Cooperation of G\textsubscript{q}, G\textsubscript{i}, and G\textsubscript{12/13} in Protein Kinase D Activation and Phosphorylation Induced by Lysophosphatidic Acid

To examine the contribution of different G-protein pathways to lysophosphatidic acid (LPA)-induced protein kinase D (PKD) activation, we tested the effect of LPA on PKD activity in murine embryonic cell lines deficient in G\textsubscript{q11} (G\textsubscript{q11} KO cells) or G\textsubscript{12/13} (G\textsubscript{12/13} KO cells) and used cells lacking rhodopsin kinase (RK cells) as a control. In RK and G\textsubscript{12/13} KO cells, LPA induced PKD activation through a phospholipase C/protein kinase C pathway in a concentration-dependent fashion with maximal stimulation (6-fold for RK cells and 4-fold for G\textsubscript{12/13} KO cells) achieved at 3 μM. In contrast, LPA did not induce any significant increase in PKD activity in G\textsubscript{q11} KO cells. However, LPA induced a significantly increased PKD activity when G\textsubscript{q11} KO cells were transfected with G\textsubscript{q11}. LPA-induced PKD activation was modestly attenuated by prior exposure of RK cells to pertussis toxin (PTx) but abolished by the combination treatments of PTx and Clostridium difficile toxin B. Surprisingly, PTx alone strikingly inhibited LPA-induced PKD activation in a concentration-dependent fashion in G\textsubscript{q12/13} KO cells. Similar results were obtained when activation loop phosphorylation at Ser-744 was determined using an antibody that detects the phosphorylated state of this residue. Our results indicate that G\textsubscript{q} is necessary but not sufficient to mediate LPA-induced PKD activation. In addition to G\textsubscript{q}, LPA requires additional G-protein pathways to elicit a maximal response with G\textsubscript{i} playing a critical role in G\textsubscript{12/13} KO cells. We conclude that LPA induces PKD activation through G\textsubscript{q}, G\textsubscript{i}, and G\textsubscript{12} and propose that PKD activation is a point of convergence in the action of multiple G-protein pathways.

Protein kinase C (PKC),\textsuperscript{1} a major target for the tumor promoting phorbol esters, has been implicated in the signal transduction pathways regulating a wide range of biological responses, including changes in cell morphology, differentiation, and proliferation (1, 2). Molecular cloning has demonstrated the presence of multiple PKC isoforms (2–5), i.e. conventional PKCs (α, β, β2, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ and λ) all of which possess a highly conserved catalytic domain.

PKD/PKCα is a serine/threonine protein kinase (6, 7) with distinct structural, enzymological, and regulatory properties (8). In particular, PKD is rapidly activated in intact cells through a mechanism that involves phosphorylation (8). Specifically, exposure of intact cells to phorbol esters, cell-permeant diacylglycerols, or bryostatin induces rapid PKD phosphorylation and activation, which is maintained during cell lysis and immunoprecipitation (8–13). Several lines of evidence, including the use of PKC-specific inhibitors and co-transfection of PKD with constitutively active PKC mutants indicate that PKD is activated through a novel PKC-dependent signal transduction pathway in vivo (9–11). The residues Ser-744 and Ser-748 in the activation loop of PKD have been identified as critical phosphorylation sites in PKD activation (14, 15). Taken together, these results suggest an important connection between PKCs and PKD and indicate that PKD can function downstream of PKC in a novel signal transduction pathway.

