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Molecular Mechanism of the Inhibition of Phospholipase C β_3 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 phosphatidylinositide (PI) turnover and intracellular Ca^{2+} release. Activation of endogenous PKC by phorbol 12-myristate 13-acetate inhibited both Gα_q-coupled (oxytocin and M1 muscarinic) and Gα_i-coupled (formyl-Met-Leu-Phe) receptor-stimulated PI turnover by 50–100% in PHM1, HeLa, COSM6, and RBL-2H3 cells expressing PLCβ_3. Activation of conventional PKCs with thymeleatoxin similarly inhibited oxytocin or formyl-Met-Leu-Phe receptor-stimulated PI turnover. The PKC inhibitory effect was also observed when PLCβ_3 was stimulated directly by Gα_q or G$\beta\gamma$ in overexpression assays. PKC phosphorylated PLCβ_3 at the same predominant site *in vivo* and *in vitro*. Peptide sequencing of *in vitro* phosphorylated recombinant PLCβ_3 and site-directed mutagenesis identified Ser^{1105} as the predominant phosphorylation site. Ser^{1105} 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 Gα_q-stimulated PLCβ_3 activity was completely abolished by mutation of Ser^{1105} to Ala. In contrast, mutation of Ser^{1105} or Ser^{26}, another putative phosphorylation target, to Ala had no effect on inhibition of G$\beta\gamma$-stimulated PLCβ_3 activity by PKC or PKA. These data indicate that PKC and PKA act similarly in that they inhibit Gα_q-stimulated PLCβ_3 as a result of phosphorylation of Ser^{1105}. Moreover, PKC and PKA both inhibit G$\beta\gamma$-stimulated activity by mechanisms that do not involve Ser^{1105}.

Stimulation of seven transmembrane receptors coupled to the Gα_q or Gα_i subunits of heterotrimeric G proteins results in activation of PLCβ[1] isoforms that hydrolyze phosphatidylinositol 4,5-bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (1, 2). IP_3 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, PLCβ_3 is ubiquitously expressed and activated by all known PLCβ activators (Gα_q, G$\beta\gamma$, and calcium) (2). Regulation of PLCβ_3 may be of great importance in many cellular processes (11–15). Insufficient expression of PLCβ_3 has been correlated with increased sensitivity to tumor formation (15, 16), whereas overexpression of PLCβ_3 seems to suppress tumor growth (17). PLCβ_3 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 PLCβ_3 or PLCβ_2 by PKA inhibits their activity and establishes a mechanism for cross-talk between Gα_q- or Gα_i-coupled and Gα_s-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, β_1, β_2, 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 Gα_q- or Gα_i-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 PLCβ_1 and PLCβ_2 by PKC has been reported (23, 24, 29, 30), the physiological relevance of these observations has not been demonstrated. PLCβ_t, a turkey PLCβ isoform with highest homology to PLCβ_2, is phosphorylated by conventional PKCs, and its catalytic activity is inhibited (29). PLCβ_3 is not phosphorylated by PKCα *in vitro* (23). Nonetheless, a correlation between PLCβ_3 phosphorylation and PKC inhibition of receptor-initiated PI turnover has been reported (21, 31).

To determine the importance of PLCβ_3 phosphorylation by PKC, we have identified the phosphorylation site on PLCβ_3 and investigated which PKC subfamily can catalyze the phospho-

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[1] The abbreviations used are: PLC, phospholipase C; PI, phosphatidylinositide; IP_3, phosphatidylinositol 1,4,5-trisphosphate; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; fMLP, formyl-Met-Leu-Phe; PMA, phorbol 12-myristate 13-acetate; Tx, thymeleatoxin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CPT-cAMP, 8-[4-chlorophenythiol]-cAMP; MES, 4-morpholineethanesulfonic acid.

