ATP Stimulates the Hydrolysis of Phosphatidylethanolamine in NIH 3T3 Cells

POTENTIATING EFFECTS OF GUANOSINE TRIPHOSPHATES AND SPHINGOSINE*

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Recently, phospholipase D-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) was shown to be stimulated by activators of protein kinase C (Kiss, Z., and Anderson, W. B. (1989) J. Biol. Chem. 264, 1483-1487), suggesting that PtdEtn metabolism may play a role in signal transduction. Here we have studied the possible regulation of PtdEtn hydrolysis by adenine and guanine nucleotides, as well as by sphingosine, both in membranes isolated from [14C]ethanolamine- or [32P]PtdEtn-prelabeled NIH 3T3 cells and in intact cells. In isolated membranes both ATP and ADP stimulated the hydrolysis of PtdEtn. Both nucleotides had maximal (~2-fold) effects at about 0.5 mM concentration. The main water-soluble product of [14C]PtdEtn hydrolysis was [14C]ethanolamine, while in [32P]PtdEtn-prelabeled membranes the nucleotides stimulated the formation of [32P]phosphatidic acid, suggesting the involvement of a phospholipase D-type enzyme. The hydrolysis-resistant analogs of ATP, such as guanosine 5’-3-O[(thio)triphosphate and guanyly-5’-y-methyl-eneadenosine 5’-triphosphate, greatly potentiated the stimulatory effects of ATP and ADP on PtdEtn hydrolysis. On the other hand, the nonphosphorylating analogs of ATP, adenyly-5’-yl β,γ-imidodiphosphate and β,γ-methylenedephosphatidylinositol 4,5-bisphosphate, failed to stimulate PtdEtn hydrolysis both in the absence and presence of guanine triphosphates. Sphingosine, while exhibiting no effect alone, had a relatively modest (1.2–1.3-fold) potentiating effect on ATP-stimulated PtdEtn hydrolysis in isolated membranes. The effect of sphingosine was mimicked by threo- and erythrosphinganines, while N-acetylsphingosine was without effect. In studies with [14C]ethanolamine-prelabeled intact NIH 3T3 cells, externally added ATP did not stimulate PtdEtn hydrolysis. In contrast, sphingosine and sphinganine had much greater stimulatory effects on PtdEtn hydrolysis in intact cells than with isolated membranes. These data indicate that PtdEtn hydrolysis may be regulated by adenine and guanine nucleotides in addition to, or in cooperation with, the activators of protein kinase C, and that sphingosine may be an additional regulator of PtdEtn hydrolysis.

Increased phospholipid metabolism appears to play a key role in the transmission of extracellular signals across the cell membrane. Thus, phospholipase C-catalyzed rapid degradation of phosphatidylinositol 4,5-bisphosphate has been shown to mediate the effects of various growth factors and oncogenes (1–11). Degradation of this phospholipid yields 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, which have second messenger functions to stimulate protein kinase C (2, 3, 12) and to release calcium from internal stores (4, 13), respectively. It is increasingly evident that stimulation of phospholipase D-mediated hydrolysis of phosphatidylcholine (PtdCho) by phorbol ester and various nucleotides also produces biologically active compounds, most notably phosphatidic acid and 1,2-diacylglycerol (14–22). Products of this pathway, however, do not induce the redistribution of intracellular calcium. Phospholipase D, which is thought to act specifically on PtdCho, also has been proposed to catalyze the formation of phosphatidylethanol from PtdCho and short chain alcohols (23–28).

Recently, we found that activators of protein kinase C, including the phorbol ester TPA, also stimulated phospholipase D-catalyzed hydrolysis of PtdEtn in several different cell types (29). Since adenine and guanine nucleotides appear to be important regulators of PtdCho hydrolysis (15, 22), it was of interest to study the effects of these nucleotides on PtdEtn hydrolysis. We used NIH 3T3 fibroblasts in this study since this cell line exhibited a strong PtdCho-hydrolyzing activity (29). Both ATP and ADP were found to stimulate PtdEtn hydrolysis in membranes isolated from [14C]ethanolamine- or [32P]PtdEtn-prelabeled NIH 3T3 cells. Guanine triphosphates and sphingosine, while having no effect in themselves, were found to potentiate the stimulatory effects of adenine nucleotides on PtdEtn hydrolysis.

