Gα13 mediates activation of the cytosolic phospholipase A2α through fine regulation of ERK phosphorylation

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$G\alpha_{13}$ mediates activation of the cytosolic phospholipase $A_2\alpha$ through fine regulation of ERK phosphorylation

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Abstract

Heterotrimeric GTP-binding (G) proteins transduce hormone-induced signals to their effector enzymes, which include several phospholipases. In particular, the G_o/G_i and G_q protein families have been shown to couple signaling to phospholipase A_2 (PLA_2), phospholipase C, and phospholipase D, while the G_{12}/G_{13} family has been linked to the activation of small GTPases of the Rho family, and hence, to phospholipase D activation. Here, we demonstrate that in CHO cells, the G_{12}/G_{13} family is also able to activate $cPLA_2\alpha$, through the activation of RhoA and, subsequently, ERK1/2. Hormone-induced arachidonic acid release increased as a consequence of $G\alpha_{13}$ overexpression, and was inhibited through inhibition of $G\alpha_{13}$ signaling. The $G\alpha_{13}$-mediated $cPLA_2\alpha$ activation was inhibited by pharmacological blockade of ERK1/2 with either U0126 or PD98059, and by RhoA inactivation with C3 toxin or a dominant-negative RhoA (N19RhoA), and was stimulated by the serine–threonine phosphatase inhibitor calyculin A. Our data thus identify a pathway of $cPLA_2\alpha$ regulation that is initiated by thrombin and purinergic receptor activation, and that signals through $G\alpha_{13}$, RhoA and ERK1/2, with the involvement of a calyculin-sensitive phosphatase.

Keywords: Arachidonic acid; $cPLA_2$; G-protein-coupled receptors; G proteins; Mitogen-activated protein kinase; Thrombin; ATP; CHO cells

1. Introduction

The heterotrimeric GTP-binding (G) proteins transduce a variety of signals from the heptahelical G-protein-coupled receptors to their intracellular effectors (reviewed in Neves et al. [1]). On the basis of the sequence similarities of their α subunits, the G proteins have been grouped into four subfamilies: G_s, $G_{i/o}$, G_q and G_{12} [1–3]. The G_{12} subfamily was the last to be identified [4] and remains to be completely characterized in terms of its coupling to downstream effectors.

G_{12} and G_{13}, the two members of the G_{12} subfamily, are ubiquitously expressed and share 67% amino-acid sequence identity [4]. G_{13} is coupled to different receptors, including thromboxane A_2, thrombin and lysophosphatidic acid receptors, and regulates diverse and complex cellular responses by activating multiple signaling pathways [5,6]; for a review, see Riobo [7]. Members of the Rho family of monomeric G-protein GTPases have been well characterized as downstream effectors in G_{13}-mediated signaling (Cdc42, Rac1 and RhoA) [8,9]. In particular, the RGS (regulators of G-protein signaling) domain of the RhoA-specific p115RhoGEF guanine nucleotide exchange factor interacts directly with G_{13} [9,10]. In this way the G_{12} subfamily, through the binding and activation of

Abbreviations: CHO, Chinese hamster ovary; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; $cPLA_2$, cytosolic phospholipase A_2; PKC, protein kinase C; CaMKII, calcium calmodulin kinase II.

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p115RhoGEF, provide a mechanism by which G-protein-coupled receptors can activate RhoA and its downstream responses.

Cytosolic phospholipase A_2 ($cPLA_2$) is a ubiquitous enzyme that hydrolyzes membrane phospholipids to arachidonic acid and lysophospholipids [11,12]. Free arachidonic acid is the substrate for the enzyme pathways that produce the leukotrienes, prostaglandins and thromboxane, which can then be released from the cell into the extracellular space, where they act as hormones, and hence activate specific receptors and cell functions [13]. These metabolites have been shown to have roles in inflammation, neurodegenerative diseases and cancer development [14–16].

The PLA_2 enzymes have been classified into four broad categories: the low-molecular-weight, secretory enzymes; the $cPLA_2$ and its paralogs, group IV enzymes; the calcium-independent, group VI enzymes; and the selective acetyl hydrolases of platelet activating factor, groups VII and VIII PLA_2. This classification is based on their structure, calcium requirements, substrate specificities, and susceptibility to pharmacologic inhibition [17]. Among the available inhibitors, low concentrations (0.5–5.0 μM) of bromoenol lactone (BEL) have been used to selectively inhibit intracellular calcium-independent PLA_2 [18], while the recently synthesised pyrrophenone is a selective inhibitor of a member of the group IV enzymes: the most abundant intracellular calcium-dependent $cPLA_2\alpha$ [19].

$cPLA_2\alpha$ is now well recognized as the crucial enzyme in hormone-induced arachidonic acid release [20–22]. This is also the case in CHO cells, where $cPLA_2\alpha$ is coupled to thrombin (PAR-1) and purinergic ($P2Y_2$) receptor activation for induction of arachidonic acid release [23–25].

Upon hormonal stimulation, $cPLA_2\alpha$ has been shown to translocate from the cytosol to the nuclear envelope, endoplasmic reticulum and Golgi membranes [26,27]. This translocation is calcium dependent and is mediated by the $cPLA_2$ amino-terminal C2 domain [28]. This domain was originally described in protein kinase C (PKC) and has now been identified in more than 60 proteins; in all cases, its presence has been related to calcium-dependent protein translocation to membranes [29,30].

Another requirement for hormone-induced $cPLA_2\alpha$ activation is the phosphorylation of its Ser505 [25,31]. Indeed, $cPLA_2\alpha$ can be phosphorylated on Ser505 by either p42/p44 mitogen-activated protein kinases (ERK1/2 MAPKs) or p38 MAPK [17]. Alternative sites of phosphorylation on $cPLA_2\alpha$ have been described for PKC, protein kinase A and calcium calmodulin kinase II (CaMKII), although only the MAPK-induced phosphorylation can result in a significant increase in $cPLA_2\alpha$ activity [11].

Due to the relevance of $cPLA_2$ in cellular functions, we have decided to define the signaling cascades that lead to its activation. Moreover, a full characterization of the diverse signaling proteins that are regulated by G_{13} is fundamental to the elucidation of the molecular basis for G_{13}-regulated cellular functions. In this study, we have examined the signal transduction pathway involved in $cPLA_2\alpha$ activation and whether it is a downstream effector of G_{13}.

