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Protein Kinase C-Promoted Inhibition of $G\alpha_{11}$-Stimulated Phospholipase C-β Activity

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ABSTRACT

The effects of protein kinase C (PKC) activation on inositol lipid signaling were examined. Using the turkey erythrocyte model of receptor-regulated phosphoinositide hydrolysis, we developed a membrane reconstitution assay to study directly the effects of activation of PKC on the activities of $G\alpha_{11}$, independent of potential effects on the receptor or on PLC-β. Membranes isolated from erythrocytes pretreated with 4β-phorbol-12β-myristate-13α-acetate (PMA) exhibited a decreased capacity for $G\alpha_{11}$-mediated activation of purified, reconstituted PLC-β1. This inhibitory effect was dependent on both the time and concentration of PMA incubation and occurred as a decrease in the efficacy of GTPγS for activation of PLC-β1, both in the presence and absence of agonist; no change in the apparent affinity for the guanine nucleotide occurred. Similar inhibitory effects were observed after treatment with the PKC activator phorbol-12,13-dibutyrate but not after treatment with an inactive phorbol ester. The inhibitory effects of PMA were prevented by coaddition of the PKC inhibitor bisindolylmaleimide. Although the effects of PKC could be localized to the membrane, no phosphorylation of $G\alpha_{11}$ occurred either in vitro in the presence of purified PKC or in intact erythrocytes after PMA treatment. These results support the hypothesis that a signaling protein other than $G\alpha_{11}$ is the target for PKC and that PKC-promoted phosphorylation of this protein results in a phosphorylation-dependent suppression of $G\alpha_{11}$-mediated PLC-β1 activation.

Various receptors transduce signals through heterotrimeric G proteins of the G_q family, resulting in activation of phospholipase C (PLC)-β isoenzymes and subsequent cleavage of membrane phosphatidylinositol(4,5)P_2 [PtdIns(4,5)P_2] to the second messengers inositol(1,4,5)P_3 [Ins(1,4,5)P_3] and diacylglycerol (Berridge and Irvine, 1987). Ins(1,4,5)P_3 initiates release of calcium from intracellular stores, and diacylglycerol, in conjunction with calcium and phospholipids, activates protein kinase C (PKC).

Agonist-induced desensitization is an important regulatory process in inositol lipid signaling (Fisher, 1995), although the precise mechanisms involved are unclear. Receptor-promoted desensitization is mimicked in several cell types by activation of protein kinase C (Orellana et al., 1985; Rittenhouse and Sasson, 1985) and is prevented by down-regulation of PKC by chronic exposure to 4β-phorbol-12β-myristate-13α-acetate (PMA; Hepler et al., 1988) or by PKC inhibitors (Galas and Harden, 1995). These observations strongly suggest that PKC plays a negative regulatory role in phosphoinositide hydrolysis, probably by causing a phosphorylation-dependent change in the activity of another component of the pathway. Potential targets of PKC-catalyzed phosphorylation in this putative negative-feedback system include the receptor, the G protein, the effector enzyme PLC-β, or other less well-defined membrane-signaling proteins. GTPγS-stimulated PLC-β activity is attenuated in membranes isolated from PMA-treated cells (Orellana et al., 1987), suggesting that PKC acts on a protein downstream of the receptor.

The turkey erythrocyte is a well-characterized model of receptor-promoted inositol phospholipid signaling and is particularly useful because the three primary proteins in the pathway, i.e., the turkey $P2Y_1$ receptor (Boyer et al., 1989; Filtz et al., 1994), the G protein $G\alpha_{11}$ (Waldo et al., 1991; Maurice et al., 1993), and the effector enzyme PLC-βt (Morris et al., 1990a,b; Waldo et al., 1996), have been identified and cloned. Desensitization of phosphoinositide hydrolysis has been described in the turkey erythrocyte. Both indirect activation of PKC by P2Y-receptor agonists and direct PKC activation by PMA attenuate agonist and GTPγS-stimulated PLC-βt activity in turkey erythrocyte membranes (Martin and Harden, 1989; Galas and Harden, 1995), suggesting that regulation of inositol phospholipid signaling occurs down-

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ABBREVIATIONS: PLC, phospholipase C; PKC, protein kinase C; PMA, 4β-phorbol-12β-myristate-13α-acetate; GTPγS, guanosine 5′-*O*-thiotriphosphate; 2-MeSATP, 2-methylthioadenosine triphosphate; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonyl fluoride.

stream of the turkey $P2Y_1$ receptor. Previous studies of desensitization in the turkey erythrocyte membrane assessed receptor and G protein-stimulated inositol lipid hydrolysis catalyzed by endogenous PLC-βt activity, making it difficult to distinguish between effects of PKC on PLC-βt and those on $G\alpha_{11}$ or other membrane-signaling proteins. Similarly, previous studies with mammalian cell preparations have failed to localize desensitizing effects of PKC activation to PLC-β, to the involved G_q family G protein, or to other less well-defined components of the inositol lipid-signaling pathway. In this study, we have established a turkey erythrocyte membrane assay with reconstituted, purified PLC-β1 that provides direct quantification of the activity of $G\alpha_{11}$. We have used this assay to demonstrate for the first time that $G\alpha_{11}$-stimulated activation of a PLC-β isoenzyme is inhibited in membranes from cells treated with PMA.

