Guanine nucleotides mediate stimulatory and inhibitory effects on cerebral-cortical membrane phospholipase C activity

Irene LITOSCH

Department of Pharmacology, University of Miami School of Medicine, Miami, FL 33101, U.S.A.

In cerebral-cortical membranes, hydrolysis-resistant guanine nucleotides exert a dual regulatory effect on phospholipase C activity. Nanomolar concentrations of guanosine 5'-[β -imido]triphosphate (p[NH]ppG) or guanosine 5'-[γ -thio]triphosphate (GTP[S]) inhibited basal phospholipase C activity, with a maximum inhibition of 30 % at 10 nM. Increasing the concentration of p[NH]ppG or GTP[S] to over 10 nM resulted in a reversal of the inhibitory effect and onset of stimulation of phospholipase C activity. These inhibitory effects were blocked by 100 μ M-guanosine 5'-[β -thio]diphosphate. GTP was relatively ineffective in producing either stimulation or inhibition of phospholipase C activity. Similarly, ATP, adenosine 5'-[β -imido]triphosphate and GDP were also ineffective. Expression of the dual effects of guanine nucleotides was affected by the Mg^{2+} concentration. At 0.3 mM- Mg^{2+} , both the inhibitory and the stimulatory components of p[NH]ppG action were evident. At 2.5 mM- Mg^{2+} , only p[NH]ppG stimulation was observed. Pertussis-toxin treatment blocked the p[NH]ppG-mediated inhibition of phospholipase C activity. These results demonstrate that non-hydrolysable guanine nucleotides exert both a stimulatory and an inhibitory effect on membrane phospholipase C activity. These effects may be mediated through distinct GTP-binding proteins.

INTRODUCTION

Considerable evidence indicates that the phosphoinositide-phospholipase C system is regulated by a guanine-nucleotide-binding protein (G-protein). In membranes, guanine nucleotides are essential to promote agonist activation of phospholipase C activity (Litosch *et al.*, 1985; Smith *et al.*, 1985; Uhing *et al.*, 1986; Straub & Gershengorn, 1986; Martin *et al.*, 1986; Guillon *et al.*, 1986). As is true of the adenylate cyclase system, hydrolysis-resistant guanine nucleotides promote activation of phospholipase C activity in membranes, suggesting that activation of the regulatory G-protein is sufficient to increase phospholipase C activity (Litosch *et al.*, 1985; Smith *et al.*, 1985; Cockcroft & Gomperts, 1985; Wallace & Fain, 1985; Uhing *et al.*, 1986; Straub & Gershengorn, 1986; Martin *et al.*, 1986; Guillon *et al.*, 1986; Gonzales & Crews, 1986; Litosch, 1987; Cockcroft & Taylor, 1987).

Various studies suggest that the phosphoinositide-phospholipase C system may also be subject to an inhibitory regulation. In cultured anterior-pituitary cells, dopamine inhibited TRH- (Simmonds & Strange, 1985; Journot *et al.*, 1987) and angiotensin II- (Enjalbert *et al.*, 1986) stimulated production of inositol phosphates. The dopamine effect on TRH action was antagonized by pertussis toxin, which inactivates G_i -like proteins, suggesting that inhibition was mediated through a G-protein (Enjalbert *et al.*, 1986). These studies measured agonist-stimulated inositol phosphate production after a relatively long incubation, and thus it remained to be determined if the inhibitory effect was a direct receptor-

mediated event or a secondary consequence of receptor activation. It was subsequently demonstrated that a major component of the dopamine effect could be attributable to an inhibition of Ca^{2+} influx (Vallar *et al.*, 1988). However, the maximal degree of inhibition induced by dopamine on TRH action could not be induced by verapamil or Ca^{2+} -free medium, suggesting that there might be both a Ca^{2+} -dependent and a Ca^{2+} -independent mechanism involved in the inhibition of phospholipase C activity. In rat hippocampal slices, excitatory amino acids inhibited carbachol-stimulated inositol phosphate production (Baudry *et al.*, 1986). Adenosine inhibited basal and TRH-stimulated inositol phosphate production in GH₃ cells through a mechanism which appeared insensitive to omission of Ca^{2+} from the medium (Delahunty *et al.*, 1988). Cumulatively, these data suggest that phospholipase C may be regulated by both inhibitory and stimulatory receptor-mediated mechanisms.

