Molecular Mechanism of the Inhibition of Phospholipase C β3 by Protein Kinase C*

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Activation of protein kinase C (PKC) can result from stimulation of the receptor-G protein-phospholipase C (PLCβ) pathway. In turn, phosphorylation of PLCβ by PKC may play a role in the regulation of receptor-mediated phosphatidylinositol (PI) turnover and intracellular Ca2+ release. Activation of endogenous PKC by phorbol 12-myristate 13-acetate inhibited both Goα-coupled (oxytocin and M1 muscarinic) and Goq-coupled (formyl-Met-Leu-Ph-e) receptor-stimulated PI turnover by 50–100% in PHM1, HeLa, COSM6, and RBL-2H3 cells expressing PLCβ3. Activation of conventional PKCs with thymeleatoxin similarly inhibited oxytocin or formyl-Met-Leu-Ph-e receptor-stimulated PI turnover. The PKC inhibitory effect was also observed when PLCβ3 was stimulated directly by Goα or Gβγ in overexpression assays. PKC phosphorylated PLCβ3 at the same predominant site in vivo and in vitro. Peptide sequencing of in vitro phosphorylated recombinant PLCβ3 and site-directed mutagenesis identified Ser1105 as the predominant phosphorylation site. Ser1105 is also phosphorylated by protein kinase A (PKA; Yue, C., Dodge, K. L., Weber, G., and Sanborn, B. M. (1998) J. Biol. Chem. 273, 18023–18027). Similar to PKA, the inhibition by PKC of Goqα-stimulated PLCβ3 activity was completely abolished by mutation of Ser1105 to Ala. In contrast, mutation of Ser1105 or Ser276, another putative phosphorylation target, to Ala had no effect on inhibition of Gβγ-stimulated PLCβ3 activity by PKC or PKA. These data indicate that PKC and PKA act similarly in that they inhibit Goqα-stimulated PLCβ3 as a result of phosphorylation of Ser1105. Moreover, PKC and PKA both inhibit Gβγ-stimulated activity by mechanisms that do not involve Ser1105.

Stimulation of seven transmembrane receptors coupled to the Goα or Goq subunits of heterotrimeric G proteins results in activation of PLCβ isoforms that hydrolyze phosphatidylinositol 4,5-bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (1, 2). IP3 binds to a receptor in endoplasmic reticulum and releases intracellular calcium from its stores. Diacylglycerol, alone or in conjunction with elevated intracellular calcium, activates PKC and initiates additional cellular responses (3). Currently, four isoforms of mammalian PLCβ have been identified and characterized (4–10). Significantly, PLCβ3 is ubiquitously expressed and activated by all known PLCβ activators (Goα, Gβγ, and calcium) (2). Regulation of PLCβ3 may be of great importance in many cellular processes (11–15). Insufficient expression of PLCβ3 has been correlated with increased sensitivity to tumor formation (15, 16), whereas overexpression of PLCβ3 seems to suppress tumor growth (17). PLCβ3 knockout mice exhibit altered response to 𝜇-opioids (11) or early embryonic lethality (18).

Phosphorylation appears to play an important role in regulating the activity of PLCβ isoforms. Phosphorylation of PLCβ3 or PLCβ2 by PKA inhibits its activity and establishes a mechanism for cross-talk between Goα+ or Goq-coupled and Goα-coupled receptors (12, 19). The inhibition of G protein-coupled receptor-mediated PI turnover or intracellular calcium release by protein kinase C has been reported (20–25). Protein kinase C is comprised of three subfamilies, the conventional (α, β1, β2, and γ), novel (δ, ε, η, μ, and θ), and atypical (ζ and λ) PKCs (3). The conventional and novel PKCs are activated subsequent to the stimulation of Goα+ or Goq-coupled receptors (3, 26). The inhibition of PI turnover by PKC may present a feedback for determining the frequency and amplitude of signals being transmitted.

