THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 7, Issue of February 14, pp. 4882–4891, 2003

Printed in U.S.A.

Cooperation of G_q, G_i, and $G_{12/13}$ in Protein Kinase D Activation and Phosphorylation Induced by Lysophosphatidic Acid*

Received for publication, October 31, 2002, and in revised form, December 10, 2002
Published, JBC Papers in Press, December 10, 2002, DOI 10.1074/jbc.M211175200

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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 $G\alpha_{q/11}$ ($G\alpha_{q/11}$ KO cells) or $G\alpha_{12/13}$ ($G\alpha_{12/13}$ KO cells) and used cells lacking rhodopsin kinase (RK cells) as a control. In RK and $G\alpha_{12/13}$ 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 $G\alpha_{12/13}$ KO cells in autophosphorylation activity) achieved at 3 μM. In contrast, LPA did not induce any significant increase in PKD activity in $G\alpha_{q/11}$ KO cells. However, LPA induced a significantly increased PKD activity when $G\alpha_{q/11}$ KO cells were transfected with $G\alpha_q$. 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 $G\alpha_{12/13}$ 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_q is necessary but not sufficient to mediate LPA-induced PKD activation. In addition to G_q, LPA requires additional G-protein pathways to elicit a maximal response with G_i playing a critical role in $G\alpha_{12/13}$ KO cells. We conclude that LPA induces PKD activation through G_q, G_i, and G_{12} and propose that PKD activation is a point of convergence in the action of multiple G-protein pathways.

Protein kinase C (PKC),[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_1, β_2, 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/PKCμ 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 cotransfection 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_s, G_i, G_q, and G_{12}. The α subunit of G_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^{2+} from internal stores and diacylglycerol that activates the classical and novel isoforms of PKC (reviewed in Ref. 17). A variety of neuropeptide 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_q and $G\alpha_q$ signaling stimulates PKD activity (20), expression of a COOH-terminal fragment of $G\alpha_q$ 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 $G\alpha_q$ but also through other G protein-mediated signaling pathways.

* This work was supported in part by National Health Institute Grants DK 55003, DK56930, DK 17294, and NCI Grant P50 CA 90388-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by National Institutes of Health National Research Service Award F32 CA84658-01A1.

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[1] The abbreviations used are: PKC, protein kinase C; LPA, lysophosphatidic acid; G proteins, guanine nucleotide-binding regulatory proteins; GPCRs, G protein-coupled receptors; GFP, green fluorescent protein; PDB, phorbol 12,13-dibutyrate; PKD, protein kinase D; RK cells, murine embryonic cell line with rhodopsin kinase gene knockout; $G\alpha_{q/11}$ KO cells, murine embryonic cell line with $G\alpha_{q/11}$ gene knockout; $G\alpha_{12/13}$ KO cells, murine embryonic cell line with $G\alpha_{12/13}$ gene knockout; PTx, pertussis toxin; PLC, phospholipase C; ERK, extracellular signal-regulated kinase.

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 Gα 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 Gα_q and PLC signaling are synergistic with Rho. Recently, we demonstrated that in addition to Gα_q, Rho- and Gα_{13}-mediated signaling can promote PKD activation in intact cells and that endogenous Rho and Gα_{13} contribute to PKD activation in response to bombesin GPCR stimulation (39). Thus, our results identified PKD as a novel downstream target in Gα_{13} and Rho signaling and indicated that GPCR stimulation promotes PKD activation via both G_q- and Gα_{13}/Rho-dependent pathways.

Lysophosphatidic acid (LPA), a major bioactive lipid of serum (40), elicits a plethora of biological responses by activation of its specific GPCRs (41–43). Thus far, three genes (*lp$_{A1}$*, *lp$_{A2}$*, and *lp$_{A3}$*) encoding LPA receptors, LP_{A1}/EDG-2, LP_{A2}/EDG-4, and LP_{A3}/EDG-7, have been identified in mammals (43–45). All LPA receptors are coupled to G_i and G_q and, at least LP_{A1} and LP_{A2} are also coupled to G_{12} (42, 45, 46). LPA induces Ras activation leading to stimulation of Raf, MEK, and the ERKs via a pertussis toxin (PTx)-sensitive pathway that involves the $\beta\gamma$ subunits of G_i (47–51) while the α subunit of this trimeric G protein mediates inhibition of adenylate cyclase activity (42, 46). LPA stimulates PLC-mediated generation of inositol 1,4,5-trisphosphate and diacylglycerol, leading to Ca^{2+} mobilization from intracellular stores and PKC activation, respectively. These PLC-dependent responses are mediated by the α subunit of G_q and/or the $\beta\gamma$ subunits of the PTx-sensitive G_i (52). LPA also promotes PTx-insensitive stress fiber formation, assembly of focal adhesions, and tyrosine phosphorylation of focal adhesion proteins (53) via activation of Gα_{13} and Rho (24, 54). Because LPA receptors are expressed in most cell types, this bioactive lipid is a prototype agonist that promotes the activation of multiple endogenous G protein pathways that are responsible for transducing LPA signals into a broad spectrum of biological responses (43, 45, 55).

LPA has also been shown to induce a rapid PKC-dependent PKD activation in intact Swiss 3T3, Rat-1, and IEC-6 cells (56, 57). Interestingly, treatment of these cells with PTx markedly attenuated PKD activation in response to LPA. These results identified the involvement of an additional G_i-dependent pathway leading to PKD activation and indicated that the endogenous G_q pathway is not sufficient to promote PKD activation in response to LPA. Because our previous studies demonstrated that PKD can be activated through G_q- and G_{12}-dependent pathways (see above) and because LPA receptors are also coupled to G_q and G_{12}, we hypothesized that LPA induces PKD through multiple G protein signal transduction pathways. Here, we tested this hypothesis using mouse embryonic cell lines deficient in G$\alpha_{q/11}$ (G$\alpha_{q/11}$ KO cells) or G$\alpha_{12/13}$ (G$\alpha_{12/13}$ KO cells), and used a cell line lacking rhodopsin kinase (RK cells), as a control. Based on our results, we conclude that LPA induces PKD activation through the cooperation of G_q with either G_i or G_{12} and that PKD activation is a point of convergence in the action of multiple G protein pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse embryonic fibroblasts were generated from genetically engineered mice (58, 59) that contained gene knockout for rhodopsin kinase, G$\alpha_{q/11}$, and G$\alpha_{12/13}$. 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.

