Hormonal regulation of phospholipase C ϵ through distinct and overlapping pathways involving G $_{12}$ and Ras family G-proteins

Grant G. KELLEY^{*1}, Sarah E. REKS^{*} and Alan V. SMRCKA[†]

^{*}Departments of Medicine and Pharmacology, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, U.S.A., and [†]Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave, Box 711, Rochester, NY 14642, U.S.A.

PLC ϵ (phospholipase C ϵ) is a novel PLC that has a CDC25 guanine nucleotide exchange factor domain and two RA (Ras-association) domains of which the second (RA2) is critical for Ras activation of the enzyme. In the present studies, we examined hormonal stimulation to elucidate receptor-mediated pathways that functionally regulate PLC ϵ . We demonstrate that EGF (epidermal growth factor), a receptor tyrosine kinase agonist, and LPA (lysophosphatidic acid), S1P (sphingosine 1-phosphate) and thrombin, GPCR (G-protein-coupled receptor) agonists, stimulate PLC ϵ overexpressed in COS-7 cells. EGF stimulated PLC ϵ in an RA2-dependent manner through Ras and Rap. In contrast, LPA, S1P and thrombin stimulated PLC ϵ by both RA2-independent and -dependent mechanisms. To determine the G-proteins that mediate the effects of these GPCR agonists, we co-expressed

constitutively active G-proteins with PLC ϵ and found that G $_{\alpha 12}$, G $_{\alpha 13}$, Rho, Rac and Ral stimulate PLC ϵ in an RA2-independent manner; whereas TC21, Rap1A, Rap2A and Rap2B stimulate PLC ϵ in an RA2-dependent manner similar to H-Ras. Of these G-proteins, we show that G $_{\alpha 12}$ /G $_{\alpha 13}$ and Rap partly mediate the effects of LPA, S1P and thrombin to stimulate PLC ϵ . In addition, the stimulation by LPA and S1P is also partly sensitive to pertussis toxin. These studies demonstrate diverse hormonal regulation of PLC ϵ by distinct and overlapping pathways.

Key words: epidermal growth factor (EGF), lysophosphatidic acid (LPA), phospholipase C ϵ , Ras, sphingosine 1-phosphate (S1P), thrombin.

INTRODUCTION

Phosphatidylinositol-specific PLC (phospholipase C) is a critical cellular enzyme that hydrolyses PtdIns(4,5) P_2 to generate Ins P_3 and diacylglycerol, which stimulate Ca²⁺ mobilization and protein kinase C respectively [1,2]. Four main classes, which include several isoforms, have been identified based on structure and regulation: PLC β 1–4, PLC γ 1–2, PLC δ 1–4 and PLC ϵ . PLC β is regulated by GPCRs (G-protein-coupled receptors) through G-proteins of the G α_q family and G $\beta\gamma$ subunits. PLC γ is regulated by receptor and non-receptor protein tyrosine kinases. The mechanism regulating PLC δ is unclear but possibly involves Ca²⁺ and G α_h , high-molecular-mass G-protein.

The newest member of this group is PLC ϵ , which, in addition to conserved PLC catalytic domains, has an N-terminal CDC25 GEF (CDC25 guanine nucleotide exchange factor) domain and two C-terminal Ras-binding RA (Ras-association) domains [3–5]. In addition to Ras [3,6], other G-proteins have been shown to stimulate PLC ϵ including G $_{\alpha 12}$ [4], TC21 [7], G $\beta\gamma$ [8] and Rap1A and Rap2B [6]. We have shown that the second RA domain (RA2) binds Ras by a typical Ras–effector interaction and that mutations in this domain inhibit binding of Ras and activation of PLC ϵ in parallel, strongly suggesting direct regulation [3]. In contrast, the mechanism by which G $_{\alpha 12}$ and G $\beta\gamma$ stimulate PLC ϵ is not known, but, unlike Ras, G $\beta\gamma$ stimulation is not inhibited by mutations in the RA2 domain [8].

EGF (epidermal growth factor) is a member of six EGF family hormones that are generated by the proteolytic cleavage of transmembrane precursors by metalloproteinases and are important mediators of proliferation and differentiation [9]. EGF binds the EGFR (EGF receptor; HER or ErbB), a RTK (receptor tyrosine

kinase), and induces homodimerization and/or heterodimerization with other ErbB family receptor members, which leads to activation and autophosphorylation of tyrosine residues that serve as docking sites for effector proteins. PLC γ 1 is one of these effectors that binds EGFR through its SH (Src homology) 2 domains and is phosphorylated at specific tyrosine residues that are necessary for its activation [10]. The Grb2–Sos complex also interacts with activated EGFR and activates Ras through the Ras GEF activity of Sos [11]. Similarly, EGFR interacts with Crk and the Rap GEF, C3G, to activate Rap1 and Rap2 [12].

In contrast to EGF, LPA (lysophosphatidic acid) [13], S1P (sphingosine 1-phosphate) [14] and thrombin [15] activate heptahelical GPCRs and are important regulators of critical cellular functions including mitogenesis and anti-apoptosis. The phospholipids LPA and S1P bind and activate LPA $_{1-3}$ and S1P $_{1-5}$ (previously EDG $_{1-8}$) receptors and thrombin activates proteinase-activated receptors, PAR $_{1,3,4}$, by cleaving the N-terminus of the receptor and unmasking an activating ligand. This group of GPCRs couples to heterotrimeric G-proteins of the G $_{i1}$, G $_{q}$ and G $_{12}$ families. Agonist activation of these receptors has been shown to stimulate PLC β isoforms through the G α subunits of the G $_{q}$ family and G $\beta\gamma$ subunits liberated from activated G $_{i1}$ /G $_{o}$ in a pertussis toxin-sensitive manner. Recently, these agonists have also been shown to be shown to transactivate the EGFR and activate the Ras/mitogen-activated protein kinase pathway [16,17].

In the present studies, we examined the hormonal regulation of PLC ϵ by these diverse agonists. We demonstrate that these RTK and GPCR-coupled agonists stimulate PLC ϵ by distinct pathways involving RA2-dependent and -independent mechanisms. EGF stimulates PLC ϵ through Ras and/or a Ras-like GTPase in a completely RA2-dependent manner. Serum, LPA, S1P and

Abbreviations used: GEF, guanine nucleotide exchange factor; PLC, phospholipase C; GPCR, G-protein-coupled receptor; RA, Ras-association; EGF, epidermal growth factor; EGFR, EGF receptor; RTK, receptor tyrosine kinase; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; GST, glutathione S-transferase; FBS, fetal bovine serum; RGS, regulators of G-protein signalling.

¹ To whom correspondence should be addressed (e-mail kelleyg@upstate.edu).

thrombin, on the other hand, require an intact RA2 domain for complete stimulation but also stimulate in an RA2-independent manner through $G_{\alpha 12}/G_{\alpha 13}$ and G_i .