The mechanisms by which PKC inhibits agonist-stimulated PI turnover have not been well defined. PKC can phosphorylate certain G protein-coupled receptors (platelet-activating factor receptor, C5A receptor) and thereby inhibit PI turnover or intracellular calcium release (reviewed in Ref. 27). PKC also appears to inhibit agonist-stimulated PI turnover at a post-receptor level (25, 28). Although phosphorylation of PLCβ1 and PLCβ2 by PKC has been reported (23, 24, 29, 30), the physiological relevance of these observations has not been demonstrated. PLCβ1, a turkey PLCβ isoform with highest homology to PLCβ2, is phosphorylated by conventional PKCs, and its catalytic activity is inhibited (29). PLCβ3 is not phosphorylated by PKC in vitro (23). Nonetheless, a correlation between PLCβ3 phosphorylation and PKC inhibition of receptor-initiated PI turnover has been reported (21, 31).

To determine the importance of PLCβ3 phosphorylation by PKC, we have identified the phosphorylation site on PLCβ3 and investigated which PKC subfamily can catalyze the phosphorylation of PLCβ3.

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‡ The abbreviations used are: PLC, phospholipase C; PI, phosphatidylinositol; IP3, phosphatidylinositol 1,4,5-trisphosphate; PKC, protein kinase C; PKA, CAM-dependent protein kinase; ML, formyl-Met-Leu-Ph-e; PMA, phorbol 12-myristate 13-acetate; Tx, thymeleatoxin; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; CPT-cAMP, 8-[4-Chlorophenylthio]-cAMP; MES, 4-morpholineethanesulfonic acid.
Inhibition of PLCβ<sub>3</sub> by PKC

In Vivo and in Vitro 32P Labeling and Isolation of PLCβ<sub>3</sub>

**Materials**—Thymeleaxtin (Tx), G<sub>q</sub> 6976, PKC catalytic fragment, PKCβ<sub>3</sub>, and PKC<sub>γ</sub> were obtained from Calbiochem, H-89 was purchased from Calbiochem, H-89 was purchased from Sigma. Lys C was obtained from Wako Bioproducts (Richmond, VA). Modified sequence grade trypsin, GeneEditor site-directed mutagenesis kit, and the gel drying film were purchased from Promega (Madison, WI). LipofectAMINE, Dulbecco's modified Eagle's medium (DMEM), phosphate-free DMEM, and all other cell culture reagents were obtained from Life Technologies, Inc. [3H]Inositol (22 Ci/mmol) and [32P]orthophosphate (5 mCi/ml) and [32P]orthophosphate (5 mCi/ml) were from Amersham Pharmacia Biotech. The RBL-2H3 cell line stably expressing fMLP receptor and fMLP were provided by Dr. D. Haviland, University of Texas, Houston. The plasmid encoding PKA catalytic subunit was provided by Dr. G. S. McKnight, Washington University (Seattle, WA).

Cloning, Site-directed Mutagenesis, and Protein Purification—PLCβ<sub>3</sub>, G<sub>α</sub>, G<sub>β</sub>, and G<sub>γ</sub> plasmids were constructed as described elsewhere (12, 32). Site-directed mutation of Ser<sup>1105</sup> to Ala was achieved elsewhere (12) using GeneEditor. All plasmid sequences were confirmed by DNA sequencing. Construction of baculovirus containing PLCβ<sub>3</sub>Ser<sup>1105</sup>→Ala (His)<sub>k</sub> and purification of the recombinant protein from Sf9 cells were carried out as described for PLCβ<sub>3</sub>(His)<sub>k</sub> (12).

**In Vivo and in Vitro 32P Labeling and Isolation of PLCβ<sub>3</sub>—For in vivo phosphorylation, COSM6 cells seeded in 6-well plates were transfected with PLCβ<sub>3</sub>(His)<sub>k</sub> plasmid and metabolically labeled with [32P]orthophosphate (0.10 mCi) in 0.5 ml of phosphate-free DMEM for 90 min. After PMA (1 μm) treatment for 30 min, cells were lysed in 500 μl of M-PER lysis buffer (Pierce) containing a mixture of protease and phosphatase inhibitors (21) and centrifuged at 15,000 × g for 5 min at 4 °C. Phosphorylated PLCβ<sub>3</sub>(His)<sub>k</sub> was isolated with nickel-nitriolactric acid resin, separated on a 7.5% SDS-polyacrylamide gel, stained with Coomassie Blue, and analyzed by autoradiography.