EXPERIMENTAL PROCEDURES

Materials—Sphingosine, erythro- and threo-dihydroxyphospholipines (sphingamines), TPA, ATP, GTP, guanyly-5’-yl imidodiphosphate (Gpp(NH)p), guanosine 5’-O-3-(thio)triphosphate (GTPS), phosphatidylethanolamine, and phosphatidylcholine were purchased from Sigma; [2-14C]ethanolamine (20 mCi/mmol), [methyl-14C]choline chloride (50 mCi/mmol), and [32P]orthophosphate (carrier-free in water) were from Amersham Corp.; tissue culture reagents were from Gibco. N-Acetylsphingosine was synthesized as described (30).

Preparation of [32P]Phosphatidylethanolamine—To prepare [32P]PtdEtn (185 mCi/mmol), lima beans (5 beans) were placed in 1.5 ml of tap water containing 5 mCi of [32P]orthophosphoric acid and incubated in the dark at room temperature for 40 h. Following incubation the beans were washed and then disrupted by Dounce homogenization in water. Labeled phospholipids were extracted from

* The abbreviations used are: PtdCho, phosphatidylcholine; GTPS, guanosine 5’-3-O-(thio)triphosphate; PtdEtn, phosphatidylethanolamine; Gpp(NH)p, guanyly-5’-yl imidodiphosphate; TPA, 12-O-tetradecanoylphorbol-13-acetate; App(NH)p, adenyly-5’-yl β,γ-imidophosphatidylinositol 4,5-bisphosphate; App(CH3)p, β,γ-methylenedadenosine 5’-triphosphate.

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this bean homogenate with 10 volumes of chloroform/methanol (1:1, v/v). [3P]PtdEtn was isolated by thin layer chromatography on Silica Gel H using a solvent system (by volume) of chloroform/methanol/concentrated ammonium hydroxide (65:25:5). [3P]PtdEtn was re-extracted from the silica gel with the above chloroform/methanol mixture. After filtration to remove the silica gel, the [3P]PtdEtn was stored in chloroform at -70 °C for 1 day prior to use. About 3% of the total [3P] activity was incorporated into PtdEtn.

**Cell Culture—** NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin (50 units/ml and 50 μg/ml, respectively), and glutamine (2.5 mM) and propranolol, leupeptin, 0.5 mM phenylmethylsulfonyl fluoride. Remaining intact cells were removed by centrifuging the homogenates at 1,000 × g for 5 min. Incubations were carried out at 37 °C for 5-30 min in a shaking water bath, and terminated by the addition of 4 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v).

**Measurement of Phospholipid Degradation during the Chase Period in Cells Prelabelled with [14C]Ethanolamine or [14C]Choline—** Phospholipid hydrolysis was determined as previously described (29), with some modifications. Briefly, NIH 3T3 cells were incubated with [2-14C]ethanolamine (0.2 μCi/ml) or [methyl-14C]choline (0.25 μCi/ml) for 48 h, washed with fresh medium, incubated for 3.0 h in fresh medium, and then harvested by gentle scraping from the dish. The harvested cells, usually pooled from 3-5 dishes (150 cm2), were again washed with fresh medium. Then, 0.3-ml aliquots (1.0-1.5 × 106 cells/ml) were incubated at 37 °C (final volume 0.32 ml) in polypropylene tubes for the presence of unlabeled ethanolamine (or 2 μM choline when appropriate), along with other agents as specified. Incubations were terminated by the addition of 4 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 2 ml of chloroform/methanol (1:1, v/v).

**Measurement of Phosphatidylethanolamine Hydrolysis in Membranes Isolated from [14C]Ethanolamine-prelabelled Cells—** Cells were grown in the presence of [2-14C]ethanolamine (0.2 μCi/ml) for 48 h to about 70-80% confluency, then washed twice in fresh medium, harvested from three dishes (150 cm2) by scraping, and disrupted by Dounce homogenization (A pestle; 100 strokes) in 5 ml of ice-cold homogenization buffer (150 mM KCI, 5 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 0.05 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Remaining intact cells were removed by centrifuging the homogenates at 1,000 × g for 5 min. The resulting supernatants were further centrifuged at 20,000 × g for 10 min at 4 °C; the membrane pellets were washed once in the upper (water/methanol) phase and in the phospholipids isolated from the lower (chloroform) phase as described (29).

