G Protein-mediated Inhibition of Phospholipase C Activity in a Solubilized Membrane Preparation*

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Phospholipase C (PLC) is an effector system utilized by hormones that mediate increases in cytosolic Ca²⁺ levels and activation of protein kinase C (1). GTP-binding proteins (G proteins) function as key intermediaries in promoting receptor-dependent regulation of phosphoinositide-specific PLC activity. Pertussis toxin has functionally identified a pertussis toxin-sensitive G protein. Adenosine (11) and dopamine (12) inhibited PLC activation due to thyrotropin-releasing hormone in GH₃ cells (11) and cultured anterior pituitary cells (12) through a pertussis toxin-sensitive mechanism. In a permeabilized rat thyroid cell line FRTL5, carbachol inhibited both basal and norepinephrine-stimulated PLC activity through a GTP- and pertussis toxin-sensitive mechanism (13). Dual regulation of PLC activity by guanine nucleotides has been demonstrated in rat cerebral cortical membranes (14). Inhibition of PLC activity occurred with nanomolar guanine nucleotide concentrations, whereas stimulation ensued with micromolar concentrations of guanine nucleotides. A similar dual regulation of effector function by guanine nucleotides has been described in the adenyl cyclase system and has been attributed to the temporal activation of inhibitory and stimulatory G proteins (15, 16).

At present, little is known concerning the mechanism(s) that mediate the inhibition of PLC activity. The present studies demonstrate that solubilized membrane preparations retain G protein-dependent inhibition of PLC activity. Inhibition of PLC activity occurs through a rapid but transient mechanism and results in a decrease in the Ca²⁺ sensitivity of PLC.

**EXPERIMENTAL PROCEDURES**

Preparation of Membranes—Membranes were prepared essentially as described previously (17). The only modification in the procedure was to omit the sucrose gradient purification step. The washed membranes obtained from the 17,000 × g centrifugation were frozen in a dry ice/alcohol bath and stored at -80°C until use. For membrane studies, 3 ml of frozen membrane was thawed, diluted with 4 ml of buffer consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 100 μM phenylmethanesulfonyl fluoride. The membranes were pelleted at 16,000 rpm for 30 min. The membranes were resuspended in the same buffer and centrifuged an additional time. The resulting pellet was resuspended in buffer to a final protein concentration of 7 mg/ml. PLC activity in membranes was determined as described under “Measurement of Phospholipase C Activity.”

Membrane Solubilization—Membranes were thawed and resuspended by vortexing in 4 volumes of buffer containing 0.1% sodium cholate, 50 mM NaCl, 1.0 mM EDTA, 10 mM Tris-HCL (pH 8.0), 1 mM dithiothreitol, and protease inhibitor mixture (17). The resuspended membranes were maintained on ice for 15 min followed by centrifugation at 32,000 × g for 30 min to pellet the membranes. The supernatant was discarded. This step was repeated twice. Preextracted membranes were brought to a final protein concentration of 10 mg/ml in preextraction buffer, and sufficient sodium cholate was added to stabilize membranes to bring the final cholate concentration to 1%. Ethylene glycol was added at 0.1%. The membranes were solubilized for 1.5-2 h at 5°C. At the end of the solubilization, the ethylene glycol concentration was increased to 10%. The solubilized mixture was centrifuged at 100,000 × g for 90 min. The clear supernatant (extract) was removed, diluted 3-fold in buffer A which con-
tained 1% cholate, 1.0 mM EDTA, 10 mM Tris (pH 8.0), 10% ethylene glycol, 1 mM dithiothreitol, and protease inhibitor mixture. The sample was applied to a 1-ml DEAE column equilibrated with buffer A. The column was washed with 2 volumes of buffer A to remove unbound protein. The bound activity was eluted with 2 ml of buffer A containing 250 mM NaCl. The solubilized preparation was stored frozen in 100-µl aliquots at -80°C. Each aliquot was thawed once for use in the designated experiments.