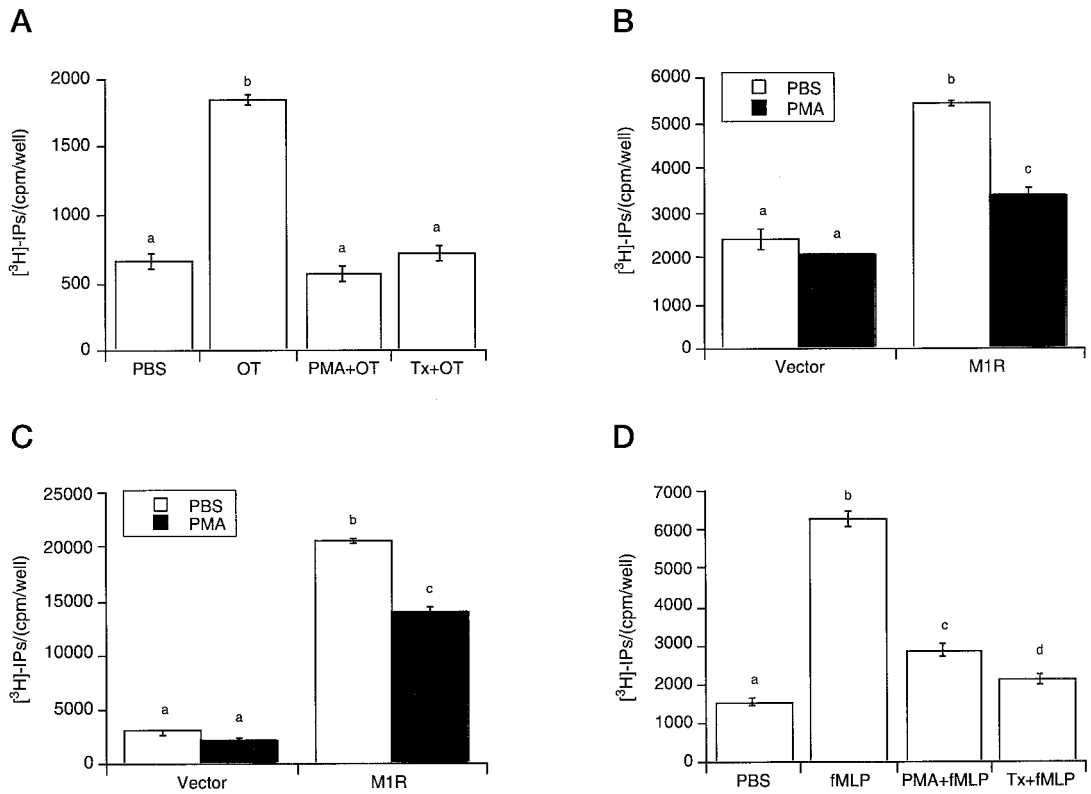

FIG. 1. **Prior treatment with PMA or Tx inhibits oxytocin (*OT*), carbachol, or fMLP-stimulated total IP production in PHM1–41 (*A*), HeLa (*B*), COSM6 (*C*) or RBL-2H3 (*D*) cells, respectively.** HeLa and COSM6 cells were transfected with (*M1R*) or without (*Vector*) a plasmid expressing M1 receptor and were stimulated with 15 μM carbachol. Where indicated, PBS was used as control reagent. Data are presented as the means ± S.E. ($n = 3$) of 1 of 2–4 similar experiments and were analyzed by analysis of variance and Duncan's test. Groups with different letters are different from each other at $p < 0.05$.

rylation. We report the identification of Ser^{1105} as the predominant PKC phosphorylation site, the involvement of conventional PKCs in this phosphorylation, and the convergence of PKC and PKA on phosphorylation and inhibition of PLCβ$_3$ by Gα$_q$. Furthermore, we find that Gβγ-stimulated PLCβ$_3$ activity is inhibited by both PKC and PKA by mechanisms that do not involve Ser^{1105} phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Thymeleatoxin (Tx), Gö 6976, PKC catalytic fragment, PKCβ$_1$, and PKCγ were obtained from Calbiochem. H-89 was purchased from Seikagaku America, Inc. (Rockville MD). PMA, CPT-cAMP (8-[4-chlorophenythiol]-cAMP), and other chemicals were 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. [^{3}H]Inositol (22 Ci/mmol) was obtained from American Radiolabeled Chemical Co. (St. Louis, MO), [^{32}P]orthophosphate (5 mCi/ml) and γ-[^{32}P]ATP (3000 Ci/mmol) 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$_3$, Gα$_q$, Gβ$_1$, and Gγ$_2$ plasmids were constructed as described elsewhere (12, 32). Site-directed mutation of Ser^{26} to Ala was achieved with the mutagenic primer (5′-CGGCGCGGGGCTAAGTTCATCAAATGG-3′) identically as described for the Ser^{1105} → Ala mutation (12) using GeneEditor. All plasmid sequences were confirmed by DNA sequencing. Construction of baculovirus containing PLCβ$_3$ Ser^{1105} → Ala-(His)$_6$ and purification of the recombinant protein from Sf9 cells were carried out as described for PLCβ$_3$ (His)$_6$ (12).