Experimental Procedures

Materials. Recombinant PLC-β1 was purified after baculovirus-promoted expression in Sf9 insect cells as previously described (Paterson et al., 1997). PtdIns(4)P was purified from bovine brain Folch fraction as described previously (Morris et al., 1990a). [^{3}H]PtdIns(4)P was purified from [^{3}H]*myo*-inositol-labeled turkey erythrocytes as described previously (Waldo et al., 1994). Phosphatidylethanolamine (bovine heart), 1,2-dioleoyl-*sn*-glycerol, and phosphatidylserine (brain) were purchased from Avanti Polar Lipids (Alabaster, AL). GTPγS, protein A-agarose, and PKC purified from rat brain were purchased from Boehringer Mannheim (Indianapolis, IN). PMA, 4α-phorbol-12,13-didecanoate, and phorbol-12,13-dibutyrate were obtained from Sigma (St. Louis, MO). 2-Methylthioadenosine triphosphate (2-MeSATP) was purchased from Research Biochemicals Inc. (Natick, MA). [^{32}P]Orthophosphate and [γ-^{32}P]ATP were purchased from DuPont NEN (Boston, MA).

Turkey Erythrocyte Treatment and Membrane Preparation. Turkey erythrocytes were collected and washed as previously described (Boyer et al., 1989). For treatment with phorbol esters, 1 ml of packed erythrocytes was resuspended in 4 ml HEPES-Dulbecco's modified Eagle's medium (DMEM), pH 7.4, and equilibrated at 37°C for 5 min. Phorbol esters were added as stocks in 100% ethanol, and an equal volume (~0.1% of total volume) of this solvent was always used as a vehicle control. Drug treatments proceeded at 37°C for 20 min unless otherwise indicated. Treated erythrocytes were centrifuged at 500*g*, the medium was aspirated, and the cells were resuspended in ice-cold lysis buffer [20 mM HEPES, pH 7.0, 145 mM NaCl, 5 mM $MgCl_2$, 50 mM NaF, 1 mM EGTA, 200 μM phenylmethylsulfonyl fluoride (PMSF), and 200 μM benzamidine] at a 1:1 ratio (v/v). Resuspended cells (2 ml) were vortexed vigorously in the presence of 1 g of glass beads (0.45 mm) for four rounds of 30 s each. Erythrocytes were cooled on ice between vortexing, and all subsequent steps were carried out at 4°C. Samples were centrifuged at 500*g* for 3 min to sediment the glass beads and unlysed cells. The supernatant was collected in a fresh tube, and the glass beads were resuspended in 1 ml of lysis buffer. The vortex and centrifugation steps were repeated to lyse the remaining cells. Glass beads were washed a final time with 1 ml of lysis buffer, vortexed, and allowed to settle for 5 min without centrifugation. The resultant supernatant was added to the previous supernatant fractions. The supernatant pool was centrifuged at 35,000*g* for 10 min, and the membrane pellet was resuspended in 1 ml of lysis buffer. After two washes in lysis buffer, the membrane pellet was resuspended in 1 ml of membrane buffer [20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 100 mM NaCl, 40 mM β-glycerophosphate, 2 mM dithiothreitol, 200 μM benzamidine, 200 μM PMSF, and 200 nM calyculin A] and homogenized with DNase I (45 mU/ml) to degrade any residual DNA. Membranes were used immediately in a reconstitution assay or frozen at −80°C for later use.

Reconstitution of Purified PLC-β1 with Turkey Erythrocyte Membranes. Receptor and G protein-regulated PLC-β1 activity was quantitated with turkey erythrocyte membranes reconstituted with purified PLC-β1 and phospholipids. Erythrocyte membranes were prepared as described above, assayed for protein concentration, and diluted in membrane buffer to 25 μg/μl, unless otherwise indicated. Phospholipid substrate was prepared as a mixture of PtdIns(4)P (5 nmol/assay), phosphatidylethanolamine (25 nmol/assay), and [^{3}H]PtdIns(4)P (~10,000 cpm/assay), dried under nitrogen, and resuspended by sonication in membrane buffer (10 μl/assay). Membranes and phospholipids were mixed at a ratio of 3:2 (v/v) and incubated on ice for 30 min. Twenty-five microliters each of radiolabeled membranes, DB buffer, and 20 mM HEPES, pH 7.0; GTPγS; or GTPγS with 2-MeSATP was mixed and preincubated at 30°C for 2 min. Drugs were prepared as 4X stocks in 20 mM HEPES, pH 7.0. Unless otherwise indicated, the assay was initiated by the addition of 25 μl of purified PLC-β1 (3 ng) in 4X enzyme buffer (80 mM HEPES, pH 7.2, 3.2 mM $MgCl_2$, 12 mM EGTA, 60 mM NaCl, 120 mM KCl, 0.8 mM EDTA, 10.6 mM $CaCl_2$, and 4 mM dithiothreitol or 4X enzyme buffer alone. The assay was at 30°C for 20 min, or as indicated in the figure legends. The reaction was terminated by the addition of 375 μl of $CHCl_3$/MeOH/concentrated HCl (40:80:1). $CHCl_3$ (125 μl) and 0.1 M HCl (125 μl) were added, and the samples were centrifuged at 1800*g*. Three hundred fifty microliters of the aqueous phase was counted by liquid scintillation spectrometry to quantitate [^{3}H]Ins(1,4)P production. All data are reported as means ± S.D. of triplicate determinations and are representative of two or three experiments.