Adenylate cyclase activity is regulated by two distinct G-proteins: a stimulatory G protein, G_s , functions to activate the cyclase activity, and G_i mediates the effects of inhibitory agonists to cause a decrease in the cyclase activity (Stryer & Bourne, 1986; Gilman, 1987). There are distinct biochemical differences between G_s and G_i . In membranes, the guanine-nucleotide sensitivity of the G_i -mediated inhibitory response is greater than the stimulatory response. Thus, in platelet membranes, inhibition of adenylate cyclase is observed at nanomolar concentrations of guanine nucleotides, whereas stimulation is observed at micromolar concentrations (Katada *et al.*, 1984a,b). Furthermore, expression of G_i effects is

Abbreviations used: p[NH]ppA, adenosine 5'-[β -imido]triphosphate; G-proteins, GTP-binding regulatory proteins involved in signal transduction; G_s , the G-protein that stimulates adenylate cyclase; G_i , the G-protein that inhibits adenylate cyclase; GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; PtdIns P_2 , phosphatidylinositol 4,5-bisphosphate; TRH, thyrotropin-releasing hormone (thyroliberin).

optimal at low Mg^{2+} concentration, whereas optimal G_s effects are seen at high Mg^{2+} (Katada *et al.*, 1984a,b).

In an effort to ascertain whether phospholipase C activity might be subject to a similar inhibitory regulation, the present studies examined the effects of guanine nucleotides on phospholipase C activation in cerebral-cortical membranes under conditions similar to those that promote expression of inhibitory effects on adenylate cyclase. Cerebral-cortical membranes were chosen since these membranes have a readily measurable $p[NH]ppG$ - and Ca^{2+} -stimulated phospholipase C activity. GTP is ineffective in stimulating phospholipase C activity, indicating that stimulation requires a non-hydrolysable GTP analogue (Litosch, 1987). Furthermore, all inhibitory effects reported thus far have been confined to tissue of nervous-system origin (Simmonds & Strange, 1985; Baudry *et al.*, 1986; Journot *et al.*, 1987). The results of the present studies demonstrate that guanine nucleotides exert a complex regulation of phospholipase C activity in cerebral-cortical membranes. Nanomolar concentrations of $p[NH]ppG$ and GTP[S] promote a dose-dependent inhibition of phospholipase C activity. This inhibition, however, is converted into a stimulatory effect as the guanine nucleotide concentration is increased to 1 μM or greater.

MATERIALS AND METHODS

Membranes from rat cerebral cortex were prepared as previously described (Litosch, 1987). In brief, tissue obtained from male rats (150–250 g, Sprague–Dawley) was homogenized in 10 vol. of homogenization buffer, containing 280 mM-sucrose, 0.5 mM-EDTA, 60 μg of soybean trypsin inhibitor/ml and 10 mM-Tris/HCl (pH 7.5) at 5 °C, by using a Brinkmann Polytron (setting 4 for 10 s). The homogenate was centrifuged at 500 g for 10 min, and the supernatant was kept. The pellet was rehomogenized and centrifuged for an additional 10 min, at 500 g . The supernatant from the two centrifugation procedures was pooled and centrifuged at 32000 g for 30 min. The resulting pellet was resuspended in 5 vol. of homogenization buffer with a Dounce homogenizer and centrifuged again at 32000 g for 30 min. The pellet was extensively washed three times and finally resuspended in appropriate buffer at 0.3 $\mu g/\mu l$. All procedures were carried out at 5 °C with freshly prepared membranes.

