Phosphorylation of Serine 1105 by Protein Kinase A Inhibits Phospholipase Cβ3 Stimulation by Gaq*

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The mechanism by which protein kinase A (PKA) inhibits Goq-stimulated phospholipase C activity of the β subclass (PLCβ) is unknown. We present evidence that phosphorylation of PLCβ by PKA results in inhibition of Goq-stimulated PLCβ activity, and we identify the site of phosphorylation. Two-dimensional phosphoamino acid analysis of in vitro phosphorylated PLCβ revealed a single phosphoserine as the putative PKA site, and peptide mapping yielded one phosphopeptide. The residue was identified as Ser1105 by direct sequencing of reverse-phase high pressure liquid chromatography-isolated phosphopeptide and by site-directed mutagenesis. Overexpression of Goq with PLCβ or PLCβ (Ser1105→Ala) mutant in COSM6 cells resulted in a 5-fold increase in [3H]phosphatidylinositol 1,4,5-trisphosphate formation compared with expression of Goq or PLCβ (Ser1105→Ala) mutant alone. Whereas Goq-stimulated PLCβ activity was inhibited by 58–71% by overexpression of PKA catalytic subunit, Goq-stimulated PLCβ (Ser1105→Ala) mutant activity was not affected. Furthermore, phosphatidylinositol turnover stimulated by presumably Goq-coupled M1 muscarinic and oxtocin receptors was completely inhibited by pretreating cells with 8-(4-chlorophenylthio) cAMP in RBL-2H3 cells expressing only PLCβ. These data establish that direct phosphorylation by PKA of Ser1105 in the putative G-box of PLCβ inhibits Goq-stimulated PLCβ activity. This can at least partially explain the inhibitory effect of PKA on Goq-stimulated phosphatidylinositol turnover observed in a variety of cells and tissues.

Ligand stimulation of seven transmembrane domain receptors coupled to Go proteins of the Goq or Goa subfamilies results in the activation of the respective heterotrimeric G protein complexes. Free Goa or Gβγ subunits activate PLCβ isoforms to catalyze the production of IP3 and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (1–3). PLCβ1,4 comprise the currently known mammalian phosphatidylinositol-specific PLCβ subfamily. Although all PLCβs are activated by Goa, PLCβ2 and PLCβ3 are also stimulated by Gβγ, primarily released from Goa (1).

Cross-talk between the G protein-PLCβ pathway and PKA has been documented in numerous studies (4–13). Although it is generally agreed that G protein-activated PLCβ activity can be inhibited by PKA (4–11), PKA can enhance the G protein-PLCβ pathway in some cases (12, 13). Because PKA can inhibit phosphatidylinositol (PI) turnover activated by both Goa (4–8) and Goa (9–11) coupled receptors, it may inhibit the stimulation of both Goa- and Gβγ-stimulated PLCβ activity. This notion is further supported by studies with the G protein activators GTPγS and AIFγ. These two compounds nonselectively activate all heterotrimeric G proteins and generate free Goa and Gβγ subunits that can stimulate PLCβs. PKA inhibition of PI turnover initiated by GTPγS or AIFγ (5, 8, 14, 15) is consistent with the inhibition of Goa- as well as Gβγ-stimulated PLCβ activity. In addition, this phenomenon also suggests that the PKA effect is distal to receptors.

Recently, the mechanism for PKA inhibition of Gβγ-stimulated PI turnover has been elucidated. Phosphorylation of PLCβ2 by PKA resulted in inhibition of Gβγ-stimulated PI turnover (10). However, in the same study, PKA apparently did not inhibit Goa15- and Goa16-stimulated endogenous PLCβ (β1 and β2) activity. More recently, Ali et al. (11) have reported phosphorylation of PLCβ2 in response to CPT-cAMP treatment in RBL-2H3 cells expressing only PLCβ2. CPT-cAMP inhibited Gβγ-stimulated PLCβ2 activated by the Goa-coupled formylmethionyleucylphenylalanine receptor but had no effect on PAF-stimulated PLCβ3 activity, presumably mediated by Goq. These studies led to the conclusions that phosphorylation of PLCβ2 and PLCβ3 by PKA could explain the inhibition of Gβγ-stimulated PI turnover by cAMP (10, 11). However, a biochemical mechanism for the inhibition by PKA of Goa-stimulated PLCβ activity observed in several systems remains to be clarified. In this study, we present evidence that phosphorylation of PLCβ3 Ser1105 by PKA results in direct inhibition of Goq-stimulated PLCβ3 activity.

