# Ca²⁺ inhibits guanine nucleotide-activated phospholipase D in neural-derived NG108-15 cells

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We have investigated the regulation of phospholipase D (PLD) activity by guanine nucleotides and Ca²⁺ in cells of the NG108-15 neuroblastoma X glioma line that were permeabilized with digitonin. The nonhydrolyzable GTP analogue guanosine-5'-O-(3-thiotriphosphate) (GTPγS) caused a nearly sixfold increase (EC$_{50}$ = 3 µM) in production of [³H]phosphatidylethanol (specific product of the PLD transphosphatidylation reaction). Other GTP analogues were less effective than GTPγS, and guanosine-5'-O-(2-thiodiphosphate) inhibited PLD activation by GTPγS. Both basal and GTPγS-stimulated PLD activities were potentiated by MgATP and Mg2+. Adenosine-5'-O-(3-thiotriphosphate) and ADP also potentiated the effect of GTPγS, but nonphosphorylating analogues of ATP had no such effect. The activation of PLD by GTPγS did not require Ca²⁺ and was independent of free Ca²⁺ ions up to a concentration of 100 nM (resting intracellular concentration). Higher Ca²⁺ concentrations (≥1 µM) completely inhibited PLD activation by GTPγS. It is concluded that elevated intracellular Ca²⁺ concentrations may negatively modulate PLD activation by a guanine nucleotide-binding protein, thus affecting receptor-PLD coupling in neural-derived cells.

## Introduction

The rapid activation of phospholipase D (PLD)[1] by extracellular signal molecules (neurotransmitters, hormones, and growth factors) has attracted considerable attention recently. Agonist-induced stimulation of PLD activity was observed in a large number of different cell types (reviewed by Loeffelholz, 1989; Billah and Anthes, 1990; Exton, 1990; Liscovitch, 1991). In all of these cases, PLD activation is triggered by Ca²⁺-mobilizing agonists that were previously known to stimulate also phosphoinositide-specific phospholipase C activity. However, the mechanism(s) of PLD activation, and its possible relationships with the activation of phospholipase C and the elevation of intracellular Ca²⁺ in stimulated cells, remained unresolved.

We recently have identified and partially characterized a neutral PLD in synaptic plasma membranes isolated from rat brain (Chalifa et al., 1990). Its neutral pH optimum and its plasma membrane localization suggest that this activity represents the enzyme that is involved in receptor signaling. However, the in vitro conditions utilized for its assay (e.g., the presence of a detergent, sodium oleate) are unsuitable for studies of physiological activation mechanisms. Neuroblastoma X glioma hybrid NG108-15 cells are a widely used neuronal cell model (Nirenberg et al., 1983; Hamprecht et al., 1985). We have demonstrated previously that phorbol esters stimulate the activity of PLD in NG108-15 cells via activation of protein kinase C (Liscovitch et al., 1987; Liscovitch, 1989). In preliminary experiments carried out with homogenates of NG108-15 cells, we have observed a small stimulatory effect of guanosine-5'-O-(3-thiotriphosphate) (GTPγS), a hydrolysis-resistant analogue of GTP, on PLD activity (Liscovitch and Chalifa, 1989). We have pursued these initial studies in NG108-15 cells that were permeabilized with digitonin. Cell permeabilization enables experimental control of the intracellular ionic environment (e.g., Ca²⁺) and allows access of membrane-impermeant metabolites (e.g., nucleotides) to their intracellular sites of action. In addition, cytosolic proteins are partially retained and, in contrast to the situation in cell homogenates, the spatial organization of the permeabilized cell is largely preserved.

In the present study we examined the hypothesis that PLD is regulated by a G protein by investigating the effects of GTPγS and other guanine nucleotides on the activity of PLD in

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[1] Abbreviations used: ATPγS, adenosine-5'-O-(3-thiotriphosphate); DMEM/BSA, Dulbecco's modified Eagles's medium with 1 mg/ml of fatty acid-free bovine serum albumin; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; G protein, guanine nucleotide-binding protein; GDPβS, guanosine-5'-O-(2-thiodiphosphate); GMP-PNP, guanosine-5'-O-(β,γ-imino)triphosphate; GTPγS, guanosine-5'-O-(3-thiotriphosphate); PLD, phospholipase D.

permeabilized NG108-15 cells. PLD activity was monitored by the formation of its unique product, phosphatidylethanol, which is exclusively produced by PLD via transphosphatidylation when ethanol acts as the phosphatidyl group acceptor (Kobayashi and Kanfer, 1987; Pai et al., 1988). Here we demonstrate that guanine nucleotides markedly stimulate PLD activity in digitonin-permeabilized NG108-15 cells; furthermore, we provide evidence that, although it is potentiated by MgATP and $Mg^{2+}$, this effect does not require $Ca^{2+}$. Rather, $Ca^{2+}$ at concentrations $\geq$ 1 $\mu$M completely inhibits guanine nucleotide activation of PLD.

## Results

NG108-15 cells, in which membrane phospholipids were metabolically prelabeled with [$^3$H]oleic acid, were incubated in a buffer that approximates the intracellular ionic composition (high in $K^+$ and low in $Na^+$ and $Ca^{2+}$). In the absence of digitonin, there was no effect of GTP$\gamma$S (25 $\mu$M) on the production of [$^3$H]phosphatidylethanol. In the presence of 20 $\mu$g/ml digitonin, however, GTP$\gamma$S caused a marked increase in the accumulation of [$^3$H]phosphatidylethanol over 30 min of incubation (Figure 1). At concentrations $\geq$ 20 $\mu$g/ml, digitonin caused permeabilization of the cell membrane as evinced by the elevated release of [$^3$H]adenine metabolites from the cells (Figure 1). At digitonin concentrations higher than 20 $\mu$g/ml there was a gradual decline in the response to GTP$\gamma$S, perhaps due to the increased leakage of high molecular weight intracellular constituents such as proteins. Low digitonin concentrations (5 and 10 $\mu$g/ml) caused a moderate elevation of [$^3$H]phosphatidylethanol accumulation in the absence of GTP$\gamma$S (not shown). The average stimulation of phospholipase D activity observed with an optimal concentration of GTP$\gamma$S (25–100 $\mu$M) over a large number of independent experiments was 5.75 $\pm$ 0.45-fold (mean $\pm$ SEM, n = 28). These results suggest that GTP$\gamma$S can stimulate PLD activity in NG108-15 cells only if it is allowed access to the interior of the cell.

