G-protein $\beta\gamma$ subunits antagonize protein kinase C-dependent phosphorylation and inhibition of phospholipase C- β_1

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Protein kinase C (PKC) isoforms phosphorylated phospholipase C- β_1 (PLC- β_1) *in vitro* as follows: PKC α $\gg$ PKC ϵ ; not PKC ζ . PLC- β_3 was not phosphorylated by PKC α . G-protein $\beta\gamma$ subunits inhibited the PKC α phosphorylation of PLC- β_1 in a concentration-dependent manner. Half-maximal inhibition occurred with 500 nM $\beta\gamma$. G-protein $\beta\gamma$ subunits also antagonized the PKC α -mediated inhibition of PLC- β_1 enzymic activity.

PKC α , in turn, inhibited the stimulation of PLC- β_1 activity by $\beta\gamma$. There was little effect of PKC α on the stimulation of PLC- β_1 by $\alpha_{q/11}$ -guanosine 5' [γ -thio]triphosphate (GTP[S]). These findings demonstrate that G protein $\beta\gamma$ subunits antagonize PKC α regulation of PLC- β_1 . Thus $\beta\gamma$ subunits might have a role in modulating the negative feedback regulation of this signalling system by PKC.

INTRODUCTION

Phosphoinositide-specific phospholipase C (PLC) constitutes part of the transmembrane signalling system that promotes an increase in cytosolic Ca^{2+} concentration and an activation of protein kinase C (PKC) in response to hormone stimulation [1,2]. PKC, a family of serine and threonine kinases, phosphorylates several cellular substrates [3] including PLC- β_1 [4]. PLC- β_1 is activated by receptors coupled to the G-protein $G_{q/11}$. Three major PLC- β isoforms, PLC- β_1 , PLC- β_2 and PLC- β_3 , have been identified and shown to be stimulated by the G-protein $\alpha_{q/11}$ and $\beta\gamma$ subunit [5–8].

Activation of PKC *in vivo* results in a partial or complete attenuation of the hormone-stimulated increase in PLC activity [9–11]. Although these observations indicate that PKC functions as part of a negative feedback control mechanism to modulate the receptor activation of PLC activity, the mechanism(s) involved are not fully understood. Inhibition of the Ca^{2+} -dependent stimulation of PLC- β_1 activity by PKC has been demonstrated *in vitro* [12]. The following studies were undertaken to characterize further the regulation of PLC- β_1 by PKC.

MATERIALS AND METHODS

PLC- β_1 , PLC- β_3 and G-protein isolation

PLC- β_1 was purified to homogeneity from bovine brain membranes as described [8], with slight modification. A 2 M KCl membrane extract was subjected to precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis overnight and purification by chromatography on Heparin Sepharose CL-6B (Pharmacia), phenyl-Sepharose (Sigma) and Fast Flow Q (Sigma). A similar protocol was used to purify PLC- β_3 from bovine heart. Heart contains predominantly PLC- β_3 with little or no PLC- β_1 [13]. PLC- β_1 and PLC- β_3 concentration was determined by using an average absorbance $A_{1\text{cm},280}^{0.1\%}$ of 1.14 [8] and Coomassie Blue staining of the purified protein after SDS/PAGE. Western blot analysis of the purified enzymes with anti-(PLC- β_1) and anti-(PLC- β_3) was performed as described [14] and established the identity of the purified PLC- β isoform [14]. $\beta\gamma$ subunits were purified from a cholate extract of bovine brain membranes as described [15,16]. $\alpha_{q/11}$ was from Dr. Paul Sternweis (University of Texas, Dallas,

TX, U.S.A.) and purified as described [17]. Purification of α_o and generation of the GDP-liganded state were performed as described [18,19]. Protein was determined by Amido Black staining [20].