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) are composed of α, β, and γ subunits and transduce external signals from heptahelical receptors to intracellular effectors (16). Mammalian G protein α subunits are classified into four subfamilies: G\textsubscript{αi}, G\textsubscript{αq}, and G\textsubscript{α12}. The α subunit of G\textsubscript{q} stimulates the β isoforms of phospholipase C (PLC), which catalyze the production of inositol 1,4,5-trisphosphate that triggers the release of Ca\textsuperscript{2+} from internal stores and diacylglycerol that activates the classical and novel isoforms of PKC (reviewed in Ref. 17). A variety of neuromodulatory agonists that signal through heptahelical receptors and couple to heterotrimeric G proteins, including bombesin, Bradykinin, endothelin, and vasopressin, induce rapid PKD activation in normal and neoplastic cells (11, 13, 18, 19). Although each of these receptors activates G\textsubscript{q} and G\textsubscript{αi} signaling stimulates PKD activity (20), expression of a COOH-terminal fragment of G\textsubscript{q} that acts in a dominant-negative fashion, attenuated (but did not eliminate) PKD activation in response to agonist stimulation of bombesin receptor (20). These results suggested that G protein-coupled receptors (GPCRs) stimulate PKD activation not only via G\textsubscript{q} but also through other G protein-mediated signaling pathways.
Many GPCRs also interact with other heterotrimeric G proteins including members of the G_{12} family, which mediate activation of the low molecular weight G proteins of the Rho subfamily (21–26) via guanine nucleotide exchange factors that directly link the G_{α} subunits to Rho (27–29). Rho plays a major role in promoting cytoskeletal responses including formation of actin stress fibers, assembly of focal adhesions, and tyrosine phosphorylation of focal adhesion proteins and has been implicated in gene expression, cell migration, proliferation, and transformation (30–32). Interestingly, a number of recent studies have suggested a convergence between Rho- and PKC-mediated signaling in yeast and mammalian cells (33–38). For example, Slater et al. (37) have demonstrated that Rho-GTP potently stimulates PKCα activity in vitro using recombinant proteins and Sagi et al. (38) reported that G_{α}s and PLC signaling are synergistic with Rho. Recently, we demonstrated that in addition to G_{αs}, Rho- and G_{α16}-mediated signaling can promote PKD activation in intact cells and that endogenous Rho and G_{α16} contribute to PKD activation in response to bombesin GPCR stimulation (39). Thus, we have identified PKD as a novel downstream target in G_{α} activation of multiple endogenous G protein pathways that are regulated by G_{q/11} (40). Cooperation of G Proteins in PKD Activation by LPA

Experimental Procedures

**Cell Culture**—Mouse embryonic fibroblasts were generated from genetically engineered mice (58, 59) that contained gene knockouts for G_{αq}, G_{α12}, and G_{α13}. Stock cultures of these cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 250 μg/ml G418 in a humidified atmosphere containing 10% CO_{2} and 90% air at 37 °C. For experimental purposes, cells were plated in 60- or 100-mm dishes at about 30% confluency in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 250 μg/ml G418 and were allowed to grow to near confluency (6–7 days) and then changed to serum-free Dulbecco’s modified Eagle’s medium for 8–24 h prior to the experiment.

**Kinase Assay of PKD**—PKD autophosphorylation was determined in an in vitro assay by mixing 20 μl of PKD immunocomplexes with 10 μl of a phosphatidylinositol mixture containing (final concentration) 100 μM γ_{32}ATP (specific activity 400–600 cpm/pmol), 30 mM Tris/HCl, pH 7.4, 10 mM MgCl_{2}, and 1 mM dithiothreitol. After 10 min of incubation at 30 °C, the reaction was stopped by washing with 1 ml of kinase buffer and then adding an equal volume of 2× SDS-PAGE sample buffer (200 mM Tris/HCl, pH 6.8, 2 mM EDTA, 5% glycerol, and 1% Triton X-100). Cell lysates were centrifuged at 15,000 × g for 10 min at 4 °C. PKD was immunoprecipitated at 4 °C for 2–4 h with the antibody that specifically recognizes the phosphorylated state of Thr-202 and Tyr-204 of ERL-1/2 (9, 10). The immuno complexes were recovered using protein A coupled to agarose.

**Immunoprecipitation**—Cultures of PKD KO cells, treated as described in the individual experiments, were washed and lysed in lysis buffer (50 mM Tris/HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 1% Triton X-100). Cell lysates were centrifuged at 15,000 × g for 10 min at 4 °C. PKD was immunoprecipitated at 4 °C for 2–4 h with the antibody that specifically recognizes the phosphorylated state of Thr-202 and Tyr-204 of ERL-1/2 (9, 10). The immuno complexes were recovered using protein A coupled to agarose.

**Western Blot Analysis for pS744/748 and ERK-2/ERK-1 Activation**—Samples of cell lysates were directly solubilized by boiling in 2× SDS-PAGE sample buffer (200 mM Tris/HCl, pH 6.8, 2 mM EDTA, 5% glycerol, and 1% mercaptoethanol), followed by SDS-PAGE analysis (9, 60). The gels were dried and the 110- or 140-kDa radioactive bands corresponding to autophosphorylated PKD or GFP-PKD was visualized by autoradiography. Autoradiographs were scanned in a GS-710 Calibrated Imaging Densitometer (Bio-Rad), and the labeled band was quantified using the Quantity One™ software program.