In Vivo and in Vitro ^{32}P Labeling and Isolation of PLCβ$_3$—For *in vivo* phosphorylation, COSM6 cells seeded in 6-well plates were transfected with PLCβ$_3$(His)$_6$ plasmid and metabolically labeled with [^{32}P] 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$_3$(His)$_6$ was isolated with nickel-nitrilotriacetic 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$_3$(His)$_6$ or PLCβ$_3$Ser^{1105} → Ala(His)$_6$ was incubated with purified constitutively active PKC fragment (0.04 μM) in the presence of 2.5 μCi of [γ-^{32}P]ATP and 100 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 $MgCl_2$) for the times specified at 30 °C. Equal amounts of PLCβ$_3$(His)$_6$ were also incubated for 40 min with purified PKCβ$_1$ or PKCγ (20 ng) in a total volume of 10 μl of reaction buffer (20 mM HEPES, pH 7.4, 100 μM $CaCl_2$, 10 mM $MgCl_2$, 100 μg/ml phosphatidylserine, 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$_3$ 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, ^{32}P-labeled PLCβ$_3$ 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$_3$ 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 in 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

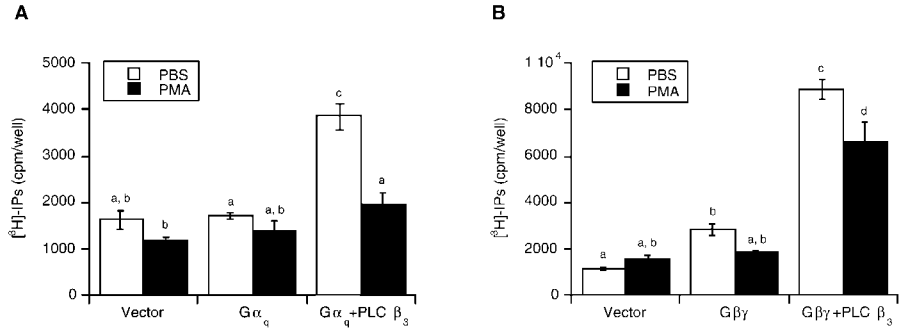

FIG. 2. **PMA inhibits $G\alpha_q$-stimulated $PLC\beta_3$ (*A*) and $G\beta\gamma$-stimulated $PLC\beta_3$ (*B*) activity in COSM6 cells transfected with plasmids expressing $G\alpha_q$, $G\beta\gamma$, and $PLC\beta_3$.** Data are presented as the means ± S.E. ($n = 3$) of 1 of 3 similar experiments 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 × *g* for 5 min, and the supernatant was discarded. The pellet was resuspended 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 2 μg of trypsin was added. The tube was incubated at 37 °C for 5 h before the addition of another 2 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 ^{32}P-labeled $PLC\beta_3(His)_6$ (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 (1.8×10^5/well) were seeded in 6-well plates and transfected 16–24 h later as described (34) with M1 receptor (1 μg), $G\alpha_q$ (0.5 μg), $G\beta_1$ (0.375 μg), $G\gamma_2$ (0.375 μg), and $PLC\beta_3$ (0.25 μg) as indicated. Empty rcCMV 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 thymeleatoxin for 30 min in PBS+ (phosphate-buffered saline (PBS) plus 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+}, and 1.0 mM glucose) containing 10 mM LiCl prior to stimulation by agonists (100 nM oxytocin, 15 μM carbachol, or 100 nM fMLP) for 30 min. Where indicated, H-89 (10 μM) or Gö 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 $PLC\beta_3$ by $G\alpha_q$ or $G\beta_1\gamma_2$, 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 $G\alpha_q$-coupled oxytocin receptor-initiated PI turnover (35) was studied in PHM1-41 cells, a human myometrial smooth muscle cell line (33). 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. 1*A*). 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 $G\alpha_q$-coupled M1 muscarinic receptor transfected into HeLa (Fig. 1*B*) or COSM6 (Fig. 1*C*) cells. In addition, PMA also significantly inhibited $G\alpha_i$-coupled fMLP receptor-initiated PI turnover (36) in RBL-2H3 cells (Fig. 1*D*) in which the only PLCβ form expressed is $PLC\beta_3$ (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, Gö 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 $G\alpha_q$- or $G\alpha_i$-coupled receptor-initiated PI turnover.