PLC- β_1 phosphorylation

PLC- β_1 was incubated at 27 °C in buffer containing 50 mM Tris/HCl (pH 7.5), 5 mM MgCl_2 , 0.4 mM CaCl_2 , 0.1 mM [γ - ^{32}P]ATP (2000 c.p.m./pmol), 0.2 $\mu\text{g}/\mu\text{l}$ phosphatidylserine (PS) or 0.2 $\mu\text{g}/\mu\text{l}$ PS plus 0.2 $\mu\text{g}/\mu\text{l}$ phosphatidylethanolamine (PE), 200 μM PMSF, 30 nM okadaic acid, 1 mM sodium orthovanadate and 1 mM dithiothreitol (DTT). Other additions included 0.06 ng/ μl (100 nM) PMA plus 1–5 nM PKC. For the control incubation, PMA was omitted and PKC was replaced by an equivalent amount of PKC dilution buffer [20 mM Tris/HCl (pH 7.5)/0.5 mM EGTA/0.5 mM EDTA/10 mM 2-mercaptoethanol/10% (v/v) glycerol/0.05% Triton X-100/400 mM NaCl]. Control experiments established that phosphorylation was dependent on the presence of PKC cofactors. At the indicated time, the incubation was terminated by the transfer of tubes to ice followed by the addition of 2 $\times$ Laemmli sample buffer and boiling for 5 min. PLC- β_1 was subjected to SDS/PAGE [7.5% or 11% (w/v) gel] performed by the method of Laemmli [21]. PLC- β_1 was detected by Coomassie Blue stain and autoradiography. The labelled band was excised from the gel and the incorporated radioactivity was determined by liquid-scintillation counting. A similar protocol was used for PLC- β_3 . For the $\beta\gamma$ experiments, PLC- β_1 was mixed with an equal volume of buffer containing G-protein $\beta\gamma$ subunit or $\beta\gamma$ buffer [1% (w/v) cholate/25 mM Hepes (pH 7.5)/1 mM EDTA/1 mM DTT] and maintained on ice for 20–30 min as described in the figure legends. The cholate concentration of the mixture was adjusted to 1%. After incubation on ice, the PLC- $\beta_1/\beta\gamma$ mixture was diluted approx. 1:5 by addition to the phosphorylation mixture containing PKC. The $\beta\gamma$ concentration in the figures indicates the final concentration in the phosphorylation assay. As indicated in the text, $\beta\gamma$ subunits were also premixed with a 3-fold excess of α_o -GDP before addition to PLC- β_1 preparation to establish the $\beta\gamma$ dependence for regulation.

Initial experiments employed rat brain PKC (Calbiochem), which consisted of a mixture of PKC isoforms, mostly the Ca^{2+} /diacylglycerol-dependent isoforms (α , β and γ). Similar results were obtained with recombinant human PKC α (Panvera) or recombinant rabbit PKC α (Gibco-BRL) and thus the data derived from these isoforms were pooled. PKC activity was determined with histone III S substrate as described [22] and experiments were performed with comparable amounts of PKC activity. PKC activity was also measured with a synthetic peptide corresponding to the PKC phosphorylation site of PLC- β_1 , APGSVKAPAK. The synthetic peptide was synthesized by the Protein Core Facility at the University of Miami School of Medicine (Miami, FL, U.S.A.). Purity was confirmed by HPLC.

PLC activity studies

PLC- β_1 was incubated for 90 min at 27 °C in incubation buffer containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl_2 , 0.4 mM CaCl_2 , 4.5 mM Na_2ATP , 5–10 nM PKC α and additions as described in the previous section. For the $\beta\gamma$ pretreatment, 7 μl of PLC- β_1 (7.5 ng/ μl) was combined with 4.8 μl of $\beta\gamma$ (150 ng/ μl) or $\beta\gamma$ buffer and incubated on ice for 30 min. A sample (5 μl) of this mixture was added to 7 μl of phosphorylation mixture and incubated in the presence or absence of PKC α as indicated in the figure legends. After incubation the sample was centrifuged briefly and placed on ice. The PLC mixture was diluted 30-fold in 25 mM Hepes, pH 7.0, and 10 μl was taken for PLC assay.

PLC determination

PLC- β_1 activity was determined essentially as described previously [12]. A sample (10 μl) of the incubated PLC- β_1 (diluted from the incubation mixture) was added to a PLC assay tube containing 50 mM Hepes, pH 7.0, pCa as indicated, BSA (250 ng per tube), and 40 μM [^3H]phosphatidylinositol 4,5-bisphosphate (labelled plus unlabelled, 15000 c.p.m. per tube), in combination with 20 μM PS and 500 μM PE. Other additions included G-protein $\beta\gamma$ subunit or $\beta\gamma$ dilution buffer; $\beta\gamma$ subunits were diluted into 0.75% cholate in 25 mM Hepes (pH 7.5)/0.1 mM EDTA/1 mM DTT. The final cholate concentration in the assay was 0.15%. Activation of $\alpha_{q/11}$ was performed by incubation for 2 h at 30 °C in 50 mM Hepes (pH 7.2)/1 mM EDTA/3 mM EGTA/5 mM MgCl_2 /2 mM DTT/100 mM NaCl/1% cholate/1 mM GTP[S]. A 5 μl sample of this mixture was added to the PLC assay tubes. Control incubations received the same buffer minus the subunit. Incubation was at 30 °C in a total incubation volume of 50 μl . Ca^{2+} concentration was set by a Ca^{2+} /EGTA buffer containing 3 mM EGTA [23–25]. Incubation was terminated by the transfer of tubes to ice followed by the addition of 200 μl of ice-cold 10% (w/v) trichloroacetic acid and 100 μl BSA (10 mg/ml). The samples were centrifuged, the supernatant was removed and the amount of released radioactivity was determined by liquid-scintillation counting.