For pertussis-toxin studies, cerebral-cortical membranes were prepared and layered on a sucrose step gradient as described by Koenig (1974), to remove mitochondrial and lysosomal membranes. The C membrane fraction was collected, washed in homogenization buffer and used immediately for studies with pertussis toxin. Membranes were incubated for 15 min in a 100 μl reaction mixture containing 50 mM-Hepes (pH 8.0), 1 mM-EDTA, 1 mM-dithiothreitol, 1 mM-ATP, 10 mM-phosphocreatine, 10 μg of creatine kinase/ml, 10 mM-thymidine, 100 μM -GTP, 2.5 mM-NAD, 30 μg of membrane protein and 4 μg of activated pertussis toxin or vehicle control as described by Katada *et al.* (1984a). During the incubation, NAD was supplemented at 2.5 mM every 5 min to maintain NAD concentrations essential for ADP-ribosylation. After 15 min, 2 ml of ice-cold 10 mM-Tris buffer (pH 8.0)/1 mM-EDTA/1 mM-dithiothreitol was added. The membranes were centrifuged for 30 min at 32000 g . The pellet was resuspended in the

Tris/EDTA/dithiothreitol buffer and used immediately for assay of phospholipase C activity.

Phospholipase C activity, in all but one set of experiments, was determined with exogenously added substrate. The following protocol was used for measurements of phospholipase C activity. $PtdInsP_2$ substrate (unlabelled plus labelled tracer) was evaporated to just dryness under a stream of N_2 . For a 500 μl reaction buffer, 2.5 μl of 0.1 % deoxycholate in 50 mM-Tris/HCl buffer (pH 7.0) was added, followed by addition of 200 μl of buffer [50 mM-Hepes (pH 6.75), 1 mM or varied $MgCl_2$ and 25 mM-LiCl]. The reaction buffer (200 μl) containing $PtdInsP_2$ was sonicated for 30–40 s in an ice bath with a micro-probe at maximum setting (Fisher Sonic Dismembrator), adjusted to the final 500 μl volume and used immediately for assay. The general assay for phospholipase C activity, except as indicated in the text, contained 25 μl of reaction buffer containing substrate (175–350 pmol at ~ 150 –85 c.p.m./pmol), plus the indicated Ca^{2+} concentration, which was set, as specified, with a Ca^{2+} -EGTA buffer system using 1 mM-EGTA (Portzehl *et al.*, 1964), 10 μl of membrane protein [3–5 μg of protein in 50 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/1 mM-dithiothreitol] and effectors to a total volume of 50 μl . Incubation was carried out at 30 °C for the indicated time. Each experiment contained a zero incubation control with all the components of the assay buffer. This zero value (50–100 c.p.m.) was subtracted from all experimental points. The incubation was terminated by the addition of 50 μl of ice-cold 100 mM-*trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid (CDTA)/1 mM-mannitol, followed by 1.25 ml of acidified methanol/chloroform (2:1, v/v), 0.5 ml of chloroform and 0.5 ml of water. The CDTA and mannitol were included to terminate the incubation and to enhance the efficiency of extraction of the inositol phosphates. The upper phase was used for analysis of inositol phosphates by chromatography on Dowex formate columns. The water-soluble fraction was composed of $InsP_1$, $InsP_2$ and $InsP_3$. The $InsP_1$, $InsP_2$ and $InsP_3$ fractions were collected as one and taken as an index of phospholipase C activity.

Phospholipase C activity was also measured in membranes containing endogenous prelabelled substrate. In these studies, the isolated cerebral cortex was chopped into fine pieces and incubated in oxygenated Krebs–Henseleit buffer containing 60 μCi of [3H]inositol (19 Ci/mmol)/ml at 37 °C to label the phosphoinositides (Gonzales & Crews, 1986). After 1 h, the medium was aspirated and the tissue homogenized in ice-cold homogenization buffer containing 100 μM -ATP. The homogenate was centrifuged at 500 g for 10 min. The resulting supernatant was centrifuged at 32000 g for 30 min. Membranes were resuspended in homogenization buffer and used immediately. Labelled membranes (approx. 200–300 μg of membrane protein/assay tube) were assayed for phospholipase C activity in incubation buffer containing 25 mM-Hepes (pH 7.0), 12 mM-LiCl, 0.3 mM- $MgCl_2$, 1 mM- NaH_2PO_4 , 100 μM - Na_2ATP and effectors as indicated in a total volume of 100–200 μl . Inositol phosphates were determined by adsorption of aqueous fraction to Dowex formate columns followed by elution with 1 M-ammonium formate/0.1 M-formic acid.