In vitro phosphorylation by PKC was carried out according to protocols provided by the vendor. Briefly, 0.8 μM purified recombinant PLCβ<sub>3</sub>(His)<sub>k</sub> or PLCβ<sub>3</sub>Ser<sup>1105</sup>→Ala(His)<sub>k</sub> was incubated with purified constitutively active PKC fragment (0.04 μM) in the presence of 2.5 μCi of [γ-<sup>32</sup>P]ATP and 10 μM ATP in a total volume of 10 μl of PKC buffer (50 mM MES, pH 6.5, 1.25 mM EGTA, 12.5 mM MgCl<sub>2</sub>) for the times specified at 30 °C. Equal amounts of PLCβ<sub>3</sub>(His)<sub>k</sub> were also incubated for 40 min with purified PKC<sub>α</sub> or PKC<sub>γ</sub>(20 ng) in a total volume of 10 μl of reaction buffer (20 mM HEPES, pH 7.4, 100 μM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 100 μg/ml phosphatidylyserine, 20 μg/ml diacylglycerol, 0.03% Triton X-100). Reactions were terminated by adding 10 μl of 2× SDS sample buffer and boiling for 5 min. Proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The phosphorylated bands were localized by autoradiography. The stoichiometry of PLCβ<sub>3</sub> phosphorylation by PKC was determined at 100 min by filter binding assay as described elsewhere (12).

Phosphoamino Acid Analysis, Peptide Mapping, and Sequencing—For two-dimensional tryptic peptide mapping and phosphoamino acid analysis, 32P-labeled PLCβ<sub>3</sub> from in vivo or in vitro phosphorylation reactions was separated by SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue, dried between two layers of drying membranes, and exposed to Biomax-MS x-ray film (Eastman Kodak Co.). PLCβ<sub>3</sub> bands were cut out and rehydrated in 50 mM ammonium bicarbonate, pH 8 (buffer A), overnight. After peeling off the drying membrane, each gel slice was boiled for 5 min in 100 μl of buffer A containing 5 mM dithiothreitol. The tube was cooled to room temperature, 50 μl of 100 mM iodoacetic acid was added, and the tube was incubated for 30 min at the dark at room temperature. The gel slice was washed again in buffer A and ground with a disposable pestle. The residual Coomassie Blue dye was removed by rinsing the gel slurry with...
Inhibition of PLCβ3 by PKC

FIG. 2. PMA inhibits Goa-stimulated PLCβ3 (A) and Gβ3-stimulated PLCβ3 (B) activity in COSM6 cells transfected with plasmids expressing Goa, Gβγ, and PLCβ3. Data are presented as the means ± S.E. (n = 3) of 1 of 3 similar experiments and were analyzed by analysis of variance and Duncan’s test. Groups with different letters are different from each other at p < 0.05.

50 μl of 50% acetonitrile in buffer A. The tube was centrifuged at 15,000 x g for 5 min, and the supernatant was discarded. The pellet was reconstituted in 50 μl of acetonitrile and incubated for 5 min. The tube was centrifuged again, and the pellet was dried in a SpeedVac for 10 min after removal of supernatant. The pellet was resuspended in 75 μl of buffer A, and 5 μg of trypsin was added. The tube was incubated at 37°C for 5 h before the addition of another 5 μg of trypsin, and the total incubation time was between 18 and 24 h. The liquid containing the digested peptides was recovered and further prepared for two-dimensional peptide mapping with a Hunter thin layer electrophoresis system (C.B.S. Scientific Co., Del Mar, CA) according to the protocol provided by the manufacturer. External markers for each dimension were included in each thin layer plate to facilitate the comparison between samples. For phosphoamino acid analysis, about 100 cpm of total tryptic peptides mixture was used. Peptide sequencing using 22p-labeled PLCβ3(His)6 (150 pmol) recombinant protein purified from SF9 cells was carried out as described elsewhere (12).