Cell Transfection and cDNA Constructs Used in Transfections—To transfect the cells, RK cells and G$\alpha_{q/11}$ KO cells were subcultured to 80–90% confluency in 10-cm dishes. All transfections and cotransfections were carried out with equivalent amounts of DNA (20 μg/dish). Transfections were carried out in Opti-MEM using LipofectAMINE 2000 reagent according to the protocol from the manufacturer (Invitrogen). Cells were used for experiments 48–72 h after of transfection. Chimeric fusion proteins between GFP and PKD (GFP-PKD) have been described previously (69, 70).

Immunoprecipitation—Cultures of RK, G$\alpha_{q/11}$, and G$\alpha_{12/13}$ 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 clarified by centrifugation at 15,000 $\times$ *g* for 10 min at 4 °C. PKD was immunoprecipitated at 4 °C for 2–4 h with the PA-1 antiserum (1:100) or with GFP antibody for GFP-PKD transfected cell lysates, as previously described (9, 10). The immune complexes were recovered using protein A coupled to agarose.

Kinase Assay of PKD—PKD autophosphorylation was determined in an *in vitro* kinase assay by mixing 20 μl of PKD immunocomplexes with 10 μl of a phosphorylation mixture containing (final concentration) 100 μM [γ-^{32}P]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, 0.1 M Na_3VO_4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol), followed by SDS-PAGE analysis (9, 60). The gels were dried and the 110- or 140-kDa radioactive band 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 mixture containing 2.5 mg/ml syntide-2 (PLARTLSVAGLPGKK), a peptide based on phosphorylation site two of glycogen synthase. After 10 min of incubation at 30 °C, the reaction was stopped by adding 100 μl of 75 mM H_3PO_4, and 75 μl of the supernatant was spotted on P-81 phosphocellulose paper. Free [γ-^{32}P]ATP was separated from the labeled substrate by washing the P-81 paper four times for 5 min, in 75 mM H_3PO_4. The papers were dried, and the radioactivity incorporated into syntide-2 was determined by Cerenkov counting.

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. Following SDS-PAGE on 8% gels, proteins were transferred to Immobilon-P membranes (Millipore) and blocked by 3–6 h incubation with 5% nonfat milk in phosphate-buffered saline, pH 7.2. Membranes were then incubated 3 h with the respective antibodies. PKD phosphorylation was determined by incubating the membrane with an antibody that specifically recognizes the phosphorylated state of serine 744 and serine 748 of PKD at a dilution of 1:1000 in phosphate-buffered saline, 0.1% Tween 20 containing 5% bovine serum albumin.

Activation of ERK-2 and ERK-1 occurs through phosphorylation of specific threonine and tyrosine residues, resulting in slower migrating forms in SDS-PAGE gels. These activated forms were monitored by using a specific antiphospho-ERK-1/ERK-2 monoclonal antibody that recognizes the phosphorylated state of Thr-202 and Tyr-204 of ERL-1/2 at a dilution of 1:1000 in phosphate-buffered saline, 0.1% Tween 20 containing 5% nonfat dried milk. The same membranes were stripped and probed in a similar fashion with goat anti-ERK-2 polyclonal antibody.

Horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5000, Amersham Biosciences) was then applied for 1 h at room temperature after washing 3 times with phosphate-buffered saline containing 0.05%

Tween. Immunoreactive bands were detected by enhanced chemiluminescence Western blotting ECL™ reagents.

Materials—[γ-^{32}P]ATP (370 MBq/ml), horseradish peroxidase-conjugated donkey anti-rabbit IgG, and enhanced chemiluminescence reagents were from Amersham Biosciences. Protein-A agarose and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride were from Roche Molecular Biochemicals. LPA, PDB, GF 109203X, GF V, U-0126, PD 098059, and *Clostridium difficile* toxin B were obtained from Sigma. Ro 31-8220, U73122, and PTx were purchased from Calbiochem (La Jolla, CA). Opti-MEM and LipofectAMINE 2000 reagent were from Invitrogen. PA-1 antiserum was raised against the synthetic peptide EEREMKALSERVSIL that corresponds to the carboxyl-terminal region of the predicted amino acid sequence of PKD, as previously described (9, 60). Phospho-PKD Ser-744/748 antibody was obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK-1/2 monoclonal antibody was obtained from New England Biolabs. Anti-G$\alpha_{q/11}$, anti-G$\alpha_{12/13}$, anti-GFP, and anti-ERK-2 polyclonal antibodies were from Santa Cruz Biotechnologies (Palo Alto, CA). Other items were from standard suppliers or as specifically indicated.