EXPERIMENTAL

Materials

Reagents used were EGF, S1P and pertussis toxin from Calbiochem (La Jolla, CA, U.S.A.); LPA from Sigma (St. Louis, MO, U.S.A.); and α -thrombin from Haematologic Technologies (Essex Junction, VT, U.S.A.). Antibodies used were anti-PLC γ 1 and anti-PLC β 1 from Upstate Biotechnologies (Lake Placid, NY, U.S.A.), anti-FLAG from Sigma, anti-Ras, anti-EGF receptor, anti-Rap1, anti-Rap2 and anti-RalA from BD Transduction Laboratories (Lexington, KY, U.S.A.), anti-HA from Roche Diagnostics (Manheim, Germany), and anti-PLC β 2, anti- $G_{\alpha 12}$ and anti- $G_{\alpha 13}$ from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-PLC ϵ used in these studies was a rabbit polyclonal antibody generated to the RA1 domain.

Plasmids

pNeoSR α II-PLC γ 1 and pNeoSR α II-PLC β 1 were generated by subcloning cDNAs from pIBI31-PLC γ 1 and pIBI30-PLC β 1 (from Sue Goo Rhee, National Institutes of Health, Bethesda, MD, U.S.A.) into pNeoSR α II (from Alfred Bothwell, Yale University, New Haven, CT, U.S.A.). The following constructs were generously provided by other investigators: pCMV- $G_{\alpha 12}$ Q229L and pCMV- $G_{\alpha 13}$ Q226L (Dianqing Wu, University of Connecticut, Farmington, CT, U.S.A.); pCEFAu5-G23V RalA (from Silvio Gutkind, National Institutes of Health); pCMV-myc p115RhoGEF (1-252; RGS domain) (where RGS means regulators of G-protein signalling; from Tohru Kozasa, University of Illinois at Chicago, Chicago, IL, U.S.A.); pCEV-TC21 and pCEV-Q72LTC21 (from Ying Huang, SUNY Upstate Medical University, Syracuse, NY, U.S.A.); pRC/CMV-EGFR (from Gordon Gill, University of California San Diego, San Diego, California, U.S.A.); pFLAG3-Rap1A, pFLAG3-Q63E Rap1A, pFLAGCMV2-RapGAP1 (from Lawrence Quilliam, Indiana University School of Medicine, Indianapolis, IN, U.S.A.); pcDNA3mycCDC42 G12V (Michael Brown, SUNY Upstate Medical University; modified construct of Marc Symons, The Picower Institute for Medical Research, Manhasset, NY, U.S.A.) and pRSV-Q61L H Ras (from Johannes Bos, University Medical Center Utrecht, Utrecht, The Netherlands). pcDNA3.1 HA-G12V Rac, pcDNA3.1 HA-G14V RhoA, pcDNA3.1-RalA, pcDNA3.1-Rap2A and pcDNA3.1-Rap2B were from the Guthrie Resource Center (Sayre, PA, U.S.A.). Mutations were introduced by site-directed mutagenesis (QuikChange Mutagenesis Kit; Stratagene, La Jolla, CA, U.S.A.) into the aforementioned plasmids to generate the following constructs: wild type pRSV-H-Ras, pcDNA3.1-Q63E Rap2A and pcDNA3.1-Q63E Rap2B. All generated constructs were sequenced. Other plasmids used in this study were described previously [3].

GST (glutathione S-transferase) pull-down assay

A GST pull-down assay was performed as described previously [3]. Briefly, transformed DH5 α *Escherichia coli* were induced with isopropyl β -D-thiogalactoside (400 μ M) for 4 h at room temperature, except GST RBD Raf-1 (where RBD is Ras-binding domain) and pGEX vector for 2 h at 37 °C, and then pelleted and stored at -70 °C for less than 1 month. GST fusion proteins

were isolated using glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions and typically 250 pmol of fusion protein was then incubated for 2 h or overnight with 200–400 μ g of a COS-7 cell lysate overexpressing the indicated G-proteins at 4 °C. Following four washes, Western blot analysis was performed using Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) for detection.

Transfection of COS-7 cells and PLC activity assay

COS-7 cells (ATCC, Manassas, VA, U.S.A.) were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FBS (fetal bovine serum). PLC activity was determined as described previously [3] with the following modifications. Cells were routinely used at passages 20–30 unless indicated; lower-passage cells give higher basal PLC ϵ activity. Cells were seeded into 24-well tissue-culture plates at a density of 4×10^5 , grown overnight and then transfected in conditioned medium with 0.25 μ g of each type of cDNA construct (unless indicated otherwise) using 1 μ l of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, U.S.A.) per well following the manufacturer's protocol. After exposure to the DNA–liposome complex overnight, the cells to be assayed for PLC activity were labelled for 24 h with 2 μ Ci of [^{3} H]myo-inositol in inositol-free, serum-free medium.

The PLC assay was initiated with the addition of reagents or vehicle where indicated and 20 mM LiCl, and incubated for 60 min unless indicated. Agonists were dissolved as follows: LPA, 0.1 % BSA in PBS; S1P, 100 % methanol; thrombin, 0.1 % poly-(ethylene glycol)/1 mg/ml BSA in PBS; and EGF, 0.1 % BSA/10 mM acetic acid. The reaction was stopped by aspirating off the incubation medium and adding 375 μ l of ice-cold 50 mM formic acid. After a 15 min incubation on ice, samples were removed, neutralized with 125 μ l of 150 mM NH $_4$ OH and diluted with 5 ml of ice-cold water. Total inositol phosphates were separated by column chromatography using AG 1-X8 200–400 μ m mesh, formate form, and quantitated by liquid scintillation counting. Expression was determined in parallel wells without radioactivity by Western blot analysis and was not significantly different within an experiment unless stated. Steady-state labelling of PtdIns was not significantly different between conditions unless stated.

Calculations and statistical analysis

Experiments were performed in duplicate except EGF experiments with S17N H-Ras and RapGAP1, which were performed in quadruplicate. A LacZ control, in which a plasmid expressing LacZ replaces the variable being examined in the transfection, was determined for each condition. In some figures the data have been reduced for comparison purposes. Basal PLC activity attributable to a given PLC isoform was determined by subtracting the c.p.m. obtained for the LacZ control from the overexpressed PLC isoform c.p.m. and is given when a data transformation is made, e.g. PLC ϵ – LacZ. For stimulation, because the basal PLC activity was different for each PLC isoform and stimulation by EGF was less than the GPCR agonists, the measure fold basal PLC isoform was used. This was calculated by first subtracting the LacZ control for each condition and then dividing the stimulated by unstimulated c.p.m., e.g. (PLC ϵ LPA – LacZLPA)/(PLC ϵ – LacZ). For inhibition, percentage of control stimulation was used. This was calculated by first subtracting the LacZ control for each condition, then subtracting the unstimulated PLC activity from the stimulated PLC activity and then dividing the c.p.m. obtained with the mutant PLC ϵ or inhibitor by the counts

Figure 1 EGF, FBS, LPA, S1P and thrombin stimulate PLC ϵ

COS-7 cells were transiently transfected with plasmids expressing PLC ϵ or LacZ, and PLC activity was determined in response to EGF, FBS, LPA, S1P and thrombin. Values are means $\pm$ S.D. from a representative of at least three similar experiments performed in duplicate.

without the mutant PLC ϵ or inhibitor $\times 100$, e.g. [(K2150EPLC ϵ LPA – LacZLPA) – (K2150EPLC ϵ – LacZ)]/[(PLC ϵ LPA – LacZLPA) – (PLC ϵ – LacZ)] $\times 100$.