Materials

PKC, purified from rat brain and containing a mixture of the Ca^{2+} /diacylglycerol-dependent isoforms, was obtained from Calbiochem. Purity was greater than 95% by SDS-PAGE (results not shown). Recombinant PKC α was obtained from Gibco-BRL (rabbit isoenzyme) or Panvera (human isoenzyme). Recombinant PKC ϵ and PKC ζ were obtained from Panvera. PS and PE were from Avanti Polar Lipids. [^3H]Phosphatidylinositol 4,5-bisphosphate was from New England Nuclear. All other reagents were from Sigma.

RESULTS

Phosphorylation of PLC- β_1 by PKC

As shown in Figure 1, PKC α produced a time-dependent phosphorylation of purified PLC- β_1 . Near-maximal levels of phosphorylation were attained after a 60–90 min incubation with PKC α . The addition of fresh ATP or PKC α after 60 min of incubation did not result in additional phosphorylation, indicating that termination of phosphorylation was not due to ATP depletion or inactivation of PKC α . The stoichiometry for phosphorylation was determined to be 1.1 mol of phosphate/mol of PLC- β_1 after 120 min of incubation, consistent with one phosphorylation site as has been reported previously [4]. Similar results were obtained with PKC purified from rat brain, which consists predominantly of a mixture of the Ca^{2+} /diacylglycerol-dependent PKC isoforms (results not shown). Little phosphorylation of PLC- β_1 was evident in the absence of added PKC but in the presence of PKC cofactors, indicating the lack of endogenous PKC activity in the PLC- β_1 preparation. The purity of the PLC- β_1 preparation, as assessed by Coomassie Blue staining, and an autoradiogram of the phosphorylated PLC- β_1 are shown in Figure 1B.

Phosphorylation of PLC- β_1 by PKC α , PKC ϵ and PKC ζ is compared in Table 1. PKC ϵ , a member of the Ca^{2+} -independent but diacylglycerol-dependent PKC isoforms, produced approx. 50% of the phosphorylation observed in the presence of PKC α . PKC ζ failed to phosphorylate PLC- β_1 . Similar results were obtained with the respective PKC isoform at 5 and 20 nM, indicating that the reason for the diminished phosphorylation relative to PKC α was not a limiting amount of PKC. These findings indicate that the Ca^{2+} /diacylglycerol-dependent PKC

Figure 1 Time-dependent phosphorylation of PLC- β_1 by PKC α

(A) PLC- β_1 (●) at 3 ng/ μl was incubated in the presence of PKC α plus PKC activators (Ca^{2+} , PS and PMA) and [^{32}P]ATP. At the indicated time, an aliquot of the incubation mixture was removed and the amount of labelled PLC- β_1 was determined as described in the Materials and methods section. At 60 min, additional ^{32}P -ATP (▽) or PKC α (▼) was added to a final concentration of 0.1 mM or 3 nM respectively, and the incubation was continued. Phosphorylation in the absence of added PKC but in the presence of PKC cofactors is also shown (■). The results shown are from one experiment although the time course for phosphorylation was repeated several times during this study. Phosphorylation in the absence of added PKC was done twice and the results shown are representative of these two experiments. (B) Lane 1, Coomassie Blue stain of PLC- β_1 subjected to SDS/PAGE [11% (w/v) gel]; lane 2, autoradiogram of phosphorylated PLC- β_1 after a 120 min incubation in the presence of PKC α plus cofactors. The position of PLC- β_1 is indicated by an arrow alongside each lane.

Table 1 PKC isoform dependence for phosphorylation of PLC- β_1 and PLC- β_3

PLC- β_1 or PLC- β_3 was incubated with the indicated PKC isoforms at 5 or 20 nM for 120 min. Phosphorylation by 5 or 20 nM PKC α was measured as described in the Materials and methods section. All incubations contained PS plus PE. Results are the means of four experiments. PKC ϵ phosphorylation was measured in the presence of PMA and the absence of Ca^{2+} . PKC ζ phosphorylation was measured in the absence of PMA or Ca^{2+} . Results with PKC ϵ and PKC ζ are the means of two experiments. Phosphorylation of PLC- β_3 is the mean of three experiments.

