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Purification and G Protein Subunit Regulation of a Phospholipase C-β from *Xenopus laevis* Oocytes*

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***Xenopus* oocytes exhibit both pertussis toxin-sensitive and -insensitive inositol lipid signaling responses to G protein-coupled receptor activation. The G protein subunits $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, $G\alpha_s$, and $G_{\beta\gamma}$ all have been proposed to function as activators of phospholipase C in oocytes. Ma *et al.* (Ma, H.-W., Blitzer, R. D., Healy, E. C., Premont, R. T., Landau, E. M., and Iyengar, R. *J. Biol. Chem.* 268, 19915–19918) cloned a *Xenopus* phospholipase C (PLC-βX) that exhibits homology to the PLC-β class of isoenzymes. Although this enzyme was proposed to function as a signaling protein in the pertussis toxin-sensitive inositol lipid signaling pathway of oocytes, its regulation by G protein subunits has not been directly assessed. As such we have utilized baculovirus-promoted overexpression of PLC-βX in Sf9 insect cells and have purified a recombinant 150-kDa isoenzyme. PLC-βX catalyzes hydrolysis of phosphatidylinositol(4,5)bisphosphate and phosphatidylinositol(4)monophosphate, and reaction velocity is dependent on Ca^{2+}. Recombinant PLC-βX was activated by both $G\alpha_q$ and $G_{\beta\gamma}$. PLC-βX exhibited a higher apparent affinity for $G\alpha_q$ than $G_{\beta\gamma}$, and $G\alpha_q$ was more efficacious than $G_{\beta\gamma}$ at lower concentrations of PLC-βX. Relative to other PLC-β isoenzymes, PLC-βX was less sensitive to stimulation by $G\alpha_q$ than PLC-β1 but similar to PLC-β2 and PLC-βT. PLC-βX was more sensitive to stimulation by $G_{\beta\gamma}$ than PLC-β1 but less sensitive than PLC-β2 and PLC-βT. In contrast PLC-βX was not activated by the pertussis toxin substrate G proteins $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$. These results are consistent with the idea that PLC-βX is regulated by α-subunits of the G_q family and by $G_{\beta\gamma}$ and do not support the idea that α-subunits of pertussis toxin-sensitive G proteins are directly involved in regulation of this protein.**

Xenopus oocytes have been used broadly to define the pharmacological and signaling selectivities of cloned, expressed G protein[1]-coupled receptors. Oocytes exhibit a Ca^{2+}-activated Cl^- current (I_{Ca-Cl}) whose mean peak current correlates with intracellular inositol(1,4,5)trisphosphate concentrations (1) and which has been utilized as a convenient, real-time readout of PLC stimulation by heterologously expressed receptors. PLC-activating receptors expressed in *Xenopus* oocytes do not always maintain a fidelity of coupling to pertussis toxin (PTX)-insensitive G proteins, *i.e.* G_q family G proteins, usually associated with activation of these receptors in mammalian cells. For example, inositol lipid hydrolysis promoted by the [Arg[8]]vasopressin (2), 5-HT_{1c} serotonin (3), m1 muscarinic (4), or α_{1b}-adrenergic (4) receptors exhibit a sensitivity to blockade by PTX in oocytes that is absent in mammalian cells. However, a PTX-insensitive PLC response also exists in *Xenopus* oocytes and can be activated by heterologously expressed m1 muscarinic (5) or mGluR1 metabotropic glutamate (6) receptors.

The G protein activator(s) of *Xenopus* oocyte PLC has not been unambiguously defined. *Xenopus* $G\alpha_{i1}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_s$ (7, 8), $G\alpha_q$, and $G\alpha_{11}$ (9) are 75–89% identical to their mammalian homologues, suggesting a similar functionality of these well conserved proteins. Moriarty *et al.* (10) reported that the endogenous muscarinic receptor of *Xenopus* oocytes was coupled to a PTX-sensitive I_{Ca-Cl} response that could be stimulated by $G\alpha_o$ but not $G\alpha_i$. $G\alpha_o$ also has been implicated in the PTX-sensitive inositol lipid signaling pathway associated with several other receptors exogenously expressed in *Xenopus* oocytes, including mGluR1, mGluR5 (11), 5-HT_{1c} (4, 12), m1 muscarinic (10, 13), and α_{1B}-adrenergic (4) receptors. Others have reported that $G\alpha_i$ mediates the oocyte inositol lipid signaling response to m1 muscarinic (3, 11), μ-opioid, κ-opioid (14), and δ-opioid (15) receptors. $G\alpha_q$ (16), $G\alpha_{11}$ (6), and $G\alpha_s$ (12) have been proposed to mediate PTX-insensitive activation of PLC by G protein-coupled receptors in oocytes.