We have examined next the accumulation of phosphatidic acid (the natural lipid product of PLD) in cells that were incubated without ethanol. GTP$\gamma$S (25 $\mu$M) caused a twofold increase in [$^3$H]phosphatidic acid levels after 5 min of incubation, from 6934 $\pm$ 479 to 13 729 $\pm$ 989 cpm/7.5 $\times$ 10$^5$ cells (mean $\pm$ SD of triplicate determinations from a typical experiment). Thus, the results obtained with phosphatidic
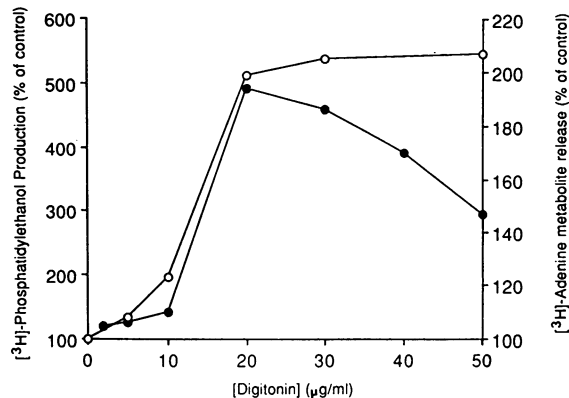


Figure 1. Dependence of NG108-15 cell permeabilization and GTP$\gamma$S-induced activation of PLD on digitonin concentration. The permeabilization of NG108-15 cells by increasing concentrations of digitonin was assessed by measuring the release of $^3$H-labeled metabolites from cells that were prelabeled with [$^3$H]adenine, as described (Lavie and Liscovitch, 1990). The release of [$^3$H]adenine metabolites (O) is expressed as the percent of medium $^3$H-radioactivity in incubations without digitonin. PLD activation was assessed by the production of [$^3$H]phosphatidylethanol as detailed under Materials and methods. [$^3$H]Phosphatidylethanol production (●) was measured in cells that were incubated either with or without GTP$\gamma$S (25 $\mu$M) in the presence of increasing concentrations of digitonin. The results are expressed as the percent of control incubations without GTP$\gamma$S. Values are the mean of duplicate determinations obtained in a representative experiment.

acid are qualitatively similar to those seen with phosphatidylethanol. The difference in the stimulated:control ratio is due to the high basal levels of phosphatidic acid which, in contrast to phosphatidylethanol, is a normal cellular constituent. In addition, phosphatidic acid is probably subject to rapid catabolism by enzymes such as phosphatidic acid phosphohydrolase, whose activity determines steady-state levels of phosphatidic acid in NG108-15 cells (cf. Lavie et al., 1990). In addition to stimulating the production of phosphatidic acid and of phosphatidylethanol, GTP$\gamma$S also stimulated the production of phosphatidylmethanol and phosphatidylpropanol in the presence of methanol and 1-propanol (100 mM), respectively (Figure 2). 2-Propanol was much less effective a substrate for PLD then 1-propanol. However, when the radioactive bands were scraped and counted it was found that the stimulation of phosphatidylalcohol production by GTP$\gamma$S was comparable ($\sim$5-fold) irrespective of the alcohol that served as the substrate for PLD. These data show the specific requirement of PLD for primary alcohols as phosphatidyl group acceptors, which is in accordance with observations of both the plant (Dawson, 1967) and mammalian (Tettenborn and Mueller, 1987) enzymes.
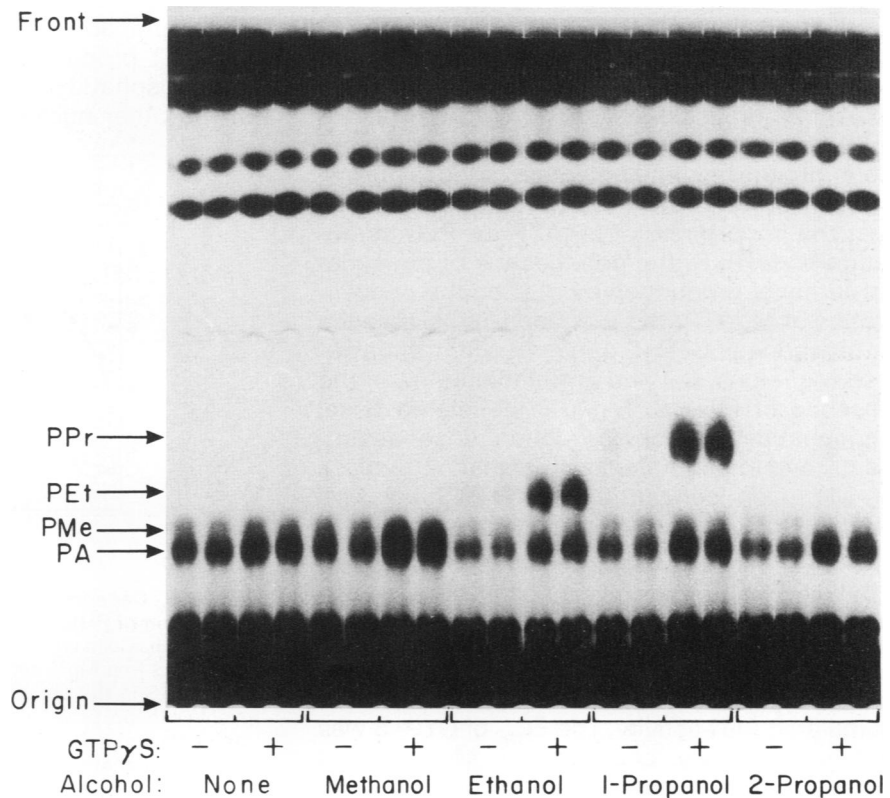
GTP$\gamma$S stimulated the production of phosphatidylethanol by PLD in a concentration-dependent manner (Figure 3A). Half-maximal activation of PLD was obtained at a GTP$\gamma$S concentration of 3 $\mu$M and maximal activation at a concentration of 100 $\mu$M. To exclude the possibility that GTP$\gamma$S acts as a thiophosphoryl group donor in a phosphorylation reaction, we tested the effects of other nonphosphorylating GTP analogues as well as GTP itself, on the formation of [$^3$H]phosphatidylethanol (Figure 3A). Among these guanine nucleotides, GTP$\gamma$S was clearly the most effective, providing the largest stimulation of PLD activity. At a maximal concentration (0.5–1 mM), guanosine-5'-$O$-($\beta$,$\gamma$-imino)triphosphate (GMP-PNP) and guanosine-5'-$O$-($\beta$,$\gamma$-methylene)triphosphate stimulated PLD activity 2.14 ± 0.12-fold and 1.26 ± 0.12-fold, respectively (mean ± SEM, n = 4) (Figure 3A). GTP did not stimulate PLD activity at this concentration range, probably because the rate of its degradation greatly exceeds the rate of its exchange with bound GDP in the absence of receptor stimulation. Guanosine-5'-$O$-(2-thiodiphosphate) (GDP$\beta$S), which is a nonhydrolyzable phosphorothioate analogue of GDP, increased PLD activity marginally when present alone but nearly completely inhibited the stimulatory ef-
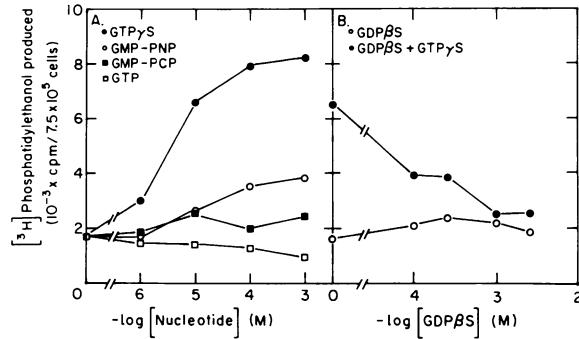
**Figure 3. Effects of GTP$\gamma$S and other GTP analogues on production of [$^3$H]phosphatidylethanol.** (A) Cells were incubated with increasing concentrations of the indicated GTP analogues in the presence of 1.8 mM MgATP. (B) Permeabilized cells were preincubated with the indicated concentrations of GDP$\beta$S for 10 min and then GTP$\gamma$S (25 $\mu$M) was added for additional 30 min of incubation. [$^3$H]Phosphatidylethanol production was measured as detailed under Materials and methods. Values are the mean of triplicate determinations obtained in a representative experiment.