In Vitro Kinase Reaction. Native $G\alpha_{11}$ from turkey erythrocytes (9 pmol) was incubated with 20 μU of PKC for 30 min at 30°C in a reaction containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 500 μM $CaCl_2$, 100 μg/ml phosphatidylserine, 20 μg/ml 1,2-dioleoyl-*sn*-glycerol, and 200 μM [γ-^{32}P]ATP (~1500 cpm/pmol) in a volume of 20 μl. Reactions were terminated by the addition of 20 μl of 2X Laemmli sample buffer. Samples were separated by SDS-polyacrylamide gel electrophoresis through 12.5% acrylamide according to the method of Laemmli (Laemmli, 1970), and the protein bands were visualized by Coomassie stain. The gel was dried and exposed to autoradiography film to detect radioactive bands.

In Vivo [^{32}P]Orthophosphate Labeling and $G\alpha_{11}$ Immunoprecipitation. Washed turkey erythrocytes were radiolabeled for 4 h in phosphate-free DMEM (4 ml/ml of packed erythrocytes) supplemented with [^{32}P]P_i (~2 mCi/ml of packed cells) at 37°C in 5% CO_2. Radiolabeled erythrocytes were centrifuged at 500*g* for 5 min and resuspended in HEPES-DMEM, pH 7.4 (4 ml/ml of packed erythrocytes). Erythrocytes were treated, and membranes were prepared with glass beads as described above. Crude membrane pellets were isolated by centrifugation at 35,000*g* for 10 min. Membranes were resuspended in cholate extraction buffer (50 mM Tris, pH 7.5, 50 μM $AlCl_3$, 10 mM $MgCl_2$, 25 mM NaF, 40 mM β-glycerophosphate, 30 μM GDP, 200 μM benzamidine, 200 μM PMSF, and 1.2% cholate) at ~2 mg of protein/ml of extraction buffer and incubated for 2 h at 4°C with mixing. Samples were centrifuged at 100,000*g* for 30 min, and the supernatants (500 μl) were diluted 1:1 in immunoprecipitation buffer (50 mM Tris, pH 8.0, 2 mM $MgCl_2$, 100 mM NaCl, 25 mM NaF, 40 mM β-glycerophosphate, 200 μM benzamidine, and 200 μM PMSF) and incubated with preimmune serum or anti-$G\alpha_{q/11}$ antiserum (117, antiserum to a synthetic peptide corresponding to the COOH-terminal sequence of both $G\alpha_q$ and $G\alpha_{11}$; 1:100 dilution; Maurice et al., 1993) overnight at 4°C. Immunoprecipitated samples were incubated with protein A-agarose beads for 2 h at 4°C and then centrifuged at 14,000*g* for 20 s. Immunoprecipitated pellets were washed twice with immunoprecipitation buffer. The washed pellets were resuspended in 70 μl Laemmli sample buffer and incubated for 10 min at 85°C.

Immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis through 12.5% acrylamide. Proteins were transferred to nitrocellulose and exposed to a PhosphoImager screen (Molecular Dynamics, Inc., Sunnyvale, CA) for quantitation of protein-associated radioactivity. $G\alpha_{11}$ was detected by Western blotting with antiserum 118 (Maurice et al., 1993) produced against the COOH-terminal of $G\alpha_{q/11}$.

Results

Activation of PKC by acute PMA treatment has been shown to inhibit agonist-and guanine nucleotide-stimulated inositol phosphate production in various intact cell and membrane assays (Orellana et al., 1985, 1987; Rittenhouse and Sasson, 1985; Hepler et al., 1988; Galas and Harden, 1995). Inositol lipid hydrolysis in these studies was catalyzed by endogenous PLC-β, and the relative effects of PMA on G protein versus effector enzyme activity therefore could not be distinguished. To resolve potential PMA effects on the G protein or other membrane-signaling molecules from those on PLC-β, we used the turkey erythrocyte model of inositol phospholipid signaling to develop an assay that directly measures G_{11} activity in situ. In this assay, turkey erythrocyte membranes are depleted of endogenous PLC-βt by successive washes, [^{3}H]PtdIns(4)P [or [^{3}H]PtdIns(4,5)P_2] substrate is incorporated into the membranes, and after 2 min incubation with GTPγS, purified PLC-β1 is reconstituted with the membranes. PLC-β1 was selected for this assay because it is much less sensitive to $\beta\gamma$-subunit activation than is PLC-βt (Boyer et al., 1992, 1994) and therefore provides a measure of $G\alpha_{11}$-stimulated PLC-β activation. [^{3}H]Ins(1,4)P_2 [or [^{3}H]Ins(1,4,5)P_3] production is quantitated after various times of incubation at 30°C with PLC-β1.

Washed membranes did not exhibit significant inositol phosphate production in the presence of GTPγS or GTPγS with 2-MeSATP (Fig. 1A), demonstrating that endogenous PLC-βt was removed by the wash procedure. Addition of exogenous purified PLC-β1 to membranes resulted in a marked increase in basal phosphoinositide hydrolysis (Fig. 1A). Moreover, whereas GTPγS had no effect in washed membranes, significant GTPγS-promoted inositol lipid hydrolysis was observed after reconstitution of PLC-β1. The small activation observed with GTPγS and 2-MeSATP in washed membranes was also markedly augmented after reconstitution of PLC-β1 (Fig. 1A). Higher concentrations of membrane protein resulted in increased inositol phosphate production (Fig. 1A).