Clapham and co-workers (5) recently suggested that PTX-insensitive activation of PLC in *Xenopus* oocytes is not transduced by $G\alpha_q$ subunits, but rather involves $G_{\beta\gamma}$ released from the G_q heterotrimer. I_{Ca-Cl} was activated by injection of purified $G_{\beta\gamma}$ and agents that bound free $G_{\beta\gamma}$ also blocked activation of I_{Ca-Cl} by m3 muscarinic receptors. Although this finding suggests a role for $G_{\beta\gamma}$ in activation of PLC, other groups observed no effect of purified $G_{\beta\gamma}$ on I_{Ca-Cl} in oocytes (17, 18).

In face of the conflicting reports of G protein subunit-mediated regulation of inositol lipid signaling in *Xenopus* oocytes, it is important to identify and to define directly the regulation of the involved *Xenopus* PLC. Iyengar and co-workers (18) cloned a frog PLC (PLC-βX) that shares high homology with previously identified mammalian and *Drosophila* PLC-β isoenzymes. The highest percent identity was with mammalian PLC-β3, which has been shown to be activated by both $G\alpha_q$ and $G_{\beta\gamma}$ (19). Injection of oocytes with antisense oligonucleotides to block expression of PLC-βX reduced the PTX-sensitive I_{Ca-Cl} response to endogenous angiotensin receptors and to heterologously expressed α_1-adrenergic and m1 muscarinic acetylcholine receptors. These results, together with previous studies

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[1] The abbreviations used are: G protein, guanine nucleotide-binding protein; PLC, phospholipase C; PTX, pertussis toxin; PtdIns(4,5)P_2, phosphatidylinositol(4,5)bisphosphate; PtdIns(4)P, phosphatidylinositol(4)monophosphate; BSA, bovine serum albumin; PAGE, polyacrylaminde gel electrophoresis; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); TBS, Tris-buffered saline.

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illustrating $G\alpha_i$- and $G\alpha_o$-promoted activation of $I_{Ca\text{-}Cl}$, led to the suggestion that PLC-βX is activated by α-subunits of the pertussis toxin-sensitive class of G proteins. To directly address the regulation of PLC-βX by G protein subunits, we have purified recombinant isoenzyme for the first time following baculovirus-mediated expression in Sf9 cells. This 150-kDa protein is Ca^{2+}-sensitive, selectively catalyzes the hydrolysis of PtdIns(4)P and PtdIns(4,5)P_2, and is activated by both $G\alpha_q$ and $G_{\beta\gamma}$. Importantly, we observed no evidence for activation of PLC-βX by either $G\alpha_o$ or $G\alpha_i$ subunits.

EXPERIMENTAL PROCEDURES

Materials—PtdIns(4)P and PtdIns(4,5)P_2 were purified from bovine brain Folch fraction I (Sigma) as described previously (20). [^{3}H]PtdIns, [^{3}H]PtdIns(4)P, and [^{3}H]PtdIns(4,5)P_2 were purified as described previously (21) from turkey erythrocytes labeled with [^{3}H]inositol.

PLC-βX cDNA was provided by Dr. Ravi Iyengar (Mt. Sinai Medical Center). $G_{\beta\gamma}$ purified from bovine brain was a generous gift from Dr. Patrick Casey (Duke University). $G_{\beta1\gamma2}$ subunits purified following overexpression in Sf9 cells as well as baculoviruses encoding $G_{\beta2}$, $G_{\gamma2}$, and C-terminal hexahistidine-tagged $G\alpha_q$ were kindly provided by Drs. John Hepler and Alfred Gilman (University of Texas Southwestern Health Sciences Center). Recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, purified from Sf9 cell cytosol following overexpression, were kindly provided by Dr. James Garrison (University of Virginia).

Recombinant, myristoylated $G\alpha_o$ subunits were purchased from Calbiochem. Phosphatidylcholine (brain), phosphatidylserine (bovine brain), phosphatidylethanolamine (bovine heart), and PtdIns (bovine liver) were purchased from Avanti Polar Lipids (Alabaster, AL). BSA and fatty acid free BSA (fraction V), benzamidine, phenylmethylsulfonyl fluoride, and Tween 20 were purchased from Sigma. Pepstatin A was purchased from Peptides International (Louisville, KY). Hydroxylapatite (Bio-Gel HTP), acrylamide, bisacrylamide, Tris, glycine, nitrocellulose, TEMED, ammonium persulfate, SDS, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium chloride were acquired from Bio-Rad. Excell 401 medium and Grace's insect culture medium were purchased from JRH Biosciences (Lenexa, KS). Fetal bovine serum was from Irvine Scientific (Santa Ana, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum, *Not*I, *Cla*I, and *Xba*I were purchased from Boehringer Mannheim. Baculogold viral DNA transfection kit and pVL1392 vector were purchased from PharMingen (San Diego, CA). Mono Q HR 5/5 column, Q-Sepharose, and heparin-Sepharose resins were purchased from Pharmacia Biotech Inc. Nickel-nitrilotriacetic acid resin was bought from Qiagen (Chatsworth, CA). Sf9 insect cells (ATCC CRL1711) were purchased from Invitrogen (San Diego, CA).