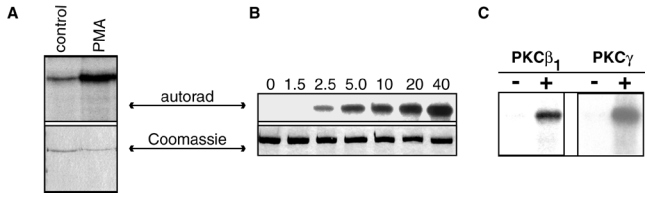

FIG. 3. *A, in vivo* ^{32}P labeling of $PLC\beta_3(His)_6$ isolated from COSM6 cells transfected with $PLC\beta_3(His)_6$ plasmid and pretreated with (*PMA*) or without (*control*) PMA. *B,* time-dependent phosphorylation of $PLC\beta_3(His)_6$ by PKC *in vitro.* Reactions were terminated at the times indicated in minutes. Coomassie Blue staining of the respective gels is shown *below* the autoradiographs. *C,* autoradiography of $PLC\beta_3(His)_6$ after incubation without (−) or with (+) purified $PKC\beta_1$ and PKCγ *in vitro*.

PKC Inhibits the Direct Stimulation of $PLC\beta_3$ by $G\alpha_q$ and $G\beta\gamma$—Because $PLC\beta_3$ 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 $PLC\beta_3$ activity. If so, PKC should inhibit the direct stimulation of $PLC\beta_3$ by $G\alpha_q$ or $G\beta\gamma$. COSM6 cells transfected with both $PLC\beta_3$ and $G\alpha_q$ plasmids exhibited a marked increase in total [^{3}H]IPs compared with transfection with either plasmid alone (Fig. 2*A*). Consistent with the prediction, pretreating cells with PMA nearly abolished $G\alpha_q$-stimulated $PLC\beta_3$ activity. Tx elicited a similar inhibitory effect on $G\alpha_q$-stimulated $PLC\beta_3$ activity (data not shown). Cotransfection of $G\beta_1\gamma_2$ and $PLC\beta_3$ into COSM6 cells also resulted in marked increase in PI turnover. This increase was significantly reduced by PMA (Fig. 2*B*), but the reduction was not of the magnitude observed for $G\alpha_q$-stimulated $PLC\beta_3$. Thus PKC inhibition of PI turnover occurs at a post-receptor level, and this effect may require the phosphorylation of $PLC\beta_3$.

Phosphorylation of $PLC\beta_3$ by PKC in Vivo and in Vitro—$PLC\beta_3$ overexpressed in COSM6 cells exhibited significant ^{32}P incorporation under basal conditions. Nonetheless, PMA induced a substantial increase in ^{32}P incorporation into $PLC\beta_3$ (Fig. 3*A*). The phosphorylation of $PLC\beta_3$ by PKC was investigated further *in vitro*. Purified recombinant $PLC\beta_3$ was incubated with catalytically active PKC fragments (a rat brain mixture of multiple PKC isoforms, including α, β, and γ) in the presence of [γ-^{32}P]ATP. As shown in Fig. 3*B*, $PLC\beta_3$ was phos-

