Phosphorylation by protein kinase C decreases catalytic activity of avian phospholipase C-β

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The potential role of protein kinase C (PKC)-promoted phosphorylation has been examined in the G-protein-regulated inositol lipid signalling pathway. Incubation of [32P]Pi-labelled turkey erythrocytes with either the P2Y1 receptor agonist 2-methylthioadenosine triphosphate (2MeSATP) or with PMA resulted in a marked increase in incorporation of methylthioadenosine triphosphate (2MeSATP) or with PMA into the G-protein-activated phospholipase C PLC-β/T. Purified PLC-β/T also was phosphorylated by PKC in vitro to a stoichiometry (mean ± S.E.M.) of 1.06 ± 0.2 mol of phosphate/mol of PLC-β/T. Phosphorylation by PKC was isoenzyme-specific because, under identical conditions, mammalian PLC-β2 also was phosphorylated to a stoichiometry near unity, whereas mammalian PLC-β1 was not phosphorylated by PKC. The effects of PKC-promoted phosphorylation on enzyme activity were assessed by reconstituting purified PLC-β/T with turkey erythrocyte membranes devoid of endogenous PLC activity. Phosphorylation resulted in a decrease in basal activity, AIF4T-stimulated activity, and activity stimulated by 2MeSATP plus guanosine 5'-[β-thio]triphosphate in the reconstituted membranes. The decreases in enzyme activities were proportional to the extent of PKC-promoted phosphorylation. Catalytic activity assessed by using mixed detergent/phospholipid micelles also was decreased by up to 60% by phosphorylation. The effect of phosphorylation on Gα2-stimulated PLC-β/T in reconstitution experiments with purified proteins was not greater than that observed on basal activity alone. Taken together, these results illustrate that PKC phosphorylates PLC-β/T in vivo and to a physiologically relevant stoichiometry in vitro. Phosphorylation is accompanied by a concomitant loss of enzyme activity, reflected as a decrease in overall catalytic activity rather than as a specific modification of G-protein-regulated activity.

Key words: inositol phospholipid, G proteins, signal transduction.

INTRODUCTION

Activation of G-protein-coupled receptors by neurotransmitters, hormones and chemoattractants elicits intracellular responses from a small number of effectors, including the membrane phospholipid-hydrolysing enzyme phospholipase C-β (PLC-β). PLC-β hydrolyses PtdIns(4,5)P2 to Ins(1,4,5)P3 and diacylglycerol, which in turn leads to the mobilization of intracellular Ca2+ and the activation of protein kinase C (PKC) [1]. The coding sequences of four mammalian isoenzymes (PLC-β1, PLC-β2, PLC-β3 and PLC-β4), a Meleagris (turkey) isoenzyme, and two Drosophila isoenzymes (NorpA and dPLC-β) of PLC-β have been identified [2].

Agonist-induced desensitization is an important regulatory process in the inositol lipid signalling pathway [3]. PKC-promoted activation of PKC also results in the desensitization of the PLC signalling response by modification of components downstream of the G-protein-coupled receptor [4–7]. PKC apparently does not phosphorylate G-proteins of the Gα family [8]. However, evidence for the PKC-promoted phosphorylation of signalling effector enzymes [9–16], including PLC-β isoforms [17–20], has been observed. Although direct demonstration of a change in enzyme activity was not reported, agonist- and PKC-induced desensitization of the platelet-activating factor receptor-coupled inositol lipid signalling response in RBL-2H3 cells was shown to occur simultaneously with phosphorylation of PLC-β3 [20]. Direct phosphorylation by PKC of PLC-β purified from bovine brain (presumably PLC-β1) also has been reported [17], but the functional consequence of phosphorylation on enzyme activity was not established. Litosch [18] also observed phosphorylation in vitro of partly purified PLC-β1 by PKC and reported that phosphorylation resulted in a decrease in Ca2+-dependent catalytic activity [19]. However, purification of PLC-β1 diminished PKC-dependent effects on catalytic activity, suggesting that an unidentified cofactor was mediating the effect of PKC on PLC-β1.