Production and Purification of Recombinant PLC-βX Baculovirus—cDNA sequence for PLC-βX was subcloned into pVL1392, a baculovirus-compatible transfection vector, in two fragments. A 1636-base pair *Not*I-*Cla*I fragment and a 3023-base pair *Cla*I-*Xba*I fragment of PLC-βX were ligated into pVL1392 linearized with *Not*I and *Xba*I. The resulting 14.3-kilobase pair plasmid (pVL1392-PLC-βX) was sequenced at the vector/PLC-βX junction sites to confirm complete insertion. PLC-βX recombinant baculovirus was constructed by co-transfection of pVL1392-PLC-βX with Baculogold viral DNA into Sf9 cells using the Baculogold transfection kit. Recombinant virus was isolated and purified through three rounds of plaque assay by standard procedures (22).

Sf9 Cell Culture—Sf9 insect cells were maintained as suspension cultures in spinner flasks (70 rpm) at 27 °C in Grace's insect medium supplemented with 10% fetal bovine serum, 3.3 g/liter yeastolate, 3.3 g/liter lactalbumin, 6.4 mM glutamine, and antibiotic/antimycotic mix.

Large Scale Infection and Expression of PLC-βX—Sf9 cells were serum-free-adapted by transfer into Excell 401 medium supplemented with antibiotic/antimycotic in a 1L Bellco stir flask, and the propeller speed was increased to 120 rpm for 400 ml of cell culture. After attaining a doubling rate of 24 h, Sf9 cells were infected at a density of 2–3 × 10^6 cells/ml using a viral multiplicity of infection of 3. Sf9 cells were collected by low speed centrifugation (500 × *g*, 5 min, 4 °C) 48 h after infection and were washed once with low pH PBS (140 mM NaCl, 40 mM KCl, 1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, pH 6.2). The resultant cell pellet was resuspended at 2 × 10^7 cells/ml in hypotonic lysis buffer consisting of 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, and protease inhibitor mixture (200 μM phenylmethylsulfonyl fluoride, 200 μM benzamidine, and 2 μM pepstatin A) and incubated 10 min on ice prior to Dounce homogenization (10 strokes). Following low speed centrifugation (500 × *g*, 5 min, 4 °C) the supernatant was cleared by ultracentrifugation (100,000 × *g*, 60 min, 4 °C) and the resultant cytosol frozen at −80 °C. Cytosol from a total of 1.3 × 10^{10} infected Sf9 cells was collected prior to purification.

Purification of PLC-βX—A 100-ml Q-Sepharose FF column (2.6 × 20 cm) was equilibrated with buffer A (25 mM Hepes, pH 7.2, 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, and protease inhibitor mixture) containing 10 mM NaCl. Sf9 cytosol (670 ml mixed 1:1 with buffer A) was applied at a flow rate of 14 ml/min. The column was washed with 2 column volumes of buffer A plus 10 mM NaCl, and protein was eluted with a 10-column volume linear gradient of 10–600 mM NaCl in buffer A. Fifteen-ml column fractions were collected and 2 μl of every other fraction was assayed for PLC activity using cholate-containing [^{3}H]PtdIns(4)P vesicles. Fractions containing PLC activity (fractions 19–33; 225 ml; 170–300 mM NaCl eluate) were pooled, diluted into 2 volumes of buffer B (25 mM Hepes, pH 7.2, 10 mM KCl, 2 mM dithiothreitol, and protease inhibitor mixture) containing 5 mM KH_2PO_4, and applied to a 50-ml hydroxylapatite column (2.6 × 10 cm) at a rate of 4 ml/min. The hydroxylapatite column was washed with 2 column volumes of buffer B plus 10 mM KH_2PO_4, and protein was eluted with a 12-column volume linear gradient of 10–600 mM potassium phosphate (168 mM KH_2PO_4, 432 mM K_2HPO_4, pH 7.2) in buffer B. Aliquots (0.5 μl) of every other fraction were assayed for PLC activity, and the fractions containing PLC activity (fractions 57–76; 150 ml; 410–540 mM phosphate eluate) were pooled and diluted in 2 volumes of buffer A. A 14-ml column of heparin-Sepharose CL-6B (1.6 × 7 cm) was equilibrated in buffer A plus 10 mM NaCl, and the pooled PLC-containing sample was applied to the column at a rate of 3 ml/min. Protein was eluted with a 280-ml linear gradient of 10–1000 mM NaCl in buffer A. Four-ml fractions were collected and 0.1 μl assayed for PLC activity. As a final concentrating step, fractions containing PLC activity (fractions 38–52; 60 ml; 500–760 mM NaCl eluate) were pooled, diluted in 6 volumes of buffer C (buffer A containing 20% glycerol), and applied to an FPLC Mono Q HR 5/5 column pre-equilibrated in buffer C plus 10 mM NaCl. Proteins were eluted with a 25-ml gradient of 10–1000 mM NaCl in buffer C. Fractions (0.5 ml) containing PLC activity (13–15) were pooled and stored as aliquots at −80 °C. PLC-βX concentration was determined by comparison with a standard protein curve on a silver-stained 7.5% SDS-PAGE gel.