To establish optimal conditions for assay of $G\alpha_{11}$-stimulated activities, PLC-β1 concentration-response and time course experiments were performed. GTPγS-stimulated inositol phosphate production increased essentially linearly up to ~5 ng of added PLC-β1 (Fig. 1B). After an initial lag of 2 to 3 min, GTPγS-stimulated inositol lipid hydrolysis proceeded linearly for up to 30 min (Fig. 2). Incubation with the $P2Y_1$-receptor agonist 2-MeSATP and GTPγS resulted in an increase in inositol phosphate production that was dependent on the concentration of 2-MeSATP (Fig. 1C; $EC_{50} = 25 \pm 1$ nM; mean ± S.E.; $n = 3$) and was comparable in agonist potency to that previously observed with turkey erythrocyte ghosts (Boyer et al., 1989). The experiments presented in Figs. 1 and 2 demonstrate that this reconstitution system can be used effectively to assess the capacity of $G\alpha_{11}$ to activate PLC-β under conditions in which the enzyme is reconstituted with membranes depleted of endogenous PLC-βt.

The effects of PMA treatment of turkey erythrocytes on $G\alpha_{11}$ activity were studied. Membranes were prepared from control or 1 μM PMA-treated turkey erythrocytes, activated with GTPγS, and reconstituted with varying concentrations of PLC-β1. PMA treatment inhibited GTPγS-stimulated phosphoinositide hydrolysis at each concentration of PLC-β1 (Fig. 1B) and time of incubation (Fig. 2) examined. PMA treatment decreased GTPγS-stimulated PLC-β1 activity in a concentration-dependent manner, with half-maximal inhibition occurring at ~50 nM PMA and maximal suppression at 1 μM (Fig. 3A). GTPγS-stimulated phosphoinositide hydrolysis decreased with time of PMA treatment, and maximal inhibition was observed within 20 min of PMA treatment (Fig. 3B). The concentration and time dependence for the

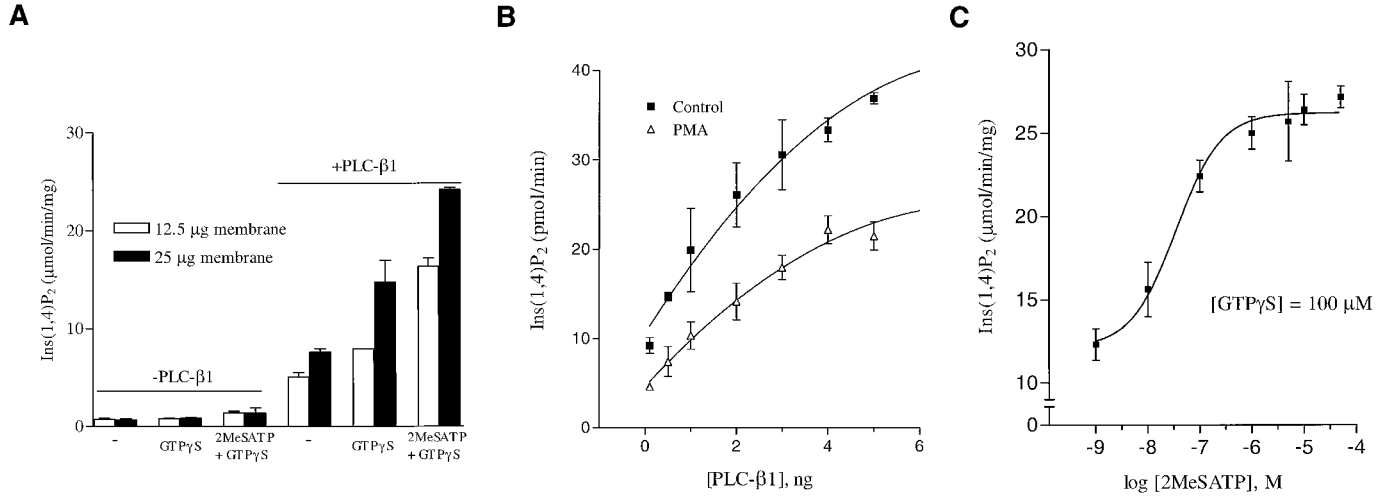

Fig. 1. Characterization of the membrane reconstitution assay. Membrane reconstitution assays were performed as described in *Experimental Procedures*. A, indicated amounts of membrane protein from vehicle-treated turkey erythrocytes were challenged with 100 μM GTPγS or 100 μM GTPγS plus 100 μM 2-MeSATP and incubated with 3 ng of purified PLC-β1 or enzyme buffer for 20 min at 30°C. B, membranes from control (■) or 1 μM PMA-treated (△) turkey erythrocytes were challenged with 100 μM GTPγS and incubated with varying concentrations of purified PLC-β1. C, membranes from control erythrocytes were stimulated in the presence of 100 μM GTPγS and varying concentrations of 2-MeSATP and assayed as described. The data are means ± S.D. of triplicate determinations and are representative of at least three experiments. The levels of [^{3}H]Ins(1,4)P_2 production in the absence of PLC-β1 were subtracted from the values presented in B.

inhibitory effects of PMA on G protein-regulated PLC activity were similar to those previously observed in studies with turkey erythrocytes (Galas and Harden, 1995) and with various mammalian cell lines (Orellana et al., 1985, 1987; Rittenhouse and Sasson, 1985; Hepler et al., 1988).

Experiments were carried out to determine whether PMA decreased the efficacy of GTPγS or the concentration of GTPγS needed for half-maximal stimulation. In the absence of 2-MeSATP, PMA treatment suppressed maximal GTPγS-stimulated activation of PLC-β1 by 35 to 50% compared with that of control membranes (Fig. 4A). The EC_{50} concentration of GTPγS was not significantly changed in membranes from PMA-pretreated erythrocytes (control, 7 ± 3 μM versus PMA treated, 13 ± 12 μM; mean ± S.E.; $n = 3$). Maximal GTPγS-stimulated inositol phosphate production in the presence of 2-MeSATP was inhibited 30 to 40% by PMA treatment, with no significant change in the EC_{50} concentration of GTPγS observed (Fig. 4B; control, 27 ± 10 nM versus PMA treated, 37 ± 13 nM,; mean ± S.E.; $n = 2$). These data indicate that PMA-induced inhibition of $G\alpha_{11}$-stimulated phosphoinositide hydrolysis results from a decrease in the apparent efficacy of GTPγS for activation of PLC-β1 rather than as a change in apparent affinity for the guanine nucleotide.