EXPERIMENTAL PROCEDURES

Materials—PLCβ3 antibody, immunoblotting, and immunoprecipitation reagents were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Lys-C was obtained from Wako Bioproducts (Richmond, VA). PKA catalytic subunit and other chemicals were purchased from Sigma. LipofectAMINE, DMEM, and all other cell culture reagents were obtained from Cellgro (Loudon, VA). 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.

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The abbreviations used are: PLC, phospholipase C; IP3, phosphatidylinositol 1,4,5-trisphosphate; PKA, cAMP-dependent protein kinase; PI, phosphatidylinositol; GTPγS, guanosine 5′-[γ-thio]triphosphate; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.
catalytic subunit plasmid was kindly provided by Dr. S. McKnight (University of Washington), and Goα plasmid by Dr. M. Simon (California Institute of Technology).

Cloning, Site-directed Mutagenesis, and Protein Purification—PLCβ3 and PLCβ3(His)6 in pCR3.1 vector (Invitrogen, San Diego, CA) and PLCβ3(His), in baculovirus (Pharmingen, San Diego, CA) were constructed from the PLCβ3 cDNA plasmid (17). Site-directed mutation of Ser1105 to Ala was achieved with the mutagenic primer (5’-AGGCCTATAACGCCCATCTCGGAGG-3’) using the GeneEditor kit (Promega, Madison, WI). All plasmid sequences were confirmed by DNA sequencing.

PLCβ3(His) was purified essentially as described for PLCβ3 (18) from the membrane fraction from Si9 cells and was 99% pure as judged by SDS-PAGE.

In Vitro Phosphorylation, Phosphoamino Acid Analysis, Peptide Mapping, and Sequencing—0.5, 1.5, or 2.5 μM purified recombinant PLCβ3(His)6 was incubated with PKA catalytic subunit at molar ratios of 20:1 or 50:1 in the presence of 1–10 μCi of [γ-32P]ATP and 100 μM ATP in a total volume of 10 μl of PKA buffer (10 mM Tris, pH 7.5, 5 mM MgCl2) for 10 min at 30 °C. For the time course study, 1.3 μM PLCβ3(His)6 was incubated with PKA at a ratio of 1:10:1. Reactions were terminated by addition of an equal volume of 2× SDS sample buffer (15) and boiling for 5 min. Proteins were separated on SDS-PAGE gels, and the phosphorylated band was localized by autoradiography.

Two-dimensional phosphoamino acid analysis and peptide mapping of in vitro [32P]labeled PLCβ3(His) bound to PVDF membranes were carried out with a Hunter thin layer electrophoresis system (CBS Scientific Company, Del Mar, CA) according to the manufacturer’s instructions. For two-dimensional peptide mapping, the membrane bound samples were digested with Lys-C (3 μg) for 24 h at 37 °C. For peptide sequencing, 150 pmol of [32P]PLCβ3(His)6 was digested with Lys-C (1 μg). The phosphopeptide separated by reverse-phase HPLC was sequenced at the microsequencing facility at Baylor College of Medicine (Houston, TX).

In Vivo 32P Labeling and Immunoprecipitation—Nearly confluent PHM1–41 immortalized myometrial cells (10-cm dish) were labeled with [32P]orthophosphate (0.33 mCi/ml) in phosphate-free DMEM containing 10% dialyzed fetal calf serum for 4 h. After the treatments indicated in the figure legends, cells were lysed in 1 ml of ice-cold lysis buffer containing a mixture of protease and phosphatase inhibitors (11) and centrifuged at 15,000 × g for 5 min at 4 °C. Phosphorylated proteins were immunoprecipitated with 4 μg of anti-PLCβ3 antibody and separated on SDS-PAGE transferred to a PVDF membrane, and analyzed by autoradiography. PLCβ3 was visualized by Western blot using anti-PLCβ3 antibody (1:1000) to normalize for sample loading.