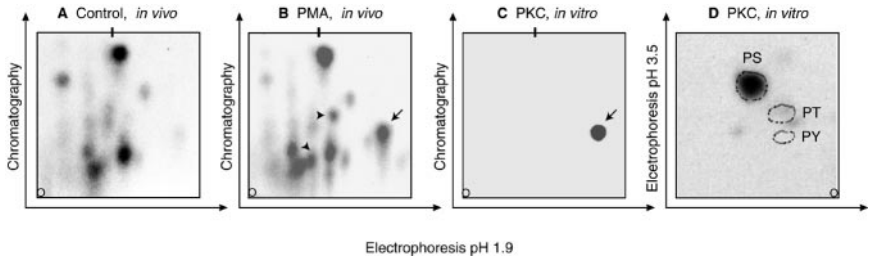

FIG. 4. **Two-dimensional tryptic peptide mapping of PLCβ_3(His)$_6$ ^{32}P-labeled *in vivo* (*A* and *B*) or *in vitro* (*C*).** Two markers were applied on each TLC plate as migration controls for each dimension. The *black markers* on the *top* of each panel indicate the position of one such marker; 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 PLCβ_3(His)$_6$ phosphorylated by PKC *in vitro*. The *dotted circles* indicate the migration positions of phosphoserine (*PS*), phosphothreonine (*PT*), and phosphotyrosine (*PY*) standards.

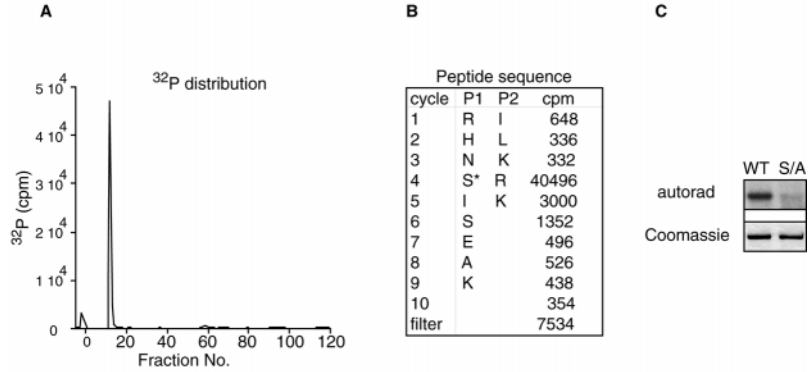

FIG. 5. *A,* ^{32}P distribution among fractions collected after reverse-phase high pressure liquid chromatography separation of Lys C-digested PLCβ_3(His)$_6$ labeled with ^{32}P *in vitro*. Fraction 12 has ~60% of the total ^{32}P. *B,* sequence of peptides in fraction 12 and associated ^{32}P. The serine residue with more than 90% of total ^{32}P loaded onto the sequencing membrane is denoted by *. *filter* represents ^{32}P left on the sequencing membrane after 10 cycles. *C, in vitro* phosphorylation by PKC (30 min at 30 °C) of recombinant wild type (*WT*) or Ser^{1105} → Ala mutant (*S/A*) PLCβ_3(His)$_6$ purified from Sf9 cells. The Coomassie Blue staining (*Coomassie*) and autoradiography (*autorad*) of the same gel are shown.

phorylated in a time-dependent manner. A stoichiometry of 0.4 mol of phosphate/PLCβ_3 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 PKCβ_1 or PKCγ also phosphorylated PLCβ_3 *in vitro*, whereas no phosphorylation of PLCβ_3 was observed in the absence of kinase (Fig. 3*C*).

Ser^{1105} Is the Predominant Phosphorylation Site for PKC—As shown by two-dimensional phosphopeptide mapping of *in vivo* ^{32}P-labeled PLCβ_3, trypsin digestion yielded multiple phosphopeptides in the basal state (Fig. 4*A*). PMA specifically induced phosphorylation on one predominant site (Fig. 4*B,* 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 PLCβ_3 on one predominant site (Fig. 4*C*, *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. 4*D*). We utilized *in vitro* phosphorylated recombinant PLCβ_3(His)$_6$ to identify the PKC phosphorylation sites. After isolation by SDS-polyacrylamide gel electrophoresis, ^{32}P-labeled PLCβ_3 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. 5*A* shows the ^{32}P distribution among these fractions. About 8% of ^{32}P was found in the follow-through (fraction −1 to −4) and appeared to be free ^{32}P as judged by phosphopeptide mapping (data not shown). Nearly 60% of the total ^{32}P was recovered in fraction 12. This fraction was subjected to peptide sequencing. Although two peptides