2. Materials and methods

Fetal bovine serum was from Biochrom KG (Berlin, Germany), and acrylamide/bis-acrylamide from Eurobio Labtek (Milan, Italy). Dulbecco's Modified Eagle's Medium (DMEM), Hank's balanced salt solution with calcium (1.3 mM) and magnesium (0.9 mM) ($HBSS^{++}$), and OptiMEM I were from Gibco BRL (Grand Island, NY, USA). Adenosine 5′-triphosphate (ATP) was from Roche Diagnostic GmbH (Indianapolis, IN, USA) and U0126 was from Promega (Madison, WI, USA). Thrombin, fatty-acid-free bovine serum albumin (BSA), sodium orthovanadate, cyclosporin A, and PD 098059 were from Sigma-Aldrich (Milan, Italy). Lipofectamine-PLUS reagent was from Invitrogen Life Technologies (Ontario, Canada). [5,6,8,9,11,12,14,15-^{3}H]-Arachidonic acid (210 Ci/mmol) was from Amersham Pharmacia (Piscataway, NJ, USA). The mouse monoclonal anti-phospho MAP kinase clone 12D4 antibody was from Upstate (Lake Placid, NY, USA). The rabbit polyclonal anti-ERK1 (K-23) and the rabbit polyclonal anti-$G\alpha_{q/11}$ antibodies were from Santa Cruz Biotechnology (San Diego, CA, USA). The rabbit polyclonal anti-$G\alpha_{13}$ C-terminal (367–377), goat anti-rabbit and -mouse IgG horseradish peroxidase conjugates, SB203580 and *Discodermia calyx* calyculin A were from Calbiochem (La Jolla, CA, USA). The rabbit polyclonal anti-phospho p38 MAP kinase (Thr 180/Tyr 182) and the rabbit polyclonal anti-p38 MAP kinase antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The Ultima Gold scintillation fluid was from Canberra-Packard, Co. (Pangbourne, UK). Pyrrophenone was generously provided by Dr. K. Seno (Shionogi Research Laboratories, Shionogi and Co. Ltd., Osaka, Japan) [19]. All other reagents were obtained from Sigma-Aldrich at the highest purities available.

2.1. Cell culture and transfections

Chinese hamster ovary (CHO) cells were grown in monolayers, at 37 °C in 95% air/5% CO_2, in DMEM supplemented with 10% fetal bovine serum, 58 μg/ml proline, 53 μg/ml L-aspartatic acid, 60 μg/ml L-asparagine, 100 U/ml penicillin, and 100 μg/ml streptomycin, 2 mM L-glutamine (growth medium). The plasmids encoding for wild-type $G\alpha_{13}$/pCMV5 and $G\alpha_q$/pCMV5 were from Dr. J. Hepler (Emony University, Atlanta, USA), those encoding for the constitutively active mutants of the Gα subunits, $G\alpha_{13}$Q226L/pCMV5 and $G\alpha_q$Q209L/pcDNA3, were obtained from Dr. D. Dhanasekaran (Temple University School of Medicine, Philadelphia, USA), those encoding for RhoA wild-type and mutants (pRK5myc), and myc-C3 toxin (mycC3/pcDNA1) were from Dr. A. Hall (University College London, UK), and those encoding for protein kinase D wild-type and the kinase inactive form (PKD-K618N) were from Dr. V. Malhotra (University of California, San Diego, USA). For the transient transfections, CHO cells were plated into 12-well plates at 1.0×10^5 cells/well. After 24 h, the cells were transfected in OptiMEM I with the Lipofectamine-PLUS method, according to the manufacturer's instructions, using 0.7 μg cDNA/well. Mock cells were transfected with the pCMV, pCMV5 and pcDNA3 empty vectors.

2.2. Arachidonic acid assay

The arachidonic acid assay was performed as previously described [32], with the following modifications: 24 h after transfection, CHO cells were washed with $HBSS^{++}$ and labelled with 0.1 μCi [^{3}H]-arachidonic acid/well in growth medium for 16 h. The cells were then washed three times with buffer A (10 mM Hepes in $HBSS^{++}$, pH 7.4, plus 0.2% [w/v] fatty-acid-free BSA) before the addition of stimuli (0.5 U/ml thrombin or 100 μM ATP, unless otherwise specified) for the indicated times at 37 °C. When necessary, before agonist stimulation, the cells were treated with inhibitors for the specified times, in buffer A. After stimulation with the agonist of choice, the [^{3}H]-arachidonic acid released by the cells was quantified by scintillation counting. The radioactivity associated to the cell content of arachidonic acid was determined by scraping the cells in 0.6 ml 0.2% [v/v] Triton X-100. In all of the arachidonic acid release experiments the amounts of radioactivity released were expressed as percentages of the total incorporated radioactivity (i.e., arachidonic acid released plus cell content). The reported inhibitions have been calculated as percentage of inhibition of agonist-stimulated [^{3}H]-arachidonic acid release after basal subtraction.

2.3. MAPK assay and immunoblotting

The activation status of ERK1/2 and p38 was determined as previously described [33], with minor modifications. CHO cells were plated into 6-well plates at 2.0×10^5 cells/well. The transfection was performed as described above, with the exception that 2 μg cDNA/well were used. After 24 h, the transfected cells were stimulated for 7 min at 37 °C with thrombin or ATP in buffer A. The reactions were terminated by aspiration of the buffer, washing with $HBSS^{++}$, and scraping into 200 μl ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 200 μM sodium orthovanadate, 40 mM β-glycerophosphate, 1% Triton X-100, 50 μM *O*-phenanthroline, 2 μM pepstatin, and 1 mM phenylmethylsulphonyl fluoride). The lysates were incubated on ice for 15 min and then centrifuged at 20,000×*g* for 5 min at 4 °C. For the immunoblotting analysis, 100 μg of the supernatant proteins were run on 10% SDS-PAGE and electroblotted onto Protran nitrocellulose membranes (Perkin Elmer Life Sciences, Boston, MA, USA). The filters were probed with the rabbit polyclonal anti-phospho p38 and anti-phospho ERK1/2 antibodies and with peroxidase-conjugated secondary antibodies. The Western blots were developed using the chemiluminescent method (ECL, Amersham Pharmacia Biotech, UK). These antibodies were then removed with stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) for 30 min at 55 °C, and the filters were probed again with the rabbit polyclonal anti-p38 or with the rabbit polyclonal anti-ERK1 (K-23) antibodies. Bands were quantified by densitometric analysis using an NIH Image 1.60. The quantification of each band associated to a phospho-specific antibody was normalized for the signal of the corresponding pan-antibody.