Measurement of Phospholipase C Activity—PLC activity was assayed as described previously (17). Five microliters of appropriately diluted solubilized preparation was added to buffer containing (final concentration) 25 mM HEPES (pH 6.75), MgCl₂ as indicated, CaCl₂ as indicated, 12 mM LiCl, and 3.5 µM phosphatidylinositol 4,5-bisphosphate (~100-150 cpm/µmol) in a final volume of 50 µl ATP and App(NH)₃ (final concentration of 0.1 mM) were included in early studies, but later it was found that omission of these nucleotides did not affect the inhibitory response. As indicated, a substrate mixture of 206 µM phosphatidylethanolamine and 3.5 µM phosphatidylinositol 4,5-bisphosphate was used in the membrane studies and G protein subunit reconstitution studies. Except where indicated, the free Ca²⁺ concentration was 500 nM and set by a Ca²⁺-EGTA buffer using 3 mM EGTA. The free Ca²⁺ concentration was determined from the total concentration by solving equilibrium binding equations (18, 19) using published stability constants for EDTA, EGTA, and ATP (20).

Incubation was performed in a microvolume of fresh buffer, and 5 µl of the membrane preparation was taken for PLC assay using mixed phospholipid vesicles. For immunoblots, protein was separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels. Western blotting was performed as described (21) using mixed monoclonal antibodies to PLC-β₁, or pooled antibodies to PLC-β₁, (mixed monoclonal specific for PLC-γ₁), or anti-PLC-β₁ (mixed monoclonal specific for PLC-γ₁) at 4°C for 2 h. Following incubation, the membranes were diluted with an equal volume of fresh buffer, and 5 µl of the membrane preparation was taken for PLC assay using mixed phospholipid vesicles. For immunoblotting, protein was separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels.

Inhibitory Regulation of Phospholipase C

Fig. 1. Time-dependent regulation of PLC activity by GTPγS and AlF₄⁻. The soluble preparation was incubated with either no addition, 1 µM GTPγS (■), 50 µM GTPγS (●), or AlF₄⁻ (○) for the time indicated. The following values are obtained for the time points of 7.5, 15, 20, and 25 min; 0 for control were 2.99 ± 0.43, 5.97 ± 0.19, 6.41 ± 0.41, 7.27 ± 2.97; for 10 µM GTPγS were 2.99 ± 0.31, 6.62, 7.16 ± 0.26, 9.18 ± 0.11; for 50 µM GTPγS were 2.45 ± 0.05, 5.04 ± 0.12, 7.34 ± 0.90; and for AlF₄⁻ were 2.40 ± 0.57, 5.60 ± 0.45, 6.62 ± 0.96, 7.77 ± 1.71 pmol, respectively. Results are from one experiment done in duplicate, and the results are shown as a percent of control activity at the respective time point. PLC assay conditions were as described under “Experimental Procedures.”
Inhibitory Regulation of Phospholipase C

FIG. 3. Dose-response curve for GTPγS-mediated inhibition of PLC activity. The soluble preparation was incubated in the presence of the indicated GTPγS concentration for 15 min. Results are shown as percent of control activity and are the mean ± S.E. of four experiments. The decrease in basal activity was significant with p < 0.025 as indicated by the asterisk.

FIG. 4. Effect of GDPβS on the inhibition due to GTPγS. The soluble preparation was incubated in the presence of 10 nM GTPγS and with or without 1 μM GDPβS for 20 min. Basal PLC activity was 1.78 pmol and 1.69 in the presence of 1 μM GDPβS. Results are shown as percent of control activity and are the mean ± S.E. of three experiments. The decrease in basal activity caused by GTPγS was significant with p < 0.025. The decrease in basal activity in the presence of GTPγS and GDPβS was not significant.

Inhibitory effects of AlF₄⁻ and GTPγS were often more sustained in stored preparations. As shown in Fig. 2, A and B, the inhibition induced by GTPγS and AlF₄⁻ was maintained for at least 25 min. The magnitude of the inhibition was greater than that of Fig. 1 and resulted in a 30% decrease in basal activity at 15 min. This increased inhibitory response was due, in part, to the reduced rate of reversal. Some reversal of inhibition was nonetheless evident at 30 min.