PLC- β isoform	PKC isoform	PKC concentration (nM) ...	Stoichiometry of phosphorylation	
			5	20
PLC- β_1	PKC- α		1.0	1.1
	PKC- ϵ		0.5	0.6
	PKC- ζ		< 0.1	< 0.1
PLC- β_3	PKC- α		< 0.1	< 0.1

isoforms are likely to have the major role in regulating PLC- β_1 activity. In contrast with these results, PKC α produced little phosphorylation of PLC- β_3 .

Effect of G-protein $\beta\gamma$ subunit on the phosphorylation of PLC- β_1 by PKC α

Preincubation of PLC- β_1 with G-protein $\beta\gamma$ subunits resulted in a decrease in the rate of phosphorylation of PLC- β_1 by PKC α (Figure 2). At 10 min of incubation, phosphorylation of PLC- β_1

by PKC α in the presence of 500 nM $\beta\gamma$ was 87% of the corresponding control value. The inhibition due to $\beta\gamma$ decreased as incubation continued. At 120 min, phosphorylation in the presence of $\beta\gamma$ was similar to that in the control (Figure 2A). These results indicated that preincubation of PLC- β_1 with $\beta\gamma$ subunits inhibited the initial rate of PLC- β_1 phosphorylation by PKC α . The diminished inhibition by $\beta\gamma$ observed with incubation suggested that the interaction between PLC- β_1 and $\beta\gamma$ was not maintained in the presence of phosphorylation mixture containing PS. PE is included in most PLC assays that measure the stimulation of activity by $\beta\gamma$ [6–8]. The effect of adding PE to the phosphorylation mixture is shown in Figure 2(B). Under these conditions, a more marked effect of $\beta\gamma$ on PKC α phosphorylation of PLC- β_1 was evident. Phosphorylation of PLC- β_1 in the presence of 500 nM $\beta\gamma$ was 51% of control at 10 min of incubation. In contrast with the results shown in Figure 2A, inhibition was maintained for the 120 min incubation. At 120 min, PLC- β_1 phosphorylation in the presence of 500 nM $\beta\gamma$ was 61% of control values. These results demonstrate that the inhibitory effects of $\beta\gamma$ are more pronounced with the inclusion of PE in the phosphorylation mixture. In a separate series of experiments, the rate of PLC- β_1 phosphorylation by PKC α in the presence of PE or PE plus PS was compared. The rate of PLC- β_1 phosphorylation in the presence of PE plus PS was $68 \pm 4\%$ (S.E.M.) of that observed in the presence of PS alone. Similar results were obtained when PKC α activity was determined with histone III S substrate. These results demonstrate that the inclusion of PE decreased PKC α activity by 30%. However, the decrease in PKC α activity did not seem to be the basis for the greater inhibitory effect of $\beta\gamma$ subunit under these conditions. The magnitude of inhibition was not affected by decreasing the rate of PLC- β_1 phosphorylation by the use of lower PKC α

Figure 2 Effect of G-protein $\beta\gamma$ subunits on the PKC α -dependent phosphorylation of PLC- β_1

PLC- β_1 (5 μl , 20 ng/ μl) was premixed with 4 μl of $\beta\gamma$ buffer (○) or 4 μl of $\beta\gamma$ at 109 ng/ μl (●) and incubated on ice for 30 min. The mixture was added to 16 μl of phosphorylation mixture and PKC α -dependent phosphorylation was subsequently initiated in the presence of PMA and either PS (A) or PS plus PE (B). The final concentration of $\beta\gamma$ in the phosphorylation assay was 500 nM. The incubation mixture was sampled at the indicated time and the label incorporated into PLC- β_1 was determined as described in the Materials and methods section. Results are one of two experiments that yielded similar results. Autoradiograms demonstrating the phosphorylation of PLC- β_1 in the absence or presence of $\beta\gamma$ under these conditions are shown.

Figure 3 Effect of preincubation with $\beta\gamma$ on the $\text{PKC}\alpha$ -dependent phosphorylation of $\text{PLC-}\beta_1$

$\text{PLC-}\beta_1$ ($7 \mu\text{l}$, $55 \text{ ng}/\mu\text{l}$) was preincubated with $7 \mu\text{l}$ of $\beta\gamma$ ($262 \text{ ng}/\mu\text{l}$) on ice for 30 min followed by the addition of $56 \mu\text{l}$ of phosphorylation mixture (∇) or mixed with $\beta\gamma$ and added immediately to the phosphorylation mixture ($\bullet$). The final concentration of $\beta\gamma$ in the phosphorylation assay was 727 nM . The phosphorylation of $\text{PLC-}\beta_1$ is shown as the percentage of the maximal control phosphorylation that received $\beta\gamma$ buffer. Phosphorylation relative to the control that received $\beta\gamma$ buffer ($\circ$) is shown. Results are means $\pm$ S.E.M. for three experiments. The inset demonstrates the effect of adding $\beta\gamma$ ($\bullet$) or $\beta\gamma$ buffer ($\circ$) after 10 min of incubation with $\text{PKC}\alpha$. Results are the means of two experiments.

concentrations (results not shown). Thus the enhancement of inhibition by inclusion of PE in the pretreatment does not seem to be due to an effect on phosphorylation itself.