RESULTS AND DISCUSSION

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, G$\alpha_{q/11}$, and G$\alpha_{12/13}$. 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-^{32}P]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. 1*A* 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 pS744/748 antibody. As shown in Fig. 1*A*, 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 I (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. 1*B* (*left panels*), exposure to either GF I 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 I 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

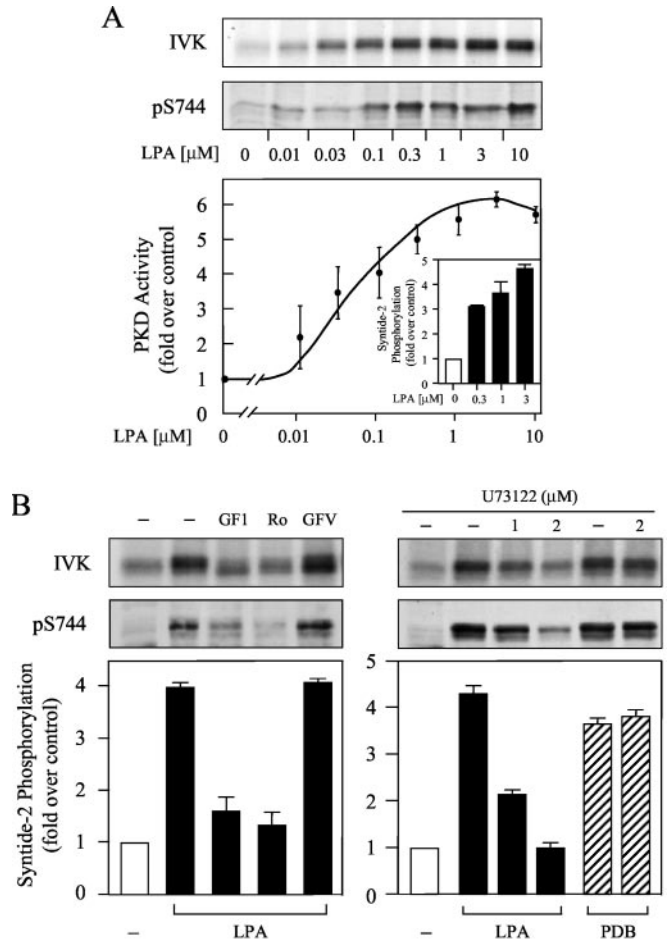

FIG. 1. **LPA activates PKD in a dose-dependent and PLC/PKC-dependent manner in RK cells.** *A*, LPA activates PKD in a dose-dependent manner. Confluent and quiescent cultures of RK cells were incubated with various concentrations of LPA for 10 min at 37 °C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. *Top panel*, *in vitro* kinase (*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*, 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. *Bottom 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 3 μM LPA. *Inset*, 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*, LPA induces PKD activation through a PLC/PKC-dependent pathway. Confluent and quiescent RK cells were incubated for 1 h with the PKC inhibitor GF-109203X (*GF1*, 3.5 μM) or Ro 31-8220 (*Ro*, 2.5 μM), or with PLC inhibitor, U73122. Control cells (−) received equivalent amounts of solvent or GFV. The cultures were subsequently stimulated for 10 min with 3 μM LPA or 200 nM PDB at 37 °C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. *Top panels*, *in vitro* kinase (*IVK*), PKD activity was determined by an *in vitro* kinase assay, as described under "Experimental Procedures." The autoradiogram shown is representative of three independent experiments. *Middle panels*, pS744, cell lysates from the experiments described as the above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using pS744/748 PKD antibody. *Bottom panels*, 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.

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. 1*B* 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. 1*B* (*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. 1*B*, *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. 1*B*).

Effect of Treatment with PTx 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 PTx in IEC-18 cells (57). Because PTx catalyzes the ADP-ribosylation and inactivation of members of the $G\alpha_i$ family (68), these results indicated that G_i contributes to PKD activation to a different degree in different cell contexts.

To assess the contribution of G_i to LPA-induced PKD activation in RK cells, cultures of these cells were treated with increasing concentrations of PTx (1–100 ng/ml) for 3 h and then challenged with 3 μM LPA for 10 min. As illustrated by Fig. 2*A*, 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. 2*B*). These results suggest that LPA induces PKD activation predominantly through G_i-independent pathways in RK cells, presumably involving $G\alpha_{q/11}$ and/or $G\alpha_{12/13}$.

LPA-induced PKD Activation Requires Functional G_q—Most cell types express multiple members of the G_q and G_{12} 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 $G\alpha_{q/11}$ and $G\alpha_{12/13}$. As illustrated by the Western blot analysis of cell lysates shown in Fig. 3*A*, $G\alpha_{q/11}$ KO cells did not express either $G\alpha_q$ or $G\alpha_{11}$ whereas the $G\alpha_{12/13}$ KO cells did not express either $G\alpha_{12}$ or $G\alpha_{13}$ 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. 3*B*). In striking contrast, addition of LPA to cultures of $G\alpha_{q/11}$ KO 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