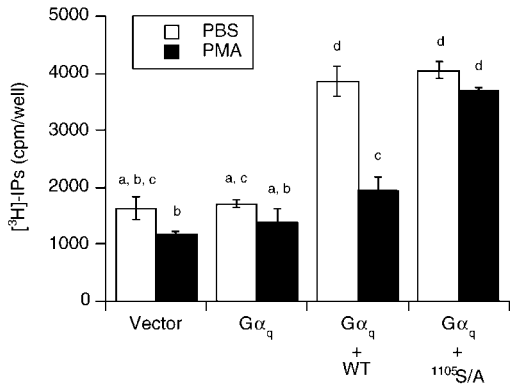

FIG. 6. **Mutation of Ser^{1105} to Ala (*S/A*) reversed the inhibition by PKC of Gα_q-stimulated PLCβ_3 in COSM6 cells transfected with plasmids expressing Gα_q and PLCβ_3 plasmids.** Data are presented as the means ± S.E. (n = 3) of 1 of 3 similar experiments and were analyzed by analysis of variance and Duncan's test. Groups with different letters are different from each other at $p < 0.05$.

were identified in this fraction, nearly 90% of the total ^{32}P was found in the fourth cycle (Fig. 5*B*). This clearly identified Ser^{1105} and not Ser^{1107} in the peptide Arg-His-Asn-Ser^{1105}-Ile-Ser-Glu-Ala-Lys as the amino acid phosphorylated. Furthermore, mutation of Ser^{1105} significantly diminished PLCβ_3 phosphorylation by PKC *in vitro* (Fig. 5*C*). This strongly argues that Ser^{1105} is the predominant site for PKC. Residual weak phosphorylation associated with Ser^{1105} → Ala mutant PLCβ_3 could indicate the presence of other minor sites. Interestingly, Ser^{1105}, unique to PLCβ_3 among the PLCβ isoforms, is preferentially phosphorylated by PKA as well (12).