Paired or unpaired Student's *t* tests were performed where appropriate. A value of *P* < 0.05 was considered significant.

RESULTS

RTK and GPCR agonists stimulate PLC ϵ

Because we have previously demonstrated that PLC ϵ is activated by Ras [3], we determined whether RTK agonists that activate Ras stimulate PLC ϵ . The data illustrated in Figure 1 demonstrate that EGF modestly stimulates PLC ϵ overexpressed in COS-7 cells. FBS also stimulated PLC ϵ (Figure 1). This stimulation was significantly 4–5-fold greater than EGF even at 1% FBS. Boiling the serum or using calf or horse serum did not significantly affect the stimulation (results not shown), indicating the response was not due to protein growth factors. In addition, inhibition by RA2 domain point mutants was different from EGF and Q61L H-Ras, suggesting a distinct mechanism of activation (discussed below, Figure 3A).

Serum contains significant amounts of the LPA (1–10 μ M) and S1P (500 nM), so we tested whether LPA or S1P would stimulate PLC ϵ . Figure 1 shows the effect of various hormones including LPA, S1P and thrombin on total inositol phosphate accumulation in the presence or absence of overexpressed PLC ϵ . LPA effectively stimulated inositol phosphate accumulation in the presence of PLC ϵ with an EC₅₀ of 3 μ M, within the range 1–100 μ M used to study its effects in other tissues [18]. Similarly, S1P also stimulated PLC ϵ . The *K*_d for S1P receptors is in the low nanomolar range [14]. The EC₅₀ for S1P in our studies was 100 nM, but S1P at concentrations as low as 10 nM stimulated PLC ϵ consistent with a receptor-mediated activation. Thrombin also stimulated and at very low concentrations with an EC₅₀ of 30 pM, consistent with activation of a PAR-1 receptor [15] previously characterized in COS-7 cells [19].

Relative activation of PLC ϵ and other isoforms of PLC

EGF is known to activate PLC γ 1 and LPA, S1P and thrombin have been postulated to activate PLC β isoforms through G_q- and G_i-family G-proteins. We compared the efficacy of these agonists to activate PLC ϵ and other PLC isoforms overexpressed in

COS-7 cells. EGF modestly stimulated PLC ϵ and PLC γ 1 to similar levels, 1.5–2-fold (Figure 2A). In an attempt to enhance this stimulation, EGFR was co-transfected with PLC ϵ or PLC γ 1; however, EGFR had disparate effects on the two PLC isoforms. EGFR markedly potentiated the response to EGF in cells expressing PLC γ 1 in the absence or presence of EGF. In contrast, EGFR alone slightly inhibited basal PLC ϵ activity and only modestly potentiated the effect of EGF.

To determine if Ras limits the EGF stimulation, cells were co-transfected with wild-type H-Ras and PLC ϵ . Figure 2(C) demonstrates that wild-type H-Ras synergistically enhanced the EGF stimulation of PLC ϵ , but not PLC γ 1. This suggests that the level of Ras protein limits the EGF stimulation of PLC ϵ in COS-7 cells and demonstrates that acute receptor activation of Ras stimulates PLC ϵ .

Unexpectedly, we found that serum, LPA, S1P and thrombin activated transfected PLC ϵ more effectively than PLC β 1 or PLC β 2 isoforms overexpressed in COS-7 cells (Figure 2E). FBS and LPA, but not S1P or thrombin, slightly stimulated PLC β 2, but significant stimulation of PLC β 1 by these agonists was not observed. This difference was not due to lack of expression (Figure 2F) or ability to activate the enzymes since AIF₄[–] effectively stimulated all three isoforms. Furthermore, wild-type G α_q selectively activated overexpressed PLC β 1, but did not potentiate the activation of PLC β 1 by these agonists, indicating that G_q is not limiting (G. G. Kelley, unpublished work). It is important to emphasize, however, that these are overexpression studies and should not be interpreted to indicate that these GPCR agonists preferentially couple to endogenous PLC ϵ . Further studies are required in this regard. What they do demonstrate, though, is that signals generated by these agonists in COS-7 cells selectively activate PLC ϵ .

Distinct dependence on the RA2 domain for RTK and GPCR agonist stimulation of PLC ϵ

We previously demonstrated that specific point mutations in the RA2 domain of PLC ϵ inhibit Ras binding and Q61L H-Ras activation of PLC in parallel [3]. Two mutants were particularly instructive: K2150A, which partially inhibited the response to Q61L H-Ras by approx. 50%, and K2150E, which almost completely inhibited the response [3]. These mutations inhibited the basal activity of PLC ϵ by about 50%, from $(8.17 \pm 0.98) \times 10^{-3}$ to $(4.06 \pm 0.56) \times 10^{-3}$ and $(4.66 \pm 0.64) \times 10^{-3}$ c.p.m. for the K2150A and K2150E mutants respectively (*P* < 0.0001; *n* = 53).

Figure 2 Comparison of agonist stimulation of PLC ϵ with PLC γ 1, PLC β 1 and PLC β 2

(A) Effect of EGFR on EGF-stimulated PLC ϵ and PLC γ 1. Cells were co-transfected with PLC ϵ , PLC γ 1 or LacZ and EGFR or LacZ and then stimulated with 20 ng/ml EGF or vehicle and PLC activity was determined (PLC ϵ , $n = 8$; PLC γ 1, $n = 3$). All values were at least $P < 0.05$ compared with control. Basal PLC ϵ and PLC γ 1 activity: PLC ϵ , $(5.9 \pm 1.3) \times 10^{-3}$; PLC γ 1, $(1.1 \pm 0.04) \times 10^{-3}$ c.p.m. (B) Immunoblot using anti-EGFR, -PLC ϵ and -PLC γ 1 antisera. (C) Effect of wild-type H-Ras on EGF-stimulated PLC ϵ and PLC γ 1. Cells were co-transfected with PLC ϵ , PLC γ 1 or LacZ and wild-type H-Ras or LacZ (PLC ϵ , $n = 5$; PLC γ 1, $n = 4$). Basal PLC ϵ and PLC γ 1 activity: PLC ϵ , $(12.1 \pm 3.1) \times 10^{-3}$; PLC γ 1, $(1.2 \pm 0.2) \times 10^{-3}$ c.p.m. (D) Immunoblot using anti-Ras, -PLC ϵ and -PLC γ 1 antisera. (E) Effect of FBS (10%), LPA (3 μ M), S1P (300 nM), thrombin, (300 pM) or AIF $_4^-$ (10 mM NaF and 10 μ M AICl $_3$) on PLC ϵ , PLC β 1 and PLC β 2. Cells were transiently transfected with plasmids expressing PLC ϵ , PLC β 1, PLC β 2 or LacZ ($n = 2-11$). * P at least < 0.05 compared with control. Basal PLC ϵ , PLC β 1 and PLC β 2 activity: PLC ϵ , $(3.8-5.5) \times 10^{-3}$; PLC β 1, $(0.3-1.3) \times 10^{-3}$; PLC β 2, $(2.2-4.4) \times 10^{-3}$ c.p.m. (F) Immunoblot using antibodies for PLC ϵ , PLC γ 1, PLC β 1 and PLC β 2 (lysate overexpressing PLC γ 1 diluted 20-fold to compare with endogenous PLC γ 1 expression). Data are means $\pm$ S.E.M. from indicated number of experiments performed in duplicate and are expressed as fold stimulation of PLC isoform (PLC ϵ , PLC γ 1, PLC β 1, PLC β 2): stimulated PLC isoform activity over basal or unstimulated activity (see the Experimental section for details).