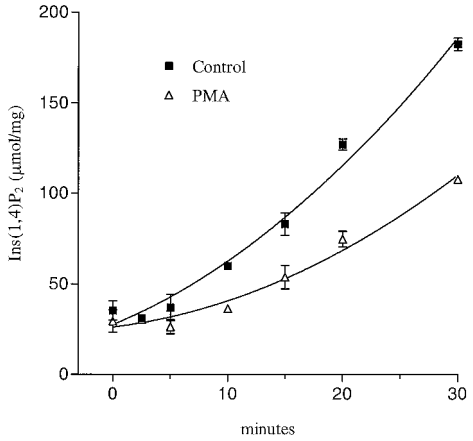

Fig. 2. Time course of GTPγS-stimulated inositol phosphate accumulation. Membranes from vehicle (■) or 1 μM PMA-treated (△) turkey erythrocytes were assayed for GTPγS-stimulated [^{3}H]Ins(1,4)P_2 production as described in *Experimental Procedures*. Membranes were challenged with 100 μM GTPγS and incubated with 3 ng of purified PLC-β1 for the indicated times. Values are means ± S.D. of triplicate determinations and are representative of three experiments.

Although PMA is a well characterized PKC activator, the observed suppression of $G\alpha_{11}$ stimulation of PLC-β1 could originate from a nonspecific effect of phorbol ester treatment. Treatment with phorbol-12,13-dibutyrate, which also activates PKC, resulted in decreases in GTPγS and GTPγS and 2-MeSATP-stimulated inositol phosphate production similar to that observed in membranes from PMA-treated cells (Fig. 5). Conversely, membranes prepared from erythrocytes treated with 4α-phorbol-12,13-didecanoate, which does not activate PKC, displayed receptor- and G protein-stimulated PLC-β1 activities equivalent to those of vehicle-treated erythrocytes (data not shown). To further establish the role of PKC in PMA-induced inhibition of $G\alpha_{11}$-activated phosphoinositide hydrolysis, the capacity of the PKC inhibitor bisindolylmaleimide to reverse the PMA-promoted suppression was examined. Membranes from erythrocytes treated with bisindolylmaleimide in the presence of PMA exhibited GTPγS-stimulated PLC-β1 activity equivalent to that of control cells (Fig. 5).

We previously demonstrated that the PLC-β present in turkey erythrocytes is a substrate for PKC (Filtz et al., 1999). However, because the experiments presented in this article were carried out with an assay that uses exogenously reconstituted PLC-β1 to assess $G\alpha_{11}$-stimulated PLC-β activity, effects of PKC on the endogenous avian PLC-β do not contribute to the phenomenon presented in Figs. 1 through 5. Therefore, to determine whether the inhibition of guanine nucleotide-stimulated inositol lipid hydrolysis was caused by a phosphorylation-dependent change in the activity of $G\alpha_{11}$, we performed in vitro kinase reactions with purified PKC and native $G\alpha_{11}$ from turkey erythrocytes. No evidence of $G\alpha_{11}$ phosphorylation by PKC was observed under conditions sufficient for PKC to promote stoichiometric phosphorylation of PLC-βt (data not shown).

Although PKC did not directly phosphorylate $G\alpha_{11}$ in vitro, we investigated the possibility that $G\alpha_{11}$ was phosphorylated in intact turkey erythrocytes in response to PMA treatment. $G\alpha_{11}$ was immunoprecipitated from [^{32}P]orthophosphate-labeled turkey erythrocytes treated with vehicle or 1 μM PMA. PMA promoted large increases in [^{32}P] incorporation into

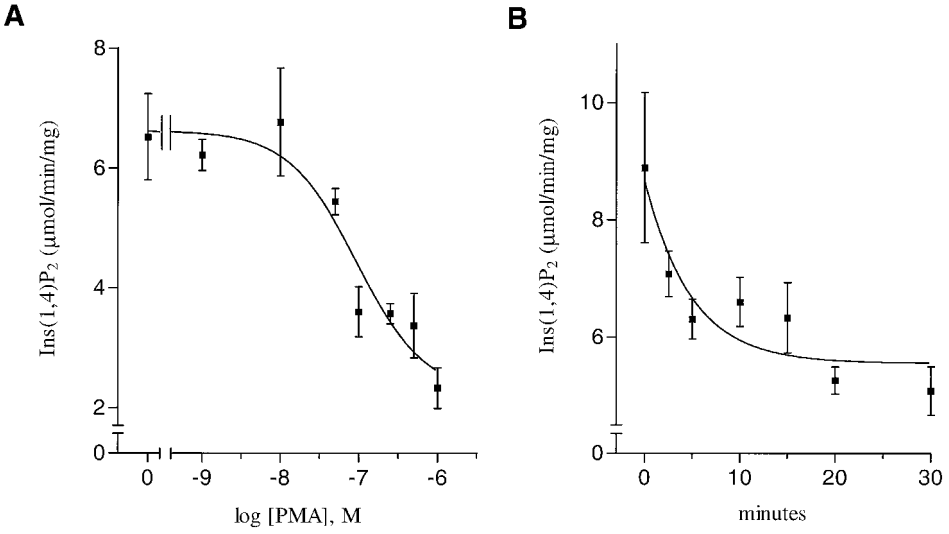

Fig. 3. Concentration and time dependence of PMA-induced inhibition of GTPγS-stimulated PLC-β1 activity. Turkey erythrocytes were treated with the indicated concentrations of PMA for 20 min (A) or 1 μM PMA for the indicated times (B) at 37°C. Membranes were challenged with 100 μM GTPγS and assayed for [^{3}H]Ins(1,4)P_2 production as described in *Experimental Procedures*. Basal levels of [^{3}H]Ins(1,4)P_2 were subtracted from the values presented in A. The data in A are means ± S.D. of triplicate determinations and representative of three experiments. The data in B are means ± S.D. from two experiments performed in triplicate.