fect of GTP$\gamma$S when both were given together (Figure 3B). Fluoride ions, tested in the 5- to 50-mM concentration range in the presence of Al$^{3+}$ (5 $\mu$M) and in the absence of ethylene glycol-bis($\beta$-aminoethyl ether)-$N$,$N$,$N'$,$N'$-tetraace-

**Figure 2. Effect of GTP$\gamma$S on production of different phosphatidyl-alcohols in permeabilized NG108-15 cells.** Cells were incubated under standard incubation conditions with various alcohols (100 mM) in the presence or absence of GTP$\gamma$S (25 $\mu$M) as indicated. After incubation the cells were extracted and the lipid extracts were separated by TLC as detailed under Materials and methods. TLC plates were sprayed with En$^3$Hance and autofluorographed. The bands corresponding to the different phosphatidyl-alcohols were tentatively identified by their R$_f$ values relative to phosphatidylethanol and phosphatidic acid standards. PA, phosphatidic acid; PMe, phosphatidylmethanol; PEt, phosphatidylethanol; PPr, phosphatidylpropanol.
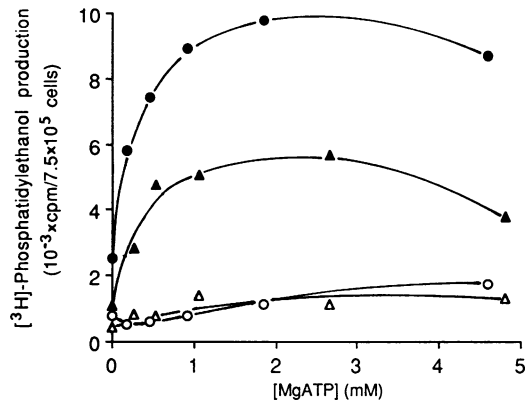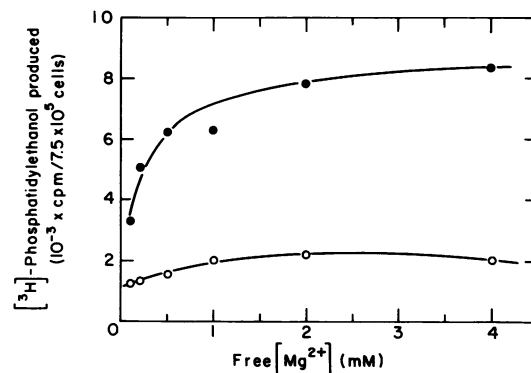
**Figure 4.** Dependence of basal and GTPγS-stimulated production of [³H]phosphatidylethanol on MgATP concentration. Cells were incubated with increasing concentrations of MgATP either without (O, △) or with (●, ▲) 25 μM GTPγS, in the presence of 0.2 mM (△, ▲) or 2.0 mM (O, ●) free $Mg^{2+}$ ions. [³H]Phosphatidylethanol production was measured as detailed under Materials and methods. Values are the mean of duplicate determinations obtained in a representative experiment.

tic acid (EGTA) and $Ca^{2+}$, failed to affect either basal or GTPγS-stimulated PLD activity in permeabilized NG108-15 cells (results not shown). The ability of GMP-PNP to activate PLD (albeit less effectively than GTPγS) as well as the inhibition by GDPβS clearly indicates that these guanine nucleotides indeed interact with a G protein to regulate PLD activity.