Purification of Turkey and Mammalian PLC-β Isoenzymes—PLC-βT (23), PLC-β1 (24), and PLC-β2 (24) were purified to homogeneity from Sf9 cells following baculovirus-mediated expression as described previously.

Purification of $G\alpha_qHis_6$—C-terminal hexahistidine-tagged $G\alpha_q$ was expressed from recombinant baculovirus and purified over a nickel-nitrilotriacetic acid column from membranes collected from Sf9 cells as described by Hepler *et al.* (25).

Phospholipase C Assay—Catalytic activity of PLC-βX was quantitated with [^{3}H]PtdIns(4)P or [^{3}H]PtdIns(4,5)P_2 as described previously (21). PLC (25 μl; 1–10 ng) in 10 mM Hepes, pH 7.0, and 2 mg/ml fatty acid-free BSA was added to 75 μl of reaction mixture on ice to give these final concentrations of the following components: 50 μM phospholipid (except when varied for kinetic analysis), 15,000 cpm [^{3}H]phospholipid, 0.5% (w/v) sodium cholate, 10 mM Hepes, pH 7.4, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 5.8 mM $MgSO_4$, and 2.1 mM $CaCl_2$ (unless otherwise indicated). Incubations were for 5 min at 30 °C and were terminated by addition of 375 μl of $CHCl_3$:CH_3OH:HCl (40:80:1). $CHCl_3$ (125 μl) and 0.1 N HCl (125 μl) were added, the samples mixed vigorously, and aqueous and organic phases separated by centrifugation for 5 min at 2000 × *g*. [^{3}H]Product release was quantitated by scintillation counting of 400 μl of the upper phase.

Reconstitution Assay—Regulation of PLC-β activity was quantitated utilizing detergent-free phospholipid vesicles reconstituted with purified PLC isoenzymes and G protein subunits. PtdIns(4)P (2 nmol), phosphatidylserine (2 nmol), phosphatidylethanolamine (8 nmol), and [^{3}H]PtdIns(4)P (15,000 cpm) were dried under nitrogen and resuspended in dialysis buffer (20 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM NaCl, and protease inhibitor mixture) plus 0.8% sodium cholate. G protein subunits (0.04–100 ng) were diluted in dialysis buffer plus 0.8% sodium cholate and mixed 1:1 (v:v) with resuspended phospholipids. The G protein/phospholipid mixture was dialyzed overnight against 2 liters of dialysis buffer (4 °C, in the dark), and 25 μl of the resultant unilamellar phospholipid vesicles were mixed with 25 μl of 4 × buffer (150 mM Hepes, pH 7.0, 300 mM NaCl, 16 mM $MgCl_2$, 8 mM EGTA, 6.4 mM $CaCl_2$, and 1.6 mg/ml BSA (fatty acid-free)), 25 μl of PLC-βX (0.3–300 ng diluted in 20 mM Hepes, pH 7.0, plus 0.4 mg/ml BSA (fatty acid-free)), and with 25 μl of Hepes (20 mM Hepes, pH 7.0), or in the case of reconstitution with Gα, with 4 × AlF_4^- (80 μM

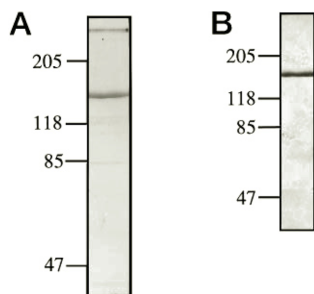

FIG. 1. **Purified PLC-βX.** PLC-βX (200 ng) purified as described was analyzed by electrophoresis on 7.5% SDS-PAGE. The gels were silver-stained (*A*) or transferred to nitrocellulose and incubated with antibody 1978 produced against the peptide FINQKQRDPRLN, a sequence of high homology in PLC-β isoenzymes. Antibody binding was detected by alkaline-phosphatase-conjugated anti-IgG and colorimetric AP substrate (*B*). Migration of molecular mass standards (kilodaltons) was as indicated.

$AlCl_3$, 40 mM NaF diluted in 20 mM Hepes, pH 7.0). Assays were incubated at 30 °C for 5 min to maintain initial rate conditions in all experiments. The reactions were terminated, and phospholipase C activity was quantitated as described above.

GTPγS-stimulated activity was assessed after preincubation of Gα-reconstituted vesicles for 1 h at 30 °C with 1 mM GTPγS. GTPγS (4 mM in 20 mM Hepes, pH 7.0) was included in lieu of 4 × AlF_4^- in these assays.

PLC activity data are shown minus basal activities (1.72–15.6 μmol/min/mg of protein) measured in the absence of G protein subunits. All assays were performed in duplicate with the number of repetitions indicated below each figure. Assays utilizing $G_{\beta\gamma}$ purified from bovine brain were repeated with recombinant $G_{\beta1\gamma2}$ purified from Sf9 cells, and no difference in relative activity between subunit preparations was detectable.