As shown in Figure 3, inhibitory effects of $\beta\gamma$ were greater for $\text{PLC-}\beta_1$ preincubated with $\beta\gamma$ before phosphorylation. Preincubation of $\text{PLC-}\beta_1$ with $\beta\gamma$ subunits on ice resulted in an immediate decrease in the subsequent phosphorylation of $\text{PLC-}\beta_1$ by $\text{PKC}\alpha$. Inhibitory effects of $\beta\gamma$ were also evident without a prior preincubation with $\text{PLC-}\beta_1$, but the magnitude of inhibition was 50 % less than that observed with preincubation. Addition of $\beta\gamma$ subunits after 15 min of phosphorylation produced a 10–15 % decrease in the rate of phosphorylation (Figure 3, inset). However, addition of $\beta\gamma$ subunits after 60 min of phosphorylation had no effect on the levels of phosphorylated $\text{PLC-}\beta_1$ (results not shown). These results demonstrate that maximal inhibition of phosphorylation occurred when $\beta\gamma$ was incubated with $\text{PLC-}\beta_1$ before addition to the phosphorylation mixture. Because $\beta\gamma$ subunits did not affect the level of fully phosphorylated $\text{PLC-}\beta_1$, the decrease in the initial rate of phosphorylation was not due to $\beta\gamma$ activation of a phosphatase. Furthermore $\beta\gamma$ did not affect $\text{PKC}\alpha$ activity, indicating that $\beta\gamma$ mediated its effects through an interaction with $\text{PLC-}\beta_1$. In the presence of 500 nM $\beta\gamma$, $\text{PKC}\alpha$ activity as assayed with histone III S substrate under standard assay conditions [22] was 104 % of that observed in the presence of $\beta\gamma$ buffer (mean of two experiments). Similarly, $\text{PKC}\alpha$ phosphorylation of a synthetic peptide, APGSVKAPAK, which corresponds to the PKC phosphorylation site on $\text{PLC-}\beta_1$, was not affected by 500 nM $\beta\gamma$. Phosphorylation in the presence of $\beta\gamma$ was $102 \pm 10 \%$ (S.E.M.) of that observed in the absence of $\beta\gamma$ (mean of three experiments). $\beta\gamma$ subunits also had no effect on the autophosphorylation of $\text{PKC}\alpha$. Autophosphorylation of $\text{PKC}\alpha$ in the presence of 500 nM $\beta\gamma$ was 106 % of that observed in the absence of $\beta\gamma$. Other studies have also reported that $\beta\gamma$ subunits do not affect PKC

Figure 4 Concentration dependence of the inhibition by $\beta\gamma$ of the phosphorylation of $\text{PLC-}\beta_1$ by $\text{PKC}\alpha$

$\text{PLC-}\beta_1$ ($5 \mu\text{l}$, $20 \text{ ng}/\mu\text{l}$) was preincubated with $4 \mu\text{l}$ of a range of $\beta\gamma$ concentrations or $\beta\gamma$ buffer for 30 min on ice, after which it was added to $16 \mu\text{l}$ of phosphorylation mixture containing $\text{PKC}\alpha$. Incubation with $\text{PKC}\alpha$ was conducted for 10 min ($\bullet$). Results shown are means $\pm$ S.E.M. for five experiments. An autoradiogram with phosphorylation of $\text{PLC-}\beta_1$ in the presence of buffer control (lane 1), 50 nM $\beta\gamma$ (lane 2), 500 nM $\beta\gamma$ (lane 3), 750 nM $\beta\gamma$ (lane 4) and $2 \mu\text{M}$ $\beta\gamma$ (lane 5) is shown. The bottom panel depicts the stimulation of $\text{PLC-}\beta_1$ by the indicated concentrations of $\beta\gamma$. PLC activity was determined as described in the Materials and methods section. Results are the means of four experiments.

activity [26,27]. The lack of an effect of $\beta\gamma$ on the phosphorylation of these substrates also indicates that $\beta\gamma$ does not serve as a competitive substrate for $\text{PKC}\alpha$. Phosphorylation of the β subunit by PKC was less than 0.02 mol of phosphate/mol of β subunit.