The permeabilization buffer utilized in these experiments included routinely MgATP (at 1.8 mM) added to sustain cellular energy-dependent processes and maintain a quasinormal phosphorylation state of cellular proteins. To test the importance of MgATP for PLD stimulation by GTPγS, the dependence of phosphatidylethanol production by PLD on the concentration of MgATP was examined in the absence or presence of GTPγS at two concentrations of free $Mg^{2+}$ ions, 0.2 and 2 mM (Figure 4). In the absence of GTPγS, MgATP stimulated basal phosphatidylethanol production dose dependently and to about the same extent, regardless of $Mg^{2+}$ ion concentration. GTPγS (25 μM) stimulated PLD activity also in the absence of MgATP, but MgATP markedly and dose dependently potentiated the response to GTPγS. MgATP (1.8 mM) elicited a 2.5 ± 0.4-fold increase (mean ± SEM of 13 experiments) in the basal PLD activity and a 3.2 ± 0.4-fold increase (mean ± SEM of 12 experiments) in the GTPγS-stimulated PLD activity. The $EC_{50}$ of GTPγS was not substantially changed by MgATP (not shown). Half-maximal stimulation of the GTPγS

response was seen at an MgATP concentration of 0.3 mM, and maximal stimulation was achieved at a concentration of 1.8 mM (Figure 4). The concentration of free $Mg^{2+}$ had no effect on either of these parameters, but GTPγS-induced production of phosphatidylethanol was greater in the presence of 2 mM $Mg^{2+}$ than in the presence of 0.2 mM $Mg^{2+}$ (Figure 4). This suggested that free $Mg^{2+}$ ions can modulate the stimulation of PLD activity by GTPγS. To examine this possibility, the concentration of free $Mg^{2+}$ ions was varied in the range of 0.1–4 mM, keeping MgATP concentration constant at 1.8 mM (Figure 5). Stimulation of PLD activity by GTPγS was augmented by $Mg^{2+}$ ions, which exhibited an apparent $EC_{50}$ of 0.1 mM and produced a maximal effect at 2 mM (Figure 5). Because MgATP was kept constant at 1.8 mM at all free $Mg^{2+}$ concentrations, these data show that the activation of PLD by GTPγS has a requirement for $Mg^{2+}$ ion in addition to that which is required to form the MgATP complex.

To gain further insight into the mechanism of MgATP action, we have tested the effects of several other nucleotides on the PLD response to GTPγS (Figure 6). All the nucleotides (tested as their $Mg^{2+}$ salts at 2 mM) elevated basal production of [³H]phosphatidylethanol somewhat. In the presence of a maximally effective concentration of GTPγS, however, only ATP, ADP, and adenosine-5'-O-(3-thiotriphosphate) (ATPγS) produced a synergistic elevation of [³H]phosphatidylethanol formation. The effects of the other nucleotides were merely additive



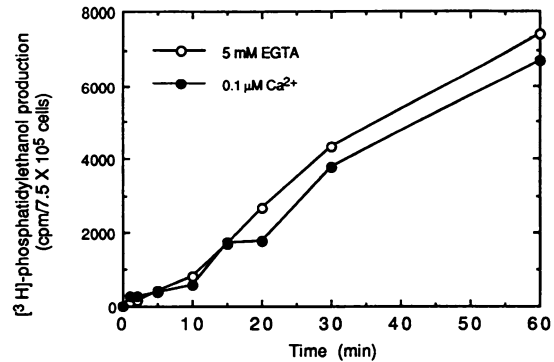**Figure 5.** Dependence of basal and GTPγS-stimulated production of [³H]phosphatidylethanol on free $Mg^{2+}$ concentration. Cells were incubated with increasing concentrations of free $Mg^{2+}$ ions either without (O) or with (●) 25 μM GTPγS in the presence of 1.8 mM MgATP. [³H]Phosphatidylethanol production was measured as detailed under Materials and methods. Values are the mean of quadruplicate determinations obtained in a representative experiment.
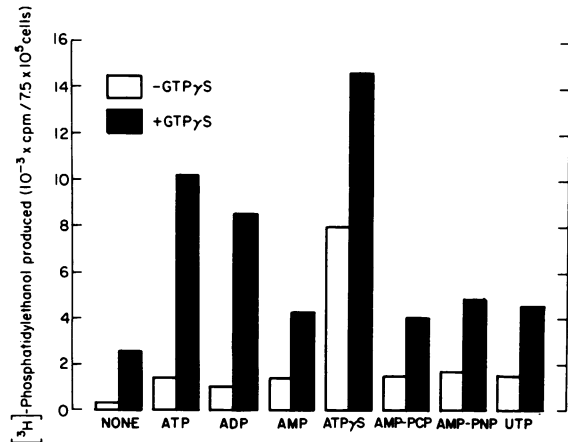
Figure 6. Effect of ATP analogues on basal and GTPγS-stimulated production of [³H]phosphatidylethanol. Cells were incubated with the indicated ATP analogues (all at a concentration of 2 mM as the $Mg^{2+}$ salt) either in the absence (□) or presence (■) of 25 $\mu$M GTPγS. [³H]Phosphati-dylethanol production was measured as detailed under Materials and methods. Values are the mean of duplicate determinations obtained in a representative experiment.