Electrophoresis, Silver Staining, and Immunoblotting—SDS-PAGE (7.5% gels) was carried out according to the method of Laemmli (26). Silver staining of gels was performed according to the method of Morrisey *et al.* (27). Following SDS-PAGE proteins were electrophoretically transferred to nitrocellulose and PLC-βX protein immunologically detected by antipeptide polyclonal antibody 1978 directed against a 12-amino acid epitope from PLC-β3 (FINQKQRDPRLN) that shares 11 of 12 amino acids with PLC-βX. In brief, nitrocellulose was blocked in TBS (20 mM Tris, pH 7.5, 500 mM NaCl) containing 3% BSA and incubated overnight with a 1:500 dilution of antibody 1978 in TBS plus 1% BSA. The nitrocellulose was washed with TBS plus 0.05% Tween 20 and incubated for 1 h with alkaline phosphatase-conjugated goat-anti rabbit IgG antibody (1:1000 dilution) in TBS plus 1% BSA. Proteins were detected by incubation with alkaline phosphatase colorimetric substrates (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride).

RESULTS

Characterization of Purified Recombinant PLC-βX—The coding sequence for PLC-βX plus 12 base pairs of 5′-untranslated sequence preceded by a *Not*I restriction endonuclease site was ligated into pVL1392, a baculovirus transfer vector containing the polyhedron promoter for overexpression in Sf9 cells. Recombinant baculovirus encoding PLC-βX was obtained following co-transfection of pVL1392-PLC-βX with Baculogold viral DNA and was purified through three rounds of plaque assay prior to large scale viral amplification. PtdIns(4)P-hydrolyzing activity was detectable in lysates from Sf9 cells infected with recombinant baculovirus, and active PLC-βX was purified to near-homogeneity from cytosol prepared from these cells. Chromatography over a hydroxylapatite column provided the most effective purification step.

A protein of approximately 150 kDa co-purified with PLC activity (Fig. 1*A*), which coincides with the deduced mass of PLC-βX of 139 kDa based on primary amino acid sequence. A final yield of 500 μg of purified 150-kDa protein was obtained from 1.3×10^{10} Sf9 cells. The purified protein preparation also contained a >240-kDa species. Only the 150-kDa species was detected by antibody 1978 (directed against a homologous epitope in PLC-β3 and PLC-βX), which suggests that the high molecular weight species is a contaminant rather than a multimer of PLC-βX (Fig. 1*B*).

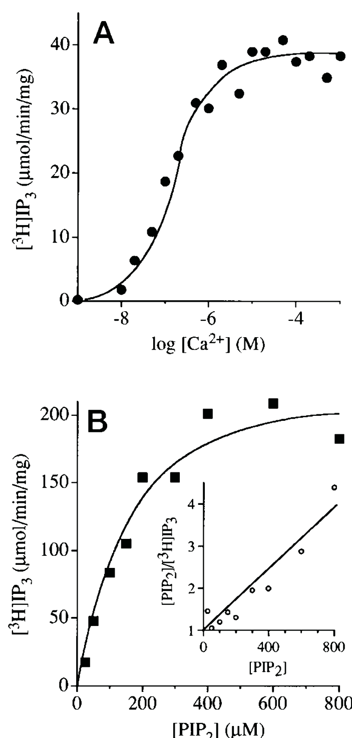

FIG. 2. **Calcium and substrate dependence of PLC-βX catalytic activity.** Purified PLC-βX (5 ng) was incubated with the indicated concentrations of Ca^{2+} in an assay containing 0.5% cholate and 50 μM PtdIns(4,5)P_2 (*A*) or with the indicated concentrations of PtdIns(4,5)P_2 in an assay containing 0.5% cholate and 0.5 μM free calcium (*B*). Each assay was incubated for 5 min at 30 °C and [³H]inositol(1,4,5)trisphosphate quantitated as described. A replot of the data by the method of Hanes is included as an *inset*. V_{max} and K_m values for PtdIns(4,5)P_2 were 259 μmol/min/mg and 216 μM, respectively. The results are representative of three similar experiments.

The dependence of purified PLC-βX on calcium and substrate concentration was studied in catalytic assays in the presence of 0.5% cholate. PLC-βX reaction velocity was dependent on Ca^{2+} concentration with a half-maximal rate observed at 0.2 μM Ca^{2+} and maximal rate obtained at 2–10 μM Ca^{2+} (Fig. 2*A*). Reaction velocity also increased as a hyperbolic function of PtdIns(4,5)P_2 concentration (Fig. 2*B*). A Hanes replot of substrate concentration *versus* substrate concentration/reaction velocity (Fig. 2*B, inset*) suggested a single enzymatic component (r = 0.91) with an apparent V_{max} of 259 μmol/min/mg of enzyme and an apparent K_m for PtdIns(4,5)P_2 of 216 μM. PLC-βX also hydrolyzed PtdIns(4)P with reaction velocity dependent on substrate concentration (data not shown). As has been observed with mammalian PLC-β isoenzymes, PLC-βX was selective for PtdIns(4)P and PtdIns(4,5)P_2 over PtdIns, catalyzing the hydrolysis of PtdIns only at high concentrations of phospholipid (>300 μM) that precluded achievement of saturation with respect to reaction velocity (data not shown). PLC-βX was inactive against phosphatidylcholine under the same assay conditions (data not shown).