Statistical analysis of the data was based on Student's *t* test for paired comparison. Each experiment represents data from one freshly prepared batch of membranes.

Approx. 20 different membrane preparations were used in these studies.

p[NH]ppG, GTP[S], GDP[S], GTP, GDP and p[NH]ppA were from Boehringer Mannheim. The concentration of guanine nucleotides used in these experiments was verified by measuring the A_{252} of a given stock solution and calculating the concentration by using the appropriate absorption coefficient. AG1-X8 (formate) resin was from BioRad. [^3H]PtdInsP₂ (2 Ci/mmol) was from New England Nuclear. [^3H]Inositol (19 Ci/mmol) was from American Radiochemical Co. Pertussis toxin was from List Biochemicals. Other reagents were from Sigma Chemical Co.

RESULTS

Phospholipase C activity in [^3H]inositol-prelabelled cerebral-cortical membranes is stimulated by hydrolysis-resistant guanine nucleotides at concentrations of 1 μM or greater (Gonzales & Crews, 1986). The effects of lower concentrations of p[NH]ppG on the system have not been studied. The data in Fig. 1 demonstrate that nanomolar concentrations of p[NH]ppG produced a dose-dependent inhibition of basal phospholipase C activity, with a maximal inhibition of 10–15% at 10 nM-p[NH]ppG. Increasing the p[NH]ppG concentration resulted in an attenuation of the inhibitory effect and the onset of a dose-dependent stimulation of phospholipase C activity at 1 μM -p[NH]ppG or greater. These results indicated that in cerebral-cortical membranes nanomolar concentrations of p[NH]ppG promoted an inhibition of phospholipase C activity and suggested that there may be dual effects of guanine nucleotides on the phospholipase C system.

Phospholipase C activity in membranes can also be measured by using exogenously added substrate (Litosch & Fain, 1985; Fülle *et al.*, 1987; Taylor & Exton, 1987; Litosch, 1987). The time course for production of inositol phosphates from hydrolysis of exogenously added PtdInsP₂ under basal conditions and in the presence of 10 nM- or 100 μM -p[NH]ppG is shown in Fig. 2. As described previously, cerebral-cortical membranes rapidly hydrolysed added PtdInsP₂ (Litosch, 1987). p[NH]ppG at 100 μM stimulated a rapid increase in phospholipase C activity, resulting in an increase in both the rate and maximal degree of hydrolysis of PtdInsP₂. Stimulatory effects were not seen with either ATP or p[NH]ppA, indicating that phospholipase C was not regulated by adenine nucleotides (95 ± 15 and 89 ± 15 , as % of control incubated for 90 s; mean $\pm$ S.E.M. of three experiments with 100 μM -ATP and p[NH]ppA respectively). However, 10 nM-p[NH]ppG inhibited both the rate and degree of PtdInsP₂ hydrolysis. Although the rate of hydrolysis under basal conditions and in the presence of p[NH]ppG was not linear with time, the effects of p[NH]ppG, as measured at 90 s, provided an index of the relative state of activation of phospholipase C.

A comparison of the effects of varied concentrations of p[NH]ppG, GTP[S] and GTP on phospholipase C activity is shown in Fig. 3. Nanomolar concentrations of p[NH]ppG or GTP[S] produced a significant dose-dependent inhibition of basal phospholipase C activity. Maximal inhibition of phospholipase C activity was observed at 1 nM-GTP[S] and at 10 nM-p[NH]ppG. Half-maximal inhibition was obtained at approx. 0.33 nM-p[NH]ppG. Increasing the p[NH]ppG or GTP[S] con-

Fig. 1. Effect of p[NH]ppG concentration on phospholipase C activity measured in [^3H]inositol-prelabelled membranes

[^3H]Inositol-prelabelled cerebral-cortical membranes were incubated with the indicated concentration of p[NH]ppG for 90 s. Basal inositol phosphate production at 90 s was 300 c.p.m. Results are means $\pm$ S.E.M. for three experiments.