2.4. Data analysis

The data are expressed as means±S.E. (or±S.D.; see figure legends) of at least three independent experiments, with each performed in triplicate. Statistical analyses were performed using the Student's *t*-test ($p<0.01$ was considered significant).

3. Results

3.1. $G\alpha_{13}$ mediates arachidonic acid release in CHO cells

CHO cells were used to investigate G-protein-mediated $cPLA_2$ activation. In these cells, it has previously been reported that thrombin (PAR-1) and purinergic ($P2Y_2$) receptors, which are both endogenously expressed in CHO cells [34–36], are able to activate calcium-dependent $cPLA_2$ through two classes of G proteins, G_i and G_q [24,37], without any involvement of the calcium-independent or secretory PLA_2s [24,37]. Thus, to further investigate the G-protein subtypes mediating the coupling of these receptors to $cPLA_2$, CHO cells were transiently transfected with expression vectors for wild-type $G\alpha_{13}$ and $G\alpha_q$, as well as with constitutively active $G\alpha_{13}$ ($G\alpha_{13}$Q226L) and $G\alpha_q$ ($G\alpha_q$Q209L). The signaling pathway leading to $G\alpha_q$-mediated $cPLA_2$ activation has been well characterized [24,38] and thus represented a reference point for this study.

The basal $cPLA_2$ activity, which was measured as arachidonic acid release into the extra-cellular medium (see Materials and methods), was not significantly affected by overexpression of wild-type $G\alpha_{13}$ and $G\alpha_q$ (the levels of overexpression for both the wild-type and constitutively active forms ranged from 2- to 4-fold the endogenous levels; Fig. 1A). However, increases in basal $cPLA_2$ activity were observed following overexpression of their constitutively active $G\alpha_{13}$Q226L and $G\alpha_q$Q209L forms, respectively (Fig. 1B, compare basal levels). This indicated that as with $G\alpha_q$, $G\alpha_{13}$ can also induce $cPLA_2$ activation in CHO cells.

We evaluated whether thrombin and purinergic receptors can activate $cPLA_2$ through $G\alpha_{13}$ (Fig. 1B). Interestingly, in cells overexpressing wild-type $G\alpha_{13}$ (that per se did not affect arachidonic acid release; Fig. 1B), the arachidonic acid release induced by thrombin and by ATP were both twice that induced by the same agonists in the mock-transfected control cells (Fig. 1B). Similar results were obtained in cells overexpressing wild-type $G\alpha_q$ (Fig. 1B). These data demonstrate a productive coupling between the stimulated thrombin and purinergic receptors and the overexpressed wild-type $G\alpha_{13}$, while confirming the coupling of these receptors to $G\alpha_q$.

In line with the general view that hormone activities are dependent on receptor interactions with GDP-bound G proteins, the overexpression of constitutively active $G\alpha_{13}$Q226L did not enhance the coupling of the thrombin and ATP receptors to $cPLA_2$ activation: the arachidonic acid release showed no further increase over that of the mock-transfected control cells upon stimulation with these agonists (Fig. 1B). The data were similar for the constitutively active $G\alpha_q$Q209L-overexpressing cells (Fig. 1B).

Since $G\alpha_{13}$ is endogenously expressed in CHO cells (see Fig. 1A, lane 1), and since hormonal stimulation of these cells with both thrombin and ATP leads to an increase in $cPLA_2$ activation when $G\alpha_{13}$ is overexpressed (Fig. 1B), this indicates that $G\alpha_{13}$ is part of the signaling in CHO cells that leads to $cPLA_2$ activation through the PAR1 and purinergic receptors.

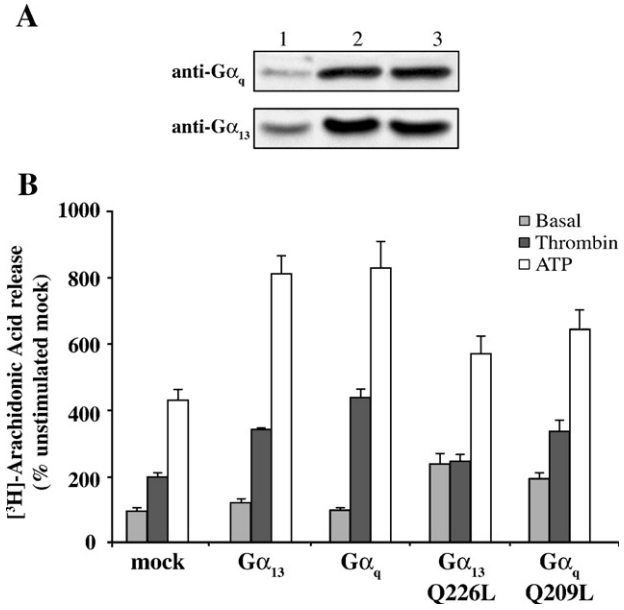

Fig. 1. [^{3}H]-Arachidonic acid release in Gα-transfected CHO cells. (A) Western blotting of Gα expression levels (see Materials and methods) showing: lanes 1, empty-vector-transfected cells (mock); lanes 2, wild-type $G\alpha_q$ and $G\alpha_{13}$ overexpression; lanes 3, constitutively active $G\alpha_q$Q209L and $G\alpha_{13}$Q226L overexpression; upper and lower panels, respectively. (B) [^{3}H]-Arachidonic acid release (15 min at 37 °C) in unstimulated (basal) and thrombin (0.5 U/ml) and ATP (100 μM) stimulated CHO cells under the indicated transfection conditions. The data are expressed as percentages of basal control (mock) release, as means±S.D. of five ($G\alpha_q$ and $G\alpha_q$Q209L) and seven ($G\alpha_{13}$ and $G\alpha_{13}$Q226L) independent experiments, each carried out in triplicate (see Materials and methods).

To test this, we used a membrane-permeable, $G\alpha_{13}$-carboxy-terminal peptide ($G\alpha_{13}$CT) [39]. The ability of this peptide to specifically disrupt the receptor-$G\alpha_{13}$ interaction has been shown previously [39–41]. As shown in Fig. 2A (see also inset), $G\alpha_{13}$CT inhibition of the interactions of $G\alpha_{13}$ with the activated PAR1 and purinergic receptors resulted, respectively, in 78% and 44% reductions in the agonist-stimulated arachidonic acid release, compared to DMSO-treated cells. As a control, a $G\alpha_s$-carboxy-terminal peptide ($G\alpha_s$CT) targeted to the $G\alpha_s$-interaction domain of the receptors was essentially ineffective, due to the lack of PAR1 and purinergic receptor coupling to $G\alpha_s$ in these cells.