The turkey erythrocyte has proved a useful model for the study of receptor-regulated inositol lipid signalling. All three of the signalling proteins, namely turkey P2Y, receptor [21,22], turkey Gα1, [23,24] and turkey PLC-β/T [25–27], of this signalling pathway have been identified and their coding sequences cloned. Desensitization of the inositol lipid signalling pathway in turkey erythrocytes has been characterized. Activation of turkey erythrocyte PKC indirectly by P2Y receptor agonists or directly by PMA resulted in decreases in agonist- and guanosine 5'-[β-thio]triphosphate (GTP[S])-stimulated PLC activity in membranes prepared from these cells [28,29]. The decreased responsiveness to GTP[S] suggested that the regulation of inositol lipid signalling occurred at a level downstream of the P2Y receptor, i.e. through the inactivation of either Gα1 or PLC-β/T. In this study we tested the hypothesis that PKC-promoted phosphorylation of PLC-β/T serves as a mechanism for desensitization of inositol lipid signalling in turkey erythrocytes and provide direct evidence for PKC-mediated phosphorylation leading to a change in PLC-β/T catalytic activity.

Abbreviations used: 2MeSATP, 2-methylthioadenosine triphosphate; DOG, 1,2-dioleoyl-sn-glycerol; PLC, phospholipase C; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; Ni-NTA, nickel-nitrosoacetic acid; DMEM, Dulbecco’s modified Eagle’s medium; GTP[S], guanosine 5'-[γ-thio]triphosphate; PKC, protein kinase C; DTT, dithiothreitol; Tos-Phe-CH2Cl, tosylphenylalanylchloromethane; Gαx-CH3Sγ, C-terminal hexahistidine-tagged Gαx.
MATERIALS AND METHODS

Materials
PKC purified from rat brain was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). PKC isoforms α, β1, β2 and γ were purchased from Calbiochem (San Diego, CA, U.S.A.). PtdIns(4)P and PtdIns(4,5)P2 were purified from bovine brain Folch fraction I as described previously [25]. [3H]PtdIns(4)P and [3H]PtdIns(4,5)P2 were purified from [3H]inositol-labelled turkey erythrocyte membranes as described previously [30]. Phosphatidylethanolamine (PtdEtn) (bovine heart), 1,2-dioleoyl-sn-glycerol (DGG) and phosphatidylserine (PtdSer) (brain) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco/BRL (Grand Island, NY, U.S.A.). 5-Bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium chloride were obtained from Bio-Rad (Hercules, CA, U.S.A.).

Materials

Protein purification
PLC-β/T was purified from SF9 cells after baculovirus-mediated expression as described previously [27]. cDNA species for C-terminal hexahistidine-tagged Gx (Gx-CHis8), hexahistidine-tagged Gβγ, Gβγ and Gγ were obtained from Dr. John Hepler and Dr. Alfred Gilman (University of Texas Southwestern Medical Health Science Center, Dallas, TX, U.S.A.). Gx-CHis8 was purified from SF9 cell membranes after baculovirus-mediated overexpression as described previously [8]. Gβγ and hexahistidine-tagged Gγ baculovirus constructs were used to infect SF9 cells simultaneously; heterotrimeric Gγ was purified by using Nt-NTA affinity chromatography, and purified Gβγ was eluted with AIF3− as described [8]. Turkey Gx11 was purified from turkey erythrocyte membranes as described previously [30].

Kinase reaction
PLC-β1, PLC-β2 and PLC-γ/T (2 pmol/10 μl) were incubated with 10 μl-units (unless otherwise indicated) of PKC (rat brain mix or purified α, β1, β2 or γ isoforms) for 10 min at 30 °C in a reaction containing 20 mM Tris, pH 7.5, 10 mM MgCl2, 100 μM CaCl2, 200 μM dithiothreitol (DTT), 200 μM AP, lipid activators [100 μg/ml PtdSer and 20 μg/ml DOG initially solubilized by sonication in 10 mM Tris (pH 7.5)/10 mM CHAPS] and (1−5) × 10−6 c.p.m. [γ-32P]ATP. Reactions were stopped with the addition of an equal volume of 2× Laemmli sample buffer [31]. Incorporation of [32P] was quantified after protein separation by SDS/PAGE [7.5% (w/v) gel], excision of the phosphorylated PLC-γ/T, gel solubilization in 27% (v/v) H2O2/0.3 M NaNH4 at 55 °C, and liquid-scintillation quantification of radioactivity in the solubilized gel slice.