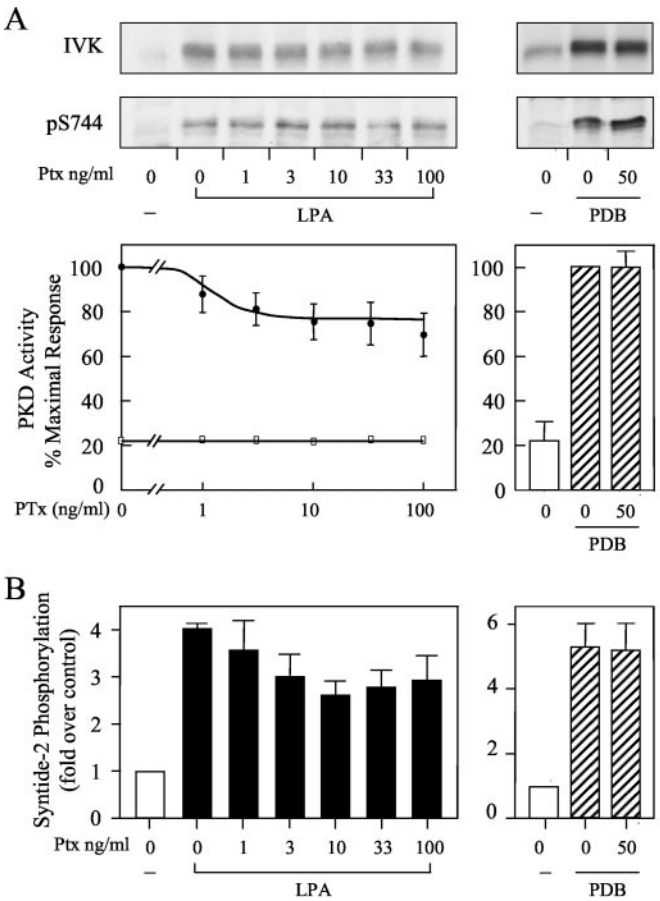

FIG. 2. **Effect of PTx on the activation of PKD induced by LPA in RK cells.** *A* and *B*, confluent and quiescent RK cells were incubated with different concentrations of PTx as indicated for 3 h. Cells were then stimulated with (+) or without (−) 3 μM LPA (*left panels*) or 200 nM PDB (*right panels*) for 10 min at 37 °C. Cultures were lysed with lysis buffer and then immunoprecipitated with PA-1 antiserum. *A*, *top panels*, *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. *Middle panels*, *pS744*, cell lysates from the experiments described as above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using pS744/748 PKD antibody. *Bottom panels*, scanning densitometry, the results shown are the values (means ± S.E. $n = 3$) of the level of PKD activation obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 3 μM LPA for 10 min at 37 °C. ●, values corresponding to PKD activity from cells incubated with PTx, and then stimulated with LPA; □, values corresponding to PKD activity from cells incubated with PTx but without LPA. *B*, 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.

on PKC and thus, bypasses the receptor-G protein interaction, induced robust stimulation of PKD activity in $G\alpha_{q/11}$ KO 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 G_q in LPA-induced PKD activation, we examined whether transfection of $G_{q/11}$ KO cells with wild type $G\alpha_q$ restores the ability of LPA to induce PKD activation. Because PKD is abundantly expressed in $G_{q/11}$ KO cells and RK cells, we used GFP-tagged PKD (GFP-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). $G_{q/11}$ KO cells were co-transfected with GFP-PKD, either alone or to-

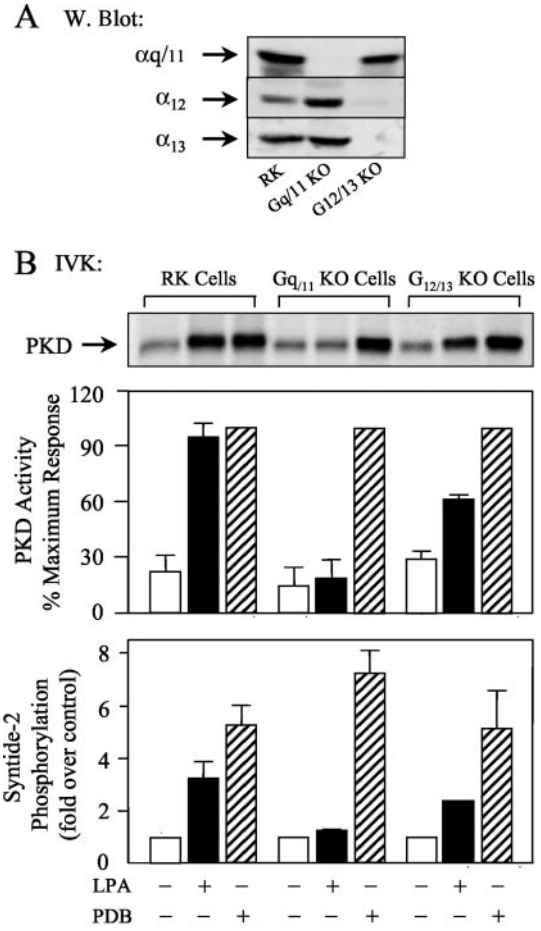

FIG. 3. **Comparison of three cell lines (RK, $G_{q/11}$ KO, or $G_{12/13}$ KO) in Gα subunit expression and PKD activation induced by LPA or PDB.** *A*, Western blot analysis (*W. Blot*) of RK, $G_{q/11}$, and $G_{12/13}$ knockout cell lysates. Confluent and quiescent cultures of the three type cells 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 $\alpha_{q/11}$, α_{12}, or α_{13}. The positions of immunoreactive Gα subunits at apparent M_r 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.

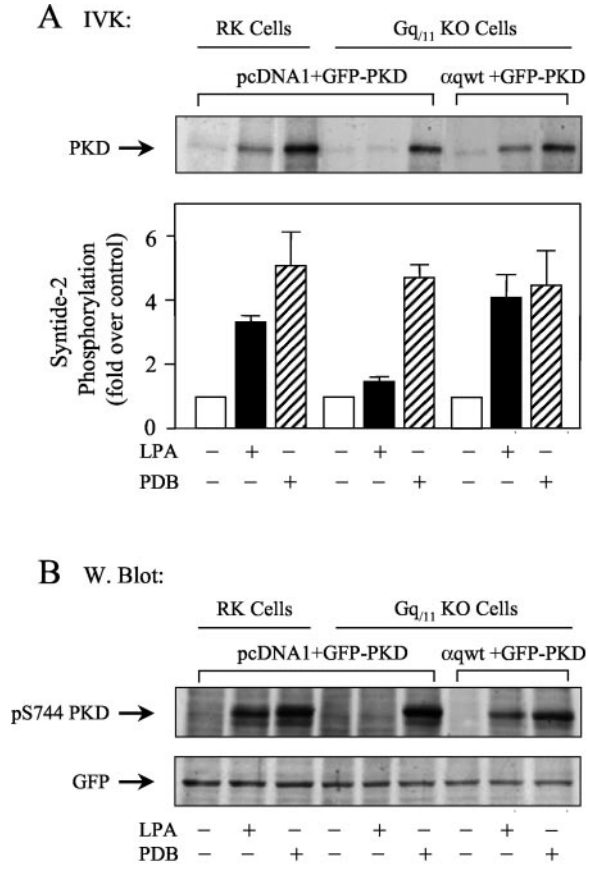

FIG. 4. **LPA induces PKD activation in $G_{q/11}$ KO cells transfected with Gα_q.** *A* and *B*, $G_{q/11}$ KO cells co-transfected with GFP-PKD and pcDNA1 or pcDNA1 encoding wild type Gα_q. The control cells (RK cells) were transfected with GFP-PKD and pcDNA1, as indicated in this figure. Three days 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*).