The next series of experiments was designed to characterize the inhibitory regulation in greater detail using preparations that were stored for at least 1 week. Most studies were conducted within a 15-min incubation period to minimize against reversal of inhibition.

The dose-response curve for GTPγS-mediated inhibition of PLC activity is shown in Fig. 3. Half-maximal inhibition was obtained with approximately 1 nM GTPγS, and maximal inhibition was evident with 1 μM GTPγS. Reversal of inhibition was evident with 10 μM GTPγS.

The effect of GDPβS which antagonizes G protein action is shown in Fig. 4. GDPβS had little effect on basal PLC activity. GDPβS, however, antagonized the inhibition caused by GTPγS.

The effect of pertussis toxin on the GTPγS-mediated inhibition of PLC activity was determined. The soluble preparation was incubated in the absence or presence of pertussis toxin for 3 h followed by measurement of PLC activity. Pertussis toxin treatment (3 μg/ml) did not affect basal PLC activity. In the control, 10 nM GTPγS produced a 20% inhibition of basal PLC activity at 10 min which was followed by a reversal of inhibition (Fig. 5). In the pertussis toxin pretreatment, GTPγS did not inhibit PLC activity within the first 10 min of incubation. However, at 15 min, GTPγS produced a 15% decrease in PLC activity. These results indicate that pertussis toxin pretreatment delayed but did not prevent GTPγS-mediated inhibition of PLC activity.

The role of G protein α subunits on the basal PLC activity was next examined. Neither α₁-GTPγS nor α₁-GDP had any major effect on PLC activity (Table I). Both α₁-GTPγS and α₁-GDP produced a 15% inhibition of PLC activity. The results shown in the right column compare the effects of GTPγS and α₁ subunits within the same experiment. A 7-min incubation with GTPγS produced a 10% inhibition of PLC activity.
could not be induced by the addition of protein kinase C treatment on the active pertussis toxin diated inhibition of PLC. The soluble preparation was incubated in the presence of heat-inactivated (○—○) or active pertussis toxin (●—●) for 3 h. An aliquot of the mixture was taken for PLC determination which was conducted in the absence or presence of 10 nM GTPyS or the time indicated. Control basal activities were 4.05 ± 0.24, 5.87 ± 0.10, and 7.94 ± 0.57 for 7.5, 10, and 15 min, respectively. The corresponding values for the pertussis toxin basal were 4.28 ± 1.06, 5.54 ± 0.70, and 9.25 ± 0.90 as the average of two experiments done in duplicate. Results are shown as percent of the respective basal activity and are the mean of two experiments performed in duplicate. The error bars indicate the range between the duplicate assays.

**Table I**

| Subunit addition | % of basal activity |
|------------------|---------------------|
| None             | 100                 |
| 10 nM GTPyS      | 94 ± 1              |
| α, GTPyS         | 98 ± 2              |
| α, GDP           | 85 ± 5              |
| α, GDP           | 84 ± 4              |

Antibodies to the three major isozymes of PLC have been used to identify PLC effector systems in membranes (7). The results shown in Table II demonstrate that pretreatment of membranes with a mixture of monoclonal antibodies to PLC-β, blocked the ability of GTPyS to inhibit PLC activity. Incubation of membranes with a polyclonal antiserum specific for PLC-γ, or a mixture of monoclonal antibodies to PLC-β, did not alter the ability of GTPyS to inhibit PLC activity. In parallel studies with solubilized preparations, Western blotting was used to identify the PLC isozymes present in the solubilized preparation. PLC-β, was the only PLC isozyme that could be detected in a Western blot against 15 μg of loaded sample using standard detection protocols or an amplified protocol (BLAST) using 0.15 μg of protein (data not shown). These results provide evidence that PLC-β, is an effector in the inhibitory response.