For the assay of PLC-γ/T activity after phosphorylation, [γ-32P]ATP was omitted from the kinase reaction and the reaction was stopped by dilution 1:50 in ice-cold Heps/BSA buffer [10 mM Heps (pH 7.0)/1 mg/ml BSA/200 mM CaCl2/10 mM β-glycerophosphate]. PLC-γ/T activity was assayed immediately after the kinase reaction.

Assay of catalytic activity of PLC-γ/T
The catalytic activity of PLC-γ/T was quantified with [3H]PtdIns(4,5)P2 as described previously [30]. PLC-γ/T (25 μl; 1–10 ng) in 10 mM Heps, pH 7.0, containing 2 mg/ml fatty-acid-free BSA was added to 75 μl of reaction mixture on ice to give final concentrations of the following components: 50 μM PtdIns(4,5)P2 (unless otherwise indicated), 10000–15000 c.p.m. [3H]PtdIns(4,5)P2, 0.5% sodium cholate, 10 mM Heps, pH 7.4, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 5.8 mM MgSO4 and 2.1 mM CaCl2. Reactions were incubated for 10 min at 30 °C and were terminated by the addition of 375 μl of chloroform/methanol/HCl (40:80:1, by vol.). Chloroform (125 μl) and 0.1 M HCl (125 μl) were added, the samples were mixed vigorously, and aqueous and organic phases were separated by centrifugation for 5 min at 2000 g. [3H]Ins(1,4,5)P3 release was quantified by scintillation counting of 350 μl of the upper phase.

Reconstitution of PLC-γ/T with turkey erythrocyte membranes
Receptor and G-protein regulation of purified PLC-γ/T was quantified by the reconstitution of purified PLC-γ/T and substrate phospholipids with turkey erythrocyte membranes. Turkey erythrocytes were collected, washed as described previously [21] and resuspended in an equal volume of lysis buffer [20 mM Heps (pH 7.0)/5 mM MgCl2/2 mM EGTA/150 mM NaCl/200 μM PMSF/200 mM benzamidine/10 μM tosylphenylalanilanylchloromethane (Tos-Phe-CH2Cl; ‘TPCK’) 200 μM pepstatin A/10 mM β-glycerophosphate/50 mM NaF]. Resuspended turkey erythrocytes (2 ml) were vortex-mixed vigorously in the presence of 1 g of glass beads (0.45 mm) for four bursts of 30 s interspersed with cooling on ice [32]. The lysate/glass bead mixture was centrifuged at 5000 g for 3 min to pellet beads and unbroken cells. The supernatant was collected in a fresh tube on ice, an additional 1 ml of lysis buffer was added to the glass beads, and the vortex-mixing and centrifugation steps repeated to complete erythrocyte lysis. Glass beads were washed a final time with 1 ml of lysis buffer and allowed to settle for 5 min without centrifugation, the supernatant was then pooled with the previous supernatant fraction. The pooled supernatant was centrifuged at 36000 g for 20 min and the resultant pellet was collected as a crude membrane fraction. Membranes were resuspended in 1 ml of lysis buffer and incubated with 46 μg-units of DNase for 30 min at room temperature, centrifuged at 36000 g for 20 min at 4 °C, and assayed for protein concentration. The final membrane suspension (25 μg/15 μl) was in DB buffer [20 mM Heps (pH 7.0)/1 mM MgCl2/100 mM NaCl/2 mM DTT/200 μM PMSF/200 μM benzamidine/10 mM microcystin-LR/10 mM β-glycerophosphate] and the membranes were stored at −80 °C. Phospholipid substrate was prepared as a mixture of PtdIns4P (5 nmol per assay), PtdEtn (25 nmol per assay) and [3H]PtdIns4P (10000–15000 c.p.m. per assay) dried under nitrogen and resuspended in 20 mM Heps, pH 7.0. Phospholipid substrate was mixed with thawed membranes at a 2:3 ratio (v/v) and incubated on ice for 1 h before assay. The resultant radio-labelled membranes (25 μl) were mixed with 25 μl of 4× membrane assay buffer [80 mM Heps (pH 7.2)/80 mM NaCl/120 mM KC1/8.8 mM EDTA/12 mM EGTA/10.64 mM CaCl2/22.8 mM MgCl2] and with 25 μl of 20 μl of lysis buffer, pH 7.0, or, for receptor or G-protein activation, with 25 μl of 4X AIF3− buffer (80 μM AlCl3/40 mM NaF diluted in 20 mM Heps, pH 7.0), 25 μl of 400 μM GTP[S] or 400 μM GTP[S] plus 40 μM 2-methylthioadenosine triphosphate (2MeSATP). Samples were preincubated for 3 min at 30 °C and then 25 μl of PLC-γ/T (2.5–20 ng per assay) was added and incubation was continued for a further 20 min at 30 °C. The reactions were terminated, and [3H]Ins(1,4,5)P3 accumulation was quantified as described above. All assays were performed in triplicate; the number of repetitions are indicated in Figure legends.