Figure 7. Time course of PLD activation by GTPγS in the absence or presence of 100 nM free $Ca^{2+}$. Cells were incubated with GTPγS in the presence of EGTA (5 mM) alone or with EGTA plus $CaCl_2$ at a concentration (2.5 mM) calculated to give a free $Ca^{2+}$ concentration of 100 nM. Incubations were terminated at the indicated time points. [³H]Phosphatidylethanol production was measured as detailed under Materials and methods. Values are the mean of duplicate determinations obtained in a representative experiment.

to that of GTPγS. Interestingly, ATPγS substantially stimulated phosphatidylethanol production even in the absence of GTPγS. A possible explanation for this atypical response could involve the in situ conversion of cellular GDP to GTPγS by a nucleoside diphosphate kinase (EC 2.7.4.6) utilizing ATPγS as thiophosphoryl group donor (Kikkawa et al., 1990).

As PLD is activated by $Ca^{2+}$-mobilizing hormones and by activators of the $Ca^{2+}$/phospholipid-dependent protein kinase C, the question whether $Ca^{2+}$ ions are required for, or influence, PLD activation has important mechanistic implications. Previous studies have provided conflicting results on the influence of $Ca^{2+}$ ions on stimulation of PLD activity by GTPγS in rat liver membranes ($Ca^{2+}$ enhances but is not absolutely required) (Bocckino et al., 1987) versus homogenates of HL-60 granulocytes ($Ca^{2+}$ in excess of 0.1 mM is absolutely required) (Anthes et al., 1989). We examined the kinetics of PLD activation by GTPγS in the presence of 5 mM EGTA and without added $CaCl_2$ (calculated free $Ca^{2+}$ concentration of 0.2 nM) and at a free $Ca^{2+}$ concentration of 100 nM (approximately the resting intracellular $Ca^{2+}$ concentration in NG108-15 cells) (cf. Noronha-Blob et al., 1988; Campbell et al., 1990; Yue et al., 1990). As shown in Figure 7, the time course of [³H]phosphatidylethanol accumulation was virtually indistinguishable under these two conditions. To further examine this issue, the PLD response to GTPγS was determined at different

free $Ca^{2+}$ concentrations (buffered with EGTA) between 0.2 nM and 1 mM (Figure 8). Basal PLD activity was not significantly affected by the variation of $Ca^{2+}$ ions concentration in this range. Activation of PLD by GTPγS was maximal in the presence of 5 mM EGTA alone (calculated free $Ca^{2+}$ concentration of 0.2 nM) and remained essentially unchanged as $Ca^{2+}$ ion levels were elevated up to 100 nM. However, higher
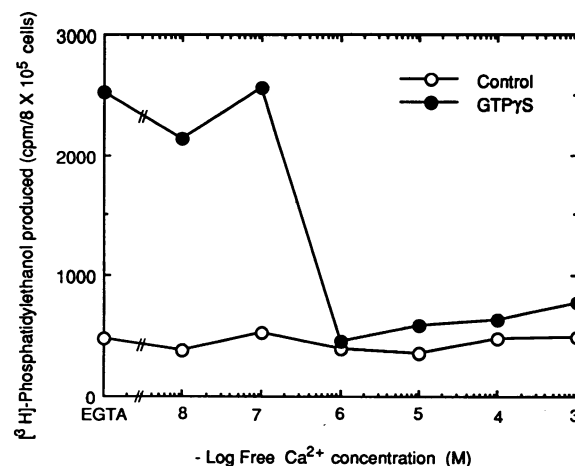


Figure 8. Dependence of basal and GTPγS-stimulated production of [³H]phosphatidylethanol on free $Ca^{2+}$ concentration. Cells were incubated with increasing concentrations of free $Ca^{2+}$ ions either without (O) or with (●) 25 $\mu$M GTPγS. [³H]Phosphatidylethanol production was measured as detailed under Materials and methods. Values are the mean of duplicate determinations obtained in a representative experiment.

$Ca^{2+}$ concentrations ($\geq 1$ $\mu M$) completely inhibited the activation of PLD by GTP$\gamma$S.

## Discussion

### Role of G protein(s) in PLD activation

The last decade has seen the emergence of guanine nucleotide-binding (or regulatory) proteins as ubiquitous switching elements in a wide variety of cellular events (see recent review by Birnbaumer et al., 1990). In many of these studies, the initial evidence for G protein involvement had been the characteristic, almost paradigmatic effect of the nonhydrolyzable GTP analogue GTP$\gamma$S. In this analogue the terminal ($\gamma$-) phosphate of GTP is substituted by a phosphorothioate. Like other phosphorothioate-substituted compounds, GTP$\gamma$S is resistant to hydrolysis by phosphatases in general and by GTPases in particular (reviewed by Eckstein, 1985). This metabolic stability confers on GTP$\gamma$S the ability to persistently activate G proteins (even in the absence of receptor stimulation) without being subject to hydrolysis by the GTPase activity of the G protein $\alpha$ subunit (see review by Spiegel and Downs, 1981).

The receptor-mediated activation of PLD has now been observed in a large number of cell types stimulated by $Ca^{2+}$-mobilizing agonists (reviewed by Loeffelholz, 1989; Billah and Anthes, 1990; Exton, 1990; Liscovitch, 1991). In the present study we examined the hypothesis that PLD is regulated by a G protein by investigating the effects of GTP$\gamma$S and other guanine nucleotides on the activity of PLD in permeabilized NG108-15 cells. The marked (nearly 6-fold) stimulation of phosphatidylethanol production by GTP$\gamma$S observed in the present study indicates that PLD is subject to regulation by one or more G protein(s). This contention is supported by the fact that PLD activity is also stimulated (albeit less effectively) by the nonhydrolyzable GTP analogue GMP-PNP and that the stimulation by GTP$\gamma$S is inhibited by the phosphorothioate GDP analogue GDP$\beta$S. Moreover, evidence was provided for the dependence of the effect of GTP$\gamma$S on $Mg^{2+}$ ions in addition to the requirement of $Mg^{2+}$ for formation of the MgATP complex. Similar characteristics were observed in other G protein-regulated systems (Spiegel and Downs, 1981; Birnbaumer et al., 1990). In this context, it is interesting to note that fluoride ions failed to stimulate PLD activity in the permeabilized NG108-15 cells. These results may be explained by a direct inhibitory effect that fluoride ions may have on PLD activity, as demonstrated in

liver plasma membranes (Bocckino et al., 1987) and rat brain synaptic membranes in vitro (Möhn, Chalifa, and Liscovitch, unpublished results).