gether with pcDNA1-Gα_q, and the RK cells transfected with GFP-PKD were used as the control. Three days after transfection, the transfected cells were stimulated without or with 3 μM LPA or 200 nM PDB. PKD was immunoprecipitated from the lysates of transfected cells with GFP antibody, and the immune complexes were incubated with [γ-^{32}P]ATP, subjected to SDS-PAGE, and analyzed by autoradiography to detect the prominent 140-kDa autophosphorylated GFP-PKD. The results presented in Fig. 4 show that $G_{q/11}$ KO cells cotransfected with wild type Gα_q and PKD exhibit a marked increase in LPA-induced PKD activity (Fig. 4*A*) and Ser-744 phosphorylation (Fig. 4*B*) compared with $G_{q/11}$ KO cells transfected with GFP-PKD alone. The increased PKD activity and phosphorylation in $G_{q/11}$ KO cells cotransfected with wild type Gα_q is comparable with that in the control RK cells transfected with GFP-PKD, which express endogenous Gα_q. These results demonstrate that a functional G$\alpha_{q/11}$ pathway is necessary for mediating LPA-induced PKD activation.

LPA-induced Maximal PKD Activation Requires Functional G$\alpha_{12/13}$—Recently, we demonstrated that Gα_{13} contributes to PKD activation through a Rho- and PKC-dependent signaling pathway and hypothesized that PKD activation is mediated by both Gα_q and Gα_{13} in response to acute bombesin receptor stimulation (39). To assess the role of G$\alpha_{12/13}$ in PKD activation induced by LPA, we examined the effect of this agonist on PKD activity in G$\alpha_{12/13}$ KO cells. As shown in Fig. 3, addition of 5 μM LPA to cultures of G$\alpha_{12/13}$ KO cells induced a lower level of PKD activation than that induced by either PDB in these cells or by LPA in RK cells. These results imply that whereas G$\alpha_{q/11}$ are necessary for LPA-induced PKD activation, G$\alpha_{12/13}$ are required for eliciting maximal LPA-induced PKD activation.