Our previous studies examining the effects of Q61L H-Ras on activation of PLC ϵ using these and additional RA2 domain mutants showed a direct parallel between mutationally induced decreases in Q61L H-Ras binding and activation of PLC ϵ [3]. We therefore utilized these mutants to determine whether EGF, FBS, LPA, S1P and thrombin were inhibited in the same manner as Q61L H-Ras. Similar to our previous studies [3], activation of K2150A PLC ϵ by Q61L H-Ras was 50% lower than for wild-type while K2150E PLC ϵ was 90% lower (Figure 3A, lower panel). Stimulation of both PLC ϵ mutants by FBS, on the other hand, was 50% lower than for wild-type (Figure 3A, upper panel). We therefore compared the effect of these PLC ϵ mutations on its ability to be activated by EGF, LPA, S1P and thrombin. Figure 3(B) shows the stimulation of each PLC ϵ mutant relative to wild-type PLC ϵ (see the Experimental section for calculation details). EGF-dependent PLC ϵ activation was inhibited by these

point mutations in the RA2 domain, 90% by the K2150A mutation and completely by the K2150E mutation, demonstrating a complete dependence on the RA2 domain. Like FBS, however, LPA-, S1P- and thrombin-dependent PLC ϵ activation were only inhibited by approx. 50% and equally by both mutations (K2150A = K2150E). Thus while EGF stimulation of PLC ϵ was completely dependent on the RA2 domain, FBS, LPA, S1P and thrombin stimulation were only partially dependent.

Ras, Rho and G $_{12}$ family G-proteins stimulate PLC ϵ by RA2-dependent and -independent mechanisms

Because the profile of inhibition of agonist stimulation of PLC ϵ by the RA2 mutations was distinct from Q61L H-Ras, other constitutively active G-proteins in the Ras, Rho and G $_{12}$ families were screened for their ability to activate PLC ϵ . The data in

Figure 3 Dependence of RTK and GPCR agonists on the Ras-binding RA2 domain of PLC ϵ

(A) Effect of RA2 domain point mutations on 10 % FBS and constitutively active Q61L H-Ras stimulation of PLC ϵ . COS-7 cells were transiently co-transfected with plasmids expressing wild-type (WT) PLC ϵ , K2150A PLC ϵ , K2150E PLC ϵ or LacZ and Q61L H-Ras or LacZ and PLC activity was determined as indicated ($n = 4$). All mutant stimulation, $P < 0.01$ compared with wild-type control. (B) Effect of RA2 domain point mutations on agonist stimulation of PLC ϵ . COS-7 cells were transfected as in (A) and stimulated with EGF (20 ng/ml), FBS (10 %), LPA (3 μ M), S1P (300 nM) and thrombin (300 pM; $n = 3-24$). Values are mean $\pm$ S.E.M. and are presented as percentage stimulation of mutant PLC ϵ relative to wild-type PLC ϵ (see the Experimental section for details). All values P at least < 0.05 compared with control. *At least $P < 0.05$ compared with corresponding K2150A mutant. Basal PLC ϵ activity: wild-type PLC ϵ , $(8.17 \pm 0.98) \times 10^{-3}$; PLC ϵ -K2150A, $(4.06 \pm 0.56) \times 10^{-3}$; PLC ϵ -K2150E, $(4.66 \pm 0.64) \times 10^{-3}$ c.p.m.; mutants $P < 0.0001$ compared with wild-type; $n = 53$. (C) Immunoblot showing expression of PLC ϵ proteins.

Figure 4(A) show that, in addition to Q61L H-Ras, Ras family members Q72L TC21, Q63E Rap1A, Q63E Rap2A, Q63E Rap2B and G23V RalA, Rho family members G14V RhoA and G12V Rac, and G₁₂ family members Q229L G_{α12} and Q226L G_{α13} stimulated PLC activity of transfected PLC ϵ . For comparison purposes, the stimulation is also presented as fold basal PLC ϵ activity (see the Experimental section for calculation details; Figure 4B). The effect of these G-proteins was not mediated by an increase in the expression of PLC ϵ (see Figure 5C). Stimulation by the Rap G-proteins was sensitive to the overexpressed protein level and only at low expression levels did they stimulate PLC ϵ (results not shown). Q229L G_{α12} and Q226L G_{α13} inhibited PLC ϵ expression but this inhibition was decreased by reducing the amount of plasmid used (results not shown). G12V CDC42 was also examined and stimulation of PLC ϵ was not observed but this G-protein also inhibited PLC ϵ expression.

We next determined whether these G-proteins that stimulate PLC ϵ were inhibited by mutations in the RA2 domain in a similar pattern to the various agonists (Figure 5A). The effect of the RA2 domain point mutations on the constitutively active G-protein stimulation of PLC ϵ could be broadly divided into two groups. The Ras family G-proteins TC21, Rap1A, Rap2B and Rap2A displayed a similar profile to H-Ras, with the activation of K2150A PLC ϵ > K2150E PLC ϵ , and the K2150E PLC ϵ mutant almost completely resistant to stimulation. A second group of

G-proteins was identified whose ability to activate PLC ϵ was unaffected by RA2 domain point mutations. These included Ral, Rho, Rac, G_{α12} and G_{α13}. The lack of effect of these mutations on the stimulation of PLC ϵ by these G-proteins was not due to a distinct molecular interaction with the RA2 domain independent of the K2150 residue because deleting the RA2 domain yielded similar results (results not shown).

To correlate activation of PLC ϵ with binding to the RA2 domain, a GST pull-down assay was performed (Figure 5B). Consistent with their RA2-dependent activation of PLC ϵ , active TC21, Rap1A, Rap2A and Rap2B bound to the RA2 domain of PLC ϵ and was precipitated with a GST-RA2 fusion protein. Furthermore, inhibition of PLC activation paralleled inhibition of binding to the RA2 domain mutants. The binding of these G-proteins was also GTP-dependent. Constitutively active, GTP-bound TC21 and Rap1A bound to a greater degree than their respective wild-type proteins, which are only partially GTP-bound under basal conditions. Constitutively active and wild-type Rap2A and Rap2B bound equally well, consistent with reports of high basal activation of Rap2 [20]; however, dominant-negative mutants showed no binding (results not shown). We also found that Rap1B (results not shown) and Rap1A (Figure 5B) bound to the RA1 domain in a GTP-independent manner, which may be responsible for the relatively low stimulatory effects of Rap1 [21,22].