MgATP was shown here to stimulate both basal and GTP$\gamma$S-stimulated PLD activity in a dose-dependent manner (Figure 4). Given the relatively high concentrations required, it is likely that the potentiation of GTP$\gamma$S-stimulated PLD activity by MgATP involves its participation in a phosphorylation reaction rather than its interaction with cell surface $P_2$-purinergic receptors. This conclusion is supported by the fact that only phosphorylating ATP analogues, i.e., ATP, ATP$\gamma$S, and ADP, synergistically potentiated PLD activation by GTP$\gamma$S. In comparison, the nonhydrolyzable ATP analogues adenosine-5'-O-($\beta,\gamma$-imino)triphosphate and adenosine-5'-O-($\beta,\gamma$-methylene)triphosphate were ineffective. The latter analogues are $P_2$ receptor agonists at the 2 mM concentration used in these experiments (Gordon, 1986). However, as ATP stimulates phosphoinositide breakdown and $Ca^{2+}$ influx in NG108-15 cells (Ehrlich et al., 1988), NG108-15 cells may express cell surface $P_2$-purinergic receptors. Thus, an ATP action on cell surface $P_2$-purinergic receptors cannot be excluded.

### Role of $Ca^{2+}$ ions in PLD activation

In permeabilized NG108-15 cells, stimulation of PLD activity ([$^3$H]phosphatidylethanol production) by GTP$\gamma$S was maximal in the presence of 5 mM EGTA and was unaffected by elevating free $Ca^{2+}$ concentrations to 100 nM (Figure 7). Similarly, the GTP$\gamma$S-induced stimulation of phosphatidic acid production in liver plasma membranes was observed in the presence of EGTA and no added $Ca^{2+}$, although at higher $Ca^{2+}$ levels PLD response was augmented (Bocckino et al., 1987). The GTP$\gamma$S-induced stimulation of [$^3$H]choline release in permeabilized endothelial cells was likewise observed when $Ca^{2+}$ was buffered at 100 nM with EGTA (Martin and Michaelis, 1989). In contrast, GTP$\gamma$S-induced stimulation of phosphatidylethanol production in HL-60 granulocytes homogenate was absolutely dependent on free $Ca^{2+}$ concentrations $\geq$ 100 $\mu M$ and was maximal at 1 mM (Anthes et al., 1989). There is thus a clear dichotomy between hepatocytes, endothelial cells, and NG108-15 cells, in which regulation of PLD activity by G proteins is observed at resting intracellular $Ca^{2+}$ concentration, and HL-60 granulocytes, where the GTP$\gamma$S response is $Ca^{2+}$-dependent and its physiological

relevance seems, at present, questionable. The ability of GTPγS to activate PLD in the virtual absence of free Ca²⁺ stands in contrast to its activation of phosphoinositide-phospholipase C, which is absolutely dependent on Ca²⁺ in several systems, including GH₃ pituitary cells (Martin *et al.*, 1986), liver membranes (Uhing *et al.*, 1986), brain membranes (Claro *et al.*, 1989), NG108-15 cell membranes (Campbell *et al.*, 1990), and permeabilized NG108-15 cells (Faiman and Liscovitch, unpublished observations). These observations demonstrate conclusively that in permeabilized NG108-15 cells the activation of PLD by GTPγS is not secondary to activation of a phosphoinositide-phospholipase C. Rather, direct regulation of PLD by a G protein is indicated.

The present study provides for the first time evidence for inhibition of GTPγS-induced stimulation of PLD activity by physiologically attainable Ca²⁺ concentrations. Ca²⁺ concentrations ca. 1 μM are within the range achieved inside stimulated cells, including NG108-15 cells (Noronha-Blob *et al.*, 1988; Campbell *et al.*, 1990; Yue *et al.*, 1990). Ca²⁺ does not have any effect on rat brain PLD activity in vitro at these concentrations (Chalifa *et al.*, 1990). It may therefore be hypothesized that intracellular Ca²⁺ concentrations play an important role in determining G protein coupling to PLD. Thus, in NG108-15 cells, receptor activation of PLD via G protein(s) may either precede or follow but not coexist with the elevation of intracellular Ca²⁺ by Ca²⁺-mobilizing agonists or by depolarizing stimuli.

At the present time there is no clue as to the identity of the G protein(s) that might be regulating PLD activity. Milligan and his associates have provided evidence for the existence of G$_s$, G$_i$2, G$_i$3, and G$_o$ in NG108-15 cells (Milligan *et al.*, 1990). However, in preliminary experiments we have found no evidence for stimulation of basal PLD activity by cholera toxin nor for inhibition of GTPγS-stimulated PLD activity by pertussis toxin. One possibility is that PLD activation is mediated by the same pertussis toxin-insensitive G protein that is thought to engage phosphoinositide-phospholipase C and that was recently suggested to be G$_{z/x}$ (Fong *et al.*, 1988) or G$_q$ (Taylor *et al.*, 1991). Be that as it may, as PLD is activated by Ca²⁺-mobilizing agonists that also are known to activate phosphoinositide-phospholipase C, the hypothesis that both PLD and phospholipase C are regulated by the same G protein is an attractive one. If this is indeed the case, the present results suggest that levels of intracellular Ca²⁺ are among the

factors that determine the coupling of the same G protein to either phospholipase C or PLD. These components of the complex signaling machinery set in action by Ca²⁺-mobilizing agonists will have to be isolated to rigorously test this hypothesis.