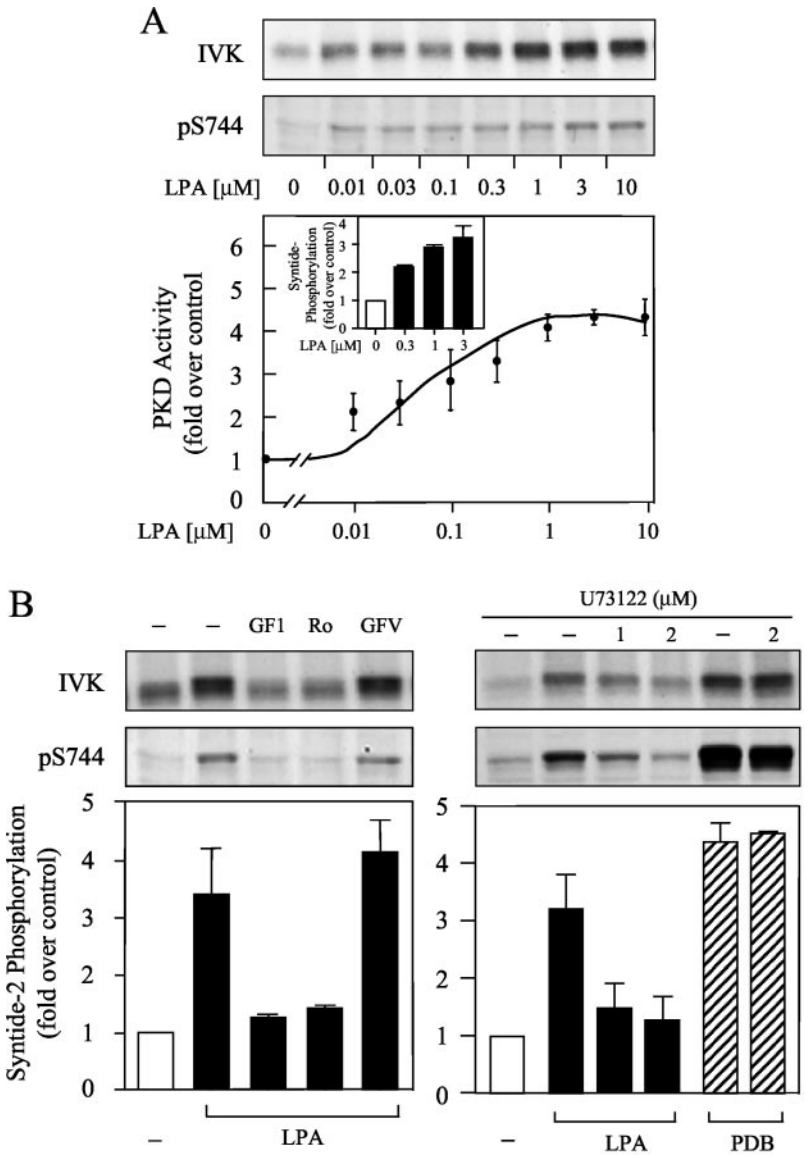

FIG. 5. **LPA activates PKD in a dose-dependent and PLC/PKC-dependent manner in $G_{12/13}$ KO cells.** *A*, LPA activates PKD in a dose-dependent manner. Confluent and quiescent cultures of $G_{12/13}$ knockout cells were incubated with various concentrations of LPA for 10 min at 37 °C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. *Top panel*, PKD activity was determined by an *in vitro* kinase (*IVK*) assay as described under "Experimental Procedures," followed by SDS-PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. *Middle panel*, *pS744*, cell lysates from the experiments described as above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using pS744/748 PKD antibody. *Bottom 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 3 μM LPA. *Inset*, 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*, LPA induces PKD activation through a PLC/PKC-dependent pathway. Confluent and quiescent $G_{12/13}$ knockout cells were incubated for 1 h with the PKC inhibitor GF-109203X (*GF1*, 3.5 μM) or Ro 31-8220 (*Ro*, 2.5 μM), or with PLC inhibitor, U73122. Control cells (−) received equivalent amounts of solvent or GFV. The cultures were subsequently stimulated for 10 min with 3 μM LPA or 200 nM PDB at 37 °C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. *Top panels*, *IVK*, PKD activity was determined by an *in vitro* kinase assay, as described under "Experimental Procedures." The autoradiogram shown is representative of three independent experiments. *Middle panels*, *pS744*, cell lysates from the experiments described as the above were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot analysis was carried out using pS744/748 PKD antibody. *Bottom panels*, 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.

The results presented in Fig. 5 were designed to examine in more detail the stimulation of PKD activity in response to LPA in $G\alpha_{12/13}$ KO cells. LPA promoted PKD activation in $G\alpha_{12/13}$ KO cells in a concentration-dependent fashion 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 $G\alpha_{12/13}$ KO cells with reduced effectiveness, even at the concentrations that induced a maximal response, and implied that the expression of $G\alpha_{12/13}$ is necessary for maximal stimulation of PKD in response to LPA. The results shown in Fig. 5*A* also demonstrate that LPA induced PKD Ser-744 phosphorylation and syntide-2 phosphorylation activity (*inset*) in a concentration-dependent manner in $G\alpha_{12/13}$ KO cells.

We verified that LPA induces PKD activation in these cells through a PKC- and PLC-dependent pathway. As shown in Fig. 5*B*, *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. 5*B*, *right*).

LPA-induced PKD Activation and Phosphorylation Requires Functional G_i in $G\alpha_{12/13}$ 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, G_q, $G\alpha_{12/13}$, and G_i (see Introduction for references). Although the effect of G_i is not prominent in LPA-induced PKD activation in RK cells, our hypothesis predicts that in $G\alpha_{12/13}$ KO cells, members of the G_q family interact with members of the G_i family in mediating LPA-induced PKD activation. To test the contribution of G_i to LPA-induced PKD activation in $G\alpha_{12/13}$ 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. 6*A*, 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. 6*B*, 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 G_i plays a critical role in promoting PKD activation in $G\alpha_{12/13}$ KO cells. We conclude that in $G\alpha_{12/13}$ KO cells, G_i cooperates with G_q in mediating the PKC/PKD phosphorylation cascade in response to LPA.

Recently, we demonstrated that in addition to $G\alpha_q$, $G\alpha_{13}$-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). In view of the results shown in Fig. 6 with $G\alpha_{12/13}$ KO cells, 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. 7*A*) and Ser-744 phosphorylation (Fig. 7*B*) 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 G_i-dependent but PKC-independent Pathways in RK, $G\alpha_{q/11}$ KO, and $G\alpha_{12/13}$ 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 $\beta\gamma$ subunits of G_i (47–51). Here, we

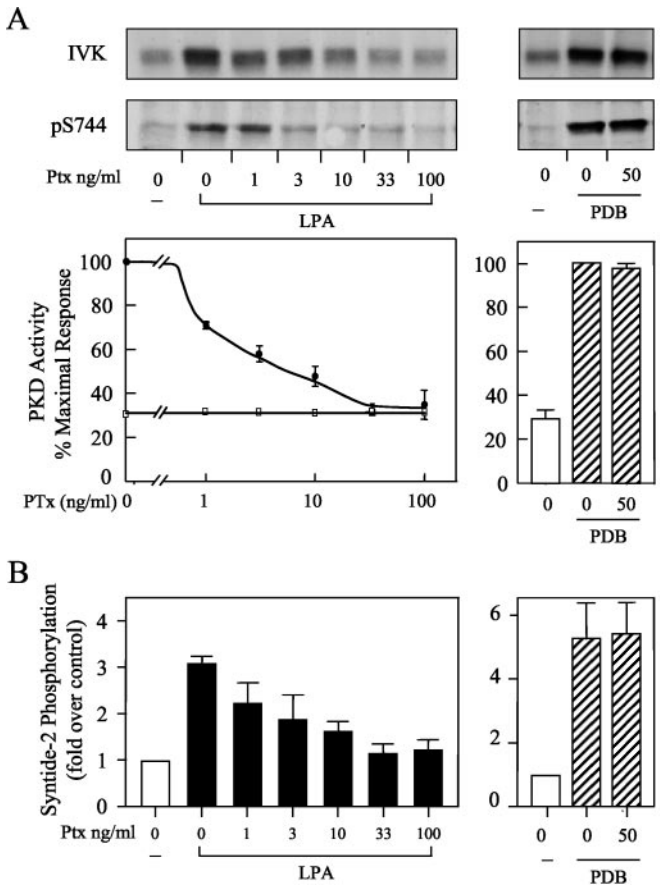

FIG. 6. **Effect of PTx on the activation of PKD induced by LPA in $G_{12/13}$ KO cells.** *A* and *B*, confluent and quiescent $G_{12/13}$ KO cells were incubated with different concentration of PTx as indicated for 3 h. Cells were then stimulated with (+) or without (−) 3 μM LPA (*left panels*) or 200 nM PDB (*right panels*) for 10 min at 37 °C. Cultures were lysed with lysis buffer and then immunoprecipitated with PA-1 antiserum. *A*, *top panels*, *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. *Middle panels*, 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. *Bottom panels*, scanning densitometry, the results shown are the values (mean ± S.E., $n = 3$) of the level of PKD activation obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 3 μM LPA for 10 min at 37 °C. ●, values corresponding to PKD activity from cells incubated with PTx, and then stimulated with LPA; □, values corresponding to PKD activity from cells incubated with PTx but without LPA. *B*, 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.

