The increased levels of cAMP appear responsible for the up-regulation of bradykinin receptors in $\alpha_{i2}/-$ embryonic fibroblasts, as suggested by the recent identification of a cAMP-responsive element in the mouse bradykinin-receptor gene [43] and by the effects of cAMP-elevating agents on bradykinin-receptor density in human fibroblasts [44].

Immunoblotting of G-protein subunits

We determined whether the alterations in signalling observed in $\alpha_{i2}/-$ mouse embryonic fibroblasts were only attributable to the lack of expression of $G_{12}\alpha$, or secondary to changes in other G-protein subunits triggered by the primary defect. Western blotting with subunit-selective antisera (Figure 8) showed that inactivation of the $G_{12}\alpha$ gene resulted in a decrease (approx.

50 %) in β -subunit levels and a small increase (≈ 25 %) in $G_{12}\alpha$, in agreement with similar observations in these cells [20]. Expression of other G-protein subunits, including $G_s\alpha$, $G_{11}\alpha$ and $G_{q/11}\alpha$, was similar in control and $G_{12}\alpha$ -deficient fibroblasts.

DISCUSSION

The results presented here indicate that $G_{12}\alpha$ plays, directly or indirectly, a central and complex role in signalling which cannot be substituted for by normal expression of other PTX substrates. The indispensable participation of $G_{12}\alpha$ includes: (1) positive coupling of LPA and thrombin-mediated activation of AA release at a step subsequent to elevation of $[Ca^{2+}]$; (2) a negative regulation (or delay) in the development of responses to bradykinin (through expression of the corresponding receptors); (3) attenuation of basal (and possibly thrombin-stimulated, as observed by Watkins et al. [45]), but not LPA-driven PLC activity. These roles differ from the less obligatory participation of $G_{12}\alpha$ in agonist-mediated inhibition of adenylate cyclase [46,47].

The requirement for α_{12} in positively coupling the responses to LPA, thrombin and A23187 hints at a locus for this PTX substrate downstream of $[Ca^{2+}]$ elevation. The observed reduction in Ca^{2+} -driven translocation of cPLA₂ may explain the alterations in agonist-mediated release of AA in α_{12} -deficient cells. However, the partial nature of this defect does not completely exclude the involvement of other forms of PLA₂ in this response or the participation of $G_{12}\alpha$ in other relevant mechanisms.

The defect in Ca^{2+} -dependent translocation of cPLA₂ in α_{12} -/- fibroblasts suggests that phenotypic changes brought about by inactivation of the α_{12} gene may result in down-regulation of accessory proteins that are required for this event. The predominant association of cPLA₂ with the perinuclear envelope as opposed to the plasma membrane in activated cells [35–37,48], in spite of the ability of the Ca^{2+} -dependent lipid-binding domain (CaLB) of cPLA₂ to bind protein-free synthetic liposomes [49], suggests that such a mechanism may indeed exist. This possibility resembles the interaction of another set of enzymes displaying CaLB domain-proteins (the C2 region-containing conventional PKC isozymes) with their receptor proteins ('RACKs'; for a review, see [50]). In this context, the recent cloning of a nuclear protein that interacts with cPLA₂ provides support to this hypothesis [51].

The elevation of cAMP levels in the α_{12} -deficient cells (Table 4) suggests a possible mechanism for the decreased release of AA triggered by LPA and thrombin in these cells. However, long-term (15–43 h) or acute treatment of control (α_{12} +/+) mouse embryonic fibroblasts with either 0.1–0.5 mM dibutyryl cAMP (Bt₂cAMP) or 1–10 μ M forskolin resulted in a 30–70 % increase in resting and LPA- or thrombin-driven release of AA (the increase in agonist-dependent release was proportionally higher than the resting activity, resulting in a 20–30 % higher stimulation by these agonists) (results not shown). Although these experiments cannot mimic the exact effect of a permanent increase in cAMP levels on the phenotype of the α_{12} -/- fibroblasts, they argue against a causative relationship between increased levels of this second messenger and impaired release of AA by LPA and thrombin in these cells. In this context, transgenic mice harbouring inducible expression of $G_{12}\alpha$ antisense RNA displayed increased protein tyrosine phosphatase activity and insulin resistance; these effects were not mimicked by increasing intracellular levels of cAMP in control animals to the values observed in the $G_{12}\alpha$ -deficient mice [22]. This cAMP-independent alteration in phosphotyrosine phosphatase activity may represent an important factor contributing to the decreased release of AA by

LPA and thrombin in $G_{12}\alpha$ -/- fibroblasts. At the same time, the coupling of LPA and thrombin receptors to the activation of low-molecular-mass GTPases (Ras and Rho families), via heterotrimeric G-proteins, influences processes such as cell proliferation and cytoskeleton-associated assembly processes in fibroblasts (reviewed in [52]). Alterations in this coupling could also influence the ability of LPA and thrombin to release AA in the $G_{12}\alpha$ -deficient cells, thus extending the list of lipid signalling pathways that are regulated following cross-talk between heterotrimeric G-proteins and low-molecular-mass GTPases.

The MAP kinases ERK1,2 act as Ser/Thr kinases for cPLA₂ and other substrates displaying the proline-rich Pro-Xaa-Ser/Thr-Pro consensus sequence. Interestingly, the normal activation of cPLA₂ in $G_{12}\alpha$ -deficient cells by thrombin or LPA (as detected by the shift in gel mobility), in spite of the decreased ability of these agonists to activate ERK2 (Figures 4A and 4B), suggests the existence of alternative MAP kinase pathways coupling receptor occupancy to phosphorylation of cPLA₂ and AA release in these cells. Recent observations on the role of p38 and Jun-N-terminal kinase in the activation of cPLA₂ [14,16] provide a precedent for these ERK2-independent pathways. Clearly, additional studies are required to examine the activation of the different MAP kinase pathways, and their corresponding upstream activators, during stimulation of control and $G_{12}\alpha$ -deficient cells.

Future studies with this system will include the comparative analysis of the activation of different MAP kinase pathways, as well as experiments aimed at correcting the different phenotypic alterations in receptor-phospholipase coupling in the α_{12} -/- fibroblasts through expression of wild-type $G_{12}\alpha$ and/or $G\beta\gamma$. At the same time, the availability of control and $G_{12}\alpha$ -deficient 129/Sv mice, both carrying a natural inactivating mutation of the group IIA (secreted) PLA₂ gene, should provide an opportunity to examine the participation of $G_{12}\alpha$ in the regulation of this enzyme by crossing $G_{12}\alpha$ -deficient 129/Sv mice with strains displaying a normal group IIA PLA₂ genotype.

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