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Reconstitution of PLC-β with purified G-protein subunits

Activation of PLC-βT by G-protein subunits was quantified by using detergent-free phospholipid vesicles reconstituted with purified proteins. PtdIns(4,5)P₂ (2 nmol per assay), PtdSer (2 nmol per assay), PtdEtn (8 nmol per assay) and [3H]-PtdIns(4,5)P₂ (10 000–15 000 c.p.m. per assay) were dried under nitrogen and resuspended in dialysis buffer [20 mM Hepes (pH 7.4)/1 mM MgCl₂/2 mM DTT/100 mM NaCl/200 μM PMSF/200 μM benzamidine] plus 0.8 % sodium cholate. G-protein subunits (0.04–100 ng) were diluted in 10 μl per assay of dialysis buffer plus 0.8 % sodium cholate and mixed 3:2 (v/v) with resuspended phospholipids. The G-protein/phospholipid mixture was dialysed overnight against 2 litres of dialysis buffer (4 °C in the dark). 25 μl of the resultant unilamellar phospholipid vesicles was mixed with 25 μl of 4 × reconstitution assay buffer [150 mM Hepes (pH 7.0)/300 mM NaCl/16 mM MgCl₂/8 mM EGTA/6.4 μM CaCl₂], 25 μl of PLC-βT [25 ng diluted in 20 mM Hepes (pH 7.0)/2 mg/ml BSA (fatty-acid-free)] and with 25 μl of 20 mM Hepes, pH 7.0, or, for stimulation of G-protein subunits, with 25 μl of 4 × AlF₃ buffer. Reactions were incubated at 30 °C for 10 min, terminated, and then [3H]Ins(1,4,5)P₃ accumulation was quantified as described above.

[32P] labelling in vivo and immunoprecipitation

Washed turkey erythrocytes were labelled overnight in air/CO₂ (19:1) with stirring (60 rev./min) at 37 °C in phosphate-free DMEM (4 ml/ml of packed erythrocytes) with [32P]P₀ (1.2 mCi/ml of packed erythrocytes). [32P]P₀-labelled erythrocytes were centrifuged for 5 min at 500 g, resuspended in DMEM buffered with 20 mM Hepes, pH 7.4 (5 ml/ml of packed erythrocytes) and equilibrated for 10 min at 37 °C. Drugs were added as indicated and incubation at 37 °C continued for 20 min. Erythrocytes were centrifuged again for 3 min at 500 g and lysed as described above. The supernatant fraction obtained after centrifugation at 36 000 g was retained as a cytosolic fraction and used for the immunoprecipitation of PLC-βT as described below.

Turkey erythrocyte cytosol (1 ml) was incubated for 1 h at 4 °C with 1 μl of anti-(PLC-βT) serum (no. 858, antiserum against native PLC-β/T [33]) on an inverting rotator. Protein A–agarose (50 μl of a 50 % suspension in 20 mM Tris/HCl, pH 7.4) was added and incubation continued for 1 h at 4 °C. Immunoprecipitation samples were centrifuged at 15 000 g for 15 s, the supernatant was aspirated and the Protein A–agarose pellet was washed twice with 1 ml of immunoprecipitation buffer [20 mM Tris (pH 7.4)/200 μM PMSF/200 μM benzamidine/2 μM pepstatin A/10 μM Tos-Phe-CH₂Cl/50 mM NaF/10 mM β-glycerophosphate/10 mM microcystin-LR] plus 1 % (v/v) BSA followed by a final 1 ml wash with immunoprecipitation buffer only. The pellet resulting from Protein A–agarose immunoprecipitation was resuspended in 50 μl of Laemmli sample buffer and incubated at 85 °C for 10 min before electrophoresis.