**DISCUSSION**

The present studies demonstrate that G proteins mediate a rapid inhibitory modulation of PLC activity *in vitro*. Evidence that inhibition of PLC activity is mediated through a G protein includes guanine nucleotide sensitivity and antagonism by GDPβS. Inhibition of PLC activity is transient, followed by a rapid reversal of inhibition and onset of stimulation (Fig. 1). The magnitude of the inhibition is affected by the rate of reversal. A decrease in the rate of reversal results in both an increased degree of inhibition and a more sustained inhibitory effect (Fig. 2). Since reversal of inhibition was followed by stimulation at 25 min, it is likely that reversal occurs as a consequence of the concurrent activation of a stimulatory G protein, but this has not been established. These results suggest, in turn, that the magnitude of the stimulatory effect as well as the time course for the onset of stimulation may be modulated by the inhibitory component. Thus in a membrane system that contains both components, the net degree of PLC inhibition and PLC stimulation will reflect a balance between the regulatory input provided by the stimulatory and inhibitory mechanisms. The presence of a dual regulatory mechanism in solubilized bovine brain membrane preparations may account for the difference in the magnitude of PLC stimulation which has been observed in the solubilized preparation as compared with studies in a
FIG. 6. Effect of \( \beta \gamma \) subunits on basal and GTP\( \gamma \)S-mediated inhibition of PLC activity. In panel A, the solubilized preparation was incubated with \( (\bullet - - \bullet) \) or without \( (\circ - - \circ) \) 200 nM purified \( \beta \gamma \) subunits. Results are the mean \( \pm \) S.E. of three experiments. In panel B, the solubilized preparation was incubated for 4 min in the absence or presence of 10 nM GTP\( \gamma \)S. After 4 min, buffer \( (\circ - - \circ) \) or \( \beta \gamma \) \( (\bullet - - \bullet) \) at a final concentration of 200 nM was added. Incubation was continued for an additional 6 min. Assay conditions utilized a mixed phospholipid substrate as described under "Experimental Procedures." Results are shown as percent of control activity and are the mean \( \pm \) S.E. of three experiments performed in duplicate. PLC activity as measured over the 6--10-min incubation was significantly decreased from basal activity in the presence of GTP\( \gamma \)S with \( p < 0.025 \). Activity measured in the presence of \( \beta \gamma \) plus GTP\( \gamma \)S was not significantly different from control.

FIG. 7. Effect of 10 nM GTP\( \gamma \)S on the Ca\( ^{2+} \) sensitivity of PLC. The solubilized preparation was incubated in the presence of the indicated Ca\( ^{2+} \) concentration for 15 min in the absence \( (\circ - - \circ) \) or presence \( (\bullet - - \bullet) \) of 10 nM GTP\( \gamma \)S. Results shown are the mean \( \pm \) S.E. of three experiments.

The present studies, however, do provide insight into the components involved in the inhibition of PLC. PLC-\( \beta_i \) is the effector in this mechanism. This conclusion is based on the observation that antibodies to PLC-\( \beta_i \) block GTP\( \gamma \)S-dependent inhibition of PLC activity (Table II) and that PLC-\( \beta_i \) is the only PLC isozyme that is detected in a Western blot of the solubilized preparation. The properties of the G protein involved in this mechanism have been partially characterized. Half-maximal inhibition is evident at 1 nM GTP\( \gamma \)S, indicating that this event is evoked by the activation of a G protein with a high affinity for guanine nucleotides (Fig. 3). \( \alpha_i \)-GTP\( \gamma \)S had little effect (Table I). The studies with \( \alpha_i \) were inconclusive since both \( \alpha_i \)-GDP and \( \alpha_i \)-GTP\( \gamma \)S produced an inhibition.

reconstituted system using purified G proteins (5, 6).

The temporal relationship between the onset of inhibition and stimulation as well as the magnitude of the inhibitory response is similar to that observed with the G protein-regulated adenylylcyclase system. Dual regulation of adenylylcyclase has been attributed to the temporal activation of an inhibitory G (G\( _i \)) and a stimulatory (G\( _s \)) G protein. However, the precise mechanisms involved in regulating adenylylcyclase have not been resolved. Controversy exists concerning the relative role of the \( \alpha \) subunit and \( \beta \gamma \) subunit in mediating the dual regulation of adenylylcyclase (2, 23, 24). Similar considerations may apply in the PLC signaling system.
of PLC activity. Since a similar degree of inhibition was obtained with both the GTP·S and GDP form of αi, the observed effect was unlikely to be a result of an activated αi that directly inhibited PLC activity. The mechanism responsible for the inhibition has not been determined.