**Exogenous substrate phosphorylation by immunoprecipitated PKD** was carried out by mixing 20 μl of the washed immunocomplexes with 20 μl of a phosphorylation reaction containing 2.5 μg/ml syntide-2 (PLARTLSVAGLPGKK), a peptide based on phosphorylation site two of rhodopsin kinase, G_{αq/11}, and G_{α12/13}. Stock cultures of these cells were grown to near confluency (6–7 days) and then maintained as described (9, 10). The immune complexes were recovered using protein A coupled to agarose.
LPA Induces PKD Activation in RK Cells through a PKC-dependent Pathway—To examine the contribution of different G protein pathways to LPA-induced PKD activation, we decided to test the effect of this agonist on PKD activity isolated from murine embryonic cell lines lacking RK, Goα11, and Gα12/13. Initially, we determined whether LPA induces PKD activation in RK cells, used as controls. Cultures of these cells were stimulated with increasing concentrations of LPA (0.01–10 μM) for 10 min and lysed. PKD was immunoprecipitated from the extracts of these cells and the immune complexes were incubated with [γ-32P]ATP, subjected to SDS-PAGE, and analyzed by autoradiography to detect the prominent 110-kDa band corresponding to autophosphorylated PKD.

The results presented in Fig. 1A show that LPA induced a marked increase in PKD autophosphorylation activity in a concentration-dependent fashion in RK cells. Half-maximal and maximal stimulation (6-fold increase) were achieved at 0.07 and 3 μM, respectively. As illustrated in the inset to Fig. 1, similar results were obtained when PKD activity in immunocomplexes was determined by phosphorylation of syntide-2 (61, 62), a synthetic peptide previously demonstrated to be an excellent substrate for PKD (6).

We previously proposed that phosphorylation of Ser-744 and Ser-748 within the PKD activation loop plays a critical role in PKD activation (14, 15, 20). Recently, a novel antibody recognizing predominantly the phosphorylated state of Ser-744 (pS744/pS748) in PKD was used in our laboratory to monitor activation loop phosphorylation in response to phorbol ester and bombesin (15). To examine whether LPA induces activation loop phosphorylation in the RK cells, lysates from these cells treated with increasing concentrations of LPA were analyzed by SDS-PAGE, followed by Western blotting with the pS744/p748 antibody. As shown in Fig. 1A, LPA induced PKD Ser-744 phosphorylation in a concentration-dependent fashion in RK cells with maximal stimulation achieved at 3 μM.

To determine whether LPA induces PKD activation and activation loop phosphorylation through a PKC-mediated pathway in RK cells, we used inhibitors that discriminate between PKCs and PKD. RK cells were treated for 1 h with the inhibitors of phorbol ester-sensitive isoforms of PKC, GF 1 (also known as GF 109203X or bisindolylmaleimide I) or Ro 31-8220 (64, 65), prior to stimulation with 3 μM LPA. As shown in Fig. 1B (left panels), exposure to either GF 1 or Ro 31-8220 prevented PKD activation (top) and Ser-744 phosphorylation (middle) induced by LPA. In contrast, the compound GFV, which is structurally related to GF 1 but biologically inactive, did not prevent PKD activation and phosphorylation in response to LPA in RK cells. Similar results were obtained when PKD activity in immunocomplexes was determined by assays of...
Cooperation of G Proteins in PKD Activation by LPA

syntide-2 phosphorylation (bottom). Previously, we demonstrated that GF I and Ro 31-8220 do not inhibit PKD activity when added directly to the in vitro kinase assay at identical concentrations to those required to block PKD activation by LPA in RK cells (9, 11). Thus, the results shown in Fig. 1B imply that GF I and Ro 31-8220 do not inhibit PKD activity directly but interfere with LPA-induced PKD activation and activation loop phosphorylation in intact RK cells by blocking PKC.