Regulation of PLC-βX by $G\alpha_q$—Hexahistidine-tagged recombinant $G\alpha_q$ was purified from Sf9 cell membranes as described above. The hexahistidine tag on the C terminus has been shown previously to not affect the regulatory activity of $G\alpha_q$ at mammalian PLC-β isoenzymes (25). $G\alpha_q$ did not activate PLC-βX in the absence of AlF_4^- (data not shown). However, in the presence of AlF_4^-, $G\alpha_q$ stimulated PLC-βX in a manner

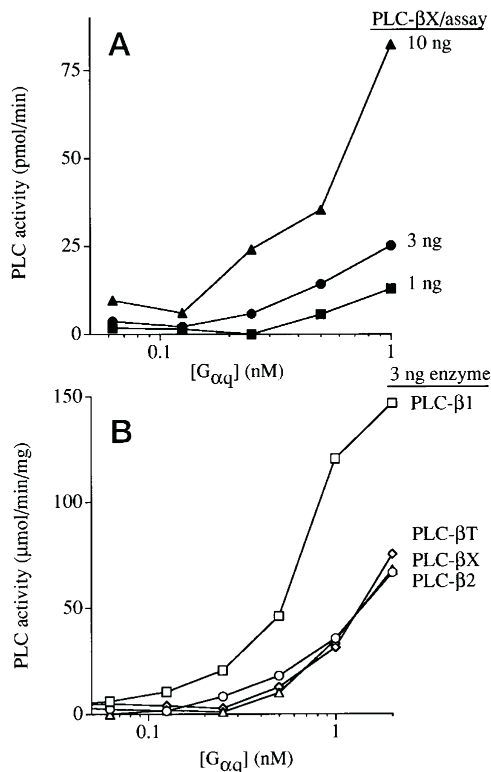

FIG. 3. **Comparative activation of PLC-βX by Gα_q.** The indicated amounts of PLC-βX enzyme (*A*) or 3 ng of the indicated PLC-β isoenzyme (*B*) were incubated with the indicated concentrations of purified Gα_q subunit activated with AlF_4^- in a reconstitution assay for 5 min at 30 °C. PLC activity was quantitated as described under "Experimental Procedures." The results are representative of two (*A*) or three (*B*) similar experiments.

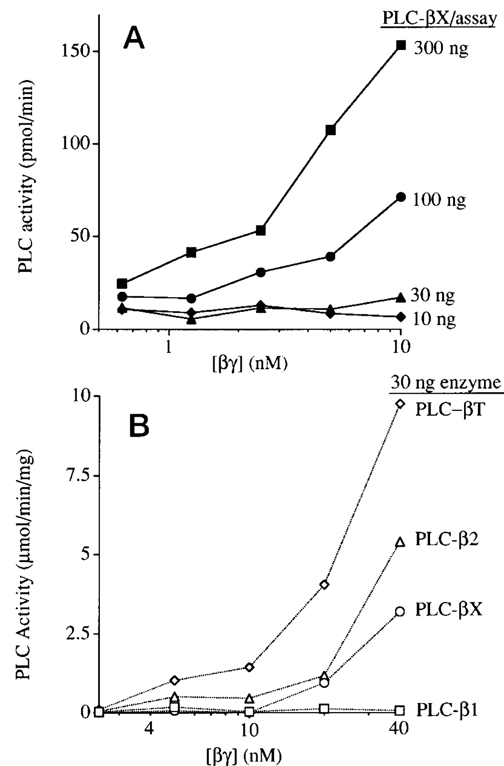

FIG. 4. **Comparative activation of PLC-βX by G$\beta\gamma$.** The indicated amounts of PLC-βX enzyme (*A*) or 30 ng of the indicated PLC-β isoenzyme (*B*) were incubated with the indicated concentrations of recombinant $G_{\beta1\gamma2}$ in a reconstitution assay for 5 min at 30 °C. PLC activity was quantitated as described under "Experimental Procedures." The results are representative of three similar experiments repeated with purified bovine brain $G_{\beta\gamma}$ and recombinant $G_{\beta1\gamma2}$.

dependent on the concentration of G protein subunit and PLC isoenzyme (Fig. 3*A*). Gα_q activation of PLC-βX was detectable at PLC concentrations of 1–10 ng/assay (67–670 pM) using 0.1–1 nM Gα_q. At equimolar PLC enzyme concentrations, PLC-β1 was stimulated by lower concentrations of Gα_q than PLC-βX, PLC-β2, and PLC-βT (Fig. 3*B*).