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-1 and ERK-2, LPA induced robust activation of the ERKs in RK, $G\alpha_{12/13}$ KO, and $G\alpha_{q/11}$ 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 in RK cells. Virtually identical results were obtained with $G\alpha_{12/13}$ KO cells and $G\alpha_{q/11}$ KO cells. Specifically, LPA pro-

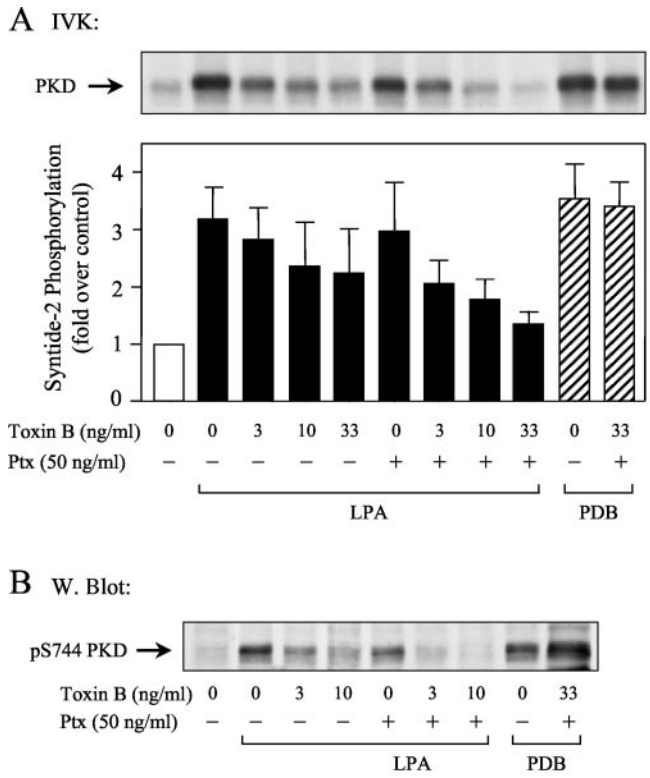

FIG. 7. **PKD activation induced by LPA in RK cells is partially blocked by *C. difficile* toxin B (*toxin B*) and almost abolished by the combination treatments of the cells with toxin B and Ptx.** *A* and *B*, confluent and quiescent RK cells were preincubated with (+) or without (−) PTx (50 ng/ml) 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.

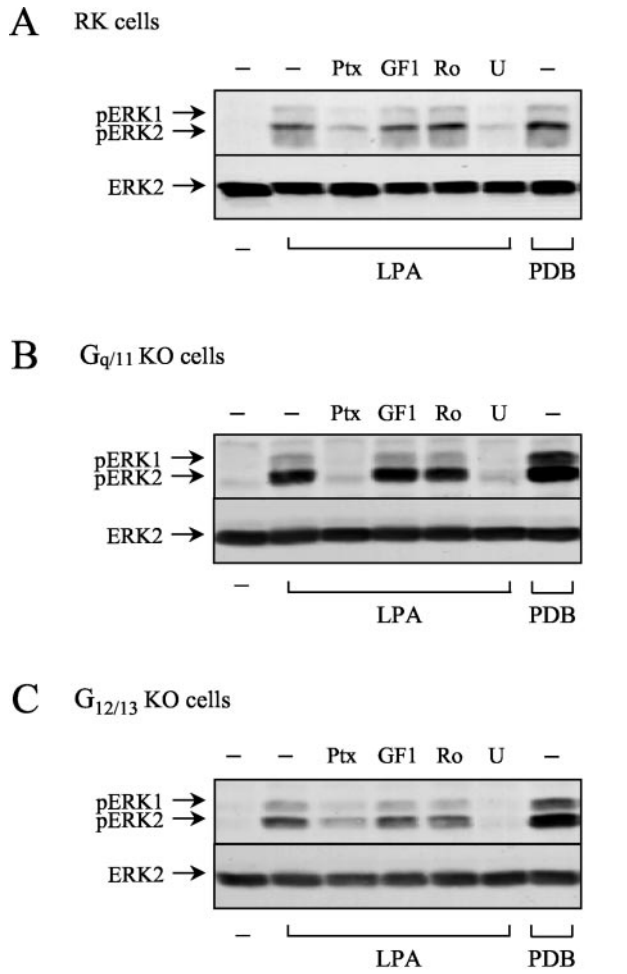

FIG. 8. **LPA induces activation of ERK-2 and ERK-1 in RK cells and $G_{q/11}$ KO or $G_{12/13}$ KO cells.** Confluent and quiescent RK cells (*A*) and $G_{q/11}$ KO cells (*B*) or $G_{12/}$13 KO cells (*C*) were incubated with PTx for 3 h or with the other inhibitors, GF-109203X (*GF1*, 3.5 μM), or 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.

moted ERK activation in both $G\alpha_{12/13}$ KO cells and $G\alpha_{q/11}$ 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 $G\alpha_q$- and $G\alpha_{13}$-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 $G\alpha_i$ 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 $G\alpha_{q/11}$ and $G\alpha_{12/13}$ 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