To determine whether LPA induces PKD activation and phosphorylation through a PLC-dependent pathway in RK cells, cultures of these cells were treated with the aminosteroid U73122, an inhibitor of PLC (66, 67), prior to stimulation with LPA. As shown in Fig. 1B (right, top and middle panels), 2 μM U73122 markedly reduced PKD activation and Ser-744 phosphorylation in response to the subsequent addition of LPA. Similar results were obtained when PKD activity in immunocomplexes was determined by assays of syntide-2 phosphorylation (Fig. 1B, right bottom panel). The inhibitory effect of U73122 was selective because treatment with this compound did not interfere with PKD activation and Ser-744 phosphorylation induced by PDB (Fig. 1B).

Effect of Treatment with PTxs on LPA-induced PKD Activation in RK Cells—Previous studies demonstrated that LPA induces PKD activation through a PTx-sensitive pathway in 3T3 (18) and IEC-6 cells (57) but through a pathway that was only partially attenuated by PTxs in IEC-18 cells (57). Because PTxs catalyze the ADP-ribosylation and inactivation of members of the Go family (68), these results indicated that Gi contributes to PKD activation to a different degree in different cell contexts.

To assess the contribution of Gi to LPA-induced PKD activation in RK cells, cultures of these cells were treated with increasing concentrations of PTxs (1–100 ng/ml) for 3 h and then challenged with 3 μM LPA for 10 min. As illustrated by Fig. 2A, treatment of RK cells with PTx at a concentration as high as 100 ng/ml attenuated only slightly PKD activation and Ser-744 phosphorylation in response to LPA (the maximal attenuation was only ~25%). Similar attenuation of PKD activation was obtained when PKD activity in immunocomplexes was determined by phosphorylation of the exogenous substrate syntide-2 rather than by autophosphorylation (Fig. 2B). These results suggest that LPA induces PKD activation predominantly through Gi-independent pathways in RK cells, presumably involving Goq11 and/or Goq12/13.

LPA-induced PKD Activation Requires Functional Gi—Most cell types express multiple members of the Go and G12 families with overlapping functions, thus rendering it difficult to analyze the contribution of these G proteins to heptahelical receptor signaling. To circumvent this problem and in view of the results presented above, we next examined whether LPA induces PKD activation in murine embryonic fibroblasts generated from double knockout mice for Goq11 and Goq12/13. As illustrated by the Western blot analysis of cell lysates shown in Fig. 3A, Goq11 KO cells did not express either Goq or Goq11 whereas the Goq12/13 KO cells did not express either Goq12 or Goq13 while, as expected, the RK cells express the α subunits of these G proteins.

In agreement with the results shown in Figs. 1 and 2, treatment of RK cells with either LPA or PDB induced a marked increase in PKD catalytic activity measured either by autophosphorylation or syntide-2 phosphorylation assays (Fig. 3B). In striking contrast, addition of LPA to cultures of Goq11KO cells did not induce any significant increase in PKD activity, as measured in immunocomplexes by either autophosphorylation or syntide-2 phosphorylation assays. PDB, which acts directly on PKC and thus, bypasses the receptor-G protein interaction, induced robust stimulation of PKD activity in Goq11KO cells, indicating that the block in LPA action in these cells is upstream of the PKC/PKD cascade.

To further confirm the requirement of functional Gi in LPA-induced PKD activation, we examined whether transfection of Goq11KO cells with wild type Goq restores the ability of LPA to induce PKD activation. Because PKD is abundantly expressed in Goq11KO cells and RK cells, we used GFP-tagged PKD (FPK-PKD) for these co-transfection experiments and GFP antibody to immunoprecipitate the ectopically expressed PKD. Previously, we demonstrated that the GFP tag does not interfere with the regulatory properties of PKD (69, 70). Goq11KO cells were co-transfected with GFP-PKD, either alone or to-
cultures of RK cells were incubated with LPA or PDB. A, Western blot analysis (W. Blot) of RK, Gq/11, and G12/13 knockout cell lysates. Confluent and quiescent cultures of the three type cells were solubilized with 2× sample buffer. The lysates were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using the antibody against αq/11, αq/12, or αq/13. The positions of immunoreactive Go subunits at apparent M₉ 43,000 are indicated by the arrows to the left, B, confluent and quiescent cultures of RK cells were incubated with (+) or without (−) 3 μM LPA or 200 nM PDB for 10 min at 37 °C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. Upper panel, IVK, PKD activity was determined by an in vitro kinase assay as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. Middle panel, scanning densitometry. The results shown are the values (mean ± S.E. n = 3) of the level of PKD activation by in vitro kinase obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 200 nM PDB. Lower panel, PKD activity in the immunocomplexes was then measured by syntide-2 phosphorylation, as described under “Experimental Procedures.” The results expressed as an increased -fold over control in phosphorylation represent the mean ± S.E. obtained from three independent experiments. B, cell lysates from the experiments described as above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot (W. Blot) analysis was carried out using pS744/748 PKD antibody (upper panel) or GFP antiserum (lower panel).