Cell Culture, Transfection, and PI Turnover—COSM6 and RBL-2H3 cells were cultured and transfected as described (4, 11) with the following modifications. COSM6 cells were transfected with a total of 1.25 μg of plasmid DNA (using empty vector recCMV as necessary) and 6 μl of LipofectAMINE in 0.75 ml of DMEM/well in 6-well plates, whereas 1.0 μg of total plasmid DNA and 5 μl of LipofectAMINE in 0.5 ml of DMEM were used to transfected RBL-2H3 cells. An equal volume of culture medium (4) containing 16% fetal calf serum was added 5 h later. The following day, cells were labeled with 6 μCi/well [3H]inositol in 1 ml of culture medium for 24 h at 37 °C. ZnSO4 (60 μM) was also included in the labeling medium to stimulate PKA catalytic subunit expression in COSM6 cells. After incubating with 10 mM LiCl for 10 (RBL-2H3) or 45 (COSM6) min, cells were treated as indicated in the figure legends and lysed by addition of ice-cold 10% trichloroacetic acid. The accumulation of [3H]IP3 was determined as described elsewhere (4).

RESULTS AND DISCUSSION

In Vitro and in Vivo Phosphorylation of PLCβ3 by PKA—We have determined previously that the PKA inhibitory effect is distal to receptor and most likely affects the coupling between Goα and PLCβ3 or PLCβ3, isoforms in pregnant human myometrial (PHM1–41) and COSM6 cell lines (4). As shown in Fig. 1A, when incubated with PKA, C-terminal (His)6-tagged PLCβ3 (PLCβ3(His)6) purified from Si9 cells was clearly a substrate for PKA in vitro. Similar results were also obtained with immunoprecipitation-purified recombinant PLCβ3 (data not shown). The phosphorylation of PLCβ3 was quite specific; neither highly purified recombinant Goα nor recombinant PLCβ1 was phosphorylated by PKA under similar conditions (data not shown), confirming previous observations (19, 20).

To quantify PKA-stimulated [32P] incorporation from [γ-32P]ATP, PLCβ3(His)6 was phosphorylated by PKA in vitro. Fig. 1C shows that the time course of [32P] incorporation approached a plateau after 15 min. A maximum ratio of 0.65 mol phosphate/mol PLCβ3 was determined by filter binding assay at the 60-min incubation point. This is consistent with a single PKA phosphorylation site in PLCβ3.

To examine whether PLCβ3 could be phosphorylated in vivo, PHM1–41 myometrial cells were labeled with [32P]orthophosphate, and PKA was activated with the cell-permeable cAMP analogue CPT-cAMP or relaxin, a hormone that increases myometrial cell cAMP (21). Fig. 1B shows that PLCβ3 immunoprecipitated from cells exposed to CPT-cAMP or relaxin exhibited increased phosphorylation. After normalizing for the amount of PLCβ3 loaded, the treatments resulted in a 2-fold increase in PLCβ3 phosphorylation. A similar fold increase in PLCβ3 phospho-phylation was recently reported in RBL-2H3 cells treated with CPT-cAMP (22). These data indicate that endogenous PLCβ3 can be phosphorylated in cells in response to elevated cAMP.

Identification of the PKA Phosphorylation Site—Two-dimensional phosphoamino acid analysis with in vitro phosphorylated PLCβ3(His)6 revealed that only serine was phosphorylated by PKA (Fig. 2A). A similar result was also obtained with non-His-tagged recombinant PLCβ3 phosphorylated by PKA in COSM6 cells (data not shown). Peptide mapping of in vitro phosphorylated PLCβ3(His)6 digested with Lys-C revealed one major phosphorylated peptide (Fig. 2B). Importantly, an increase in phosphorylation of the same peptide (indicated by the arrow) was also detected in endogenous PLCβ3 in PHM1 (Fig. 2, D versus C) and COSM6 cells (Fig. 2, F versus E) treated with CPT-cAMP as well as in overexpressed PLCβ3 in COSM6 cells coexpressing PKA catalytic subunit (Fig. 2, H versus G). In the case of overexpressed PLC and PKA (Panels G, H), there appears to be phosphorylation of another site in the basal state that decreases when the PKA site is phosphorylated. We are in
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To confirm the phosphorylation site and avoid contamination with endogenous PLCβ3, His-tagged PLCβ3 (Ser1105 → Ala) mutant was constructed. This mutant was overexpressed in COSM6 cells, purified on a Ni-NTA column and phosphorylated by PKA in vitro. As shown in Fig. 3B, mutation of Ser1105 to Ala reduced PKA phosphorylation of PLCβ3 by ~90%. The small residual phosphorylation probably represents background, because it was also seen in extracts from cells transfected with empty vector and processed similarly (data not shown). We conclude from these studies that PKA phosphorylates PLCβ3, Ser1105 both in vivo and in vitro. Notably, this PKA phosphorylation site is not present in the corresponding sequences (20) of PLCβ1 or PLCβ2 (Fig. 3A).