## Materials and methods

### Cell culture and labeling

NG108-15 cells (passage 17–28) were routinely cultured as previously described (Liscovitch, 1989). Before experiments, cells were subcultured at a concentration of 15–25 × 10³ cells/cm² in 75-cm² flasks and grown to subconfluence (usually 2–3 d). The medium was then replaced with Dulbecco's modified Eagles's medium containing 1 mg/ml of fatty acid-free bovine serum albumin (DMEM/BSA) and [³H]oleic acid (50 μCi/75-cm² flask), and the cells were allowed to incorporate this precursor into membrane lipids for 20–24 h.

### Assay of PLD activity in permeabilized cells

PLD activity was determined by measuring the transfer of phospholipid phosphatidyl moieties, metabolically prelabeled with [³H]oleic acid, into either phosphatidic acid (hydrolase activity) or, in the presence of ethanol, into phosphatidylethanol (phosphatidyl transferase activity). For permeabilization, NG108-15 cells were detached by sharply striking the culture flasks on the side, suspended in DMEM/BSA, and allowed to recover at a concentration of ∼1 × 10⁶ cells/ml for 3 h at 37°C. (This recovery period was important for obtaining a reproducibly large activation of phospholipase D.) The cells were then centrifuged (1000 rpm, 8 min), resuspended in Dulbecco's phosphate-buffered saline (without Ca²⁺ and Mg²⁺), recentrifuged, and resuspended in buffer A, which contained 20 mM Na-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2; 135 mM KCl; 5 mM NaHCO₃; 2.0 mM MgCl₂; 1.5 mM CaCl₂; 5 mM EGTA; 5.6 mM glucose; 2 mM ATP (Mg²⁺ salt); and 15 μM BSA. Unless otherwise indicated, ethanol was present at a concentration of 0.5% (vol/vol, 85 mM).

The concentrations of free Ca²⁺ (routinely 40 nM), free Mg²⁺ (1.8 mM), MgATP (1.8 mM), and free ATP (0.18 mM) were calculated by utilizing the IBM-PC Version 4.0 of Calcon, a cation-ligand binding program, assuming the concentration of Ca²⁺ in deionized-distilled water to be 10 μM. The same program was used to calculate the amount of MgCl₂, ATP, and CaCl₂ needed to achieve the indicated MgATP, free Mg²⁺, and free Ca²⁺ in Figures 4–8. (It should be noted that elevating free Ca²⁺ in the range shown in Figure 8 had a negligible effect on calculated GTPγS levels.)

The incubations were carried out in 12 × 75-mm glass tubes in a total volume of 0.4 ml at 37°C. Incubations were started by adding the cell suspension (0.3 ml in buffer A) into tubes with 0.1 ml of buffer A containing digitonin (final concentration of 20 μg/ml) and the tested agent(s). Routinely, incubations were terminated after 30 min by adding 1.5 ml of ice-cold methanol/chloroform/conc. HCl/0.5 M EDTA (100:50:1:1.5) and vortex-mixing.

Phase separation was accomplished by sequential addition of 0.5 ml chloroform and 0.5 ml of 0.1 N HCl. The lower chloroform phase was collected and evaporated by centrifugation under vacuum in a Speed-Vac (Savant, Farmingdale, New York) concentrator. Lipid extracts were separated by thin-layer chromatography, and [³H]phosphatidylethanol was quantitated by liquid scintillation spectrometry as previously

described (Liscovitch and Amsterdam, 1989). (A fairly typical example of the separation of phosphatidyl alcohols by this TLC method is shown in Figure 2.) [³H]Phosphatidic acid was quantitated by liquid scintillation spectrometry after its isolation by 180° 2-dimensional TLC (Bocckino *et al.,* 1987).

## Assay of [³H]adenine release from permeabilized cells

The release of [³H]adenine metabolites from digitonin-permeabilized NG108-15 cells was carried out as previously described (Lavie and Liscovitch, 1990).

## Materials

Digitonin (>99% pure) was obtained from Merck (Darmstadt, Germany) and was kept at 4°C as an 8-mg/ml stock solution in dimethylsulfoxide. Analogues of guanine and adenine nucleotides were supplied by Boehringer (Mannheim, Germany). [9,10-³H]Oleic acid (8.9 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). [2-³H]Adenine (9.8 Ci/mmol) was purchased from Nuclear Research Centre—Negev (Beer Sheva, Israel). All other biochemicals were obtained from Sigma (St. Louis, MO).

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## References

Anthes, J.C., Eckel, S., Siegel, M.I., Egan, R.W., and Billah, M.M. (1989). Phospholipase D in homogenates from HL-60 granulocytes: implications of calcium and G protein control. Biochem. Biophys. Res. Commun. *163*, 657–664.

Billah, M.M., and Anthes, J.C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. *269*, 281–291.

Birnbaumer, L., Abramowitz, J., and Brown, A.M. (1990). Receptor-effector coupling by G proteins. Biochim. Biophys. Acta *1031*, 163–224.

Bocckino, S.B., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987). Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. J. Biol. Chem. *262*, 15309–15315.

Campbell, M.D., Subramanian, S., Kotlikoff, M.I., Williamson, J.R., and Fluharty, S.J. (1990). Cyclic AMP inhibits inositol polyphosphate production and calcium mobilization in neuroblastoma X glioma NG108-15 cells. J. Pharmacol. Exp. Ther. *38*, 282–288.

Chalifa, V., Möhn, H., and Liscovitch, M. (1990). A neutral phospholipase D activity from rat brain synaptic plasma membranes. Identification and partial characterization. J. Biol. Chem. *265*, 17512–17519.

Claro, E., Wallace, M.A., Lee, H.-M., and Fain, J.N. (1989). Carbachol in the presence of guanosine 5'-O-(3-thiotriphos-

phate) stimulates the breakdown of exogenous phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate and phosphatidylinositol by rat brain membranes. J. Biol. Chem. *264*, 18288–18295.

Dawson, R.M.C. (1967). The formation of phosphatidylglycerol and other phospholipids by the transferase activity of phospholipase D. Biochem. J. *102*, 205–210.