PLC-βt under these conditions (Filtz et al., 1999; data not shown), but no increase in [^{32}P] incorporation into $G\alpha_{11}$ occurred (data not shown). These results are consistent with the hypothesis that a signaling protein present in washed turkey erythrocyte membranes other than $G\alpha_{11}$ is the target for PKC and that PKC-promoted phosphorylation of this protein results in an activity change that is responsible for the inhibition of inositol phospholipid hydrolysis observed with PMA treatment.

The results presented thus far illustrate that activation of PKC in turkey erythrocytes reduces the capacity of $G\alpha_{11}$ to promote inositol lipid hydrolysis. However, as mentioned above, we previously reported that PKC promotes phosphorylation of PLC-βt in vivo and that in vitro phosphorylation of PLC-βt by PKC results in a decrease in the basal catalytic activity of the enzyme (Filtz et al., 1999). Therefore, experiments were carried out to determine whether these two effects of PKC activation were additive by quantitating $G\alpha_{11}$-regulated PLC-β activity after reconstitution of phosphorylated PLC-βt with membranes from PMA-treated erythrocytes. As with PLC-β1, reconstitution of PLC-βt conferred G protein- and receptor-regulated phosphoinositide hydrolysis to washed turkey erythrocyte membranes. Phosphorylation of PLC-βt by PKC in vitro resulted in a decrease in its capacity to be activated by addition of GTPγS after reconstitution with control membranes (Table 1), in agreement with our previous results (Filtz et al., 1999). Furthermore, as was observed with reconstitution of PLC-β1, a decrease in G protein-promoted inositol lipid hydrolysis was observed in membranes from PMA-treated erythrocytes after reconstitution of exogenous purified PLC-βt (Table 1). Moreover, GTPγS-stimulated PLC-β activity observed after reconstitution of phosphorylated PLC-βt with PMA-treated membranes was lower than the GTPγS-stimulated activity observed after either reconstitution of unphosphorylated PLC-βt with membranes from PMA-treated erythrocytes or after reconstitution of phosphorylated PLC-βt with control membranes (Table 1). It is somewhat surprising that a greater reduction in activity was not observed by combining the effects of PKC-promoted phosphorylation of PLC-βt with PMA treatment of erythrocytes. This result may be partially explained by the greater sensitivity of PLC-βt to activation by G$\beta\gamma$. We have previously reported (Filtz et al., 1999) with purified proteins that activation of PLC-βt by G$\beta\gamma$ surmounts the decrease in basal activity induced by the phosphorylation of PLC-βt, and the contribution of G$\beta\gamma$ activation of PLC-βt may therefore confound these experiments. Although there was not a strong additive effect of PMA treatment and phosphorylation of PLC-βt, these experiments suggest that activation of PKC results in modification of both a membrane-signaling protein and the effector enzyme and that this dual modification may be responsible for desensitization observed with PMA treatment of intact cells.

Discussion

Receptor- and G protein-promoted PLC-β activity is attenuated in response to short-term PMA treatment of turkey erythrocytes and several mammalian cell types (Orellana et al., 1985; Rittenhouse and Sasson, 1985; Galas and Harden, 1995), which has led to the hypothesis that PKC plays a negative regulatory role in phosphoinositide hydrolysis. Previous studies in the turkey erythrocyte have measured endogenous PLC-βt activity, making it difficult to distinguish between effects of PKC on PLC-βt and those on G_{11} or other membrane-signaling proteins. We have developed a membrane reconstitution assay that permits investigation of the effects of PKC on membrane-signaling proteins under conditions that make no assumptions about specific membrane targets. This assay was used to show that activation of PKC in erythrocytes results in an attenuation of $G\alpha_{11}$-stimulated activation of purified PLC-β1. Because the assay involves use of exogenous phosphoinositide substrate, reconstitution of purified PLC-β1 into membranes from control or PKC-activated erythrocytes, and activation of signaling with GTPγS, our results point to a modification of a membrane-signaling molecule other than the receptor, the PLC enzyme, or its substrate. Therefore, we conclude that activation of PKC promotes a change in the activity of $G\alpha_{11}$ or of a protein that directly regulates $G\alpha_{11}$ activity.