Inhibition of Gq-stimulated PLCβ3 Activity by PKA—The C terminus of PLCβ3 is critical for activation by Gqα (23). Deletion studies have identified a P-box (Thr1103 to Glu1109) and a G-box (Lys1101 to Leu1112) in this region. The P-box is essential for both PLCβ1 association with the cell membrane and its activation by Gqα, whereas the G-box is involved in association with Gqα subunit (24). Ser1105 of PLCβ3 falls in a region analogous to the G-box of PLCβ1. We therefore hypothesized that phosphorylation of Ser1105 by PKA might cause interference with Gqα-PLCβ3 association and thereby inhibit Gq-stimulated PLCβ3 activity. To test this, PI turnover was studied in COSM6 cells transfected with Gqα and PLCβ3 or PLCβ3 (Ser1105 → Ala) mutant in the absence and presence of PKA. As shown in Fig. 4A, transfection of empty vector (reCMV), Gqα, PLCβ3 or PLCβ3 (Ser1105 → Ala) mutant alone had no effect on basal PI turnover, suggesting that the proteins are primarily in their inactive forms under these conditions (25). Cotransfection of Gqα with PLCβ3 produced a 5-fold increase in [3H]IP3, presumably because of the increased activation of PLCβ3 by Gqα as reported previously (26). Notably, the PLCβ3 (Ser1105 → Ala) mutant was as effective as wild type at stimulating PI turnover. This indicates that the substitution of Ala for Ser1105 did not have a major effect on catalytic activity or G protein coupling. Importantly, when PKA catalytic subunit was also coexpressed, Gqα-stimulated [3H]IP3 formation associated with wild type PLCβ3 was inhibited by ~58%, whereas no inhibition was observed with the PLCβ3 (Ser1105 → Ala) mutant. Increas-

Fig. 3. A, the sequence of the phosphopeptide isolated from PLCβ3(His)6 phosphorylated by PKA and the alignment of this sequence with the corresponding sequences in PLCβ1 and PLCβ2 (26). The position of [32P]phosphoserine1105 is designated by an asterisk. Ser and Thr are not found in comparable positions in either PLCβ1 or PLCβ2. B, in vitro phosphorylation by PKA of overexpressed wild type (lanes W) and Ser1105 → Ala mutant (lanes M) PLCβ3(His)6 purified from COSM6 cells. Protein bands (Coomassie) and their phosphorylation states (Autorad) are shown.
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More evidence in support of this contention was obtained in the RBL-2H3 cell line expressing only \( \text{PLC}\beta_{3} \) (11). RBL-2H3 cells were transfected with the M1 muscarinic and oxytocin receptors shown to couple in other cell types to \( \text{PLC}\beta \) through \( \text{Go}_{q} \) proteins (26–29). Stimulation with the respective ligands resulted in a 4-fold increase in \[^{3}H\text{IP}_{3}\] presumably through the coupling of the receptors to endogenous \( \text{Go}_{q} \) and \( \text{PLC}\beta_{3} \). Importantly, pretreating cells with CPT-cAMP, previously shown to activate endogenous \( \text{PKA} \) and result in \( \text{PLC}\beta_{3} \) phosphorylation (11), completely inhibited M1 and oxytocin receptor-stimulated PI turnover (Fig. 4C). These data are consistent with our previous data in COSM6 and PHM1–41 cells (4). In myometrial membranes, oxytocin-stimulated PI turnover was determined to be essentially completely \( \text{Go}_{q} \)-mediated (28).

Based on the position of Ser\textsuperscript{1105} in the enzyme, we hypothesize that its phosphorylation by \( \text{PKA} \) may perturb the association of \( \text{PLC}\beta_{3} \) with \( \text{Go}_{q} \). However, we do not know at present how Ser\textsuperscript{1105} phosphorylation affects the kinetic properties of \( \text{Go}_{q}/\text{PLC}\beta_{3} \) coupling. It is also not yet clear what relationship this phosphorylation has to the reported inhibition of \( \text{G}^{B_{q}} \)-stimulated \( \text{PLC}\beta_{3} \) activity by \( \text{PKA} \) (11). These questions are under study. Interestingly, Ser\textsuperscript{394}, one of the two putative PKA phosphorylation sites in \( \text{PLC}\beta_{2} \), is located in the P-box. It has been suggested that \( \text{PKA} \) phosphorylation of that site may interfere with the membrane association of \( \text{PLC}\beta_{3} \) (10). The close proximity of Ser\textsuperscript{1105} to the P-box may allow \( \text{PKA} \) to affect the membrane association of \( \text{PLC}\beta_{3} \) as well.