**Measurement of Phosphatidylcholine Formation in Membranes Isolated from [3H]Phosphatidylcholine-prelabelled Cells—** Liposomes were prepared by sonication (3 min) of a mixture of [3H]PtdCho/PtdEtn/PtdCho/phosphatidylycerine (1:2:2, molar ratio) using a microcrustacase cell disintegrator (Kentex). Cells were incubated with [3H]PtdEtn-containing liposomes (1.25 μC/ml) for 48 h. After the labeling period, nonincorporated radioactivity was removed by thorough washing with fresh pronase-streptomycin solution composed of chloroform/ethanol/methanol (1:1:1, v/v) mixture. Phospholipid hydrolysis was estimated by measuring the radioactivity present in individual phospholipids and in the upper (water/methanol) phase. Radioactivity present in individual phospholipids and in the phospholipids isolated from the lower (chloroform) phase as described (29).

**RESULTS**

**Effects of Adenine and Guanine Nucleotides and of Sphingosine on the Hydrolysis of [14C]PtdEtn in Isolated Membranes—** The effects of adenine and guanine nucleotides and sphingosine were first studied in membranes prepared from [14C]ethanolamine-prelabelled NIH 3T3 cells. Unlabeled ethanolamine and its derivatives were present in the incubation medium to inhibit possible re-esterification of the hydrolyzed 14C label by contaminating phospholipid-synthesizing enzymes. Addition of 0.5 mM ATP to membranes significantly stimulated the formation of water-soluble [14C]ethanolamine products without a detectable lag period (Fig. 1). At a nearly optimal concentration (15 μM), sphingosine alone had no effect (not shown), while this concentration of sphingosine potentiated the effect of ATP on PtdEtn hydrolysis 1.2-1.3-fold (Fig. 1). The stimulatory effect of sphingosine, an inhibitor of protein kinase C (31), suggested that the stimulatory effect of ATP was not mediated by protein kinase C. H7, another inhibitor of protein kinase C and of cAMP-dependent protein kinase (32), also failed to inhibit the effect of ATP on phosphatidylethanolamine hydrolysis (data not shown). The stimulatory effects of ATP and sphingosine on the formation of water-soluble [14C]ethanolamine products were accompanied by a proportional loss of [14C]PtdEtn from the membrane. Treatment of membranes with these agents for 30 min decreased the amount of membrane-bound [14C]PtdEtn by 14% (data not shown).

**Half-maximal or maximal (2.1-fold) stimulatory effects of ATP on [14C]PtdEtn hydrolysis in [14C]ethanolamine-prelabelled membranes required 40 or 500 μM concentrations of nucleotide, respectively (Fig. 1). The presence of an ATP-regenerating system did not significantly reduce the concentration of ATP required for maximal stimulation (data not shown). Sphingosine (15 μM) had a similar potentiating effect at each ATP concentration tested (Fig. 1).**

**The hydrolysis-resistant GTP analog, GTPγS, had little stimulatory effect on PtdEtn hydrolysis when added to membranes alone. However, this guanine nucleotide significantly potentiated the stimulatory effect of ATP. As shown in Fig. 3, GTPγS had a detectable potentiating effect at concentrations as low as 10 μM, while a maximal (1.7-fold) enhancement of the ATP effect required 1 μM guanine nucleotide. Gpp(NH)p, another nonhydrolyzable GTP analog, similarly potentiated the ATP effect on PtdEtn hydrolysis (data not shown).**

**Measurement of Phosphatidic Acid Formation in Membranes Isolated from [14C]Phosphatidylethanolamine-prelabelled Cells—** Liposomes were prepared by sonication (3 min) of a mixture of [3H]PtdEtn/PtdEtn/PtdCho/phosphatidylycerine (1:2:2, molar ratio) using a microcrustacase cell disintegrator (Kentex). Cells were incubated with [3H]PtdEtn-containing liposomes (1.25 μC/ml) for 48 h. After the labeling period, nonincorporated radioactivity was removed by thorough washing with fresh pronase-streptomycin solution composed of chloroform/ethanol/methanol (1:1:1, v/v) mixture. Phospholipid hydrolysis was estimated by measuring the radioactivity present in the upper (water/methanol) phase as described (29).
ADP appeared to be almost as effective as ATP, while the effect of GTPrS either in the presence (Fig. 3) or in the absence of ATP, were assayed for their ability to enhance the formation of ethanolamine (Table I). Half and maximal stimulation (\(-1.3\text{-}10^{-5}\text{M}\)) of phosphatidyethanolamine hydrolysis required about 5 or 20 \(\mu\text{M}\) ATP plus 15 \(\mu\text{M}\) sphingosine (M). Each point represents the mean \(\pm\) S.E. of four determinations. Similar results were obtained in two other experiments.