In addition, to demonstrate the involvement of $cPLA_2\alpha$ in this system, we used pyrrophenone, a potent and selective inhibitor of $cPLA_2\alpha$ [19]. Pyrrophenone (0.5 μM) almost completely inhibited thrombin- and ATP-induced arachidonic acid release in both the mock and $G\alpha_{13}$ transfectants (Fig. 2B), implying that the specific PLA_2 isoform involved here is $cPLA_2\alpha$. In line with this observation, bromoenol lactone (BEL, 5 μM), used at a concentration that specifically inhibits the calcium-independent PLA_2 [18], had no effect on this hormone-stimulated arachidonic acid release.

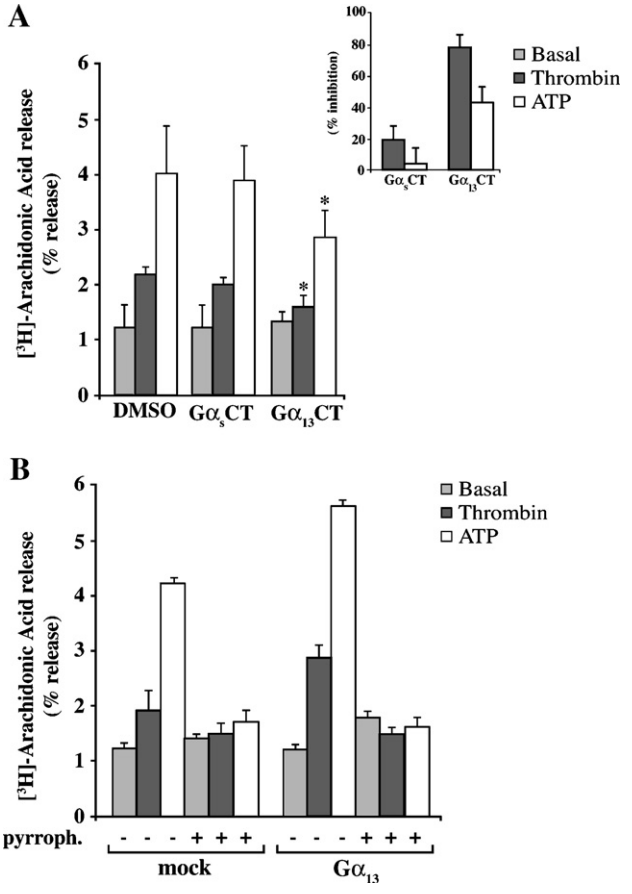

Fig. 2. Thrombin- and ATP-induced [^{3}H]-arachidonic acid release is regulated by $G\alpha_{13}$ through $cPLA_2\alpha$ activation. (A) [^{3}H]-Arachidonic acid release was measured in CHO cells pretreated with DMSO (as the peptide solvent) and the $G\alpha_s$CT and $G\alpha_{13}$CT peptides (50 μM; 30 min) and then stimulated with thrombin (0.1 U/ml) and ATP (10 μM) for 15 min at 37 °C. The data are given as percentages of release (see Materials and methods) and are the means ± S.D. of three independent experiments, each performed in triplicate. Inset: same data expressed as percentage inhibition (see Materials and methods). ∗Significantly different from DMSO-treated control cells ($p<0.01$). (B) Preincubation with pyrrophenone (0.5 μM) for 15 min at 37 °C completely blocked thrombin- (0.1 U/ml) and ATP (10 μM)-induced activation of [^{3}H]-arachidonic acid release (15 min at 37 °C) in both mock- and $G\alpha_{13}$-transfected cells. The data are given as percentages of the [^{3}H]-arachidonic acid release (see Materials and methods) and are means ± S.D. of three independent experiments, each performed in triplicate.

3.2. RhoA and ERK1/2 are involved in $G\alpha_{13}$-dependent arachidonic acid release

It has previously been reported that thrombin and purinergic receptors can induce ERK1/2 activation via $G\alpha_q$ in CHO cells, and that the resulting phosphorylation of $cPLA_2\alpha$ on Ser505 is essential for its activation [25]. For this reason, we examined whether the ERKs are part of the signaling pathway downstream of $G\alpha_{13}$, and thus part of this $cPLA_2\alpha$ activation. Moreover, since $cPLA_2\alpha$ can also be phosphorylated and activated by p38 MAP kinase [17], a well-defined $G\alpha_{13}$ effector [42], we also examined whether p38 is part of the pathway of this $G\alpha_{13}$-mediated $cPLA_2\alpha$ activation.

The role of these kinases was initially investigated using U0126, a potent inhibitor of MAPK kinase (MEK)1/2 and ERK1/2 [43,44]. In $G\alpha_{13}$ transfectants, pre-treatment with U0126 resulted in about a 70% reduction in both thrombin- and ATP-induced arachidonic acid release (that is greater than in mock-transfected cells; Fig. 3A), implying that ERK1/2 has a role in this cascade initiated by PAR-1 and $P2Y_2$ receptor activation. Similar results were obtained with a different MAPK inhibitor PD 098059 (50 μM) where thrombin- and ATP-induced arachidonic acid release were reduced by 54% and 72%, respectively, in the $G\alpha_{13}$ transfectants. Quite surprisingly, SB203580 (a p38 inhibitor) did not inhibit this arachidonic acid release (Fig. 3A), thus apparently excluding the involvement of p38 in this signaling cascade.

The direct evaluation of ERK1/2 and p38 activation in CHO cells was also performed by Western blotting, using an antibody that specifically recognizes the phosphorylated (active) forms of these kinases. Here, thrombin and ATP increased the active ERK1/2 levels to 2-fold, on average, compared to unstimulated cells (Fig. 3B). This effect was completely abolished by U0126 (Fig. 3B), in line with the similar effects on hormone-stimulated arachidonic acid release (see above, and Fig. 3A). Moreover, the levels of active p38 were also increased by both thrombin and ATP (Fig. 3B), an effect that was largely inhibited by the p38 inhibitor SB203580, but not affected by U0126 (Fig. 3B). However, the treatment with SB203580 not only blocked p38 activation, but also increased the ERK1/2 phosphorylation (Fig. 3B), thus providing an alternative explanation for the inability of SB203580 to inhibit arachidonic acid release initiated by PAR-1 and $P2Y_2$ receptor activation (Fig. 3A): an inhibitory effect of SB203580 on arachidonic acid release would be counteracted, and thus masked, by this "side effect" of further ERK1/2 activation.