Immunoprecipitated proteins were resolved by SDS/PAGE [7.5 % (w/v) gel] by the method of Laemmli [31]. Proteins were transferred electrophoretically to nitrocellulose and exposed to a PhosphorImager screen (Molecular Dynamics) for quantification of 32P incorporation by comparison with a 32P standard curve exposed simultaneously. PLC-βT was detected immunologically by polyclonal antiserum no. 246 produced against denatured PLC-β/T [33]. In brief, nitrocellulose was blocked in TBS [20 mM Tris/HCl (pH 7.5)/500 mM NaCl] plus 3 % (w/v) BSA and incubated for 1 h with a 1:500 dilution of antibody no. 246 in TBS plus 1 % (w/v) BSA. Nitrocellulose was washed with TBS plus 0.05 % (v/v) Tween 20 and incubated for 1 h with alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody (1:2000 dilution) in TBS plus 1 % (w/v) BSA. Proteins were detected by incubation with alkaline phosphatase colorimetric substrates (5-bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium). Quantification of PLC-βT immunoprecipitated for stoichiometric calculations was accomplished by a digital densitometric comparison with a standard curve of purified PLC-βT on the same blot.

Calculation of the specific radioactivity of the γ-phosphate of [32P]ATP in vivo

Calculation of the specific radioactivity of the γ-phosphate of [32P]ATP from [32P]PP₃-treated turkey erythrocytes was determined by the method of Garrison et al. [34]. In brief, 1 ml of washed and packed turkey erythrocytes was radiolabelled overnight with [32P]PP₃, as described above. 32P-labelled erythrocytes (1–10 μl) were lysed by the addition of 0.5 ml of 5 % trichloroacetic acid on ice. Samples were centrifuged at 750 g for 15 min to remove precipitated material, and the supernatant was extracted three times with 3 ml of diethyl ether to remove trichloroacetic acid. The samples were neutralized with 10 μl of 1 M Tris/HCl, pH 8.0. The ATP content of the samples was determined by luciferin/luciferase assay as described by Watt et al. [35]. The intracellular ATP concentration obtained by luciferin/luciferase assay after measuring the mean turkey erythrocyte cell volume (134 fl) was 1.2 mM. This value is in good agreement with the previously published value of 1 mM ATP in turkey erythrocytes [36].

32P-labelled nucleotides were separated on a C18 column by HPLC as described by Lazarowski et al. [37]; radioactivity co-migrating with the ATP pool was quantified by scintillation spectrometry. Half of the 32P-labelled sample was incubated with 3 units/ml hexokinase in high-glucose DMEM for 30 min at 37 °C to hydrolyse ATP completely to ADP and P₃. The specific radioactivity of the γ-phosphate of ATP was calculated as the difference in free [32P]P₃ between the hexokinase-treated and untreated samples.

RESULTS

Incubation of [32P]P₃ prelabelled turkey erythrocytes with the PKC activator PMA resulted in an increase in phosphorylation of PLC-βT (Figure 1). The effect of PMA was concentration-dependent and was not mimicked by 4x-phorbol 12,13-didecanoate, a phorbol ester that does not activate PKC. The

Figure 1  Phosphorylation of PLC-β/T in vivo

Turkey erythrocytes were treated as described in the Materials and methods section with vehicle, 10 μM 2MeSATP, the indicated concentrations of PMA, or 4x-phorbol 12,13-didecanoate (Pdd) for 20 min at 37 °C. Cells were lysed and PLC-β/T was immunoprecipitated as described in the Materials and methods section. Immunoprecipitated proteins were separated by SDS/PAGE and were detected by immunoblotting with anti-(PLC-β/T) antibodies (A) or exposed for 3P detection with a PhosphorImage (B). The 135 kDa protein species co-migrating with purified PLC-β/T is presented from a representative experiment with similar results obtained at least three times.
The concentration of PKC (Figure 2, top panel). Maximal phosphate incorporation was detected with a PhosphorImager after SDS/PAGE (top panel, inset) or quantified as described in the Materials and methods section and presented as mol of phosphate incorporated per mol of PLC-βT. Representative results are shown and similar results were obtained in four experiments. Middle panel: PLC-βT (βT), PLC-β2 (β2) and PLC-β1 (β1) were incubated for 10 min at 30 °C in kinase reactions in the presence (+) or absence (−) of 10 μ-units (μU) of PKC. Proteins were subjected to SDS/PAGE and detected by Coomassie staining (A). 32P incorporation was detected with a PhosphorImager screen (B). Bottom panel: PLC-βT (10 ng), preincubated in the absence (open bars) or presence (filled bars) of 10 μM 2MeSATP (△) for 20 min at 30 °C. Lower panel: purified PLC-βT (10 ng), preincubated in the absence (open bars) or presence (filled bars) of 10 μ-units of PKC, was added to turkey erythrocyte membranes and incubated in the presence of vehicle (basal), AlF4− or 100 μM GTP[S] (GTP[S]) plus 10 μM 2MeSATP (▲) for 20 min at 30 °C. The activity of PLC-βT is shown as pmol of Ins(1,4)P2 (IP2) released per mg of PLC-βT. Results are means ± S.D. for triplicate determinations and are representative of three experiments.