PKD alone. The increased PKD activity and phosphorylation in Gq/11 KO cells cotransfected with wild type Goq is comparable with that in the control RK cells transfected with GFP-PKD, which express endogenous Goq. These results demonstrate that a functional Goq/11 pathway is necessary for mediating LPA-induced PKD activation.

**Fig. 4.** LPA induces PKD activation in Gq/11 KO cells transfected with Gαq. A and B, Gq/11 KO cells co-transfected with GFP-PKD and pcDNA1 or pcDNA1 encoding wild type Goq. The control cells (RK cells) were transfected with GFP-PKD and pcDNA1, as indicated in this figure. Three days after transfection, the cultures were left unstimulated (−) or stimulated (+) either with 3 μM LPA or with 200 nM PDB for 10 min and lysed. A, upper panel, the lysates were immunoprecipitated with GFP antibody and PKD activity in the immunocomplexes was determined by an in vitro kinase assay (IVK) as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. A representative autoradiogram of three independent experiments is shown. Lower panel, PKD activity in immunocomplexes was measured by syntide-2 phosphorylation. The results expressed as an increased -fold over control in phosphorylation represent the mean ± S.E. obtained from three independent experiments. B, cell lysates from the experiments described as above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot (W. Blot) analysis was carried out using pS744/748 PKD antibody (upper panel) or GFP antiserum (lower panel).

**Fig. 3.** Comparison of three cell lines (RK, Gq/11 KO, or G12/13 KO) in Go subunit expression and PKD activation induced by LPA or PDB. A, Western blot analysis (W. Blot) of RK, Gq/11, and G12/13 knockout cell lysates. Confluent and quiescent cultures of the three type cells were solubilized with 2× sample buffer. The lysates were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using the antibody against αq/11, αq/12, or αq/13. The positions of immunoreactive Go subunits at apparent M₉ 43,000 are indicated by the arrows to the left, B, confluent and quiescent cultures of RK cells were incubated with (+) or without (−) 3 μM LPA or 200 nM PDB for 10 min at 37 °C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. Upper panel, IVK, PKD activity was determined by an in vitro kinase assay as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. Middle panel, scanning densitometry. The results shown are the values (mean ± S.E. n = 3) of the level of PKD activation by in vitro kinase obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 200 nM PDB. Lower panel, PKD activity in the immunocomplexes was then measured by syntide-2 phosphorylation, as described under “Experimental Procedures.” The results expressed as an increased -fold over control in phosphorylation represent the mean ± S.E. obtained from three independent experiments, each performed in duplicate. Gfp-PKD were used as the control. Three days after transfection, the cultures were left unstimulated (−) or with 200 nM PDB.
The results presented in Fig. 5 were designed to examine in more detail the stimulation of PKD activity in response to LPA in G12/13 KO cells. LPA promoted PKD activation in G12/13 KO cells in a concentration-dependent manner with half-maximal and maximal stimulation achieved at 0.07 and 3 μM. The maximal increase in PKD autophosphorylation activity (4-fold) was lower than that achieved by LPA in RK cells (6-fold). These results confirmed that LPA stimulated PKD activation in G12/13 KO cells with reduced effectiveness, even at the concentrations that induced a maximal response, and implied that the expression of G12/13 is necessary for maximal stimulation of PKD in response to LPA. The results shown in Fig. 5 also demonstrate that LPA induced PKD Ser-744 phosphorylation and syntide-2 phosphorylation activity (inset) in a concentration-dependent manner in G12/13 KO cells.

We verified that LPA induces PKD activation in these cells through a PKC- and PLC-dependent pathway. As shown in Fig. 5B, left, exposure to either GF I or Ro 31-8220 prevented PKD...
activation and Ser-744 phosphorylation induced by LPA whereas the biologically inactive analog GFV did not prevent PKD activation in response to LPA. Similarly, treatment with the PLC inhibitor U73122 (66, 67) prevented PKD activation induced by LPA but not by PDB, as shown by assays of autophosphorylation, Western blot analysis, or syntide-2 phosphorylation (Fig. 5B, right).