The concentration dependence for the inhibition of $\text{PLC-}\beta_1$ phosphorylation by $\beta\gamma$ is shown in Figure 4(A). In the presence of 30 nM $\text{PLC-}\beta_1$, half-maximal inhibition of phosphorylation occurred with approx. 500 nM $\beta\gamma$. An almost complete inhibition of phosphorylation was evident with $2 \mu\text{M}$ $\beta\gamma$. The inclusion of a 3-fold excess of $\alpha_o\text{-GDP}$, which binds $\beta\gamma$ subunits and deactivates $\beta\gamma$ -mediated effects, largely prevented the inhibition due to 500 nM $\beta\gamma$. In paired experiments, $\text{PLC-}\beta_1$ phosphorylation in the presence of 500 nM $\beta\gamma$ was 55 % of control, whereas in the presence of 500 nM $\beta\gamma$ plus $1.5 \mu\text{M}$ $\alpha_o\text{-GDP}$ the phosphorylation was 89 % of control. For comparison, the effect of a range of $\beta\gamma$ concentrations on $\text{PLC-}\beta_1$ activity is shown in Figure 4(B). Consistent with other studies [6,8], $\beta\gamma$ subunits stimulated an approx. 40 % increase in $\text{PLC-}\beta_1$ activity. The onset of $\beta\gamma$ -mediated inhibition of phosphorylation and stimulation of $\text{PLC-}\beta_1$ activity occurred over a comparable concentration range.

Effect of $\beta\gamma$ and $\text{PKC}\alpha$ on $\text{PLC-}\beta_1$ enzymic activity

The functional consequence of the inhibition of $\text{PLC-}\beta_1$ phosphorylation by $\beta\gamma$ was next determined by measuring the effects of $\beta\gamma$ and $\text{PKC}\alpha$ on the enzymic activity of $\text{PLC-}\beta_1$. $\text{PLC-}\beta_1$ was

Figure 5 Effect of preincubation with $\beta\gamma$ on the regulation of PLC- β_1 enzymic activity by PKC α

PLC- β_1 was preincubated with $\beta\gamma$ (●) or $\beta\gamma$ buffer (○) on ice for 30 min. The PLC- β_1 was then incubated in the absence (A) or presence (B) of PKC α for 90 min as described in the Materials and methods section. After incubation, the mixture was diluted approx. 1:300 for assay of activity at the indicated pCa. For comparison the results from the control are shown in (B) with a dotted line. Results are means $\pm$ S.E.M. for four experiments.

Table 2 Effect of PKC α on the stimulation of PLC- β_1 activity by $\beta\gamma$ and $\alpha_{q/11}$

PLC- β_1 was incubated in the absence or presence of PKC α for 90 min as described in the Materials and methods section. The incubated PLC- β_1 was diluted and added to a PLC assay tube. In the first series of experiments, the incubation tube contained 200 nM $\beta\gamma$ or $\beta\gamma$ buffer. Incubation was for 7–15 min at pCa 5.5 at 30 °C. Results are means $\pm$ S.E.M. for four experiments. In a separate series of experiments, PLC activity was determined in the presence of 7 nM $\alpha_{q/11}$ -GTP[S] or buffer. Incubation was conducted at a pCa of 6.6 at 30 °C. Results are the means of two experiments with similar findings. Under these assay conditions, PLC activity for control basal sample and with 200 nM $\beta\gamma$ was 18 and 22 pmol respectively. For the PKC-treated enzyme, values for the basal sample and with 200 nM $\beta\gamma$ were 13 and 15 pmol respectively. Because the specific activity of PLC- β_1 varied slightly over the course of these experiments, the incubation time and PLC- β_1 concentration were adjusted to provide a consistent level of product generation under linear assay conditions, i.e. pmol. Consequently the results are expressed as pmol of product generated.

Pretreatment	PLC activity (pmol)	
	Basal	+ $\beta\gamma$
Control	44.6 $\pm$ 1.9	61.5 $\pm$ 5.0
+ PKC α	35.1 $\pm$ 3.5	41.1 $\pm$ 2.6

Pretreatment	PLC activity (pmol)	
	Basal	+ $\alpha_{q/11}$ -GTP[S]
Control	17.7	50.0
+ PKC α	10.8	36.3

incubated with or without $\beta\gamma$ for 30 min followed by incubation in the absence or presence of PKC α for 90 min. After incubation, PLC- β_1 was diluted and assayed for activity. As shown in Figure 5(A), co-incubation of PLC- β_1 with $\beta\gamma$ subunit had little effect on control activity. PKC α decreased the Ca²⁺-dependent stimulation of PLC- β_1 (Figure 5B). PLC- β_1 preincubated with $\beta\gamma$ was less affected by PKC α . These results demonstrate that pre-incubating PLC- β_1 with $\beta\gamma$ antagonized the PKC α -mediated inhibition of PLC- β_1 .