Having established that PLC-βT was phosphorylated in vivo after the activation of PKC, we examined directly the capacity of purified brain PKC to phosphorylate PLC-βT. Purified PLC-βT was incubated with PKC in the presence of DOG and PtdSer. PKC-promoted phosphorylation of PLC-βT was observed and increased with time of incubation (results not shown) or concentration of PKC (Figure 2, top panel). Maximal phosphate incorporation was achieved within 10 min at 30 °C with a concentration of 1 μ-unit of PKC/μl of reaction. A stoichiometry of phosphorylation (mean ± S.E.M.) near unity (1.06 ± 0.20 mol phosphate/μmol PLC-βT; n = 6) was obtained under optimal conditions. Phosphorylation was minimal in the absence of PKC (Figure 2, middle panel), indicating that little or no endogenous kinase activity was present in the preparation of PLC-βT. Phosphorylation also was PLC-β isoenzyme-specific. Whereas PLC-βT and mammalian PLC-β2 were phosphorylated to a stoichiometry of approx. 1 mol of phosphorylation (mean ± S.E.M.) near unity (1.06 ± 0.20 mol phosphate/μmol PLC-βT; n = 6) was obtained under optimal conditions. Phosphorylation was minimal in the absence of PKC (Figure 2, middle panel), indicating that little or no endogenous kinase activity was present in the preparation of PLC-βT. Phosphorylation also was PLC-β isoenzyme-specific. Whereas PLC-βT and mammalian PLC-β2 were phosphorylated to a stoichiometry of approx. 1 mol/μmol, under identical reaction conditions PLC-β1 remained essentially unphosphorylated (Figure 2, middle panel).

The preparation of brain PKC consisted of a mixture of PKC isoforms. However, a comparison of the capacity of several Ca2+-
dependent and diacylglycerol-dependent PKC isoforms (α, β1, β2 and γ) to phosphorylate PLC-β1 and PLC-βT suggested that little specificity for substrate exists among the PKC isoforms (Figure 2, bottom panel). All four purified PKC isoforms phosphorylated PLC-β1 to a stoichiometry near unity (0.7–0.9 mol/mol), whereas none of these PKC isoforms phosphorylated PLC-β1 to a stoichiometry greater than 0.03 mol/mol. The brain PKC preparation was therefore used in all subsequent experiments.

A reconstitution assay was established to determine directly the effect of phosphorylation on the activity of PLC-βT. The assay involved the quantification of the activity of purified PLC-βT after reconstitution with turkey erythrocyte membranes devoid of endogenous PLC-β activity but apparently retaining a full complement of receptor and G-protein activators. As illustrated in Figure 3 (upper panel), inositol lipid hydrolysis was proportional to the amount of exogenous PLC-βT reconstituted. Activity of the reconstituted enzyme also was stimulated by GTP[S] alone or by the P2Y<sub>1</sub> receptor agonist 2MeSATP in the presence of GTP[S]. Phosphorylation of PLC-βT to a stoichiometry of approx. 1 mol/mol resulted in a decrease in reconstitution of AlF<sub>4</sub>-stimulated or 2MeSATP-plus-GTP[S]-stimulated phospholipase activity (Figure 3, lower panel) compared with non-phosphorylated PLC-βT. The phosphorylation-induced decrease in PLC activity occurred at all concentrations of PLC-βT tested (results not shown). Importantly, not only were phosphorylation-promoted decreases in G-protein-stimulated and receptor-stimulated activity of phosphorylated PLC-βT observed, but similar decreases in basal PLC activity occurred.