Regulation of PLC-βX by $G_{\beta\gamma}$—Bovine brain $G_{\beta\gamma}$ activated PLC-βX in a manner dependent on the concentration of G protein subunit and PLC-βX (Fig. 4*A*). Marked activation by 1–10 nM $G_{\beta\gamma}$ required 30–300 ng/assay (2–20 nM) of PLC-βX. This represents an approximately 30-fold increase in PLC-βX concentration and a 10-fold increase in G protein subunit relative to the concentrations necessary to detect Gα_q-promoted increases in enzyme activity. At equimolar PLC enzyme concentrations, PLC-βX required a higher concentration of $G_{\beta\gamma}$ for stimulation than PLC-β2 and PLC-βT, but less than PLC-β1 (Fig. 4*B*). For each PLC-β isoenzyme tested, $G_{\beta\gamma}$ was less efficacious than Gα_q for stimulation of PLC-β catalytic activity.

Lack of Regulation of PLC-βX by Gα_i or Gα_o Subunits—Based on the contention that PLC-βX functions as the effector enzyme in the PTX-sensitive inositol lipid signaling pathway of *Xenopus* oocytes (18), we determined the capacity of recombinant Gα_o, Gα_{i1}, Gα_{i2}, and Gα_{i3} to activate PLC-βX in a reconstitution assay. None of these PTX-substrate α-subunits stimulated PLC-βX activity at concentrations of α-subunit up to 30 nM and with concentrations of PLC-βX as high as 100 ng/assay. This lack of activity was observed irrespective of whether AlF_4^- or preincubation with 1 mM GTPγS was utilized for G protein activation (Fig. 5*A*).

To verify that purified recombinant Gα_o, Gα_{i1}, Gα_{i2}, and Gα_{i3} were fully functional under our assay conditions, the ability of these G protein α-subunits to attenuate $G_{\beta\gamma}$-stimulated PLC activity was tested. Gα_i and Gα_o subunits have been shown previously to bind to $G_{\beta\gamma}$ in the absence of GTPγS and to inhibit $G_{\beta\gamma}$-subunit-promoted activation of PLC-βT (28). The capacity of 3 nM $G_{\beta\gamma}$ to activate PLC-βT was attenuated by a 10-fold molar excess of Gα_o, Gα_{i1}, Gα_{i2}, or Gα_{i3} subunit (Fig. 5*B*). Under these same assay conditions Gα_o, Gα_{i1}, Gα_{i2}, and Gα_{i3} were ineffective as activators of PLC-βX.

DISCUSSION

Baculovirus-promoted expression of PLC-βX has provided a means to obtain this isoenzyme in purified form for the first time and to examine directly its regulation by G protein subunits. We previously have applied this approach to generate large amounts of purified PLC-β1, PLC-β2, and PLC-βT (23, 29). These three recombinant PLC-β isoenzymes exhibit physical, catalytic, and regulatory properties that are indistinguishable from their native counterparts. Thus, there is strong reason to expect that recombinant PLC-βX exhibits properties that reflect with high fidelity those of the native form of this isoenzyme. Purified PLC-βX exhibited a Ca^{2+} sensitivity and phospholipid substrate specificity that were very similar to those observed with other PLC-β isoenzymes. The apparent K_m of PLC-βX against PtdIns(4,5)P_2 also was similar to that previously observed for recombinant PLC-β1 and PLC-β2 (29), and the V_{max} of the recombinant *Xenopus* isoenzyme was as high as any reported to date for PLC isoenzymes.

The relatively high sequence homology between PLC-βX and the other PLC-β isoenzymes cloned to date suggests that the overall *in vivo* function of the *Xenopus* isoenzyme is likely similar to the signaling activity of the previously characterized mammalian and avian isoenzymes. Thus, it is not surprising that the PLC-βX isoenzyme was activated both by G protein

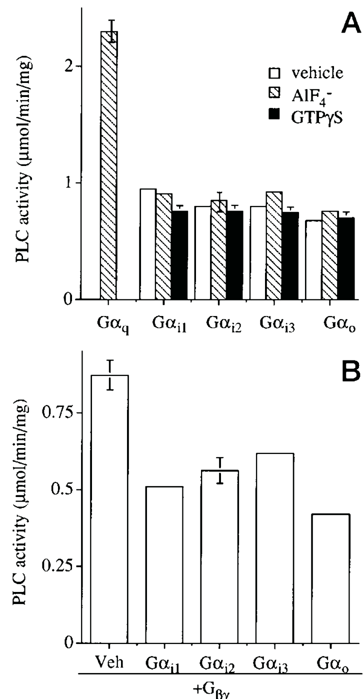

FIG. 5. **Lack of activation of PLC-βX by α-subunits of the G_i/G_o family.** PLC-βX (100 ng) was incubated with 30 nM recombinant $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ subunits or with 3 nM $G\alpha_q$ subunits in the presence of vehicle, 20 μM $AlCl_3$, and 10 mM NaF (AlF_4^-) or 1 mM GTPγS in a reconstitution assay for 5 min at 30 °C (*A*). PLC-βT (30 ng) was incubated with 30 nM recombinant $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ subunits or vehicle and 3 nM bovine brain $G_{\beta\gamma}$ in a reconstitution assay for 5 min at 30 °C (*B*). PLC activity was quantitated as described. Data are shown ± range of duplicate determinations. The results are representative of three similar experiments repeated with purified bovine brain $G_{\beta\gamma}$ and recombinant $G_{\beta1\gamma2}$.