LPA-induced PKD Activation and Phosphorylation Requires Functional Gq in G12/13 KO Cells—We hypothesized that PKD activation and activation loop phosphorylation are elicited by LPA and other GPCR agonists through interaction of complementary G protein pathways, namely, Gq, G12/13, and G11 (see Introduction for references). Although the effect of Gq is not prominent in LPA-induced PKD activation in RK cells, our hypothesis predicts that in G12/13 KO cells, members of the Gi family interact with members of the Gq family in mediating LPA-induced PKD activation. To test the contribution of Gq to LPA-induced PKD activation in G12/13 KO cells, cultures of these cells were treated with increasing concentrations of PTx (1–100 ng/ml) and PKD activity in immunocomplexes was measured by either autophosphorylation or syntide-2 phosphorylation assays.

As shown in Fig. 6A, treatment with PTx dramatically inhibited both PKD activation and Ser-744 phosphorylation elicited by LPA in a concentration-dependent fashion. Indeed, treatment with 30–100 ng/ml PTx completely abolished PKD activation and Ser-744 phosphorylation in response to LPA. In contrast, treatment with 50 ng/ml PTx did not interfere with PKD activation and Ser-744 phosphorylation induced by PDB in these cells. As illustrated in Fig. 6B, similar results were obtained when PKD activity in immunocomplexes was determined by assays of syntide-2 phosphorylation. These findings contrast with those shown in Fig. 2 with RK cells (PTx inhibited LPA-induced PKD activation by only ∼25%) and indicate that G11 plays a critical role in promoting PKD activation in G12/13 KO cells. We conclude that in G12/13 KO cells, G11 cooperates with Gq in mediating the PKC/PKD phosphorylation cascade in response to LPA.

Recently, we demonstrated that in addition to Gqα, G11α-mediated signaling contributes to PKD activation in bombesin-stimulated cells through endogenous Rho (39). C. difficile toxin monoglucosylates the threonine residue at position 35 in Rac and Cdc-42 and threonine 37 in Rho and thereby inactivates these small G proteins (71, 72). Here, we hypothesized that inactivation of Rho GTPases with C. difficile toxin B in RK cells should enhance the sensitivity of LPA-induced PKD activation to treatment with PTx. To test this hypothesis, RK cells were treated with PTx either with or without C. difficile toxin B and then stimulated with either LPA or PDB, as indicated in Fig. 7. Treatment with C. difficile toxin B markedly enhanced the ability of PTx to inhibit LPA-induced PKD activation (Fig. 7A) and Ser-744 phosphorylation (Fig. 7B) in RK cells. These results provide an independent line of evidence indicating that PKD activation induced by LPA is mediated by cooperation of multiple G protein pathways.

LPA Induces Activation of Mitogen-activated Protein Kinases via Gq-dependent but PKC-independent Pathways in RK, Gαq111 KO, and Gα12/13 KO Cells—To substantiate that the different profiles of PKD activation in response to LPA in the three cell lines used in our study is specific and reflects the participation of multiple G proteins, we examined other biological effects induced by LPA in these cells.

In most cell types, LPA stimulates Ras activation leading to stimulation of Raf, MEK, and the ERKs via a PTx-sensitive pathway that involves the βγ subunits of Gq (47–51). Here, we determined the effect of PTx on LPA-induced ERK activation in these three types of cells. As shown by Western blot analysis using an antibody that detects the dually phosphorylated and active ERK and active ERK-1 and ERK-2, LPA induced robust activation of the ERKs in RK, Gα12/13 KO, and Gαq111 KO cells (Fig. 8). Treatment with PDB also induced strong ERK activation in the three cell lines, indicating the existence of a PKC-dependent pathway.