Fig. 2. Time course for basal and p[NH]ppG-modulated phospholipase C activity in cerebral-cortical membranes

Membranes (3 μg) were assayed for phospholipase C activity in the absence or presence of 100 μM - or 10 nM-p[NH]ppG for the indicated time in incubation buffer containing 0.3 mM-MgCl₂, 12 mM-LiCl, 1 μM -Ca²⁺ (Ca-EGTA), 25 mM-HEPES buffer (pH 6.75) and 7 μM -PtdInsP₂ at 30 °C. Results are shown in c.p.m. and are from one experiment done in triplicate with 10 nM- or 100 μM -p[NH]ppG. Phospholipase C activity measured in the presence of 10 nM-p[NH]ppG was significantly less than basal, with * $P < 0.05$.

Fig. 3. Effect of p[NH]ppG, GTP[S] and GTP concentrations on phospholipase C activity in membranes

Membranes (3–4.5 μ g) were assayed for phospholipase C activity in buffer containing 0.3 mM-MgCl₂, 12 mM-LiCl, 1 μ M-Ca²⁺ (Ca-EGTA), 25 mM-Hepes (pH 7.0) and 7 μ M-PtdInsP₂ for 90 s at 30 °C in the absence or presence of the indicated nucleotide concentrations. Basal phospholipase C activity was 5.3 ± 0.4 pmol/ μ g (mean $\pm$ S.E.M. for five experiments). Results are means $\pm$ S.E.M. for five experiments for p[NH]ppG and GTP[S], and four experiments for GTP. Phospholipase C activity was significantly less than basal, with * P < 0.05, ** P < 0.01 (based on Student's t test for paired comparison).

centration to greater than 10 nM resulted in a progressive attenuation of the net inhibitory effect. At concentrations greater than 1 μ M, both p[NH]ppG and GTP[S] stimulated phospholipase C activity. In contrast with p[NH]ppG and GTP[S], GTP did not markedly inhibit phospholipase C activity within the concentration range 0.1–100 nM.

A comparison of the effects of various nucleotides on phospholipase C activity is shown in Table 1. GDP[S] at 100 μ M had little effect on basal phospholipase C activity, but antagonized the inhibition by 3.3 nM-p[NH]ppG. GDP, ATP or p[NH]ppA did not induce a similar

inhibition of phospholipase C activity, indicating that the inhibitory effects were specific for the hydrolysis-resistant GTP analogues.

The next series of experiments was designed to ascertain whether the inhibitory and stimulatory effects of p[NH]ppG could be dissociated by Mg²⁺, which affects G-protein-mediated responses (Gilman, 1987). In these experiments, 0.2 mM-EDTA was used to chelate trace amounts of Mg²⁺ which might be present in the substrate, and Mg²⁺ was subsequently added to attain the indicated concentration. EDTA alone markedly decreased basal phospholipase C activity (46 ± 3 % of basal; mean $\pm$ S.E.M. of three experiments), presumably owing to chelation of bivalent cations essential for supporting phospholipase C activity. p[NH]ppG had little effect on phospholipase C activity under these conditions (results not shown). In the presence of 0.3 mM-Mg²⁺, basal phospholipase C activity was increased. Under these conditions, both the stimulatory and inhibitory effects of p[NH]ppG were apparent. In this series of experiments, a maximal inhibition of 20 % was observed at 10 nM-p[NH]ppG and a 50 % stimulation was obtained with 10 μ M-p[NH]ppG. Increasing the Mg²⁺ concentration to 2.5 mM resulted in a further increase in the apparent basal phospholipase C activity. Inhibitory effects on phospholipase C activity were not observed at nanomolar concentrations of p[NH]ppG in the presence of 2.5 mM-MgCl₂. These results indicated that net expression of the inhibitory and stimulatory effects of p[NH]ppG on phospholipase C was affected by the Mg²⁺ concentration (Fig. 4).