Figure 4 Ras, Rho and G₁₂ family members stimulate PLCε

COS-7 cells were transiently transfected with plasmids expressing PLCε or LacZ and constitutively active G-protein (250 ng/well in 24-well plates except Rap1A, 2A and 2B, 10–100 ng/well, and Ral, G_{α12} and G_{α13}, 30 ng/well) or vector as indicated and PLC activity was determined ($n = \text{at least } 3$). **(A)** Values are mean $\pm$ S.E.M. from the indicated number of experiments performed in duplicate. **(B)** For comparison and statistical analysis, values are expressed as fold stimulation of PLCε co-expressed with G-protein relative to PLCε co-expressed with LacZ (see the Experimental section for details). All values at least $P < 0.05$ compared with paired control except for Rap1B. See Figure 5 for expression data.

Effects of FBS, LPA, S1P and thrombin are partially mediated by the G₁₂ family of heterotrimeric G-proteins

Because LPA, S1P and thrombin couple to the G₁₂ family of heterotrimeric G-proteins, which are endogenously expressed in COS-7 cells (results not shown), and the profile of inhibition of Q229L G_{α12} and Q226L G_{α13} by the RA2 mutants was similar, we determined whether G_{α12}/G_{α13} mediated the response of these agonists. The RGS domain of p115RhoGEF binds and activates the GTPase activity of G_{α12} and G_{α13} and has been used to inhibit signalling pathways mediated by these G-proteins [23].

Figures 6(A) and 6(B) show that co-expression of the RGS domain with PLCε markedly inhibited LPA, S1P and thrombin stimulation of PLCε in a dose-dependent manner. In contrast to its effects on these agonists, the RGS domain had no effect on the ability of EGF, Q61L H-Ras or Q229L G_{α12} to stimulate PLCε (Figure 6B). It is likely that the RGS domain did not inhibit Q229L G_{α12} because of the relatively high level of overexpression of the G_{α12} subunit and its inability to stimulate the GTPase activity of the mutant.

We next determined whether wild-type G_{α12} or G_{α13} would potentiate the stimulatory effects of LPA, S1P and thrombin on PLCε. Wild-type G_{α12} potentiated the response to LPA and

Figure 5 Effect of RA2 domain point mutations on agonist and G-protein-stimulated PLC activity

(A) COS-7 cells were transiently transfected and PLC activity was determined as in Figure 3(A) ($n = 3-16$). All values P at least < 0.05 compared with control except G α_{13} , Ral, Rho and Rac. * P at least < 0.05 compared with corresponding K2150A mutant. (B) G-protein binding to the RA1 and RA2 domains of PLC ϵ . Pull-down assays were performed with lysates of COS-7 cells overexpressing indicated G-proteins and purified GST and GST fusion proteins of the Ras-binding domain of Raf-1 (RafRBD), PLC ϵ -RA1 domain (RA1), wild-type (RA2) and mutant PLC ϵ -RA2 domains (RA2-K2150A, RA2-K2150E; representative of at least two experiments). Immunoblot analysis was performed using anti-RalA, anti-HA (TC21), anti-Rap1 and anti-Rap2 antibodies. Equivalent amounts of wild-type and active G-protein were used for binding in each pair (results not shown). No binding was observed to S17N Rap2A or S17N Rap2B (results not shown). (C) Immunoblot showing expression of G-proteins and effects on PLC ϵ expression (H-Ras has been shown previously [3]).

thrombin but not S1P or EGF (Figure 6D). Attempts were made to determine whether wild-type G α_{13} also potentiated the response to these agonists and in particular S1P; however, even at low levels, wild-type G α_{13} inhibited expression of PLC ϵ by greater than 70% (see Figure 6E). In contrast to the synergy observed with G α_{12} , wild-type H-Ras did not potentiate LPA, S1P or thrombin (Figure 6D).

LPA and S1P stimulation are partially sensitive to pertussis toxin

Because G β and G γ subunits co-expressed with PLC ϵ stimulate PLC ϵ [8] and LPA, S1P and thrombin also couple to the G β /G γ

family of G-proteins, we determined whether part of the stimulation of FBS, LPA, S1P or thrombin is mediated by activation of G β (G β but not G α is expressed in COS-7 cells [24]). Pertussis toxin inhibits G β activation and G β γ release by ADP-ribosylating G α_i . Pre-incubation with pertussis toxin, 100 μ g/ml overnight, had no effect on PLC ϵ expression or basal PLC ϵ activity but modestly inhibited LPA (3 μ M; $35 \pm 5\%$, $P < 0.01$; $n = 4$) and S1P (300 nM; $31 \pm 4\%$, $P < 0.02$; $n = 4$) stimulation of PLC ϵ . In contrast, pertussis toxin did not inhibit thrombin (300 pM) or EGF (20 ng/ml). Since G α_i does not stimulate PLC ϵ [4], these studies are consistent with G β γ mediating at least part of the effects of LPA and S1P on PLC ϵ .

Figure 6 $G_{\alpha 12}/G_{\alpha 13}$ family partially mediates FBS, LPA, S1P and thrombin stimulation of PLC_{ϵ}

(A) Effect of the RGS domain of p115RhoGEF on agonist stimulated PLC_{ϵ} activity. COS-7 cells were transiently transfected with plasmids expressing PLC_{ϵ} or LacZ and vector or RGS domain, stimulated with LPA (3 μ M), S1P (300 nM) and thrombin (300 pM), and PLC_{ϵ} activity was determined (representative of two experiments). Values are corrected for a decrease in PLC_{ϵ} expression (0–28 %) and reduced steady-state labelling (5–29 %). (B) COS-7 cells were transfected as in A (RGS, 250 ng/well) except Q61L H-Ras, Q229L $G_{\alpha 12}$ (30 ng/well) or LacZ was added as indicated and FBS, 10 %, was used in addition to agonists used in A ($n = 3$ –7). Values are means $\pm$ S.E.M. from the indicated number of experiments performed in duplicate and are expressed as percent stimulation of PLC_{ϵ} co-expressed with RGS relative to PLC_{ϵ} co-expressed with LacZ (see the Experimental section for details). * P at least < 0.01 compared with control stimulation. Basal PLC_{ϵ} activity: PLC_{ϵ} , 6.90 ± 1.65 ; PLC_{ϵ} + RGS domain, 7.29 ± 1.22 , corrected for decrease in PLC_{ϵ} expression, 18 %, and decrease in steady-state labelling, 28 %, $P =$ not significant; $n = 9$. (C) Immunoblot showing expression of PLC_{ϵ} and RGS domain. (D) COS-7 cells were transfected with PLC_{ϵ} or LacZ and wild-type $G_{\alpha 12}$ (10 ng/well) or wild-type H-Ras and stimulated with LPA (3 μ M), S1P (300 nM), thrombin (300 pM) or EGF (20 ng/ml); $G_{\alpha 12}$ $n = 4$ paired experiments). Basal PLC_{ϵ} activity, $(3.24 \pm 0.76) \times 10^{-3}$ c.p.m.; wild-type H-Ras $n = 3$ paired experiments. Basal PLC_{ϵ} activity: $(4.09 \pm 1.47) \times 10^{-3}$ c.p.m. * P at least < 0.05 compared with calculated additive value marked with dashed line. (E) Immunoblot showing effect of indicated G-proteins on PLC_{ϵ} expression.