Previous studies have demonstrated the phosphorylation of certain G protein α subunits. Carlson et al. (1989) and Lounsbury et al. (1991) reported that $G\alpha_z$ is phosphorylated in vitro by PKC and in platelets after PMA treatment. Ko-

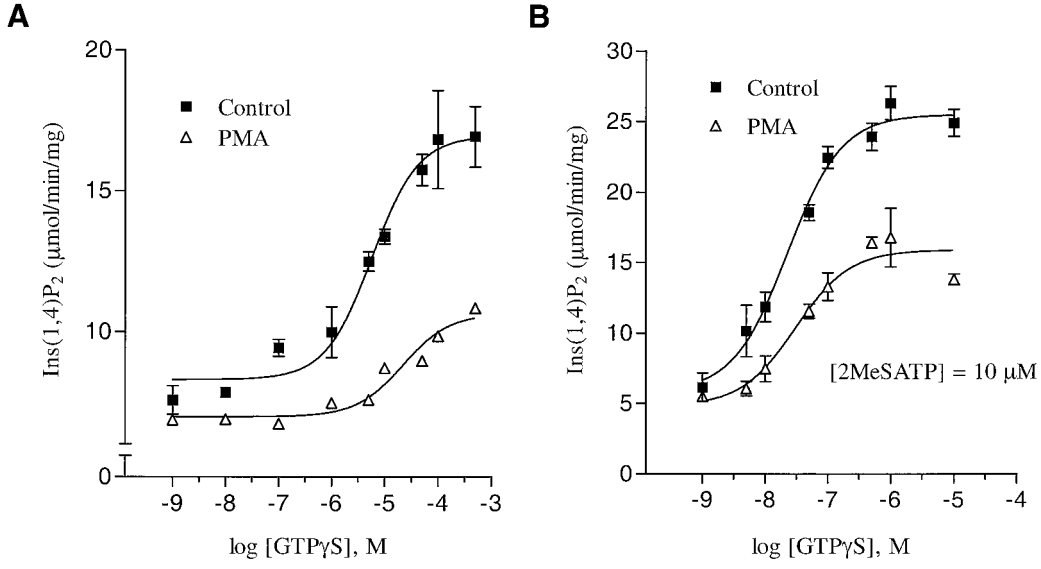

Fig. 4. Effect of PMA treatment on the efficacy of GTPγS for the stimulation of inositol phosphate production in the presence and absence of agonist. Membranes from vehicle-treated (■) or 1-μM-PMA-treated (△) turkey erythrocytes were challenged with the indicated concentrations of GTPγS alone (A) or GTPγS plus 10 μM 2-MeSATP (B) and assayed as described in *Experimental Procedures*. Values are means ± S.D. of triplicate determinations and are representative of at least two experiments.

zasa and Gilman (1996) demonstrated that $G\alpha_{12}$ is phosphorylated by purified PKC and in National Institutes of Health 3T3-G12 cells treated with PMA. Phosphorylation of both $G\alpha_z$ (Fields and Casey, 1995) and $G\alpha_{12}$ (Kozasa and Gilman, 1996) by PKC inhibits their interactions with G protein $\beta\gamma$ subunits and thereby induces a functional consequence that may alter cellular signaling activity of these G proteins. Despite the precedent for phosphorylation of G protein α subunits, we observed no phosphorylation of $G\alpha_{11}$ in vitro by PKC or in PMA-treated erythrocytes. These results are consistent with the findings of Kozasa and Gilman (1996) and Lounsbury et al. (1993) for $G\alpha_q$. Although our reconstitution assay localizes the effect of PKC to a protein at or near the level of $G\alpha_{11}$, these results support the conclusion that this membrane-signaling protein is not $G\alpha_{11}$. A $G\alpha_{11}$-signaling cohort is potentially the substrate for PKC, and regulators of G protein signaling (RGS proteins) are strong candidates (Dohlman and Thorner, 1997; Berman and Gilman, 1998).

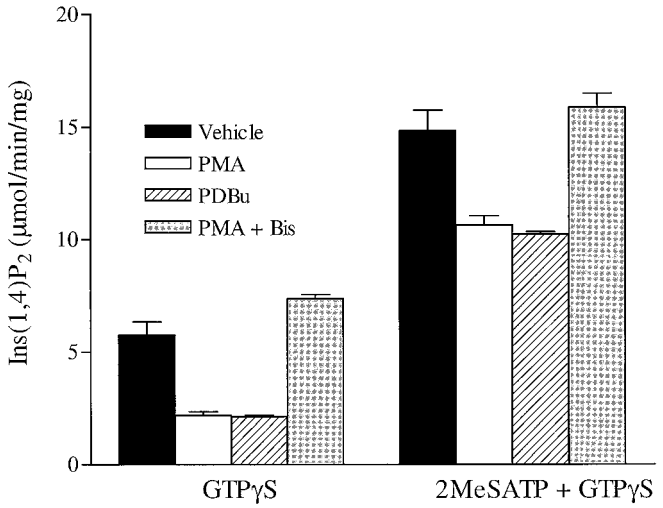

Fig. 5. PKC involvement in the PMA-induced suppression of $G\alpha_{11}$-stimulated PLC-β1 activity. Membranes from turkey erythrocytes treated with vehicle (black columns), 1 μM PMA (open columns), 1 μM phorbol-12,13-dibutyrate (PDBu, hatched columns), or 1 μM PMA plus 10 μM bisindolylmaleimide (gray columns) were challenged with 10 μM GTPγS alone or with 10 μM GTPγS in the presence of 10 μM 2-MeSATP and assayed for [^{3}H]Ins(1,4)P_2 production as described in *Experimental Procedures*. Basal levels of [^{3}H]Ins(1,4)P_2 production were subtracted from the values presented. The data are means ± S.D. of triplicate determinations and are representative of two experiments.