Based on the ubiquitous expression of \( \text{PLC}\beta_{3} \) (20), our observations could explain the inhibition of \( \text{Go}_{q} \)-stimulated PI turnover by \( \text{PKA} \) observed in a variety of cells and tissues (4–11). However, the basis for the complete inhibition of \( \text{Go}_{q} \)-stimulated PI turnover by \( \text{PKA} \) in cells expressing both \( \text{PLC}\beta_{1} \) and \( \text{PLC}\beta_{3} \) (4, 15) cannot be adequately addressed without knowing the cellular localization and relative contributions of these two isoenzymes to total PI turnover. Our data apparently contradict the reported inability of \( \text{PKA} \) to inhibit \( \text{Go}_{q} \)-coupled PAF receptor-stimulated \( \text{PLC}\beta_{3} \) activity in RBL-2H3 cells (11). The reason for this discrepancy is unknown at present but may reflect differences in the nature of specific receptor/G protein coupling in that cell line, differences in experimental design, or some other as yet unknown factor. Consistent with the findings reported here, we have found that coexpression of \( \text{PKA} \) catalytic subunit inhibits carbachol-stimulated PI turnover in COSM6 cells cotransfected with M1 muscarinic receptor and \( \text{Go}_{q} \) (4). In contrast, \( \text{Go}_{15} \) and \( \text{Go}_{16} \)-stimulated endogenous \( \text{PLC}\beta_{1} \) (\( \beta_{1} \) and \( \beta_{3} \)) activity was not inhibited by \( \text{PKA} \) in COS7 cells (10). It is unclear how effectively the various G proteins stimulate \( \text{PLC}\beta_{3} \) versus \( \text{PLC}\beta_{1} \) in these cells. In reconstitution assays, \( \text{Go}_{16} \) appears to be as effective as \( \text{Go}_{q} \) in stimulating \( \text{PLC}\beta_{1} \) but less effective than \( \text{Go}_{q} \) in stimulating \( \text{PLC}\beta_{3} \) (30). The relative contribution of \( \text{PLC}\beta_{3} \) versus \( \text{PLC}\beta_{1} \) to PI turnover and possible preferential coupling of \( \text{Go}_{q} \) subfamily isoforms to \( \text{PLC}\beta_{3} \) may account for some of the observed differences.

In summary, the data presented here establish a direct relationship between \( \text{PKA} \)-stimulated phosphorylation of Ser\textsuperscript{1105} and inhibition of \( \text{PLC}\beta_{3} \) activity. This can at least partially explain the inhibitory effect of \( \text{PKA} \) on \( \text{Go}_{q} \)-coupled receptor-stimulated PI turnover observed in a variety of cells and tissues.

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**Fig. 4.** A, \( \text{PKA} \) inhibits \( \text{Go}_{q} \)-stimulated \( \text{PLC}\beta_{3} \) but not mutant \( \text{PLC}\beta_{3} \) (Ser\textsuperscript{1105} → Ala) activity. COSM6 cells were transiently transfected with empty vector (rcCMV) or plasmids expressing \( \text{Go}_{q} \) (0.5 \( \mu \text{g} \)), \( \text{PLC}\beta_{3} \) (P\textsubscript{3}, 0.25 \( \mu \text{g} \)), or \( \text{PLC}\beta_{3} \) (S/A) mutant (P\textsubscript{3} S/A, 0.25 \( \mu \text{g} \)) as indicated in the absence (open bars) or presence (filled bars) of cotransfected \( \text{PKA} \) catalytic subunit plasmid (0.5 \( \mu \text{g} \)). Data represent duplicate determinations in one of two experiments; error bars give the range of duplicates. B, inhibition of \( \text{Go}_{q} \)-stimulated \( \text{PLC}\beta_{3} \), as in A, in the presence of 0.5, 0.65, or 0.75 \( \mu \text{g} \) of \( \text{PKA} \) catalytic subunit plasmid DNA. The control cells were treated with PBS, oxytocin, or carbachol only. Data represent duplicate determinations in one of two experiments; error bars give the range of duplicates.
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