ATP and sponginosine did not increase the \([^{14}\text{C}]\) content of phosphatidyethanolamine in isolated membranes. The effects of varying concentrations of ATP on the hydrolysis of \([^{32}\text{P}]\)phosphatidyethanolamine in isolated membranes were determined in the absence (■) or presence (▲) of 15 \(\mu\text{M}\) sponginosine. Each point represents the mean \(\pm\) S.E. of four determinations. Similar results were obtained in another experiment.

The effects of varying concentrations of ATP on phosphatidyethanolamine degradation. Both GTPrS and sponginosine potentiated the stimulatory effect of ATP, but they remained ineffective in the presence of the ATP analogs (Table I). Adenosine (10–500 \(\mu\text{M}\)) or cyclic AMP (0.1–10 \(\mu\text{M}\)) failed to alter the rate of phosphatidyethanolamine hydrolysis in isolated membranes.

Next, the specificity of sponginosine action was tested by comparing the effects of various sponginosine analogs, including three- and erythrosphingosines and N-acetylsphingosine, with that of sponginosine on the ATP-dependent hydrolysis of phosphatidyethanolamine (Fig. 4) and its derivatives (not shown). No significant effect on phosphatidyethanolamine hydrolysis in the absence of ATP. In the presence of ATP half- and maximal (\(-1.3\text{-}10^{-5}\text{M}\)) stimulation of sponginosine required about 5 or 20 \(\mu\text{M}\) concentrations, respectively. Three- and erythrosphingosines, also effective inhibitors of protein kinase C (33–35), similarly enhanced the stimulatory effect of ATP on phosphatidyethanolamine hydrolysis (Fig. 4). On the other hand, N-acetylsphingosine, an ineffective inhibitor of protein kinase C (33–35), failed to modify the rate of phosphatidyethanolamine hydrolysis (Fig. 4).

Stimulation of phospholipase D-mediated hydrolysis of phosphatidyethanolamine by ATP, in contrast to ATP-stimulated hydrolysis of phosphatidyethanolamine in membranes isolated from \([^{14}\text{C}]\)choline-prelabeled NIH 3T3 cells. As indicated in Table II, ATP had only a marginal stimulatory effect on the hydrolysis of preformed \([^{14}\text{C}]\)phosphatidyethanolamine; this effect of ATP was not enhanced by sponginosine.

ATP and sponginosine did not increase the \([^{14}\text{C}]\) content of phosphatidyethanolamine in the \([^{14}\text{C}]\)choline-prelabeled membranes, indicating that they had no effect on the methylation of phosphatidyethanolamine. Also, these compounds failed to significantly modify the formation of \([^{14}\text{C}]\)lyso-phosphatidyethanolamine, suggesting that a phospholipase A-type enzyme was not involved in this increased degradation of phosphatidyethanolamine. Addition of choline or serine (1–10 mM) to \([^{14}\text{C}]\)phosphatidyethanolamine-prelabeled membranes did not result in increased release of \([^{14}\text{C}]\)ethanolamine under the present conditions (i.e., in the absence of calcium), indicating that an active base-exchange mechanism was not present in these membranes (data not shown). Together, these data indicated that the nucleotide-stimulated hydrolysis of phosphatidyethanolamine was catalyzed by either a phospholipase C- or D-type enzyme, or by a hitherto unknown mechanism. A better understanding of the mechanism of action of nucleotides and sponginosine required selective utilization of \([^{32}\text{P}]\)phosphatidyethanolamine to determine whether these agents increased the formation of phosphatidyethanolamine. For this purpose, cells were prelabeled with \([^{32}\text{P}]\)phosphatidyethanolamine, and membranes were prepared as in the previous experiments. Although the uptake of this phospholipid by cells was an inefficient process, this procedure ensured the necessary specific labeling of membranes with \([^{32}\text{P}]\)phosphatidyethanolamine. Thus, of the total \(32\text{P}\) activity taken up by these cells about 80% was associated with phosphatidyethanolamine, with the remaining radioactivity being equally distributed between phosphatidyethanolamine and lyso-phosphatidyethanolamine. As shown in Table III, ATP stimulated the formation of \([^{32}\text{P}]\)phosphatidyethanolamine from \([^{32}\text{P}]\)phosphatidyethanolamine. The combined effects of ATP and GTPrS or sponginosine on phosphatidyethanolamine formation were higher than the effect of ATP alone (Table III).