Cell Culture, Transfection, and PI Turnover—HeLa, COSM6, and RBL-2H3 cells were cultured as described for PHM1-41 cells (33). HeLa and COSM6 cells (1.8 x 10^6/well) were seeded in 6-well plates and transfected 16–24 h later as described (34) with M1 receptor (1 μg), Goa (0.5 μg), Gβγ (0.375 μg), Gβγ (0.375 μg), and PLCβ3 (0.25 μg) as indicated. Empty pCMV vector was added to bring the total amount of plasmid DNA to 1.25 μg per well. For effects of endogenous PKC on agonist-stimulated PI turnover, near confluent PHM1 and RBL-2H3 cells (12-well plates) and COSM6 and HeLa cells (6-well plates) were treated with 1 μM PMA or 100 ng/ml thymoleptol for 30 min in PBS+ (phosphate-buffered saline (PBS) plus 1.2 mM CaCl₂, 1.0 mM MgCl₂, and 1.0 mM glucose) containing 10 mM LiCl prior to stimulation by agonists (100 nM oxytocin, 15 μM carbacol, or 100 nM fMLP) for 30 min. Where indicated, H-89 (10 μM) or Go 6976 (8 μM) were added to PHM1-41 cells. After 15 min, PMA or CPT-cAMP were added, followed by oxytocin 15 min later. For direct stimulation of PLCβ3, by Goa or Gβγ, transfected COSM6 cells were first treated with 1 μM PMA for 30 min in PBS+ followed by addition of 20 mM LiCl for 30 min. Cells were lysed, and total IPs were determined as described (19).

RESULTS

PKC Inhibits Oxytocin, M1 Muscarinic, and fMLP Receptor-initiated PI Turnover—The effect of activation of endogenous PKC on predominantly Goa-coupled oxytocin receptor-initiated PI turnover (35) was studied in PHM1-41 cells, a human myometrial smooth muscle cell line (35). Stimulation of PHM1 cells with 100 nM oxytocin significantly increased the production of total IPs. Pretreating cells with 1 μM PMA completely inhibited this increase (Fig. 1A). The PMA effect was not specific to the oxytocin receptor or to PHM1-41 cells. A similar inhibitory effect of PMA was also evident with Goa-coupled M1 muscarinic receptor transfected into HeLa (Fig. 1B) or COSM6 (Fig. 1C) cells. In addition, PMA also significantly inhibited Goa-coupled fMLP receptor-initiated PI turnover (36) in RBL-2H3 cells (Fig. 1D) in which the only PLCβ form expressed is PLCβ3 (21). This occurred under conditions where the fMLP receptor has been shown not to be phosphorylated by PKC (37). These observations, together with those previously reported (21, 31), establish that the PKC inhibitory effect on G protein-coupled receptor-initiated PI turnover is a general mechanism and that the PKC effect can occur at a post-receptor level.

To investigate the potential role of specific PKCs in this process, the effect of Tx, a specific activator of conventional PKCs (38), was compared with PMA, which activates both conventional and novel PKCs (38), in PHM1-41 and RBL-2H3 cell lines. In both cases, Tx was as effective as PMA in inhibiting oxytocin or fMLP-stimulated PI turnover at the concentration tested (Fig. 1, A and D). In addition, Go 6976, an inhibitor of conventional PKC (39), was able to reverse the PMA inhibitory effect by ~50% at a concentration of 4 μM (data not shown). These data provide evidence that conventional PKCs are capable of inhibiting Goa or Goa-coupled receptor-initiated PI turnover.