Basal, GTP[S]-stimulated, and 2MeSATP-plus-GTP[S]-stimulated PLC activities also were quantified in reconstitution assays performed with PLC-βT phosphorylated to various stoichiometries (0–0.9 mol/mol) by PKC. As illustrated in Figure 4, the phosphorylation-dependent decrease in PLC-βT activity was proportional to the concentration of PKC in the kinase reaction and to the amount of phosphate incorporated. By accumulating results over eight experiments, the decreases in GTP[S]-stimulated or 2MeSATP-plus-GTP[S]-stimulated PLC-βT activities were fully accounted for by a decrease in basal PLC-βT activity, i.e. the maximal PKC-promoted decrease in activity was essentially the same in all three activity measurements.

The phosphorylation-promoted decrease in basal PLC-βT activity observed in membrane reconstitution assays was examined more directly by measuring catalytic activity with inositol lipid substrate/Na<sup>+</sup> cholate mixed micelles. The catalytic
activity of PLC-βT against substrate in mixed detergent micelles also was decreased after phosphorylation in the presence of PKC (Figure 5, upper panel). The decrease in catalytic activity of PLC-βT was dependent on the time of incubation with PKC (Figure 5, lower panel) and the concentration of PKC (results not shown) and achieved a maximal decrease in activity of approx. 60% of control levels.

The effects of PKC-promoted phosphorylation on PtdIns(4,5)P₂, substrate concentration dependence were determined in assays with phospholipid/detergent mixed micelles. The difference in catalytic activity between phosphorylated and non-phosphorylated PLC-βT was greatest at low substrate concentrations and diminished at higher substrate concentrations (Figure 6), suggesting that phosphorylation decreased enzyme affinity for substrate rather than inhibited maximal reaction velocity. Single-site kinetics were observed for unphosphorylated PLC-βT, as has been demonstrated previously [38]. In contrast, more complex substrate kinetics, indicated by the non-linear Hanes regression curve (Figure 6, inset), were observed after phosphorylation of PLC-βT. No change in Ca²⁺ dependence was detected on PKC-promoted phosphorylation of PLC-βT (results not shown).

The effect of PKC-induced phosphorylation of PLC-βT on its regulation by G-protein subunits also was studied after reconstitution of the enzyme with purified Gα subunits in unilamellar phospholipid vesicles. PKC-promoted phosphorylation resulted in a decrease in PLC-βT-catalysed activity both in the absence and presence of AlF₃-activated Gα (Figure 7, upper panel). This finding was consistent with the phosphorylation-dependent decrease in basal activity observed in the phospholipid/detergent mixed-micelle assay. Surprisingly, a phosphorylation-dependent decrease in PLC-βT activity was not detected in assays with Gβγ (Figure 7, lower panel), suggesting that the activation of PLC-βT by Gβγ surmounts the decrease in basal activity induced by the phosphorylation of PLC-βT.

**DISCUSSION**

Guanine nucleotide-stimulated PLC activity is markedly attenuated in membranes prepared from PMA-pretreated cells, which has led to the hypothesis that the PKC-promoted modification of Gα or PLC-β is an important regulatory component of inositol lipid signalling [3,6]. A similar phenomenon occurs in turkey erythrocytes [29]; we have shown here that PLC-βT is phosphorylated to a physiologically important stoichiometry in vivo as a consequence of activation of PKC. Moreover, purified PLC-βT is an excellent substrate for four Ca²⁺-dependent and

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**Figure 6** Phosphorylation-induced change in substrate dependence of PLC-βT

PLC-βT (20 ng), preincubated in the absence (○) or presence (●) of 10 μ-units of PKC, was incubated with phospholipid/detergent mixed micelles; the bulk PtdIns(4,5)P₂ (PIP₂) concentration was varied as indicated. Reactants were incubated for 10 min at 30 °C and PLC-βT activity was quantified as μmol of Ins(1,4,5)P₃ (IP₃) produced/min per mg of PLC-βT. A replot of the data by the method of Hanes is shown in the inset. Apparent kinetic parameters were approx. 60 μM at 91 μmol/min per mg respectively for samples without PKC, and 102 μM and 108 μmol/min per mg respectively for samples without PKC. Similar results were obtained four times.