To better characterize the stimulatory effect of ATP on phosphatidyethanolamine degradation, a variety of adenine nucleotides, including the nonhydrolyzable analogs of ATP, were assayed for their ability to enhance the formation of ethanolamine (Table I). ADP appeared to be almost as effective as ATP, while App(NH)p and App(CH)p were without effect on phospholipid degradation. Both GTPrS and sponginosine potentiated the stimulatory effect of ADP, but they remained ineffective in the presence of the ATP analogs (Table I). Adenosine (10–500 \(\mu\text{M}\)) or cyclic AMP (0.1–10 \(\mu\text{M}\)) failed to alter the rate of phosphatidyethanolamine hydrolysis in isolated membranes.

The effects of ATP and sponginosine on phosphatidyethanolamine hydrolysis in intact cells. If ATP stimulates phosphatidyethanolamine hydrolysis through externally located purinergic receptors, as has been shown for phosphatidyethanolamine hydrolysis (22), then the addition of ATP to intact cells would be expected to enhance the rate of phosphatidyethanolamine hydrolysis. Addition of ATP (10–500 \(\mu\text{M}\)) to \([^{14}\text{C}]\)ethanolamine-prelabeled cells for 10–90 min failed to significantly stimulate the release of \([^{14}\text{C}]\)ethanolamine from the phospholipid-bound form (data not shown). These data would appear to indicate that purinergic receptors are not involved in mediating the ATP effect of phosphatidyethanolamine hydrolysis.
**Table I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Formation of [{(^{14})C}]ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No sphingosine</td>
</tr>
<tr>
<td></td>
<td>dpm/100 (\mu)g protein/20 min</td>
</tr>
<tr>
<td>None</td>
<td>3,250 ± 95</td>
</tr>
<tr>
<td>GTP(\gamma)S (1 (\mu)M)</td>
<td>3,690 ± 28</td>
</tr>
<tr>
<td>ATP (500 (\mu)M)</td>
<td>6,440 ± 210</td>
</tr>
<tr>
<td>ADP (500 (\mu)M)</td>
<td>3,680 ± 49</td>
</tr>
<tr>
<td>ADP (500 (\mu)M)</td>
<td>6,660 ± 73*</td>
</tr>
<tr>
<td>App(NH)(_4)p (600 (\mu)M)</td>
<td>3,240 ± 51</td>
</tr>
<tr>
<td>App(CH)(_2)p (500 (\mu)M)</td>
<td>3,350 ± 113</td>
</tr>
<tr>
<td>GTP(\gamma)S (1 (\mu)M) + ATP (500 (\mu)M)</td>
<td>10,530 ± 235*</td>
</tr>
<tr>
<td>GTP(\gamma)S (1 (\mu)M) + ADP (500 (\mu)M)</td>
<td>9,250 ± 81*</td>
</tr>
<tr>
<td>GTP(\gamma)S (1 (\mu)M) + App(NH)(_4)p (500 (\mu)M)</td>
<td>4,060 ± 56</td>
</tr>
<tr>
<td>GTP(\gamma)S (1 (\mu)M) + App(CH)(_2)p (500 (\mu)M)</td>
<td>3,990 ± 110</td>
</tr>
</tbody>
</table>

*Significantly different from the control value (\(p < 0.01\)).

*Significantly different from the value obtained after 20 min incubation in the absence of sphingosine, and in the presence of the corresponding nucleotides (\(p < 0.05\)).

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**Figure 4.** Concentration-dependent effects of various sphingosine derivatives on the hydrolysis of \([{\(^{14}\)C}]\)phosphatidylethanolamine in isolated membranes. The effects of varying concentrations of sphingosine (O), threosphinganine (A), erythrosphinganine (B), and N-acetylsphingosine (E) on the hydrolysis of \([{\(^{14}\)C}]\)PtdEtn in isolated membranes were determined in the presence of 0.5 mM ATP. The effect of sphingosine in the absence of ATP also is shown (O). Each point represents the mean ± S.E. of four determinations.