Treatment of RK cells with PTx markedly inhibited ERK activation in response to LPA, whereas exposure to GF I or Ro 31-8220 did not interfere with ERK activation, indicating that LPA induces ERK through a PTx-sensitive, PKC-independent pathway in RK cells. Virtually identical results were obtained with Gα12/13 KO cells and Gαq111 KO cells. Specifically, LPA pro-
Cooperation of G Proteins in PKD Activation by LPA

FIG. 7. PKD activation induced by LPA in RK cells is partially blocked by C. difficile toxin B (toxin B) and almost abolished by the combination treatments of the cells with toxin B and Ptx. A and B, confluent and quiescent RK cells were preincubated with (+) or without (−) Ptx (50 ng/ml) for 3 h and then treated with different concentrations of toxin B as indicated for 1.5 h. Cells were then stimulated with (+) or without (−) 3 μM LPA or 200 nM PDB for 10 min at 37 °C. Cultures were lysed with lysis buffer and then immunoprecipitated with PA-1 antiserum. A, upper panel, in vitro kinase (IVK), immunoprecipitates were subjected to in vitro kinase assay, SDS-PAGE, and autoradiography. The autoradiograph shown is representative of three independent experiments. Lower panel, PKD activity in the immunocomplexes was measured by syntide-2 phosphorylation, as described under "Experimental Procedures." The results expressed as an increased-fold over control in phosphorylation represent the mean ± S.E. obtained from three independent experiments, each performed in duplicate. B, pS744, cell lysates from the experiments described above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using pS744/748 PKD antibody.

FIG. 8. LPA induces activation of ERK-2 and ERK-1 in RK cells and G_q/11 KO or G_{12/13} KO cells. Confluent and quiescent RK cells (A) and G_q/11 KO cells (B) or G_{12/13} KO cells (C) were incubated with Ptx for 3 h or with the other inhibitors, GF-109203X (GF1, 3.5 μM), or Ro 31–8220 (Ro, 2.5 μM), or U0126 (U, 2.5 μM), as indicated, for 1 h. Control cells (−) received an equivalent amount of solvent. Cells were then stimulated with (+) or without (−) 3 μM LPA or 200 nM PDB for 10 min at 37 °C. Cultures were lysed with lysis buffer. Cell lysates from the experiments were analyzed by SDS-PAGE and transferred to Immobilon membranes. A–C, upper panels, Western blot analysis with specific antiphospho-ERK-1/2 monoclonal antibody; lower panels, the Western blot was also probed for total ERK by ERK-2 polyclonal antibody.

Promoted ERK activation in both G_{12/13} KO cells and G_{q/11} KO cells through a G_i-dependent but PKC-independent pathway (Fig. 8). These results demonstrate that the lack of expression of these G proteins does not interfere with other biological effects of LPA mediated by G_i and provide further support to the conclusion that LPA utilizes different G protein pathways to activate parallel phosphorylation cascades leading to PKD and ERK activation in RK cells.

Concluding Remarks—LPA promotes a broad range of biological responses and multiple molecular events in target cells (42). Consistent with the stimulation of multiple signaling pathways, LPA has been shown to activate several heterotrimeric G proteins including G_q, G_i, and G_{13} in Swiss 3T3 cells (54). PKC-dependent PKD activation has been identified as an early event in the action of LPA in intact Swiss 3T3, Rat-1, and IEC-6 cells (56, 57). Because our previous studies indicated that G_q and G_{13}-mediated signaling can promote PKD activation in intact cells (20, 39), it was surprising that treatment of these cells with Ptx markedly inhibited PKD activation in response to LPA (56, 57). Because Ptx catalyzes the ADP-ribosylation and inactivation of members of the G_q family (68), these results identified the involvement of an additional G_i-dependent pathway leading to PKD activation in response to LPA. However, other results also demonstrated that LPA induces PKD activation through a pathway that was only partially attenuated by Ptx in IEC-18 cells (57) indicating that G_i contributes to PKD activation to different degrees in different cell contexts. In the present study, we tested the hypothesis that LPA induces PKD activation through multiple G protein signal transduction pathways, including G_q, G_i, and G_{13}, using mouse embryonic cell lines deficient in G_{q/11} and G_{12/13}, and using a cell line deficient in rhodopsin kinase (RK cells), as a control.