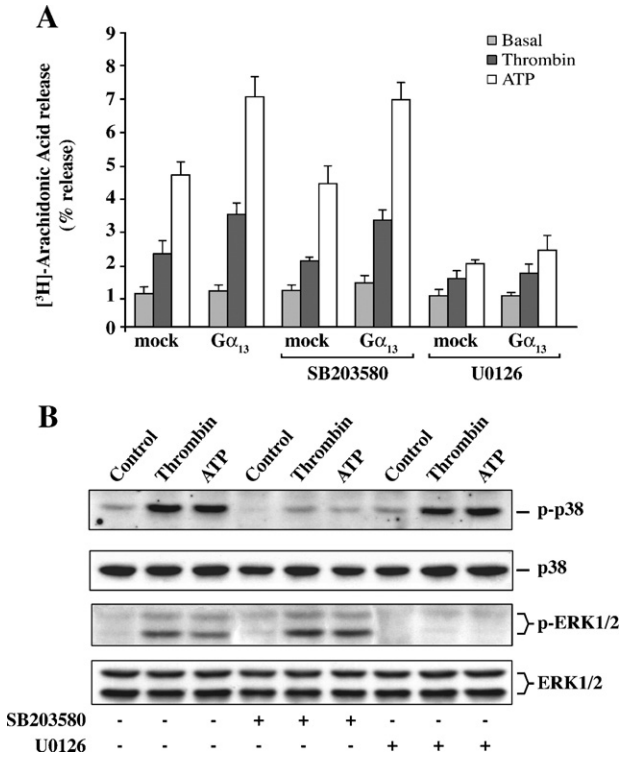

Fig. 3. [3H]-Arachidonic acid release and MAPK phosphorylation are inhibited by U0126 in CHO cells. (A) [3H]-Arachidonic acid release was measured in CHO cells pretreated for 10 min with SB203580 (10 μM) and U0126 (10 μM) and then stimulated with thrombin (0.5 U/ml) and ATP (100 μM) for 15 min at 37 °C. The data are given as percentages of release (see Materials and methods) and are means±S.E. of three independent experiments, each performed in triplicate. (B) Western blotting of a filter labeled with the anti-phospho p38 (p-p38), anti-p38 (p38), anti-phospho ERK1/2 (p-ERK1/2) and anti-ERK1/2 (ERK1/2) antibodies (see Materials and methods). ERK1/2 and p38 activities were evaluated after 7 min thrombin (0.5 U/ml) and ATP (10 μM) stimulation in control and SB203580- (10 μM) and U0126 (10 μM)-preincubated (10 min) CHO cells. The Western blot shown is representative of five independent experiments.

Therefore, to further analyze the putative role of p38 in this $G\alpha_{13}$-mediated signaling that leads to $cPLA_2\alpha$ activation, we focused on the monomeric RhoA GTPase, a signaling molecule that has been described as being upstream of p38 [45] and that is activated by $G\alpha_{13}$. For this purpose, we overexpressed the *Clostridium botulinum* ADP-ribosyltransferase C3 toxin, which catalyzes mono-ADP-ribosylation of the Rho proteins [46] and thus prevents the interactions of these Rho proteins with their effectors. In $G\alpha_{13}$-transfectants, the overexpression of C3 toxin led to inhibition of hormone-mediated activation of p38 (60% and 35% inhibition of the p38 phosphorylation levels, with thrombin and ATP, respectively; Fig. 4A), but also of ERK1/2 (77% and 30% inhibition of the ERK2 phosphorylation level; Fig. 4A), thus implying that both these kinases are downstream of RhoA signaling. Under the same experimental conditions, arachidonic acid release was inhibited upon thrombin and ATP stimulation (by 73% and 49%, respectively; Fig. 4B; see also inset). This relevant role of RhoA was confirmed in arachidonic acid release experiments using CHO cells transfected with the constructs coding for monomeric RhoA GTPase, wild type ($RhoA_{wt}$), and the dominant-positive (L63RhoA) and dominant-negative (N19RhoA) RhoA mutants. The thrombin-stimulated arachidonic acid release, calculated as % of the unstimulated transfected cells, was 145%, 166% and 70%, for $RhoA_{wt}$, L63RhoA and N19RhoA, respectively.

Moreover, the use of a variety of pharmacological tools led us to exclude the possible involvement in $G\alpha_{13}$ signaling to $cPLA_2\alpha$ of other kinases, previously demonstrated to be involved in $cPLA_2\alpha$ phosphorylation and activation [17]. Among the RhoA effectors downstream of $G\alpha_{13}$, there is the Jun N-terminal kinase (JNK) [42,45]; however, the JNK inhibitor SP600125 (30 μM) did not affect arachidonic acid release and p38 and ERK phosphorylation here, thus excluding a role for JNK in this signaling cascade. In addition, CHO cells were transfected either with the control vector or with $G\alpha_{13}$, and then pretreated for 15 min with wortmannin (phosphoinositide 3-kinase inhibitor; 100 nM),