α-subunits of the G_q class of G proteins and by $G_{\beta\gamma}$. In both cases stimulation of PLC-βX required higher concentrations of G protein subunits than some of the other PLC-β isoenzymes. Although not studied here, PLC-β3, which has the highest percent identity to the *Xenopus* isoenzyme, previously was shown to be activated by both G protein subunits of the $G\alpha_q$ class and by $G_{\beta\gamma}$ (19). The physiological significance of activation of PLC-β isoenzymes by $G_{\beta\gamma}$ is not unequivocally known, but widespread acceptance exists for the concept that certain mammalian PLC-β isoenzymes are activated as a consequence of release of $G_{\beta\gamma}$ from PTX-sensitive G proteins.

The results of Iyengar and co-workers using injection of purified $G\alpha$ proteins or antisense mRNA (4, 10, 13, 18) suggest that $G\alpha_o$ is prominently involved in pertussis toxin-sensitive activation of the inositol lipid signaling pathway of *Xenopus* oocytes by several G protein-linked receptors. Other laboratories have implicated $G\alpha_i$ (3, 11, 14, 15) in this signaling pathway in *Xenopus* oocytes. Thus, the observation of Ma *et al.* (18) that antisense-promoted inhibition of expression of PLC-βX results in a concurrent loss of pertussis toxin-sensitive, G protein-coupled receptor-stimulated, $I_{Ca\text{-}Cl}$ not only implicated this isoenzyme in the receptor-regulated pathway in oocytes but led to the suggestion that PLC-βX may exhibit a novel activating response to $G\alpha_o$ and/or $G\alpha_i$. The results reported here are inconsistent with the proposed activating role for α-subunits in a PTX-sensitive pathway involving PLC-βX in *Xenopus* oocytes. Neither $G\alpha_o$ nor any of the $G\alpha_i$ isoforms activated PLC-βX under conditions in which these proteins were shown to interact effectively with $G_{\beta\gamma}$. Thus, PLC-βX is not directly regulated by α-subunits of the PTX-sensitive class of G proteins. Although it could be argued that *Xenopus* G protein α-subunits may exhibit functional activities distinct from the mammalian counterparts used in the current study, this is unlikely based on the 90% homology between mammalian and frog G proteins and the lack of any evidence for differences in the physiological activity of species homologues of G protein subunits.

Iyengar and co-workers (18) have reported that $G_{\beta\gamma}$ is not involved in the PTX-sensitive inositol lipid signaling response of *Xenopus* oocytes. However, Stehno-Bittel *et al.* (5) recently reported conflicting results showing that $G_{\beta\gamma}$ promoted activation of $I_{Ca\text{-}Cl}$ in oocytes and suggested that $G_{\beta\gamma}$ released from G_q family heterotrimers mediate PTX-insensitive activation of the inositol lipid signaling pathway. Since PLC-βX is activated by $G_{\beta\gamma}$, this isoenzyme could function as the effector protein in such a pathway. PLC-βX clearly was more sensitive to activation by $G\alpha_q$ than by $G_{\beta\gamma}$ under our assay conditions. In fact, all purified PLC-β isoenzymes characterized to date are more sensitive to activation by $G\alpha_q$ than $G_{\beta\gamma}$ *in vitro* in spite of *in vivo* evidence suggesting that PLC-β2 is primarily stimulated by $G_{\beta\gamma}$. However, comparison of the relative sensitivity to activation of PLC-β isoenzymes *in vitro* may not be entirely reflective of activation *in vivo*, since *in vitro* studies have routinely employed AlF_4^- or GTPγS for α-subunit activation rather than the native GTP activator. PLC-β isoenzymes impart substantial GTPase activity to $G\alpha_q$ class subunits (30, 31) and may apear more potently stimulated by AlF_4^--bound $G\alpha$ than by natural GTP-activated $G\alpha$. Complete insight into the relative activation of PLC-β isoenzymes by G protein subunits awaits the characterization in progress of the relative GTPase activities of PLC-β isoenzymes as well as identification of other factors that may impinge on PLC-β activity *in vivo*.

In summary, we have purified PLC-βX and have examined its relative activation by G protein subunits *in vitro*. Contrary to suggestions from previous work on inositol lipid signaling in *Xenopus* oocytes, this PLC-β isoenzyme is not activated by G protein subunits that are PTX substrates. Instead PLC-βX appears to be regulated similarly to other PLC-β isoenzymes (recombinant or native) purified thus far. Our data on PLC-βX, together with those of Ma *et al.* (18) on inhibition of signaling in oocytes by antisense oligonucleotides based on the PLC-βX coding sequence, make important a clear resolution of the effects of $G_{\beta\gamma}$ on inositol lipid signaling in this model system.

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