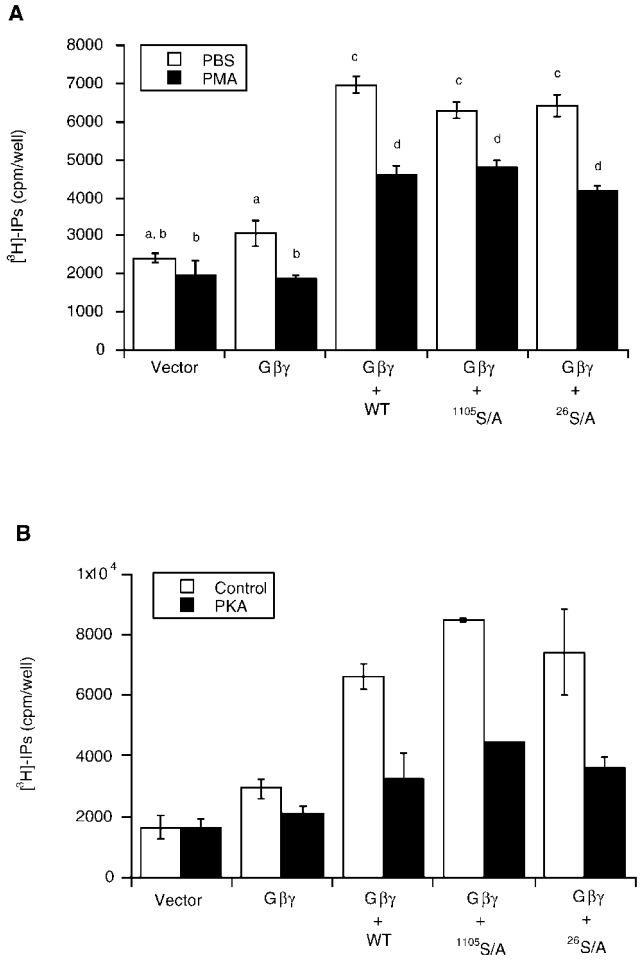

FIG. 7. **Inhibition of Gβγ-stimulated Ser^{1105} → Ala and Ser^{26} → Ala mutant PLCβ$_3$ activity by PKC (*A*) or PKA (*B*) in COSM6 cells transfected with plasmids expressing Gβγ, wild type (*WT*), Ser^{1105} → Ala or Ser^{26} → Ala mutant PLCβ$_3$.** *A,* data are presented as the means ± S.E. ($n = 3$) 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 $p < 0.05$. *B,* plasmid encoding the PKA catalytic subunit was cotransfected into COSM6 cells (*filled bars*), and its expression was induced with 60 μM $ZnSO_4$ for 24 h after transfection. Data represent the mean of duplicate determinations (range denoted by the *error bars*) in 1 of 2 similar experiments.

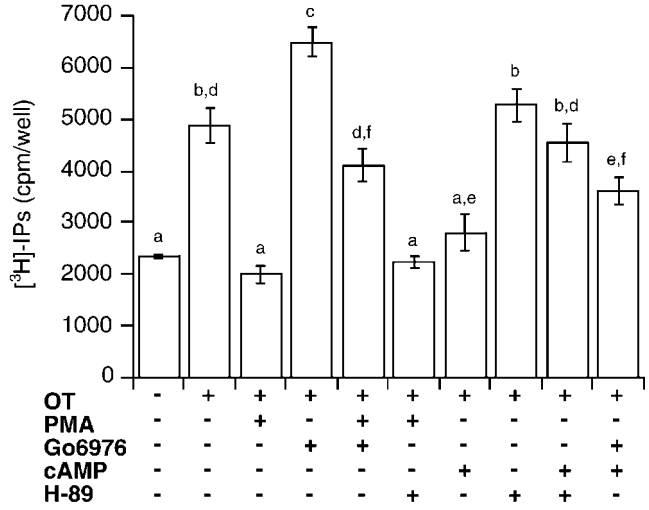

FIG. 8. **Inhibition of oxytocin-stimulated total IP production in PHM1–41 cells by PKC or PKA represents independent pathways.** Data are presented as the means ± S.E. ($n = 3$) from 1 of 2 similar experiments and were analyzed by analysis of variance and Duncan's test. Groups with different letters are different from each other at $p < 0.05$.

Functional Analysis of Phosphorylation of PLCβ$_3$ by PKC Versus PKA—We have previously shown that phosphorylation by PKA of Ser^{1105} is required for inhibition of Gα$_q$-stimulated PLCβ$_3$ activity by PKA. The finding that PKC also phosphorylates Ser^{1105} suggested that the same mechanism was utilized by PKC. To test this hypothesis, the Ser^{1105} → Ala mutant PLCβ$_3$ was cotransfected with Gα$_q$ into COSM6 cells, and the effect of PMA was evaluated. As shown before (12), the Ser^{1105} → Ala mutant PLCβ$_3$ was as effective as the wild type enzyme in coupling to Gα$_q$ (Fig. 6). Importantly, PMA inhibited Gα$_q$-stimulated wild type PLCβ$_3$ activity but had no effect on Gα$_q$-stimulated Ser^{1105} → Ala PLCβ$_3$ activity. These data unequivocally identify phosphorylation of Ser^{1105} by PKC as responsible for PKC inhibition of Gα$_q$-stimulated PLCβ$_3$ activity.

We also investigated the effect of mutating Ser^{1105} on PKC inhibition of Gβγ-stimulated PLCβ$_3$ activity. The Ser^{1105} → Ala mutant PLCβ$_3$ was as effective as wild type PLCβ$_3$ in coupling to Gβ$_1$γ$_2$ (Fig. 7*A*). However in contrast to Gα$_q$-stimulated PLCβ$_3$ activity, PKC inhibited Gβ$_1$γ$_2$-stimulated Ser^{1105} → Ala mutant PLCβ$_3$ activity to the similar degree as it did the wild type PLCβ$_3$. Thus Ser^{1105} is not absolutely required for PKC inhibition of Gβ$_1$γ$_2$-stimulated PLCβ$_3$ activity. The N-terminal region of PLCβ$_3$ appears to contribute to its interaction with Gβγ (40). We had identified Ser^{26} in the peptide Arg-Arg-Gly-Ser-Lys as a potential phosphorylation site in this region. However, there was no effect of mutating Ser^{26} to Ala on PKC inhibition of Gβγ-stimulated PLCβ$_3$ activity (Fig. 7*A*).