RA2-dependent stimulation of LPA, S1P and thrombin

The partial inhibition of serum, LPA, S1P and thrombin stimulation of PLC_{ϵ} by RA2 domain point mutations suggests that stimulation by these agonists is a composite of RA2-dependent and -independent inputs. LPA, S1P and thrombin have also been shown to transactivate the EGFR [16,17,19], which would be expected to generate an RA2-dependent signal similar to EGF. Figure 7(A) shows that AG1478, a specific EGFR kinase inhibitor, potently inhibited EGF and EGF + wild-type Ras. AG1478, however, only slightly inhibited LPA-stimulated activity by 10 % [$(7.35 \pm 0.56) \times 10^{-3}$ to $(6.60 \pm 0.53) \times 10^{-3}$ c.p.m., $P < 0.02$; $n = 5$] and had no significant effect on S1P or thrombin-stimulated PLC_{ϵ} activity, indicating that EGFR transactivation does not have a major role in the stimulation of PLC_{ϵ} by these agonists in COS-7 cells.

Ras and Rap GEFs are also activated by second messengers, Ca^{2+} and diacylglycerol, generated from stimulation of PLC [16,25]. We therefore utilized dominant-negative S17N H-Ras,

which inhibits Ras-dependent pathways by preventing GEFs from exchanging GDP with GTP, and RapGAP1, a GTPase-activating protein for Rap1 and to a lesser extent Rap2 [26]. Dominant-negative Ras significantly inhibited EGF and EGF + wild-type H-Ras by 43 and 93 % respectively but had no significant effect on LPA, S1P or thrombin stimulation of PLC_{ϵ} (Figure 7B). RapGAP1, on the other hand, significantly inhibited activation of PLC_{ϵ} by all agonists (Figure 7C). LPA, S1P and thrombin activation of PLC_{ϵ} were inhibited by approx. 30 % and EGF was inhibited by 54 %, suggesting that Rap mediates at least part of the RA2-dependent stimulation by these agonists.

EGF, LPA, S1P or thrombin do not interact synergistically to stimulate PLC_{ϵ}

Because EGF activates PLC_{ϵ} by a distinct pathway from LPA, S1P or thrombin, we determined whether these agonists would interact synergistically to stimulate PLC_{ϵ} . Table 1 lists the fold stimulation above PLC_{ϵ} control for each agonist alone and in combination and

Figure 7 RA2-dependent agonist stimulation

(A) Effect of AG1478 on agonist-stimulated PLC activity. COS-7 cells were transiently transfected with plasmids expressing PLC ϵ or LacZ and wild-type H-Ras or LacZ and PLC activity was determined as indicated. Transfected cells were incubated with AG1478 (0.1–1 μ M), added 10 min prior to start of the experiment and then FBS (10%), LPA (3 μ M), S1P (300 nM), thrombin (300 pM) or EGF (20 ng/ml) was added ($n = 3$ –4). * P at least < 0.05 compared with control. Basal PLC ϵ activity: PLC ϵ , $(5.54 \pm 1.35) \times 10^{-3}$; PLC ϵ + AG1478, $(5.37 \pm 1.16) \times 10^{-3}$ c.p.m.; P = not significant; $n = 5$. (B) Effect of dominant-negative S17N H-Ras on agonist-stimulated PLC activity. COS-7 cells were transiently transfected as in A except S17N H-Ras (125 ng/well) or wild-type H-Ras (125 ng/well) or LacZ was used as the second plasmid and PLC activity was determined as indicated ($n = 4$ –6). * P at least < 0.01 compared with control. Basal PLC ϵ activity: PLC ϵ , $(3.34 \pm 1.22) \times 10^{-3}$; PLC ϵ + S17N H-Ras, $(2.96 \pm 1.22) \times 10^{-3}$ c.p.m.; P = not significant, $n = 6$. (C) Effect of RapGAP1 on agonist-stimulated PLC activity. COS-7 cells were transiently transfected as in B except RapGAP1 (25 ng/well) or LacZ was used as the second plasmid and PLC activity was determined as indicated ($n = 4$ –6). * P at least < 0.05 compared with EGF + wild-type H-Ras and thrombin. Basal PLC ϵ activity: PLC ϵ , $(1.68 \pm 0.35) \times 10^{-3}$; PLC ϵ + RapGAP1, $(1.28 \pm 0.27) \times 10^{-3}$ c.p.m.; P = not significant, $n = 6$. (D) Immunoblot showing expression of RapGAP1, Ras and PLC ϵ . Values are means $\pm$ S.E.M. from the indicated number of experiments performed in duplicate and are expressed as percentage stimulation of PLC ϵ in the presence of AG1478, S17N H-Ras or RapGAP1 as indicated relative to stimulation of PLC ϵ in the absence of each regulator (see the Experimental section for details).

Table 1 Hormonal and GTPase interactions

COS-7 cells were transfected with PLC ϵ or LacZ and with vector, Q61L H-Ras and/or Q229L G α_{12} (30 ng/well) as indicated. Cells were stimulated with LPA (3 μ M), S1P (300 nM), thrombin (300 pM) and/or EGF (20 ng/ml) and total inositol phosphates were measured. Values are fold stimulation above control and are means $\pm$ S.E.M. from the indicated number of experiments performed (n) in duplicate. First and second refer to the agents listed under condition. Combined is the value obtained in the presence of both agents. The expected value by adding the response to both stimulators is given in parentheses in the combined column. P shows the P value comparing the observed combined value with the expected calculated value; NS, not significant.

Condition	Fold stimulation above control			P	n
	First	Second	Combined		
LPA + S1P	1.09 ± 0.11	0.58 ± 0.07	1.86 ± 0.37 (1.67 ± 0.15)	NS	3
LPA + thrombin	1.42 ± 0.21	0.88 ± 0.20	2.29 ± 0.39 (2.29 ± 0.40)	NS	5
S1P + thrombin	0.83 ± 0.26	0.64 ± 0.09	1.48 ± 0.38 (1.48 ± 0.71)	NS	4
LPA + EGF	1.50 ± 0.24	0.41 ± 0.12	2.08 ± 0.38 (1.91 ± 0.32)	NS	11
S1P + EGF	0.83 ± 0.26	0.45 ± 0.20	1.23 ± 0.59 (1.28 ± 0.37)	NS	4
Thrombin + EGF	0.69 ± 0.9	0.50 ± 0.16	1.19 ± 0.28 (1.18 ± 0.21)	NS	5
61L H-Ras + Q229L G α_{12}	4.18 ± 1.1	3.45 ± 0.84	4.33 ± 1.40 (7.63 ± 1.95)	< 0.03	3

the calculated additive value. With each combination, EGF plus LPA, S1P or thrombin, the combined response was not greater than the calculated additive response. Varying the time or dose

gave similar results (results not shown). These studies indicate that although the mechanisms of activation are distinct they do not interact synergistically to stimulate PLC ϵ . Similar results

were obtained for the combinations of LPA, S1P and thrombin. Furthermore, the combination of Q61L H-Ras and Q229L $G_{\alpha 12}$ was not greater than either G-protein alone also suggesting non-interacting activation pathways.