PKC Inhibits the Direct Stimulation of PLCβ3 by Goa and Gβγ—Because PLCβ3 is present in all four cell lines mentioned above and can be phosphorylated by PKC, at least in RBL-2H3 cells (21), it is highly possible that PKC inhibits PI turnover by decreasing PLCβ3 activity. If so, PKC should inhibit the direct stimulation of PLCβ3 by Goa or Gβγ. COSM6 cells transfected with both PLCβ3 and Goa plasmids exhibited a marked increase in total [3H]IPs compared with transfection with either plasmid alone (Fig 2A). Consistent with the prediction, pre-treating cells with PMA nearly abolished Goa-stimulated PLCβ3 activity. Tx elicited a similar inhibitory effect on Goa-stimulated PLCβ3 activity (data not shown). Cotransfection of Gβγ and PLCβ3 into COSM6 cells also resulted in marked increase in PI turnover. This increase was significantly reduced by PMA (Fig. 2B), but the reduction was not of the magnitude observed for Goa-stimulated PLCβ3 activity. Thus PKC inhibition of PI turnover occurs at a post-receptor level, and this effect may require the phosphorylation of PLCβ3.

Phosphorylation of PLCβ3 by PKC in Vivo and in Vitro—PLCβ3 were expressed in COSM6 cells exhibited significant 32P incorporation under basal conditions. Nonetheless, PMA induced a substantial increase in 32P incorporation into PLCβ3 (Fig. 3A). The phosphorylation of PLCβ3 by PKC was investigated further in vitro. Purified recombinant PLCβ3 was incubated with catalytically active PKC fragments (a rat brain mixture of multiple PKC isotypes, including α, β, and γ) in the presence of [γ-32P]ATP. As shown in Fig. 3B, PLCβ3 was phos-
Inhibition of PLCβ3 by PKC

**FIG. 4.** Two-dimensional tryptic peptide mapping of PLCβ3(His)6 32P-labeled *in vivo* (A and B) or *in vitro* (C). Two markers were applied on each TLC plate as migration controls for each dimension. The black markers on the top of each panel indicate the position of one such marker; others outside of the displayed region were also used in lining up the plates. “O” depicts the sample origin. The predominant PKC-stimulated phosphorylation site is indicated by the arrows (B and C) and the minor sites by the arrowheads. Longer exposure of C revealed some minor sites as well. D, two-dimensional phosphoamino acid analysis of PLCβ3(His)6 phosphorylated by PKC in *in vitro*. The dotted circles indicate the migration positions of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) standards.

**FIG. 5.** A. 32P distribution among fractions collected after reverse-phase high pressure liquid chromatography separation of Lys C-digested PLCβ3(His)6 labeled with 32P *in vitro*. Fraction 12 has ~60% of the total 32P. B, sequence of peptides in fraction 12 and associated 32P. The serine residue with more than 90% of total 32P loaded onto the sequencing membrane is denoted by *. C, 32P distribution among fractions collected after reverse-phase high pressure liquid chromatography separation of Lys C-digested PLCβ3(His)6 purified from Sf9 cells. The Coomassie Blue staining (Coomassie) and autoradiography (autorad) of the same gel are shown.

**FIG. 6.** Mutation of Ser1105 to Ala (S/A) reversed the inhibition by PKC of Gαq-stimulated PLCβ3 in COSM6 cells transfected with plasmids expressing Gαq and PLCβ3 plasmids. Data are presented as the means ± S.E. (n = 3) of 1 of 3 similar experiments and were analyzed by analysis of variance and Duncan’s test. Groups with different letters are different from each other at p < 0.05.

phosphorylated in a time-dependent manner. A stoichiometry of 0.4 mol of phosphate/PLCβ3 was achieved after incubation with PKC for 100 min under these conditions. In similar experiments, no phosphorylation was seen in the absence of PKC (data not shown). Purified PKCβ3 or PKCy also phosphorylated PLCβ3 in *in vitro*, whereas no phosphorylation of PLCβ3 was observed in the absence of kinase (Fig. 3C).

**Ser**1105** Is the Predominant Phosphorylation Site for PKC**—As shown by two-dimensional phosphopeptide mapping of *in vitro* 32P-labeled PLCβ3, trypsin digestion yielded multiple phosphopeptides in the basal state (Fig. 4A). PKA specifically induced phosphorylation on one predominant site (Fig. 4B, indicated by the arrow). Minor sites increased by PMA were also present (indicated by arrowhead). We cannot exclude the contribution of incomplete digestion by trypsin to this pattern.