It was possible that the inability of 10 nM-p[NH]ppG to inhibit phospholipase C activity at 2.5 mM-Mg²⁺ was due to the increased basal activity, which might result from the displacement of a trace amount of activating Ca²⁺ from substrate or membrane sites, as has been reported in other studies using similar assay conditions

Table 1. Effect of GDP[S], GDP, ATP and p[NH]ppA on phospholipase C activity

Membranes were incubated with 3.3 nM-p[NH]ppG, -GDP, -ATP or -p[NH]ppA for 90 s. GDP[S] was present at 100 μ M. Results are means $\pm$ S.E.M. for four determinations done in duplicate with p[NH]ppG, GDP[S] or p[NH]ppG + GDP[S], and three determinations in duplicate with GDP, ATP and p[NH]ppA. Basal phospholipase C activity was 2.5 pmol/ μ g. The decrease in basal activity caused by 3.3 nM-p[NH]ppG was significantly different from control (* P < 0.025).

Nucleotide	Phospholipase C activity (% of basal)
p[NH]ppG	77 $\pm$ 4*
GDP[S]	101 $\pm$ 4
p[NH]ppG + GDP[S]	101 $\pm$ 6
GDP	100 $\pm$ 12
ATP	120 $\pm$ 7
p[NH]ppA	95 $\pm$ 10

Fig. 4. Effect of Mg^{2+} concentration on the dose-dependent inhibition and activation of phospholipase C activity by $p[NH]ppG$

Membranes were incubated in the presence of 0.2 mM-EDTA and 0.3 mM- or 2.5 mM- Mg^{2+} with the indicated $p[NH]ppG$ concentrations for 90 s. Basal phospholipase C activity was 2 pmol/ μg at 0.3 mM- Mg^{2+} and 5 pmol/ μg at 2.5 mM- Mg^{2+} . Results are means $\pm$ S.E.M. for three paired experiments, and are expressed as percentage of basal activity at the respective Mg^{2+} concentration.

(Irvine *et al.*, 1984). That this was not the case is suggested by the data in Table 2. Phospholipase C activity was activated to an extent comparable with that seen with 2.5 mM- Mg^{2+} by addition of 50 μM - Ca^{2+} , and the effects of 10 nM- and 100 μM - $p[NH]ppG$ on phospholipase C activity were determined. The data in Table 2 demonstrate

Table 2. Effect of Ca^{2+} on modulation of phospholipase C activity by 10 nM- and 100 μM - $p[NH]ppG$

Membranes were assayed for phospholipase C activity in the absence of added Ca^{2+} or with 50 μM added Ca^{2+} , with the indicated $p[NH]ppG$ concentration in the presence of 0.2 mM-EDTA and 0.3 mM- Mg^{2+} . Incubation was for 90 s at 30 $^{\circ}C$ with 5 μg of membrane protein. Results are means $\pm$ S.E.M. for three experiments: significantly less than basal, with $*P < 0.010$, $**P < 0.025$ (based on Student's *t* test for paired comparison).

Condition	Phospholipase C activity (pmol)	
	No added Ca^{2+}	+ 50 μM - Ca^{2+}
Basal	17.5 $\pm$ 2.6	25.1 $\pm$ 6.6
+ 10 nM- $p[NH]ppG$	14.9 $\pm$ 2.1*	20.9 $\pm$ 1.1**
+ 100 μM - $p[NH]ppG$	46.2 $\pm$ 0.6	55.5 $\pm$ 18.9

Fig. 5. Effect of pertussis-toxin treatment on $p[NH]ppG$ modulation of phospholipase C activity

Membranes (3 μg) were incubated for 15 min in the presence of pertussis toxin (PT; 4 $\mu g/100 \mu l$) or toxin buffer control with constant supplementation by NAD. Membranes were washed and then assayed for phospholipase C activity. Ca^{2+} concentration was set at 0.3 μM with a Ca -EGTA buffer system. Mg^{2+} was present at 0.3 mM. Basal phospholipase C activity after a 10 min incubation was 1.4 ± 0.1 pmol (control) and 1.1 ± 0.2 pmol (pertussis-toxin treated). Results are means $\pm$ S.E.M. for three paired experiments. Phospholipase C activity was significantly less than basal activity, with $*P < 0.05$, $**P < 0.005$.