In contrast to ATP, sphingosine was an effective stimulator of PtdEtn hydrolysis in intact cells. In the experiment shown in Fig. 5, 10, and 20 \(\mu\)M concentrations of sphingosine added to intact cells stimulated the hydrolysis of \([{\(^{14}\)C}]\)PtdEtn by 2- and 2.4-fold, respectively. At these concentrations, sphingosine also appeared to stimulate phospholipid hydrolysis without a significant lag period. At 5 \(\mu\)M concentration, however, a stimulatory effect of sphingosine could be observed only after a 10-min lag period (Fig. 5).

In the intact cell system (Fig. 6), in contrast to the membrane system (Fig. 4), sphingosine was more effective than sphingosines in stimulating PtdEtn hydrolysis. The difference between the potencies of sphingosine and erythrosphinganine was especially striking. Among other possibilities, differential uptake of various sphingosine derivatives by cells might account for the different results in intact cells. N-Acetylphosphogosine, which had no effect on PtdEtn hydrolysis in isolated membranes (Fig. 4), also was without effect in intact cells (Fig. 6).

Next, the effects of sphingosine on basal (unstimulated) and TPA-stimulated hydrolysis of PtdEtn and PtdCho (29) were compared using cells prelabeled with either \([{\(^{14}\)C}]\)ethanolamine or \([{\(^{14}\)C}]\)choline. Apart from small differences in the concentration dependence and the maximal effects, sphingo-
sine similarly stimulated the hydrolysis of both phospholipids in the unstimulated cells (Table IV). In the presence of TPA, however, sphingosine exhibited selective effects on the hydrolysis of these two phospholipids. At concentrations of 5–10 μM, sphingosine clearly inhibited the stimulatory effect of TPA on PtdCho hydrolysis. Conversely, TPA and sphingosine showed nearly additive stimulatory effects on PtdEtn hydrolysis (Table IV).

**DISCUSSION**

In several cell types ATP has been shown to stimulate phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (36–42). The ATP analog App(3’), a P2Y purinergic receptor agonist, mimicked the effect of ATP on phospholipase C activation. Similarly, both ATP and App(3’),pGpp were found to stimulate phospholipase D-mediated hydrolysis of PtdCho (15, 22). In the above studies the ATP effects were synergistic with that of guanosine triphosphates. In the present study our results show that ATP also stimulates the degradation of PtdEtn in isolated membranes. In addition, the effect of ATP was enhanced by guanosine triphosphates. However, the effect of ATP on PtdEtn hydrolysis was not detectable in intact cells, and was not mimicked by App(3’),pGpp. Furthermore, in isolated membranes only the hydrolysis of PtdEtn, but not that of PtdCho, was significantly stimulated by ATP alone or by sphingosine in the presence of ATP. At present, we cannot rule out the possibility that NIH 3T3 cells contain specific P2 purinergic receptors which can be activated by ATP (but not App(3’),pGpp) only in the presence of another factor. Both ATP and ADP were potent stimulators of PtdEtn hydrolysis. Although these data would appear to argue against the involvement of a phosphorylation mechanism, it is still possible that small amounts of ADP might be converted to ATP to serve as a substrate in the phosphorylation process involving a protein kinase or another type of kinase.

It is well established that phorbol esters and various nucleotides stimulate the degradation of PtdCho by a phospholipase D-mediated mechanism (14–22), resulting in the formation of phosphatidic acid or, in the presence of short chain alcohols, phosphatidylalcohols (23–28). Phorbol esters were also shown to stimulate the hydrolysis of PtdEtn, presumably through a phospholipase D-mediated mechanism (29). Here we show that in membranes ATP and GTPγS stimulate the formation of phosphatidic acid and ethanolamine from PtdEtn, consistent with the involvement of a phospholipase D-type enzyme. These observations suggest that in NIH 3T3 cells the degradation of both PtdCho and PtdEtn by phospholipase D-type enzymes may be regulated by adenine and guanine nucleotides as well as by the activity state of protein kinase C.