TABLE 1

Reconstitution of PLC-βt with turkey erythrocyte membranes

PLC-βt was incubated in the presence or absence of 10 μU PKC for 10 min at 30°C as described in *Experimental Procedures*. Reaction mixtures were diluted in 10 mM HEPES/BSA (1 mg/ml) to a final PLC-βt concentration of 20 ng/25 μl. After challenge with 100 μM GTPγS, 25 μg of membranes from control erythrocytes were reconstituted with 20 ng of unphosphorylated or phosphorylated PLC-βt. Similarly, 25 μg of membranes from erythrocytes treated with 1 μM PMA were reconstituted with unphosphorylated or phosphorylated PLC-βt. Samples were incubated for 20 min at 30°C and assayed for [^{3}H]Ins(1,4)P_2 production. Ins(1,4)P_2 production in the absence of reconstituted PLC-βt or GTPγS stimulation was 0.19 ± 0.09 and 0.27 ± 0.23 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for control and PMA-treated membranes, respectively. Values are means ± S.D. of triplicate determinations and are representative of two experiments.

	GTPγS-stimulated Ins(1,4)P_2	
	Control	PMA
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$	
PLC-βt (−PKC)	3.22 ± 0.18	1.74 ± 0.07
PLC-βt (+PKC)	1.97 ± 0.05	1.47 ± 0.10

RGS2 has been shown to interact with and inhibit GTPγS-promoted signaling by α subunits of the G_q family of G proteins (Heximer et al., 1997). Other RGS proteins, e.g., RGS4 and GAIP (Hepler et al., 1997), also promote GTP hydrolysis by G_q family members and block GTPγS-stimulated activation of PLC-β by $G\alpha_q$, albeit at significantly higher concentrations than with RGS2. Glick et al. (1998) and Wang et al. (1998) recently reported that PKC-promoted phosphorylation of $G\alpha_z$ inhibited the GTPase-activation protein (GAP) activity of a G_z-selective RGSZ1 protein RGS_{Z1}, thereby demonstrating the sensitivity of an RGS-G protein interaction to protein phosphorylation state. Although the effects of PMA treatment on $G\alpha_{11}$-stimulated activation of PLC-β1 were studied under conditions where RGS proteins are unable to act as GAPs, the capacity of some RGS proteins to potentially act as effector anatagonists and inhibit GTPγS-promoted signaling leaves open the possibility that PMA induces a phosphorylation-dependent change in the activity of an RGS protein. The role of RGS2 and other RGS proteins in regulating inositol lipid signaling in the turkey erythrocyte and the effects of PKC on RGS activity are currently being examined in our laboratory.

Other putative membrane targets for PKC include G protein β and γ subunits. By using PLC-β1, which is much less sensitive to G$\beta\gamma$ than PLC-βt, we have attempted to focus solely on the effects of PKC on $G\alpha_{11}$-promoted PLC-β activity. However, phosphorylation of either Gβ or Gγ could alter the affinity of the $\beta\gamma$ dimer for $G\alpha_{11}$, perhaps inhibiting signaling by promoting a higher-affinity interaction of G$\beta\gamma$ and $G\alpha_{11}$. $G\gamma_{12}$ was previously shown to be phosphorylated in vitro by PKC, whereas $G\gamma_1$, γ_2, γ_3, and γ_7 are apparently not PKC substrates (Morishita et al., 1995; Yasuda et al., 1998). We have not formally investigated the phosphorylation state of G$\beta\gamma$ in turkey erythrocytes after PMA treatment. However, no significant change in the apparent affinity for GTPγS was observed in concentration-response experiments, suggesting that there is also no change in the affinity of G$\beta\gamma$ for $G\alpha_{11}$. Thus, the data from GTPγS concentration-response experiments suggest that it is unlikely that G$\beta\gamma$ phosphorylation accounts for the attenuation of inositol lipid signaling seen with PKC activation.

PKC-promoted loss of the activity of $G\alpha_{11}$ potentially could be explained by a phorbol ester-induced decrease in membrane association of this protein. For example, Ransnas et al. (1989, 1992) have reported that receptor-promoted activation of G_s in S49 lymphoma cells results in an increase in cytosolic $G\alpha_s$ and a corresponding decrease in membrane-associated G protein. Moreover, Mitchell et al. (1993) and Mullaney et al. (1993) previously demonstrated in CHO cells expressing the human m_1 muscarinic receptor that chronic agonist treatment produced a significant loss of membrane $G\alpha_{q/11}$. In contrast to these findings, Western blot analysis of membranes from PMA-treated turkey erythrocytes revealed no decrease in immunologically detectable $G\alpha_{11}$ compared with the levels observed in control membranes (data not shown). Additionally, equivalent amounts of $G\alpha_{11}$ were immunoprecipitated from identical starting amounts of membrane from control and PMA-treated erythrocytes, further suggesting that the inhibition of $G\alpha_{11}$-mediated activation of PLC-β1 in membranes from PKC-activated cells is not the result of changes in membrane-associated $G\alpha_{11}$ protein.

In contrast to the absence of PKC-catalyzed $G\alpha_{11}$ phos-

phorylation observed in this study, we previously reported that PLC-βt is phosphorylated in intact turkey erythrocytes in response to PMA and 2-MeSATP treatment and in vitro by PKC (Filtz et al., 1999). Phosphorylation of PLC-βt results in a decrease in basal catalytic activity (Filtz et al., 1999). The results presented here illustrate for the first time that PKC also promotes an alteration of a membrane-signaling protein other than the receptor or $G\alpha_{11}$. Thus, PKC-promoted desensitization of phosphoinositide hydrolysis in avian erythrocytes stems from a dual modification of the effector enzyme and another membrane-signaling protein. The turkey erythrocyte model should continue to be useful in identifying the membrane target for PKC and for investigating further the relative functional consequences of phosphorylation on PLC-βt-catalyzed phosphoinositide hydrolysis.

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