DISCUSSION

Our studies demonstrate that RTK and GPCR agonists diversely regulate PLC ϵ . We show that EGF stimulates PLC ϵ through an endogenous EGFR by a mechanism completely dependent on the Ras-binding RA2 domain. We previously demonstrated that constitutively active Q61L H-Ras activates PLC ϵ by binding to the RA2 domain [3] and the present studies suggest that the EGF stimulation is partially mediated by Ras because dominant-negative S17N H-Ras partially inhibited and wild-type H-Ras markedly potentiated the EGF stimulation. Ras, however, does not appear to be the sole G-protein that mediates the effects of EGF under the conditions studied because point mutations in the RA2 domain inhibited the EGF stimulation differently to Q61L H-Ras. In particular, EGF was more sensitive to the K2150A mutation. Of the G-proteins examined, the effect of the RA2 domain mutations on the stimulation of PLC ϵ by Rap, and in particular Rap1A or Rap2B, was most similar to EGF. EGF has been previously shown to activate Rap in COS-7 cells [27] and we demonstrated that RapGAP1 partially inhibited the EGF stimulation of PLC ϵ , consistent with a role for Rap participating in the activation. These results are consistent with recent studies by Kataoka and co-workers [6], who overexpressed PLC ϵ and a mutant PDGF receptor in BaF3 cells and demonstrated that PDGF stimulation of PLC ϵ was inhibited by dominant negative Ras and Spa1, a Rap GAP. Thus RTK activation of PLC ϵ by EGF and PDGF appears to be dually mediated by both Ras and Rap.

LPA, S1P and thrombin also stimulated PLC ϵ through endogenous receptors. This stimulation was greater than for EGF, although, in the presence of wild-type H-Ras the stimulation of PLC ϵ by EGF approached the levels observed with these agonists. Serum also stimulated PLC ϵ , probably through high concentrations of LPA and S1P in serum. Unlike EGF, however, mutations in the RA2 domain only partially inhibited the stimulatory effects of these agonists, indicating a distinct RA2-independent mechanism of activation. In an attempt to elucidate this pathway we identified a group of G-proteins that stimulate PLC ϵ in a predominantly RA2-independent manner, determined by stimulation of RA2 domain point mutants, which included $G_{\alpha 12}$, $G_{\alpha 13}$, RalA, RhoA and Rac. The mechanism by which these G-proteins stimulate PLC ϵ is not known; however, several lines of evidence presented here suggest that endogenous receptor activation of $G_{\alpha 12}$ and/or $G_{\alpha 13}$ functionally mediates the stimulation of PLC ϵ by LPA, S1P and thrombin in COS-7 cells. First, the profile of inhibition by mutations in the RA2 domain was similar; secondly, the RGS domain of p115RhoGEF, which binds and inactivates $G_{\alpha 12}$ and $G_{\alpha 13}$ [23], inhibited the stimulation of PLC ϵ ; and thirdly, wild-type $G_{\alpha 12}$ potentiated the stimulation of PLC ϵ induced by LPA and thrombin.

$G_{\alpha 12}$ and $G_{\alpha 13}$ are known to activate Rho by stimulating the exchange activity of Rho GEFs [28], and both $G_{\alpha 12}$ and $G_{\alpha 13}$ have been shown to stimulate Rho in COS-7 cells [29]. Thus, it is possible that the stimulatory effects of these G-proteins are mediated through activation of Rho, which we have demonstrated stimulates PLC ϵ in an RA2-independent manner similar to $G_{\alpha 12}$ and $G_{\alpha 13}$. On the other hand, we also observed differences in the stimulation of PLC ϵ by active $G_{\alpha 12}$, $G_{\alpha 13}$ and RhoA. This was particularly apparent for Q229L $G_{\alpha 12}$, which stimulated PLC ϵ more than Q226L $G_{\alpha 13}$, and was more dependent on the RA2

domain than either Q226L $G_{\alpha 13}$ or G14V RhoA. Thus, while it is likely that part of the stimulatory response to $G_{\alpha 12}$ and $G_{\alpha 13}$ is mediated by Rho, a component may be Rho-independent.

We also found that the effects of LPA and S1P, but not thrombin, were partially sensitive to pertussis toxin. This suggests that these agonists stimulate PLC ϵ through activation of G_i and probably through $G\beta\gamma$ since $G\alpha_i$ does not stimulate PLC ϵ [4]. Interestingly, a similar differential sensitivity of LPA and thrombin to pertussis toxin has been noted previously in COS-7 cells for transactivation of the EGFR [19]. The mechanism by which $G\beta\gamma$ stimulates PLC ϵ is not known, but it is RA2-independent [8] and appears distinct from its regulation of PLC β isoforms because purified $G\beta\gamma$ failed to stimulate PLC ϵ but markedly stimulated PLC $\beta 2$ in an *in vitro* reconstitution assay [3].

In addition to the RA2-independent stimulation, our studies also suggest that a component of the LPA, S1P and thrombin stimulation of PLC ϵ is dependent on an intact RA2 domain. Whether these GPCR agonists stimulate a G-protein that binds to the RA2 domain, or simply require a G-protein to be bound to the domain to activate the enzyme, remains to be determined. However, these agonists have been shown to activate Ras or Rap GEFs by mechanisms involving transactivation of RTKs, Ca^{2+} /calmodulin or Ca^{2+} /diacylglycerol [25,30]. While many studies have shown that LPA and thrombin activate the Ras/mitogen-activated protein kinase pathway in COS-7 cells through transactivation of the EGFR [19,31,32], we found that AG1478 inhibited the LPA response by only 10% and had no effect on S1P or thrombin stimulation under conditions where the EGF stimulation was completely inhibited. Furthermore, wild-type H-Ras potentiated the stimulatory response to EGF but not to LPA, S1P or thrombin and dominant negative S17N H-Ras did not significantly inhibit the stimulation. On the other hand RapGAP1 inhibited the stimulation by these GPCR agonists, suggesting a role for Rap. Since Ca^{2+} and diacylglycerol activate certain Rap GEFs [16,19,25], a parallel activation of another PLC isoform or stimulation of PLC ϵ by these agonists could activate Rap. The CDC25 domain has been reported to be a Rap1A GEF [33] and we have made similar observations (A. V. Smrcka and G. G. Kelley, unpublished work). Thus, another intriguing possibility is that this domain mediates part of the Rap- and RA2-dependent activation of PLC ϵ by these agonists.

Our studies demonstrate that LPA, S1P, thrombin and EGF couple to PLC ϵ in COS-7 cells. While differential coupling of these receptors to PLC ϵ and other PLC isoforms will require studies manipulating endogenous isoforms in multiple cell lines, these findings are significant because they raise the possibility that PLC ϵ may regulate critical cellular functions previously thought to be mediated by other PLC isoforms [1,2]. Furthermore, given the diverse regulation of PLC ϵ , it is likely that other pathways will be identified that regulate PLC ϵ . In this regard, $\beta 2$ -adrenergic, M_3 muscarinic and prostaglandin E_1 receptor-mediated activation of cAMP has been shown to stimulate PLC ϵ through activation of the Rap GEF, Epac1 and Rap2B [34,35].