that 10 nM- $p[NH]ppG$ inhibited the Ca^{2+} -activated phospholipase C by 20%. This degree of inhibition was comparable with that obtained in membranes incubated in the absence of added Ca^{2+} . Under similar conditions, 100 μM - $p[NH]ppG$ stimulated phospholipase C activity by approx. 150% whether membranes were incubated in the absence or presence of 50 μM added Ca^{2+} . Thus the inability of 10 nM- $p[NH]ppG$ to attenuate phospholipase C activity at 2.5 mM- Mg^{2+} could not be solely attributed to the observed increase in basal catalytic activity.

Pertussis toxin has been used as a tool to identify G-protein-mediated responses. As shown in Fig. 5, membranes incubated in buffer control for 15 min in the absence of pertussis toxin demonstrated a dose-dependent inhibition of phospholipase C activity in the presence of nanomolar concentrations of $p[NH]ppG$, with a 40% inhibition at 10 nM- $p[NH]ppG$. Pertussis-toxin treatment blocked the inhibitory effects of nanomolar concentrations of $p[NH]ppG$. These results indicate that the inhibitory effects of $p[NH]ppG$ are mediated through a pertussis-toxin-sensitive mechanism.

DISCUSSION

The present studies demonstrate that hydrolysis-resistant guanine nucleotides exert both stimulatory and inhibitory effects on membrane-associated phospholipase C activity. Stimulation of phospholipase C activity occurs

at p[NH]ppG and GTP[S] concentrations greater than $1\ \mu\text{M}$. These results agree with published studies demonstrating a dose-dependent activation of cerebral-cortical membrane phospholipase C activity by p[NH]ppG and GTP[S] that is apparent at $1\ \mu\text{M}$ or greater (Gonzales & Crews, 1986) or p[NH]ppG (Litosch, 1987).

In addition, the present studies demonstrate that guanine nucleotides promote inhibition of phospholipase C activity. These effects were apparent whether phospholipase C activity was determined with endogenous labelled substrate or with exogenous added substrate, indicating that the effect was independent of the assay protocol used. The maximal degree of inhibition observed in this study was found to vary from 20 to 40% throughout the course of this study with exogenously added substrate. A 10–15% inhibition was observed with prelabelled membranes. The smaller response observed with prelabelled membranes may be a consequence of the different protocol used to prepare these membranes to ensure retention of sufficient substrate for the subsequent assay. Since the measured inhibition reflects the net effect of both stimulatory and inhibitory inputs into the system, this may account for some of the variability in the magnitude of the inhibitory response observed with different membrane preparations. The measured inhibitory effects are clearly within the range reported for adenylate cyclase. In rat liver membranes, half-maximal inhibition of forskolin-stimulated adenylate cyclase occurred at $0.1\ \text{nM}$ -p[NH]ppG and corresponded to an approx. 35–45% inhibition of the measured response (Heyworth *et al.*, 1984; Itoh *et al.*, 1984). In rat cerebral-cortical membranes, a maximal inhibitory effect of approx. 20% of basal adenylate cyclase activity was observed in response to $10\ \text{nM}$ -p[NH]ppG (Perez-Reyes & Cooper, 1986). Furthermore, the dose-response curve for guanine-nucleotide-mediated inhibition of phospholipase C is similar to that described for the adenylate cyclase system, with half-maximal inhibition at approx. $0.33\ \text{nM}$ -p[NH]ppG. There is little reason, however, to expect that the magnitude of the effects reported for adenylate cyclase and phospholipase C should be similar, since the putative G-protein involved in regulation of phospholipase C is not likely to be the one involved in adenylate cyclase regulation, although this cannot be definitely ruled out at present.