The effect of PKC α on the stimulation of PLC- β_1 activity by $\beta\gamma$ is shown in Table 2. PLC- β_1 was incubated with or without PKC α for 90 min followed by an assay of activity in the absence or presence of 200 nM $\beta\gamma$. Subunits $\beta\gamma$ produced a 40 % increase in control PLC- β_1 activity. Consistent with the results of Figure 5, PKC α decreased basal PLC- β_1 activity. Stimulation by $\beta\gamma$ was also decreased as a consequence of PKC α treatment.

In a separate series of experiments the effect of PKC α on $\alpha_{q/11}$ -GTP[S] stimulation was determined. The addition of 7 nM $\alpha_{q/11}$ -GTP[S] stimulated an increase in the control and PKC-treated PLC- β_1 activity. PKC did not inhibit the degree of stimulation due to the α subunit. Nevertheless the maximal attained activity was less for the PKC-treated PLC- β_1 , possibly as a consequence of the decreased basal activity.

DISCUSSION

Activation of PKC by phorbol ester results in an inhibition of the agonist-stimulated increase in PLC activity in many cells. PKC phosphorylates PLC- β_1 *in vivo* as well as *in vitro* [6], suggesting that PKC has a role in mediating negative feedback inhibition of PLC- β_1 . Insight into a possible mechanism of action comes from studies that have shown inhibition of the Ca²⁺-dependent stimulation of PLC- β_1 by brain PKC isoenzymes [12]. Because brain PKC consists of a mixture of PKC isoforms, the identity of the PKC isoform responsible for the inhibition was not known. Differences in substrate preference by PKC isoforms have been demonstrated with other proteins [28–30]. The present studies demonstrate that PLC- β_1 is phosphorylated by PKC isoforms with the following effectiveness: PKC α $\gg$ NPKC ϵ ; not PKC ζ . Thus PKC α , a member of the Ca²⁺/diacylglycerol-dependent PKC isoforms, is likely to have a major role in regulating PLC- β_1 . This is consistent with studies that have demonstrated translocation of PKC α to membranes on the stimulation of human neuroblastoma cells by agonist [31]. PLC- β_3 was poorly phosphorylated by PKC α . The lack of PLC- β_3 phosphorylation is consistent with the absence of the PKC phosphorylation sequence present in PLC- β_1 .

The PKC α -mediated phosphorylation of PLC- β_1 was inhibited by G-protein $\beta\gamma$ subunits. Inhibitory effects of $\beta\gamma$ were best observed after preincubation of $\beta\gamma$ with PLC- β_1 before addition to the phosphorylation mixture and on the inclusion of PE in the phosphorylation mixture. The greater inhibitory effect with preincubation suggests that occupancy of the $\beta\gamma$ -binding site produces a steric hindrance or a conformational change in PLC- β_1 that interferes with subsequent PKC α binding. PKC α binding and phosphorylation, in turn, might block $\beta\gamma$ binding as suggested by the diminished stimulation of the PKC α -inhibited PLC- β_1 by $\beta\gamma$ (Table 2).

Inclusion of PE with PS facilitated the regulation of PLC- β_1 by $\beta\gamma$. PE did not affect the magnitude of inhibition through an effect on the rate of phosphorylation. One possibility is that the presence of PE stabilizes $\beta\gamma$ -PLC- β_1 interactions that are disrupted by PS. Consistent with this, the stimulation of PLC- β_1 activity by $\beta\gamma$ has not been observed when PLC activity is assayed in the presence of PS concentrations that are used to activate PKC (results not shown). PS and $\beta\gamma$ have been shown to bind to similar regions on effector proteins, and allosteric regulation of $\beta\gamma$ binding to effector proteins by PS has been reported [32,33]. Furthermore $\beta\gamma$ binding to PS has been demonstrated [34]. These findings indicate that phospholipids modulate $\beta\gamma$ action and their role in modulating the inhibition of phosphorylation by $\beta\gamma$ is under study.

Inhibition of PLC- β_1 phosphorylation by $\beta\gamma$ was concentration-dependent. In the presence of 30 nM PLC- β_1 ,

2 μM $\beta\gamma$ produced an almost complete inhibition of phosphorylation. A 50% inhibition of phosphorylation was apparent in the presence of 500 nM $\beta\gamma$. The concentration dependence for the inhibition of phosphorylation by $\beta\gamma$ is comparable with that for the stimulation of PLC- β_1 activity by $\beta\gamma$ (Figure 4). A similar concentration dependence for the stimulation of PLC- β_1 by $\beta\gamma$ has been shown in other studies [35,36]. These results suggest that the effects of $\beta\gamma$ on phosphorylation and enzymic activity occur through occupancy of similar $\beta\gamma$ -binding sites.