In the face of the inability of mutation of Ser^{1105} and Ser^{26} to reverse the effect of PKC on Gβγ-stimulated PLCβ$_3$ activity, we examined the effect of mutation of these residues on PKA-mediated inhibition as well. As seen in Fig. 7*B*, PKA inhibited Gβγ-stimulated PLCβ$_3$ activity. Mutation of Ser^{1105} or Ser^{26} also had no effect on inhibition by PKA of Gβγ-stimulated PLCβ$_3$ activity.

Inhibition of Oxytocin-stimulated Total IP Production in PHM1-41 Cells by PKC or PKA Represents Independent Pathways—Phosphoryation of Ser^{1105} by PKC or PKA suppressed Gα$_q$-stimulated PLCβ$_3$ activity. This fact raised the interesting possibility that PKC activation might lead to PKA activation, resulting in indirect phosphorylation of PLCβ$_3$ 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, Gö 6976, 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 Gα$_q$-coupled (oxytocin and M1 muscarinic) and Gα$_i$-coupled receptor (fMLP) receptor-initiated PI turnover in four different cell lines expressing PLCβ$_3$. 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 Gα$_q$-coupling to PLCβ$_3$ to PI turnover. We have also demonstrated in co-transfection assays that the PKC inhibitory effect occurred at the G protein-PLCβ$_3$ level, and we have provided direct evidence to support the hypothesis that phosphorylation of PLCβ$_3$

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

The use of *in vitro* phosphorylated PLCβ_3 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, Ser^{1105}, that is also phosphorylated by PKA (12). The marked reduction of *in vitro* phosphorylation of the Ser^{1105} → Ala PLCβ_3 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 Ser^{1105} to Ala reversed completely the inhibition of Gα_q-stimulated PLCβ_3 activity by PKC. This provides conclusive evidence for the direct inhibition of PLCβ_3 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 Ser^{1105} underscores the importance of Ser^{1105} in the regulation of Gα_q-stimulated PLCβ_3 activity in diverse cellular processes and suggests possible redundancy for the inhibition of PLCβ_3 activity by these two kinases. In addition, these data also argue that the effect of PKC or PKA targets PLCβ_3 and not G protein or proteins involved in the production of substrate phosphatidylinositol 4,5-bisphosphate, as mutation of Ser^{1105} can completely reverse the inhibition by PKC or PKA of Gα_q-stimulated PLCβ_3 activity.

In marked contrast, Ser^{1105} does not appear to be critical for inhibition of G$\beta\gamma$-stimulated PLCβ_3 activity by either PKC or PKA. Ser^{26} was also not required, although the N-terminal region of PLCβ_3 appears to contribute to its interaction with G$\beta\gamma$ (40). At present the mechanism for the inhibition of G$\beta\gamma$-stimulated PLCβ_3 activity by PKC or PKA remains unknown. It is unlikely that G$\beta_1\gamma_2$ 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 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 PLCβ_3. This conclusion is supported by *in vitro* phosphorylation of PLCβ_3 by the constitutively active PKC fragment and by purified PKCβ_1 and PKCγ. The wide distribution of conventional PKCs (26) and PLCβ_3 (2) in tissues correlates well with the generality of the PKC inhibitory effect on receptor-initiated PI turnover.

We conclude that conventional PKCs phosphorylate PLCβ_3 and inhibit Gα_q- and G$\beta\gamma$-stimulated PLCβ_3 activity. PKC and PKA act similarly in that they inhibit Gα_q-stimulated PLCβ_3 as a result of phosphorylation of Ser^{1105}. Moreover, PKA and PKC both inhibit G$\beta\gamma$-stimulated activity by mechanisms that do not involve Ser^{1105}.

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[2] C. Yue and B. M. Sanborn, unpublished observations.

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