**In vitro**, PKC phosphorylated PLCβ3 on one predominant site (Fig. 4C, arrow). The migration of this peptide relative to the standards was identical to those observed in digests after *in vivo* phosphorylation. The phosphorylation occurred exclusively on serine residues (Fig. 4D). We utilized *in vitro* phosphorylated recombinant PLCβ3(His)6 to identify the PKC phosphorylation sites. After isolation by SDS-polyacrylamide gel electrophoresis, 32P-labeled PLCβ3 was digested with Lys C instead of trypsin to achieve more complete cleavage and fewer peptides (12). The digestion mixture was separated by reverse-phase high pressure liquid chromatography, and fractions were recovered and counted. Fig. 5A shows the 32P distribution among these fractions. About 8% of 32P was found in the follow-through (fraction −1 to −4) and appeared to be free 32P as judged by phosphopeptide mapping (data not shown). Nearly 60% of the total 32P was recovered in fraction 12. This fraction was subjected to peptide sequencing. Although two peptides

were identified in this fraction, nearly 90% of the total 32P was found in the fourth cycle (Fig. 5B). This clearly identified Ser1105 and not Ser1107 in the peptide Arg-His-Asn-Ser1105-Leu-Ser-Glu-Ala-Lys as the amino acid phosphorylated. Furthermore, mutation of Ser1105 significantly diminished PLCβ3 phosphorylation by PKC *in vitro* (Fig. 5C). This strongly argues that Ser1105 is the predominant site for PKC. Residual weak phosphorylation associated with Ser1105 → Ala mutant PLCβ3 could indicate the presence of other minor sites. Interestingly, Ser1105, unique to PLCβ3 among the PLCβ isoforms, is preferentially phosphorylated by PKA as well (12).
stimulated Ser1105

(6)

mutant PLC

b

Ala mutant PLC

PLC

b

Ser1105

Ser1105

6

mutant PLC

versus PKA—

We have previously shown that phosphorylation by PKA of Ser1105 is required for inhibition of Gβγ-stimulated PLCβ3 activity. The N-terminal region of PLCβ3 appears to contribute to its interaction with Gβγ (40). We had identified Ser26 in the peptide Arg-Arg-Gly-Ser-Lys as a potential phosphorylation site in this region. However, there was no effect of mutating Ser26 to Ala on PKC inhibition of Gβγ-stimulated PLCβ3 activity (Fig. 7A).

In the face of the inability of mutation of Ser1105 and Ser26 to reverse the effect of PKC on Gβγ-stimulated PLCβ3 activity, we examined the effect of mutation of these residues on PKA-mediated inhibition as well. As seen in Fig. 7B, PKA inhibited Gβγ-stimulated PLCβ3 activity. Mutation of Ser1105 or Ser26 also had no effect on inhibition by PKA of Gβγ-stimulated PLCβ3 activity.

Inhibition of Oxytocin-stimulated Total IP Production in PHM1-41 Cells by PKC or PKA Represents Independent Pathways—Phosphorylation of Ser1105 by PKC or PKA suppressed Goγ-stimulated PLCβ3 activity. This fact raised the interesting possibility that PKC activation might lead to PKA activation, resulting in indirect phosphorylation of PLCβ3 at the PKA site or vice versa. We addressed this possibility in PHM1-41 cells. As shown in Fig. 8, H-89, a specific PKA inhibitor, reversed the inhibition by cAMP but did not affect the inhibition by PMA of oxytocin-stimulated PI turnover. Similarly, Go6976, a specific PKC inhibitor, significantly diminished the inhibitory effect of PMA but not of cAMP on oxytocin-stimulated PI turnover. These data indicate that PKC and PKA exert their inhibitory effects independent of each other.