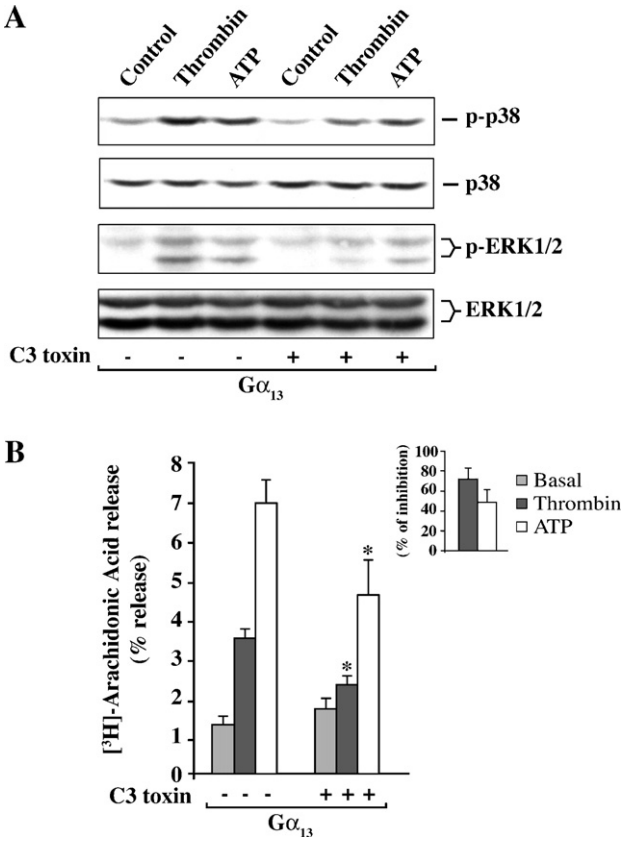

Fig. 4. C3 toxin inhibits hormone-mediated stimulation of ERK1/2, p38 and arachidonic acid release. (A) ERK1/2 and p38 MAPK activities were evaluated in $G\alpha_{13}$-transfectants in the presence or absence of overexpressed C3 toxin, with thrombin (0.5 U/ml) and ATP (10 μM) stimulation for 7 min at 37 °C (see Materials and methods). The Western blot shown is representative of two independent experiments. (B) C3-toxin-dependent inhibition of [3H]-arachidonic acid release upon thrombin (0.5 U/ml) and ATP (10 μM) stimulation for 7 min at 37 °C. The data are given as percentages of release (see Materials and methods) and are means ±S.E. of three independent experiments, each performed in triplicate. Inset: the same data expressed as percentage inhibition of agonist-stimulated [3H]-arachidonic acid release under this C3-toxin treatment. ∗Significantly different from control $G\alpha_{13}$-transfected cells ($p<0.01$).

H-89 (protein kinase A inhibitor; 50 μM), KN62 (CaMKII inhibitor; 20 μM), RO 31-8220 (PKC inhibitor; 5 μM). Wortmannin, H-89 and KN62 did not affect receptor-stimulated arachidonic acid release in either mock- or $G\alpha_{13}$-transfected cells. The PKC inhibitor RO 31-8220 inhibited hormone-stimulated arachidonic acid release by about 20%. However, since in $G\alpha_{13}$-transfected cells the extent of inhibition was not modified with respect to the mock-transfected cells, we can conclude that PKC is part of the $G\alpha_q$ pathway to $cPLA_2\alpha$ activation [47,48]. As $G\alpha_{13}$ can activate PKC via either RhoA or the kinase pyk2, which can then lead to PKC phosphorylation of PKD [1], to exclude a role of this PKC/PKD pathway in $cPLA_2\alpha$ activation by $G\alpha_{13}$, we transfected CHO cells with wild-type PKD and with a kinase-dead PKD, but these overexpressions were completely ineffective.

In summary, the data presented exclude the involvement in $G\alpha_{13}$-mediated $cPLA_2\alpha$ activation of JNK, phosphoinositide 3-kinase, protein kinase A, CaMKII, PKC and PKD and demonstrate that when activated, $G\alpha_{13}$ signals through RhoA and ERK1/2 to regulate $cPLA_2\alpha$.

3.3. Phosphatase involvement in $G\alpha_{13}$-dependent arachidonic acid release

Regulation of the MAPKs involves a dynamic interplay between various kinases and phosphatases. The ERKs are activated by phosphorylation on their threonine and tyrosine residues and are inactivated upon dephosphorylation by tyrosine phosphatases, serine–threonine phosphatases, and dual-specificity phosphatases [49]. The serine–threonine phosphatase PP2A can dephosphorylate MEKs and ERKs in vitro, and inhibition of PP2A leads to activation of these kinases [50]. Thus, we wanted to determine whether a phosphatase is involved in this $G\alpha_{13}$-dependent arachidonic acid release, by measuring $cPLA_2\alpha$ activity in the presence of phosphatase inhibitors.

CHO cells (empty vector-transfected, mock) or the $G\alpha_{13}$-transfectants were pretreated for 15 min with a mix of