**Figure 7** Reconstitution of phosphorylated PLC-βT with purified G-protein subunits

PLC-βT (20 ng) was preincubated in the absence (○) or presence (●) of 10 μ-units of PKC as described in the Materials and methods section. Upper panel: control and phosphorylated PLC-βT were reconstituted with the indicated concentrations of Gαq as described in the Materials and methods section. Lower panel: control (open bars) and phosphorylated PLC-βT (filled bars) were reconstituted with the indicated concentrations of Gβγ as described in the Materials and methods section. Incubations were for 10 min at 30 °C; the activity of PLC-βT was quantified as μmol of Ins(1,4,5)P₃ (IP₃) produced/min per mg of PLC-βT. Results are means ± S.D. for triplicate determinations and are representative of three experiments.
diacylglycerol-dependent PKC isoforms; the results provide direct demonstration of an effect of phosphorylation on the activity of a PLC-β isoenzyme.

The membrane reconstitution assay applied in this study permitted the quantification in situ of the activities of phosphorylated PLC-β/T under conditions that make no assumptions a priori about the mode by which phosphorylation regulates enzyme activity. The phosphorylation-induced decrease in basal as well as P2Y1-receptor- and guanine nucleotide-stimulated PLC activities in the reconstitution assay strongly suggest that the overall catalytic activity of PLC-β/T is decreased as a consequence of phosphorylation. This view is supported by a similar phosphorylation-induced decrease in activity measured in an assay of catalytic activity with detergent and phospholipid micelles. Results from studies in which PKC-phosphorylated PLC-β/T was reconstituted with Gαz also are consistent with this conclusion because no effect occurred on Gαz-stimulated PLC-β/T activity beyond that measured in the absence of G-protein and attributable to a decrease in PLC-β/T catalytic activity. Therefore we conclude that phosphorylation inhibits the overall catalytic activity of PLC-β/T rather than selectively modifying regulation by, for example, a G-protein subunit, as has been suggested for the regulation of PLC-β2 by cAMP-dependent protein kinase [39].

The lack of effect of PKC-promoted phosphorylation of PLC-β/T on Gβγ-stimulated activity is puzzling. The activating effects of Gβγ apparently overcome the effect of phosphorylation on basal catalytic activity. Although z-subunits of the Gα family of G-proteins are probably more important than is Gβγ for activation of PLC-β in the pertussis toxin-insensitive inositol lipid signalling response, this idea has not been formally proven. Turkey Gαz, purified on the basis of its capacity to activate PLC-β/T in the pertussis toxin-insensitive inositol lipid signalling pathway, was not a PKC substrate under the conditions used in these experiments [27].

None of the assays currently available for measurement of PLC-β activity provide a complete portrait of the physiological regulation of PLC-β enzymes. Reconstitution assays with purified G-proteins lack G-protein modulators such as regulators of G-protein signalling proteins, e.g. regulators of G-protein signalling (RGS)-proteins, remains a possibility as a mechanism for regulation of inositol lipid signalling. In other studies with turkey erythrocyte membranes devoid of endogenous PLC-β activity we have separated the effects of phosphorylation on PLC-β activity from the effects on membrane-bound regulators of PLC-β/T. These preliminary experiments have revealed that the treatment of turkey erythrocytes with PMA also results in a decreased capacity of GTP[S] to promote the activity of exogenously reconstituted PLC-β1 in membranes prepared from these cells [38, 39].

Although the results reported here demonstrate directly a phosphorylation-induced decrease in the activity of a PLC-β isoenzyme, the phosphorylation of G-proteins or G-protein-related proteins, e.g. regulators of G-protein signalling (RGS)-proteins, remains a possibility as a mechanism for regulation of inositol lipid signalling. In other studies with turkey erythrocyte membranes devoid of endogenous PLC-β activity we have separated the effects of phosphorylation on PLC-β activity from the effects on membrane-bound regulators of PLC-β/T. These preliminary experiments have revealed that the treatment of turkey erythrocytes with PMA also results in a decreased capacity of GTP[S] to promote the activity of exogenously reconstituted PLC-β1 in membranes prepared from these cells [38, 39].

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