DISCUSSION

We have presented evidence that PKC inhibits Goγ-coupled (oxytocin and M1 muscarinic) and Goγ-coupled receptor (IMLR) receptor-initiated PI turnover in four different cell lines expressing PLCβ3. The response to endogenous PKC activation by PMA differs in order of magnitude between cell lines and the state of the receptor (endogenous or transfected). This variation may reflect differences in relative membrane permeability of PMA and the localization and abundance of the PKC isoforms responsible or the relative contribution of Goγ-coupling to PLCβ3 to PI turnover. We have also demonstrated in cotransfection assays that the PKC inhibitory effect occurred at the G protein-PLCβ3 level, and we have provided direct evidence to support the hypothesis that phosphorylation of PLCβ3 inhibition of Gβγ-stimulated PLCβ3 activity. The Ser1105 region of PLCβ3 appears to contribute to its interaction with Gβγ (40). We had identified Ser26 in the peptide Arg-Arg-Gly-Ser-Lys as a potential phosphorylation site in this region. However, there was no effect of mutating Ser26 to Ala on PKC inhibition of Gβγ-stimulated PLCβ3 activity (Fig. 7A).

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is involved in the PKC inhibitory effect on Goq-coupled activation.

The use of in vitro phosphorylated PLCb3 for identifying the PKC phosphorylation site is supported by the demonstration that a similar site was phosphorylated by PKC in vivo and in vitro. PKC phosphorylates predominantly one residue, Ser1105, which is also phosphorylated by PKA (12). The marked reduction of in vitro phosphorylation of the Ser1105 → Ala PLCb3 mutant further corroborates this finding. However, the remaining weak phosphorylation associated with this mutant indicates that PKC may phosphorylate other minor sites as well.

Mutation of Ser1105 to Ala reversed completely the inhibition of Goq-stimulated PLCb3 activity by PKC. This provides conclusive evidence for the direct inhibition of PLCb3 by PKC, a response identical to that seen previously for PKA (12). We also demonstrated that the inhibitory effect of PKC occurs in the absence of PKA inhibition, suggesting that it is not a consequence of indirect PKA activation. The convergence of PKC and PKA on Ser1105 underscores the importance of Ser1105 in the regulation of Goq-stimulated PLCb3 activity in diverse cellular processes and suggests possible redundancy for the inhibition of PLCb3 activity by these two kinases. In addition, these data also argue that the effect of PKC or PKA targets PLCb3 and not G protein or proteins involved in the production of substrate phosphatidylinositol 4,5-bisphosphate, as mutation of Ser1105 completely reverses the inhibition by PKC or PKA of Goq-stimulated PLCb3 activity.

In marked contrast, Ser1105 does not appear to be critical for inhibition of GDPγ-stimulated PLCb3 activity by either PKC or PKA. Ser266 was also not required, although the N-terminal region of PLCb3 appears to contribute to its interaction with GDPγ (40). At present the mechanism for the inhibition of GDPγ-stimulated PLCb3 activity by PKC or PKA remains unknown. It is unlikely that Gβ1γ2 is the direct target for the inhibitory effects of PKA or PKC as these proteins are not phosphorylated by PKC or PKA in vitro.2 Identification of PKA or PKC minor phosphorylation sites in some proteins or nucleotides may help to solve this question. Alternatively, the mechanism may involve phosphorylation of other molecules indirectly involved in the coupling (12).

The effects of a conventional PKC-specific activator and an inhibitor indicate that conventional PKCs are capable of phosphorylating PLCb3. This conclusion is supported by in vitro phosphorylation of PLCb3 by the constitutively active PKC fragment and by purified PKCb3 and PKCy. The wide distribution of conventional PKCs (26) and PLCb3 (2) in tissues correlates well with the generality of the PKC inhibitory effect on receptor-initiated PI turnover.

We conclude that conventional PKCs phosphorylate PLCb3 and inhibit Goq and GDPγ-stimulated PLCb3 activity. PKC and PKA act similarly in that they inhibit Goq-stimulated PLCb3 as a result of phosphorylation of Ser1105. Moreover, PKA and PKC both inhibit GDPγ-stimulated activity by mechanisms that do not involve Ser1105.

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