In the adenylate cyclase system, Mg^{2+} has been shown to affect G-protein regulation. In platelet membranes, GTP[S] in the concentration range $10\ \text{nM}$ – $1\ \mu\text{M}$ inhibited adenylate cyclase activity when the assay was conducted at $2\ \text{mM}$ - MgCl_2 . However, in the presence of $12\ \text{mM}$ - MgCl_2 , concentrations of GTP[S] greater than $0.1\ \mu\text{M}$ stimulated adenylate cyclase (Katada *et al.*, 1984b). This difference appears to reflect the properties of the purified proteins, since G_i has a greater affinity for Mg^{2+} and guanine nucleotides than does G_s (Bokoch *et al.*, 1984). Similarly, in the present studies, inhibitory effects were best observed at low Mg^{2+} concentrations. Inhibition was not observed at $2.5\ \text{mM}$ - Mg^{2+} (Fig. 4), indicating that Mg^{2+} modulates the expression of the inhibitory effects. Rock & Jackowski (1987) have reported that EDTA stimulates phospholipase C activity in unwashed platelet membranes. In the present studies, however, EDTA produced a marked inhibition of phospholipase C activity, and previous studies have demonstrated that phospholipase C activity is activated by Ca^{2+} (Litosch, 1987). Similar findings have been reported in studies with

liver membranes (Taylor & Exton, 1987). In addition, Banno *et al.* (1988) have also demonstrated that EDTA and EGTA inhibit phospholipase C activity purified from platelet membranes, indicating a Ca^{2+} requirement for the enzyme.

It has not been possible to achieve a complete separation of the responses, as has been true of the adenylate cyclase system, by using pertussis toxin. Pertussis toxin, which inactivates G_i function in many cells (Gilman, 1987), blocked the inhibition of phospholipase C in response to nanomolar concentrations of p[NH]ppG. This suggests that the inhibitory effects were mediated through a pertussis-toxin-sensitive mechanism (Fig. 5). The pertussis-toxin sensitivity of the stimulatory component of p[NH]ppG action could not be ascertained, owing to the decreased stimulatory response observed in membranes incubated under these conditions. The inhibitory response caused by p[NH]ppG was of a consistently greater magnitude in the incubated control membranes than that observed throughout the course of the studies. This appears, however, to reflect an effect of the prior 15 min incubation with buffer, since unincubated membranes exhibited a dose-response curve similar to that of Fig. 3 (results not shown).

Several studies indicate that in many tissues, including brain, there are several G_i subtypes, G_{i1} , G_{i2} and G_{i3} (Itoh *et al.*, 1986; Michel *et al.*, 1986; Nukada *et al.*, 1986; Jones & Reed, 1987). The biochemical properties of these distinct G-proteins have yet to be defined with regard to their nucleotide and Mg^{2+} sensitivity as well as their effector specificity. It remains to be demonstrated whether the stimulatory and inhibitory effects reported in the present work are mediated through distinct GTP-binding proteins linked to the regulation of phospholipase C activity. The pertussis-toxin sensitivity of the inhibitory effects seen in the present studies suggests that a G_i -like protein may be recruited which interacts either directly or indirectly with the phospholipase C system. Ascertaining whether these inhibitory interactions occur in intact systems or are primarily expressed in cell-free systems will necessitate pharmacological characterization of this response and assessment of the cell types which exhibit this response.

In summary, the data in the present study indicate that there are two effects of guanine nucleotides on phospholipase C, one leading to stimulation and the other to inhibition of phospholipase C activity. In cells which contain both pathways, the net effect on phospholipase C would reflect activation of both pathways. Elucidation of the nature of the receptor linked to these pathways as well as the cell types which exhibit this response should provide information as to the mechanism by which guanine nucleotides exert these effects on phospholipase C activity and the significance of these dual effects on cell function.

Note added in proof (received 17 May 1989)

Since submission of this paper, it has become apparent that Mg^{2+} affects substrate binding to membranes (Litosch, 1989). The increased association of substrate to membranes may account, in part, for the apparent Mg^{2+} stimulation of basal phospholipase C activity observed in the present studies.

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