Pertussis toxin catalyzes the ADP-ribosylation of a cysteine residue located 4 amino acids from the carboxyl terminus of the G protein α subunit and blocks GTP as well as receptor-mediated interactions in many systems (15, 23). Pertussis toxin sensitivity has been used to identify G protein-mediated events. GTP·S dissociates the ADP-ribosylated G protein heterotrimer to the ADP-ribosylated α subunit and βγ (24). Thus, in most systems, pertussis toxin does not block the effects of G proteins that have been activated by GTP·S. In the studies shown on Fig. 5, it was possible to detect a pertussis toxin effect within the first 10 min of incubation. At 15 min, GTP·S initiated an inhibitory response in the pertussis toxin-treated sample. Since subunit dissociation by activating ligands such as GTP·S is temperature-dependent, the use of low temperatures and relatively short incubation times may have allowed maintenance of the pertussis toxin-induced modification for a limited time. Although these studies suggest that a pertussis toxin-sensitive G protein is involved in the mediation of the inhibitory effect, final proof will require purification of a pertussis toxin substrate and reconstitution of PLC regulation. These results, for instance, cannot exclude other effects of pertussis toxin which might include potentiation of the stimulatory component or interference in the ability of the PLC to be inhibited by a G protein or a G protein-controlled mechanism.

The data in Fig. 6 demonstrate that the addition of βγ subunits attenuated the GTP·S-mediated inhibition of PLC activity. Since GTP·S produces a persistent activation of G protein α subunits, the addition of βγ subunits might not be expected to reverse the GTP·S effect. There are at least two possible explanations for these results. First, the G protein may not be a typical G protein, and the addition of excess βγ subunits may be sufficient to deactivate the signal. Alternatively, the effects of βγ may be indirect. There is increasing evidence indicating that βγ subunits modulate effector systems. βγ modulates adenylcyclase activity through a direct as well as an indirect mechanism. The indirect mechanism is dependent on the presence of α subunits but is not a result of the ability of βγ to associate with α subunits and cause deactivation of the α subunit. Inhibition as well as activation of adenylcyclase by βγ occur and the observed effects are dependent on the type of adenylcyclase as well as the presence of α, (25, 26). βγ subunits activate a liver cytosolic PLC activity (27) and a turkey erythrocyte cytosolic PLC activity (28) with only marginal effects on PLC-γ1 (27, 28). βγ activation of basal PLC activity was not observed under the conditions used in the present studies. Thus, the attenuation of GTP·S dependent inhibition of PLC by βγ is not likely to be a result of a βγ-induced stimulation of basal activity. However, other indirect effects that depend on the presence of activated α subunits cannot be excluded.

These studies also demonstrate that PLC activity is negatively modulated in vitro through a G protein-controlled mechanism. Inhibition of PLC activity is associated with a decrease in the Ca2+ sensitivity of PLC as well as the maximal activity of PLC. This effect contrasts with the observed increase in Ca2+ sensitivity which has been demonstrated in response to G protein activation (6, 14, 29, 30). Since PLC-β1 is the effector for the GαiGβγ stimulatory pathway and the inhibitory pathway, these studies suggest that G proteins regulate PLC activity through a shared mechanism that remains to be identified. In the present studies, inhibition of PLC activity could not be induced by activation of protein kinase C or protein kinase A, indicating that the effects were not mediated through phosphorylation.

In summary, these studies have identified a G protein-dependent mechanism that functions to negatively modulate PLC activity in solubilized bovine brain membranes. The mechanism involved in promoting G protein-dependent inhibition of PLC activity and the regulation of this inhibitory response remain to be determined. Given the complexity of G protein-effector interactions, it is possible that both direct and indirect mechanisms may be involved in this effect.

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