G-protein $\beta\gamma$ subunits modulate the activity of diverse proteins including adenylate cyclase, PLC, ion channels and kinases [37]. This diversity in effectors suggests that different protein interactions are involved in mediating the effects of $\beta\gamma$. Numerous proteins, including the β -adrenergic kinase and PLC- γ , contain a pleckstrin homology (PH) domain, which is thought to bind $\beta\gamma$ subunits [38]. A motif in adenylate cyclase type 2 that participates in the binding of $\beta\gamma$ and is conserved in K^+ channels and β -adrenergic receptor kinases, but is not found in PLC- β , has been identified [39]. These findings indicate the existence of several $\beta\gamma$ -binding sequences. The PH domain is located in the N-terminal domain of PLC- β isoforms [40], which is distal from the PKC phosphorylation site, Ser⁸⁸⁷ in the C-terminal region [4]. However, it is likely that $\beta\gamma$ interacts with PLC- β_1 through multiple contact points and can thereby affect events distal to the primary binding site. Full elucidation of the mechanisms by which $\beta\gamma$ blocks the phosphorylation of PLC- β_1 by PKC will be facilitated by the identification and kinetic characterization of the respective binding sites.

G-protein $\beta\gamma$ subunits also inhibit PKC phosphorylation of α_x [26] and α_{12} [41]. A 1:10 molar ratio of α_x to $\beta\gamma$ [26] and a 1:1 molar ratio of α_{12} to $\beta\gamma$ [41] was required to produce maximal inhibition of PKC phosphorylation. Although the molar ratio derived for PLC- β_1 is much greater, i.e. approx. 1:100, this value is consistent with the apparent affinities of this protein for $\beta\gamma$ subunits that have been derived from functional studies.

The inhibition of phosphorylation by $\beta\gamma$ has functional consequences on PLC- β_1 activity. PKC α inhibits PLC- β_1 by decreasing the Ca^{2+} -dependent stimulation of enzymic activity (Figure 5). Inhibition by PKC α was antagonized by $\beta\gamma$ subunits. Previous studies have shown that brain PKC inhibits the Ca^{2+} -dependent stimulation of partly purified PLC- β_1 while having a smaller effect on purified PLC- β_1 [12]. In the present studies, inhibition by PKC α was clearly evident with purified PLC- β_1 . The use of different experimental conditions, including substrate composition, PKC α isoform as well as a longer incubation with PKC α might have resulted in a greater inhibitory effect of PKC α on purified PLC- β_1 in this study.

The ability of $\beta\gamma$ subunits to antagonize the phosphorylation of PLC- β_1 by PKC and the inhibition of PLC- β_1 activity by PKC might have important physiological implications. Receptor stimulation results in the activation and stimulation of PLC- β_1 activity by G_q . The resultant increase in Ca^{2+} and diacylglycerol levels activates PKC. PKC phosphorylates receptors, which can lead to the inactivation of signalling at the receptor level [42]. PKC also phosphorylates PLC- β_1 , resulting in a decreased stimulation by Ca^{2+} as well as by $\beta\gamma$. A decrease in Ca^{2+} stimulation has important consequences because Ca^{2+} is required for enzymic activity and maximal hormone stimulation of PLC- β_1 . Consistent with this, the results in Table 2 demonstrate that although the percentage stimulations of the control and PKC α -treated PLC- β_1 by $\alpha_{q/11}$ -GTP[S] are comparable, the maximal enzymic activity attained was less for the PKC-treated enzyme. Similarly, PKC decreased the maximal activity attained in response to stimulation by $\beta\gamma$. The physiological consequence of a diminished stimulation by $\beta\gamma$ remains to be determined as $\beta\gamma$ produces only a modest

stimulation of PLC- β_1 *in vitro* and no detectable stimulation *in vivo* [43,44]. G-protein $\beta\gamma$ subunits, however, can markedly decrease PLC- β_1 phosphorylation. This could delay the onset of negative feedback inhibition by PKC and prolong the activation of PLC- β_1 .

In summary, the present studies have identified a new role for $\beta\gamma$ in the modulation of PLC- β_1 activity. The ability of $\beta\gamma$ subunits to antagonize the PKC α -dependent inhibition of PLC- β_1 activity might afford an important mechanism to modulate desensitization of this signalling pathway.

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