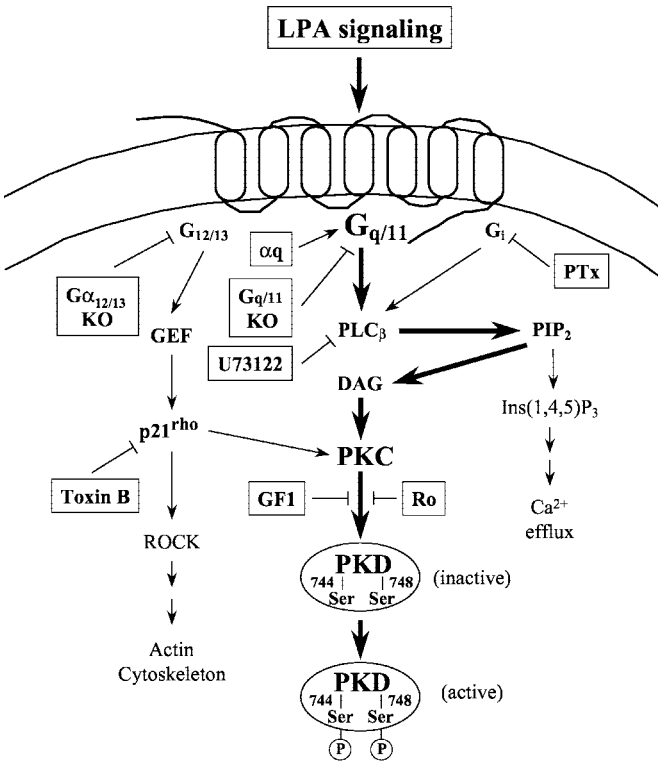

FIG. 9. **Signal transduction pathways involved in LPA-induced PKD activation.** The scheme proposes a model that envisages that PKD activation in response to LPA receptor signaling is mediated through interaction of complementary G protein pathways, namely, $G_{q/11}$, G_i, and $G_{12/13}$, and also illustrates the molecular, cellular, and pharmacological approaches used in this study. As shown in this scheme, $G\alpha_{q/11}$ plays a crucial role (expressed by *thick arrows*) in mediating LPA-induced PKD activation through a PLC- and PKC-dependent pathway, which is clearly demonstrated by the fact that LPA-induced PKD activation and phosphorylation were completely blocked (⊣) in $G_{q/11}$ knockout ($G_{q/11}$KO) cells and that transfection of $G\alpha_q$ into the $G_{q/11}$ KO cells restores (→) LPA-induced PKD activation to the similar level as the control cells. However, LPA induced maximal PKD activation requires the synergistic interaction of pathways initiated by G_q, G_{12}, and G_i. $G_{12/13}$ mediates PKD activation through Rho and PKC, and G_i mediates PKD activation through PLC and PKC. In $G_{12/13}$ knockout ($G_{12/13}$ KO) cells, or in the RK cells treated with *C. difficile* toxin B (which inactivates Rho GTPases), or with G_i inhibitor, PTx, *i.e.* when either $G_{12/13}$/Rho pathway alone or G_i pathway alone is blocked, LPA induces a reduced but substantial PKD activation. But, treatment of $G_{12/13}$ KO cells with PTx, or a combination treatment of RK cells with Ptx and *C. difficile* toxin B, which resulted in the block of both $G_{12/13}$ and G_i pathways, completely prevented LPA-induced PKD activation. In addition, this scheme also shows that LPA-induced signal transduction pathways initiated by G_q, G_{12}, and G_i leading to PKD activation is PLC and PKC-dependent, which has been verified by the fact that in either RK cells or $G_{12/13}$ KO cells, LPA induced-PKD activation and phosphorylation could be blocked (⊣) by U73122 (PLC inhibitor) or GF1 or Ro 31-8220 (*Ro*) (selective inhibitors of PKC). Therefore, PKC/PKD activation is a point of convergence in the action of multiple G protein pathways. (Also see the text for details and abbreviations). *Ins(1,4,5)P*$_3$, inositol 1,4,5-trisphosphate.

Rho GTPases, the effectors of the G_{12} pathway, had little effect on PKD activation by LPA because the remaining G protein pathways (in this case G_q and G_i) are sufficient to mediate PKD activation in response to LPA. An important result substantiating our hypothesis is that treatment of RK cells with both *C. difficile* toxin B and PTx completely prevented LPA-induced PKD activation, indicating that endogenous G_q appears necessary but not sufficient to mediate this response.

The salient features of this model are corroborated by the results obtained in the cell lines deficient in either $G\alpha_{q/11}$ or $G\alpha_{12/13}$. A crucial role of $G\alpha_{q/11}$ in mediating LPA-induced PKD activation is clearly demonstrated by the fact that LPA-induced PKD activation was completely abrogated in $G\alpha_{q/11}$ KO cells and that transfection of $G\alpha_q$ into the $G_{q/11}$ KO cells restored LPA-induced PKD activation to the similar level as the control cells. In $G\alpha_{12/13}$ KO cells, LPA induced substantial PKD activation, a response predicted by the model to be mediated by cooperation of G_q and G_i. In line with this interpretation and in sharp contrast to the results obtained with RK cells, treatment of $G\alpha_{12/13}$ KO cells with PTx dramatically inhibited PKD activation in response to LPA. These results are also consistent with the notion that endogenous $G\alpha_{q/11}$ is not sufficient to mediate PKD activation in response to LPA. Thus, our model integrates the PTx-sensitive and -insensitive effects obtained in $G\alpha_{12/13}$ KO cells and RK cells with the striking suppression of LPA-induced PKD activation in $G\alpha_{q/11}$ KO cells and leads to the notion that LPA induces PKD activation through the synergistic interaction of pathways initiated by G_q, G_{12}, and G_i. 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-κB by LPA is mediated by G_q and G_i pathways (73) and the activation of the serum response element is mediated by cooperative effects between G_i and $G\alpha_{13}$/Rho pathways (74). Furthermore, recent genetic studies indicate that the G_q-mediated signaling pathway functionally interacts with the G_{12}-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 G_q, G_i, and G_{12}. In this manner, PKD may constitute an early point of convergence of G_q, G_{12}, and G_i 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. Sinnett-Smith, Cliff Hurd, and Osvaldo Rey for helpful discussions and careful reading of the manuscript.

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