In summary, our studies demonstrate diverse hormonal regulation of PLC ϵ . Activation of a RTK by EGF stimulates PLC ϵ in an RA2-dependent manner mediated by Ras and Rap. In contrast, activation of GPCRs by LPA, S1P and thrombin stimulate PLC ϵ in an RA2-independent manner mediated by G_{12}/G_{13} and G_i and in an RA2-dependent manner through Rap. The mechanism by which G-proteins, $G_{\alpha 12}/G_{\alpha 13}$, Rho, Rac and Ral, stimulate PLC ϵ in an RA2-dependent manner remains to be determined.

We are grateful to Johannes L. Bos, Marc Symons, Gordon Gill, Tohru Kozasa, Lawrence Quilliam, Silvio Gutkind, Dianqing Wu, Ying Huang, Michael Brown, Sue Goo Rhee and Alfred Bothwell for constructs. We especially thank Lawrence Quilliam for helpful

discussions and Joanne Ondrako for excellent technical assistance. These studies were supported by the National Institutes of Health DK56294 (to G. G. K.).

REFERENCES

- Rebecchi, M. J. and Pentylala, S. N. (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol. Rev.* **80**, 1291–1335
- Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70**, 281–312
- Kelley, G. G., Reks, S. E., Ondrako, J. M. and Smrcka, A. V. (2001) Phospholipase C(epsilon): a novel Ras effector. *EMBO J.* **20**, 743–754
- Lopez, I., Mak, E. C., Ding, J., Hamm, H. E. and Lomasney, J. W. (2001) A novel bifunctional phospholipase c that is regulated by G α 12 and stimulates the Ras/mitogen-activated protein kinase pathway. *J. Biol. Chem.* **276**, 2758–2765
- Song, C., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohe, M., Wu, D., Satoh, T. and Kataoka, T. (2001) Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. *J. Biol. Chem.* **276**, 2752–2757
- Song, C., Satoh, T., Edamatsu, H., Wu, D., Tadano, M., Gao, X. and Kataoka, T. (2002) Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase C epsilon. *Oncogene* **21**, 8105–8113
- Murphy, G. A., Graham, S. M., Morita, S., Reks, S. E., Rogers-Graham, K., Vojtek, A., Kelley, G. G. and Der, C. J. (2002) Involvement of phosphatidylinositol 3-kinase, but not RalGDS, in TC21/R-Ras2-mediated transformation. *J. Biol. Chem.* **277**, 9966–9975
- Wing, M. R., Houston, D., Kelley, G. G., Der, C. J., Siderovski, D. P. and Harden, T. K. (2001) Activation of phospholipase C-epsilon by heterotrimeric G protein betagamma-subunits. *J. Biol. Chem.* **276**, 48257–48261
- Riese, D. J., 2nd and Stern, D. F. (1998) Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* **20**, 41–48
- Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1989) EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* **57**, 1101–1107
- Buday, L. and Downward, J. (1993) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* **73**, 611–620
- Kao, S., Jaiswal, R. K., Kolch, W. and Landreth, G. E. (2001) Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells. *J. Biol. Chem.* **276**, 18169–18177
- Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S. and Tigyi, G. (2002) International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol. Rev.* **54**, 265–269
- Kluk, M. J. and Hla, T. (2002) Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 72–80
- Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D. and Plevin, R. (2001) Proteinase-activated receptors. *Pharmacol. Rev.* **53**, 245–282
- Kranenburg, O. and Moolenaar, W. H. (2001) Ras-MAP kinase signaling by lysophosphatidic acid and other G protein-coupled receptor agonists. *Oncogene* **20**, 1540–1546
- Kim, J. H., Song, W. K. and Chun, J. S. (2000) Sphingosine 1-phosphate activates Erk-1/-2 by transactivating epidermal growth factor receptor in rat-2 cells. *IUBMB Life* **50**, 119–124
- van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L. and Moolenaar, W. H. (1993) Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1257–1261
- Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* **16**, 7032–7044
- Ohba, Y., Mochizuki, N., Matsuo, K., Yamashita, S., Nakaya, M., Hashimoto, Y., Hamaguchi, M., Kurata, T., Nagashima, K. and Matsuda, M. (2000) Rap2 as a slowly responding molecular switch in the Rap1 signaling cascade. *Mol. Cell Biol.* **20**, 6074–6083
- Shima, F., Okada, T., Kido, M., Sen, H., Tanaka, Y., Tamada, M., Hu, C. D., Yamawaki-Kataoka, Y., Kariya, K. and Kataoka, T. (2000) Association of yeast adenyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation. *Mol. Cell Biol.* **20**, 26–33
- Hu, C. D., Kariya, K., Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S. and Kataoka, T. (1995) Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. *J. Biol. Chem.* **270**, 30274–30277
- Shi, C. S., Sinnarajah, S., Cho, H., Kozasa, T. and Kehrl, J. H. (2000) G13alpha-mediated PYK2 activation. PYK2 is a mediator of G13alpha-induced serum response element-dependent transcription. *J. Biol. Chem.* **275**, 24470–24476
- van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Koch, W. J. and Lefkowitz, R. J. (1996) G(o)-protein alpha-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. *J. Biol. Chem.* **271**, 1266–1269
- Zwartkruis, F. J. and Bos, J. L. (1999) Ras and Rap1: two highly related small GTPases with distinct function. *Exp. Cell Res.* **253**, 157–165
- Rubinfeld, B., Munemitsu, S., Clark, R., Conroy, L., Watt, K., Crosier, W. J., McCormick, F. and Polakis, P. (1991) Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21rap1. *Cell* **65**, 1033–1042
- Zwartkruis, F. J., Wolthuis, R. M., Nabben, N. M., Franke, B. and Bos, J. L. (1998) Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. *EMBO J.* **17**, 5905–5912
- Schmidt, A. and Hall, A. (2002) Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* **16**, 1587–1609
- Gohla, A., Harhammer, R. and Schultz, G. (1998) The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho. *J. Biol. Chem.* **273**, 4653–4659
- Quilliam, L. A., Rebhun, J. F. and Castro, A. F. (2002) A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog. Nucleic Acid Res. Mol. Biol.* **71**, 391–444
- Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature (London)* **402**, 884–888
- Pierce, K. L., Tohgo, A., Ahn, S., Field, M. E., Luttrell, L. M. and Lefkowitz, R. J. (2001) Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J. Biol. Chem.* **276**, 23155–23160
- Jin, T. G., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C. D. and Kataoka, T. (2001) Role of the CDC25 homology domain of phospholipase Cepsilon in amplification of Rap1-dependent signaling. *J. Biol. Chem.* **276**, 30301–30307
- Evellin, S., Nolte, J., Tysack, K., vom Dorp, F., Thiel, M., Weernink, P. A., Jakobs, K. H., Webb, E. J., Lomasney, J. W. and Schmidt, M. (2002) Stimulation of phospholipase C-epsilon by the M3 muscarinic acetylcholine receptor mediated by cyclic AMP and the GTPase Rap2B. *J. Biol. Chem.* **277**, 16805–16813
- Schmidt, M., Evellin, S., Weernink, P. A., von Dorp, F., Rehmann, H., Lomasney, J. W. and Jakobs, K. H. (2001) A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat. Cell Biol.* **3**, 1020–1024

Received 8 September 2003; accepted 20 October 2003

Published as BJ Immediate Publication 20 October 2003, DOI 10.1042/BJ20031370