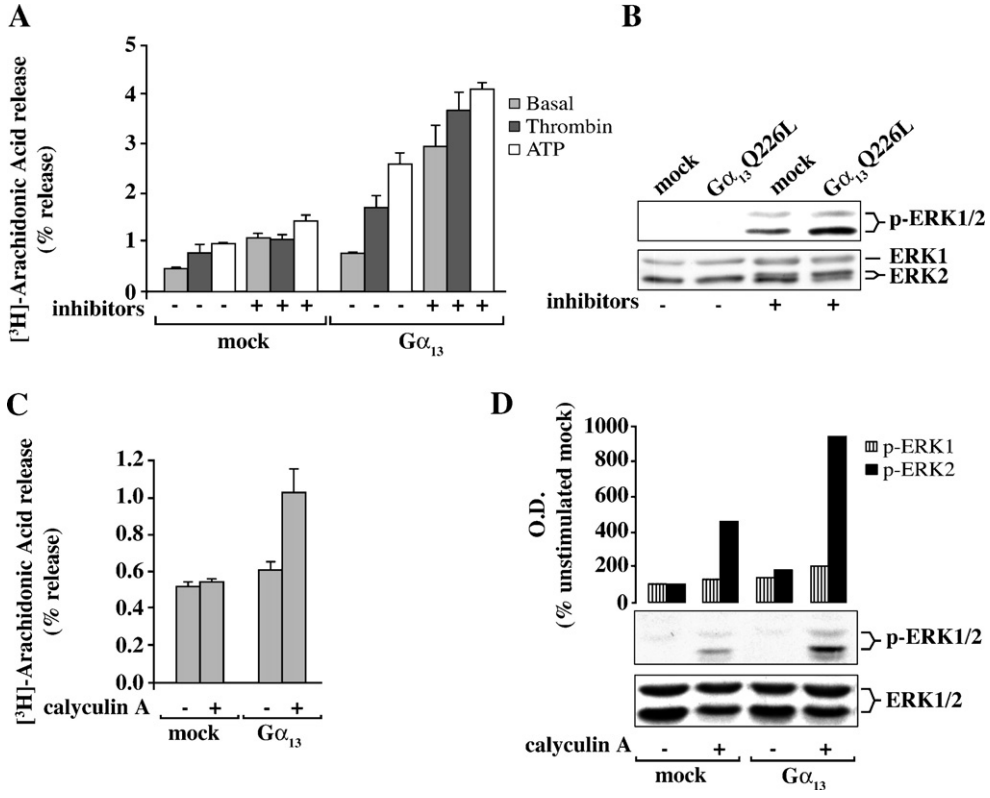

Fig. 5. Phosphatase inhibitors stimulate arachidonic acid release and ERK1/2 phosphorylation in the $G\alpha_{13}$-transfectants. (A) [^{3}H]-Arachidonic acid release after 10 min preincubation with a mixture of phosphatase inhibitors (1 μM cyclosporine, 50 nM calyculin A, 1 mM orthovanadate), in mock and $G\alpha_{13}$ transfectants upon thrombin (0.5 U/ml) and ATP (10 μM) stimulation for 7 min at 37 °C. The data are given as percentages of release (see Materials and methods) and are means ± S.E. of three independent experiments, each performed in duplicate (see Materials and methods). (B) Western blotting of a filter labeled with anti-phospho ERK1/2 and then with anti-ERK1/2 antibodies (see Materials and methods). The ERK1/2 activities were evaluated in control and phosphatase-inhibitor-pretreated (10 min) CHO cells. Note that the anti-ERK1/2 antibody recognized two different bands of ERK2 only when the cells were pre-treated with the phosphatase inhibitors, with the upper band corresponding to phosphorylated ERK2. The Western blots shown are representative of three independent experiments. (C) [^{3}H]-Arachidonic acid release in mock- and $G\alpha_{13}$-transfected CHO cells after 10-min preincubation with calyculin A (50 nM). The data are given as percentages of release (see Materials and methods) and are means ± S.D. within a single experiment that is representative of three independent experiments, each performed in triplicate (see Materials and methods). (D) Western blotting and densitometric quantification of phosphorylation levels of ERK1/2 in mock-transfected and $G\alpha_{13}$-overexpressing CHO cells pretreated for 10 min with calyculin A (50 nM) (see Materials and methods). The data are given as percentages of release (see Materials and methods). The Western blots and quantification shown are representative of three independent experiments.

phosphatases inhibitors: cyclosporine (the calcineurin or PP2B inhibitor; 1 μM); calyculin A (a PP1, PP2A and PP5 inhibitor; 50 nM); and orthovanadate (a generic tyrosine phosphatase inhibitor; 1 mM). Following subsequent stimulation with thrombin and ATP, there were pronounced increases in the basal and agonist-stimulated arachidonic acid release as a consequence of $G\alpha_{13}$ overexpression in the presence of this inhibitor cocktail (Fig. 5A); this clearly indicated a phosphatase regulation of this $cPLA_2\alpha$ activity. An even more evident effect was seen following ERK phosphorylation in CHO cells transfected with the constitutively active $G\alpha_{13}Q226L$. There was a clear shift from non-phosphorylated to phosphorylated ERK2 in the presence of the phosphatase inhibitors (Fig. 5B); importantly, this phosphorylated to non-phosphorylated ratio was increased with $G\alpha_{13}Q226L$ transfection, indicating that there is a $G\alpha_{13}Q226L$-mediated ERK phosphorylation when the phosphatase activity is blocked.

These inhibitors were then tested individually under the same experimental conditions. The effect of calyculin A alone appeared more specifically related to $G\alpha_{13}$-mediated $cPLA_2\alpha$ regulation (Fig. 5C) than that seen for cyclosporine and orthovanadate (data not shown). As illustrated in Fig. 5C, the basal arachidonic acid release in the mock-transfected cells was not affected by calyculin A pretreatment, while an increase in arachidonic acid release to twice the basal levels was seen with $G\alpha_{13}$ overexpression. This thus indicates that the phosphatases that are inhibited by calyculin A are specifically regulated by $G\alpha_{13}$ and involved in this $G\alpha_{13}$-mediated $cPLA_2\alpha$ regulation. In line with these data, the ERKs phosphorylation was also stronger as a consequence of calyculin A pretreatment of $G\alpha_{13}$-overexpressing CHO cells (Fig. 5D).

In conclusion, our findings demonstrate that $G\alpha_{13}$-mediated $cPLA_2\alpha$ activation in CHO cells is under the control of both RhoA, which activates the MAPKs, and a serine–threonine phosphatase. These data thus support a role for this phosphatase activity downstream of $G\alpha_{13}$ and upstream of ERK1/2, which will also be involved in the fine regulation of the ERK pathway (see Fig. 6, for a schematic representation of this signaling pathway).

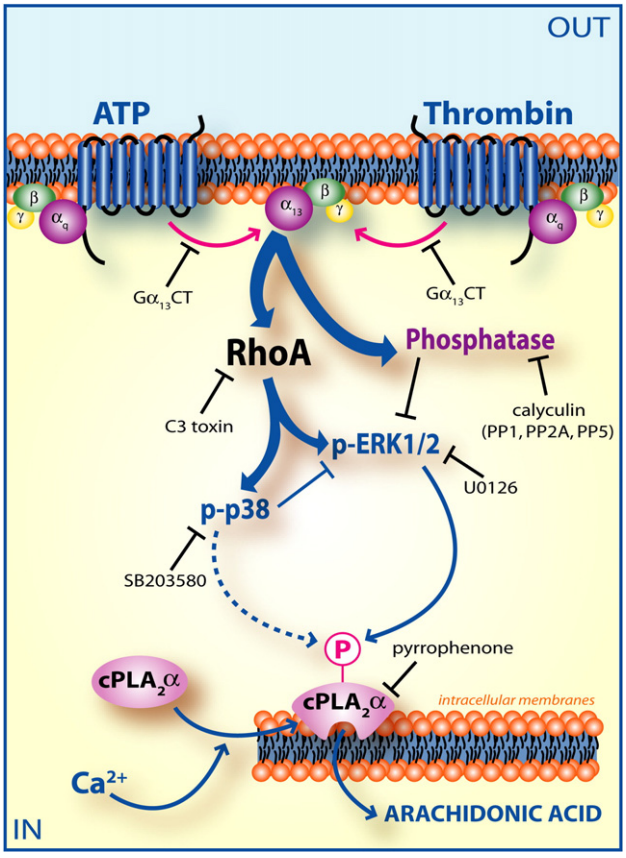

Fig. 6. Schematic representation of the signaling cascade from thrombin and ATP receptors to arachidonic acid production in CHO cells. The thrombin (PAR-1) and purinergic ($P2Y_2$) receptor signaling that leads to $cPLA_2\alpha$ activation involves both $G\alpha_q$ and $G\alpha_{13}$ activation. $G\alpha_{13}$ activates RhoA and consequently ERK1/2 and p38. Of these kinases, it is mainly ERK1/2 that phosphorylates $cPLA_2\alpha$. In the presence of an increase in intracellular calcium, the membrane-associated phosphorylated $cPLA_2\alpha$ induces the release of arachidonic acid. At the same time, $G\alpha_{13}$ activates a calyculin-sensitive phosphatase (probably PP5) that switches off ERK1/2 activation, thus allowing the strict modulation of ERK1/2. The attenuation of a number of these events by our interference with the interactions between the receptors and $G\alpha_{13}$ (e.g., $G\alpha_{13}CT$) and between RhoA and its effectors (e.g., C3 toxin), and with the inhibition of the MAPKs (e.g., U0126, SB203580), the phosphatase (e.g., calyculin A) and $cPLA_2\alpha$ (e.g., pyrrophenone) all led to the elucidation of the entire pathway. See text for details.