Eckstein, F. (1985). Nucleoside phosphorothioates. Annu. Rev. Biochem. *54*, 367–402.

Ehrlich, Y.H., Snider, R.M., Kornecki, E., Garfield, M.G., and Lenox, R.H. (1988). Modulation of neuronal signal transduction systems by extracellular ATP. J. Neurochem. *50*, 295–301.

Exton, J.H. (1990). Signaling through phosphatidylcholine breakdown. J. Biol. Chem. *265*, 1–4.

Fong, H.K.W., Yoshimoto, K.K., Eversole-Cire, P., and Simon, M.I. (1988). Identification of a GTP-binding protein $\alpha$ subunit that lacks an apparent ADP-ribosylation site for pertussis toxin. Proc. Natl. Acad. Sci. USA *85*, 3066–3070.

Gordon, J.L. (1986). Extracellular ATP: effects, sources and fate. Biochem. J. *233*, 309–319.

Hamprecht, B., Glaser, T., Reiser, G., Bayer, E., and Propst, F. (1985). Culture and characteristics of hormone-responsive neuroblastoma X glioma hybrid cells. Methods Enzymol. *109*, 316–341.

Kikkawa, S., Takahashi, K., Takahashi, K.-I., Shimada, N., Ui, M., Kimura, N., and Katada, T. (1990). Conversion of GDP into GTP by nucleoside diphosphate kinase on the GTP-binding proteins. J. Biol. Chem. *265*, 21536–21540.

Kobayashi, M., and Kanfer, J.N. (1987). Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. J. Neurochem. *48*, 1597–1603.

Lavie, Y., and Liscovitch, M. (1990). Activation of phospholipase D by sphingoid bases in NG108-15 neural-derived cells. J. Biol. Chem. *265*, 3868–3872.

Lavie, Y., Piterman, O., and Liscovitch, M. (1990). Inhibition of phosphatidic acid phosphohydrolase activity by sphingosine: dual action of sphingosine in diacylglycerol signal termination. FEBS Lett. *277*, 7–10.

Liscovitch, M. (1989). Phosphatidylethanol biosynthesis in ethanol-exposed NG108-15 neuroblastoma X glioma hybrid cells. Evidence for activation of a phospholipase D phosphatidyl transferase activity by protein kinase C. J. Biol. Chem. *264*, 1450–1456.

Liscovitch, M. (1991). Signal-dependent activation of phosphatidylcholine hydrolysis: role of phospholipase D. Biochem. Soc. Trans. *19*, 402–407.

Liscovitch, M., and Amsterdam, A. (1989). Gonadotropin-releasing hormone activates phospholipase D in ovarian granulosa cells. Possible role in signal transduction. J. Biol. Chem. *264*, 11762–11767.

Liscovitch, M., Blusztajn, J.K., Freese, A., and Wurtman, R.J. (1987). Stimulation of choline release from NG108-15 cells by 12-O-tetradecanoylphorbol 13-acetate. Biochem. J. *241*, 81–86.

Liscovitch, M., and Chalifa, V. (1989). Phospholipase D: signal-dependent activation in brain and neural-derived cells. J. Neurochem. *52* (suppl.), S167A.

Loeffelholz, K. (1989). Receptor regulation of choline phospholipid hydrolysis. A novel source of diacylglycerol and phosphatidic acid. Biochem. Pharmacol. *38*, 1543–1549.

Martin, T.F.J., Lucas, D.O., Bajallieh, S.M., and Kowalchyck, J.A. (1986). Thyrotropin-releasing hormone activates a Ca²⁺-dependent phosphoinositide phosphodiesterase in permeable GH₃ cells. GTPγS potentiation by a cholera and pertussis toxin insensitive mechanism. J. Biol. Chem. *261*, 2918–2927.

Martin, T.W., and Michaelis, K. (1989). P₂-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. J. Biol. Chem. *264*, 8847–8856.

Milligan, G., McKenzie, F.R., McClue, S.J., Mitchell, F.M., and Mullaney, I. (1990). Guanine nucleotide binding proteins in neuroblastoma X glioma hybrid NG108-15 cells. Regulation of expression and function. Int. J. Biochem. *22*, 701–707.

Nirenberg, M., Wilson, S., Higashida, H., Rotter, A., Krueger, K., Busis, N., Ray, R., Kenimer, J.G., and Adler, M. (1983). Modulation of synapse formation by cyclic adenosine monophosphate. Science *222*, 794–799.

Noronha-Blob, L., Richard, C., and U'Prichard, D.C. (1988). Voltage-sensitive calcium channels in differentiated neuroblastoma X glioma hybrid (NG108-15) cells: characterization by quin 2 fluorescence. J. Neurochem. *50*, 1381–1390.

Pai, J.-K., Siegel, M.I., Egan, R.W., and Billah, M.M. (1988). Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. J. Biol. Chem. *263*, 12472–12477.

Spiegel, A.M., and Downs, R.W., Jr. (1981). Guanine nucleotides: key regulators of hormone receptor-adenylate cyclase interaction. Endocr. Rev. *2*, 275–305.

Taylor, S.J., Chae, H.Z., Rhee, S.G., and Exton, J.H. (1991). Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. Nature *350*, 516–518.

Tettenborn, C.S., and Mueller, G.C. (1987). Phorbol esters activate the pathway for phosphatidylethanol synthesis in differentiating HL-60 cells. Biochim. Biophys. Acta *931*, 242–250.

Uhing, R.J., Prpic, V., Jiang, H., and Exton, J.H. (1986). Hormone-stimulated polyphosphoinositide breakdown in rat liver plasma membranes. Roles of guanine nucleotides and calcium. J. Biol. Chem. *261*, 2140–2146.

Yue, T.L., Gleason, M.M., Lysko, P.G., and Feurstein, G. (1990). Effect of endothelins on cytosolic free calcium concentrations in neuroblastoma NG108-15 and NCB-20 cells. Neuropeptides *17*, 7–12.