Based on the results presented in this study, we propose a model that envisages that LPA induces PKD activation through G_q acting synergistically with G_{12} and G_i. Thus, inhibition of G_i signaling in RK cells by treatment with Ptx only induces a modest attenuation of PKD activation in response to LPA because the remaining G protein pathways (G_q and G_{12}) cooperate to mediate a substantial response to LPA. Similarly, treatment of RK cells with C. difficile toxin B that inactivates...
PKD activation was completely abrogated in Goq/11 KO cells and that transfection of Goq into the Goq/11 KO cells restored LPA-induced PKD activation to the similar level as the control cells. In Goq12/13 KO cells, LPA induced substantial PKD activation, a response predicted by the model to be mediated by cooperation of Goq and Gi. In line with this interpretation and in sharp contrast to the results obtained with RK cells, treatment of Goq12/13 KO cells with PTx dramatically inhibited PKD activation in response to LPA. These results are also consistent with the notion that endogenous Goq, is not sufficient to mediate PKD activation in response to LPA. Thus, our model integrates the PTx-sensitive and -insensitive effects obtained in Goq12/13 KO cells and RK cells with the striking suppression of LPA-induced PKD activation in Goq/11 KO cells and leads to the notion that LPA induces PKD activation through the synergistic interaction of pathways initiated by Goq, Go12, and Gi. A corollary of these results is that the level of expression of the different α subunits of heterotrimeric G proteins can be identified as one of the important molecular elements that determine the influence of cell context on agonist-induced PKD activation.

Recent work from other laboratories has shown that transcriptional responses induced by LPA also result from synergistic effects between parallel G protein signaling pathways. For example, the stimulation of the transcription factor NF-xB by LPA is mediated by G, and Gi pathways (73) and the activation of the serum response element is mediated by cooperative effects between Gi and Go13/Rho pathways (74). Furthermore, recent genetic studies indicate that the G,-mediated signaling pathway functionally interacts with the G12-mediated signaling pathway to promote embryonic survival (63).

Here, we propose that PKD activation, which precedes the transcriptional responses, is also mediated by cooperative effects between events initiated by Goq, Go12, and Gi signaling in LPA-treated cells. The scheme shown in Fig. 9 summarizes the multiple G protein signal transduction pathways involved in LPA-induced PKD activation proposed in this study and also illustrates the molecular, cellular, and pharmacological approaches used in our experiments.

**Acknowledgments**—We are very grateful to Dr. Melvin I. Simon, Division of Biology, California Institute of Technology, for the generous gift of fibroblast cell lines. We also thank Steven H. Young for assistance in cell transfection and J. Sinnott-Smith, Cliff Hurd, and Osvaldo Rey for helpful discussions and careful reading of the manuscript.

**REFERENCES**

1. Nishizuka, Y. (1992) Science 258, 607–614
2. Newton, A. C. (1995) J. Biol. Chem. 270, 28485–28498
3. Dekker, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73–77
4. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329–343
5. Nishizuka, Y. (1995) FASEB J. 9, 484–486
6. Vlachova, A. M., Sinnott-Smith, J., Van Lint, J., and Rozengurt, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8572–8576
7. Johannes, F. J., Prestle, J., Eis, S., Oberhagemann, P., and Pfizenmaier, K. (1994) J. Biol. Chem. 269, 6140–6148
8. Rozengurt, E., Sinnott-Smith, J., and Zagura, J. L. (1997) Biochem. Soc. Trans. 25, 565–571
9. Zagura, J. L., Sinnott-Smith, J., Van Lint, J., and Rozengurt, E. (1996) EMBO J. 15, 6220–6230
10. Yuan, J., Bae, D., Cantrell, D., Nel, A. E., and Rozengurt, E. (2002) Biochem. Biophys. Res. Commun. 291, 444–452
11. Zagura, J. L., Waldron, R. T., Sinnott-Smith, J., and Rozengurt, E. (1997) J. Biol. Chem. 272, 23952–23960
12. Iglesias, T., and Rozengurt, E. (1996) J. Biol. Chem. 271, 410–416
13. Abedi, H., Rozengurt, E., and Zachary, J. (1998) FEBS Lett. 437, 209–212
14. Iglesias, T., Waldron, R. T., and Rozengurt, E. (1998) J. Biol. Chem. 273, 27662–27667
15. Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2001) J. Biol. Chem. 276, 32068–32075
16. Offermanns, S., and Simon, M. I. (1996) Cancer Surv. 27, 177–198
17. Eton, J. H. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 481–509
18. Paulucci, L., and Rozengurt, E. (1999) Cancer Res. 59, 572–577
19. Chia, T., and Rozengurt, E. (2001) Am. J. Physiol. 280, C929–C942
20. Yuan, J., Slice, L., Walsh, J. H., and Rozengurt, E. (2000) J. Biol. Chem. 275, 32608–32615