4. Discussion

In this study, we have demonstrated that in addition to the well-defined G_q pathway, the heterotrimeric G protein G_{13} mediates PAR-1 and $P2Y_2$ receptor-induced activation of $cPLA_2\alpha$, a key enzyme in several biological responses, including inflammation and cell proliferation [14,16]. This productive coupling between G_{13} and $cPLA_2\alpha$ was demonstrated by the overexpression of wild-type $G\alpha_{13}$ and constitutively active $G\alpha_{13}Q226L$, the treatment with a membrane-permeable peptide that is able to interfere with receptor-$G\alpha_{13}$ interactions, and thus with signaling mediated by endogenous $G\alpha_{13}$, and the use of a selective inhibitor of $cPLA_2\alpha$.

Intracellular activation of $cPLA_2\alpha$ is tightly regulated by multiple pathways, including those that control intracellular calcium concentrations, protein phosphorylation and $cPLA_2\alpha$ levels [11]. In CHO cells, as in many other cell systems, PAR-1 and $P2Y_2$ receptor-induced stimulation of $cPLA_2$ has been shown to be coupled both to G_q and to the pertussis-toxin-sensitive G_i family. The G_q-mediated activation of $cPLA_2$ is due to phospholipase C activation and the subsequent production of diacylglycerol and inositol 1,4,5-trisphosphate, which in turn activate PKC and mobilize intracellular calcium, respectively [35,48]. The increased calcium levels act on the C2 domain of $cPLA_2$, causing $cPLA_2$ translocation to membranes, where its substrates are located.

Here we have shown that stimulation of CHO cells with the PAR-1 and $P2Y_2$ receptor agonists thrombin and ATP, respectively, leads to an increase in $cPLA_2\alpha$ activity through RhoA-mediated ERK activation. The definition of this pathway is demonstrated by the use of the MEK inhibitors U0126 and PD 098059, and by the C3 toxin block of ERK1/2

phosphorylation and, consequently, of hormone-mediated arachidonic acid release in $G\alpha_{13}$ transfectants.

$G\alpha_{13}$ activates, as a virtually obligatory intermediate, the low-molecular-weight monomeric G protein RhoA [8,9]. More recently, $G\alpha_{12/13}$-mediated RhoA activation has been linked to the MAPK pathways; indeed, Gallagher et al. demonstrated that RhoA-GTP can directly interact with MEK kinase 1 (MEKK1) and stimulate its kinase activity; this activation of MEKK1 by RhoA is of a similar magnitude to that seen for the other kinases regulated by RhoA [51]. From these observations, it appears likely that the $G\alpha_{13}$ activation of RhoA signaling pathways contributes to MEKK1 signaling pathways.

Moreover, we explored the possibility that this $G\alpha_{13}$-mediated activation of $cPLA_2\alpha$ also occurs through p38. Indeed, p38 is among the $G\alpha_{13}$ effectors, and it has been shown to be involved in $cPLA_2$ phosphorylation and activation. Accordingly, in NIH3T3 cells overexpressing the $serotonin_{2A}$ receptor, the involvement of $G\alpha_{12/13}$ in $cPLA_2$ activation was shown on the basis of inhibition of hormone-induced arachidonic acid release by both the p38 inhibitor SB202190 and C3 toxin, thus leading to the hypothesis of a $G\alpha_{12/13}$-RhoA-p38 pathway. Our data show that the C3 toxin does indeed inhibit thrombin- and ATP-stimulated arachidonic acid release in $G\alpha_{13}$ transfectants, along with an inhibition of p38 phosphorylation. However, the C3 toxin inhibited ERK1/2 phosphorylation as well, thus implying that RhoA is upstream not only of p38, but also of ERK1/2. Moreover, despite the clear activation of p38 by thrombin and ATP, the p38-selective inhibitor SB203580 did not inhibit arachidonic acid release.

Several studies have suggested that there is cross-talk between the p38 MAPK pathway and the ERK and JNK pathways in a variety of eukaryotic cells; in particular, a p38 MAPK-mediated inhibition of ERK activity has been reported [52–54], even if the cellular mechanism underlying this cross-talk remains to be fully elucidated. The SB203580-dependent increase in ERKs phosphorylation in our study (see Fig. 3B) indicates that there is cross-talk between the p38 MAPK and ERK pathways also in CHO cells. From these data, we cannot exclude a contribution of p38 in the $G\alpha_{13}$-mediated activation of $cPLA_2\alpha$. On the contrary, we can exclude the involvement of the other kinases (see results) previously demonstrated to be involved in $cPLA_2\alpha$ activation [17].

The novel aspect of this $G\alpha_{13}$-mediated pathway is the fine regulation of ERK1/2 that is activated via RhoA but inactivated by a calyculin-sensitive, Ser/Thr phosphatase. In the $G\alpha_{13}$ transfectants, the extent of ERK1/2 activation was lower and less reproducible than in the $G\alpha_q$ transfectants, but it became clearly detectable, in particular, with the Ser/Thr phosphatase inhibitor calyculin A. On the basis that regulation of the ERKs is under the control of dual-specificity phosphatases [49,50] and that $G\alpha_{12/13}$ specifically interacts with the Ser/Thr phosphatase 5 (PP5) [55], we speculate that the Ser/Thr phosphatase involved in the pathway delineated here would be PP5 that is indeed calyculin-sensitive [55].

Importantly, our data can reconcile the controversial results in the literature concerning $G\alpha_{13}$ activation of ERK1/2. It has been reported that $G\alpha_{13}$ either has no effect on or inhibits ERK1/2 [56], and also that $G\alpha_{13}$ can stimulate the ERKs [57,58] or enhance the extent and duration of agonist-dependent ERK activation [59]. This $G\alpha_{13}$-dependent activation of a phosphatase may well be responsible for these different levels of ERK regulation that have been described.

In conclusion, we have demonstrated a novel activity of $G\alpha_{13}$ that involves MAPK-dependent activation of $cPLA_2\alpha$, and we have shown that in CHO cells, both the PAR-1 and $P2Y_2$ receptors signal through this $G\alpha_{13}$-mediated pathway. More importantly, we have demonstrated the new role of a calyculin-sensitive, Ser/Thr phosphatase in this signaling pathway. These findings are thus directly relevant to our understanding of receptor regulation of $cPLA_2\alpha$ through the heterotrimeric G proteins.

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