In erythrocytes (43–48) and fibroblasts (49) phospholipids appear to be asymmetrically distributed in the cell membrane. Of the major phospholipids, PtdEtn and PtdCho are preferentially localized in the inner and outer layers of cell membrane, respectively. Evidently, these phospholipids can be hydrolyzed by the same type of phospholipase only if the hydrolyzing enzyme is itself bimodally distributed. Available evidence suggests that phospholipase D may be bimodally distributed in membranes (50). Hydrolysis of PtdEtn and PtdCho on the opposite sides of plasma membrane would be expected to play different roles in signal transduction. Clearly, a better understanding of the physiological function of phospholipase D-mediated hydrolysis of these phospholipids requires the localization of the site of such hydrolysis.

In isolated membranes sphingosine, similarly to GTPγS, potentiated the stimulatory ATP effect on PtdEtn hydrolysis. Sphinganine, but not N-acetylsphingosine, mimicked the stimulatory effect of sphingosine, with threosphinganine exhibiting the largest effect. Interestingly, threosphinganine

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**TABLE IV**

**Effect of sphingosine on the hydrolysis of [14C]phosphatidylethanolamine in NIH 3T3 cells.** Cells were pulse-labeled with [3H]ethanolamine, washed, and reincubated (chase period) in the presence of increasing concentrations of sphingosine (O), threosphinganine (A), erythrosphinganine (M), or N-acetylsphingosine (*). At the beginning of the chase period, the [14C] content of PtdEtn was 342,000 dpm/106 cells. Erythrocytes (43–48) and fibroblasts (49) phospholipids are hydrolyzed by the same type of phospholipase only if the hydrolyzing enzyme is itself bimodally distributed. Available evidence suggests that phospholipase D may be bimodally distributed in membranes (50). Hydrolysis of PtdEtn and PtdCho on the opposite sides of plasma membrane would be expected to play different roles in signal transduction. Clearly, a better understanding of the physiological function of phospholipase D-mediated hydrolysis of these phospholipids requires the localization of the site of such hydrolysis.

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**FIG. 5.** Time-dependent effect of sphingosine on the hydrolysis of [14C]phosphatidylethanolamine in NIH 3T3 cells. Cells were pulse-labeled with [3H]ethanolamine, washed, and reincubated (chase period) in the presence of sphingosine (5–20 μM) for 3–60 min as described under "Experimental Procedures." Each point represents the mean of four incubations with experimental errors less than 6% in each case. At the beginning of the chase period, the [14C] content of PtdEtn was 485,000 dpm/106 cells.

**FIG. 6.** Concentration-dependent effects of various sphingosine derivatives on the hydrolysis of [14C]phosphatidylethanolamine in NIH 3T3 cells. Cells were pulse-labeled with [3H]ethanolamine, washed, and reincubated (chase period) in the presence of varying concentrations of sphingosine (O), threosphinganine (A), erythrosphinganine (M), or N-acetylsphingosine (*). At the beginning of the chase period, the [14C] content of PtdEtn was 342,000 dpm/106 cells.
also appears to be a somewhat better inhibitor of protein kinase C compared to the erythro stereoisomer (34), while N-acetyl sphingosine is not an inhibitor of protein kinase C activity (33–35). These data reveal similar specificity of sphingo sine effects on protein kinase C activity and PtdEtn hydrolysis. Sphingosine and sphingosines had even greater effects on PtdEtn hydrolysis in intact cells than in membranes. In preliminary experiments, however, externally added ATP did not modify the stimulatory effect of sphingosine on PtdEtn hydrolysis. Thus, the ATP dependence of the sphingo sine effect on PtdEtn hydrolysis in intact cells remains to be established. Sphingosine has numerous effects in cells (Ref. 51, and references therein), most of them occurring at 5–20 μM concentrations. The question as to whether cells contain sufficient amounts of sphingosine to serve as a regulatory agent has not yet been fully settled. Nonetheless, it is conceivable that sphingosine might serve as a regulator of phospholipid hydrolysis under pathological conditions characterized by high sphingosine levels (52).

In conclusion, the data presented show for the first time that adenine and guanine nucleotides are potential regulators of PtdEtn hydrolysis. This would suggest that PtdEtn hydrolysis, similarly to agonist-stimulated hydrolysis of PtdCho and phosphatidylinositol 4,5-bisphosphate, may play an important role in transmembrane signalling.

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