Phosphatidic Acid May Stimulate Membrane Receptors Mediating Adenylate Cyclase Inhibition and Phospholipid Breakdown in 3T3 Fibroblasts*

Toshikiko Murayama and Michio U†
From the Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Incubation of 3T3 fibroblasts with phosphatidic acid (PA) from egg lecithin or with thrombin resulted in decreases in cellular cAMP due to inhibition of adenylate cyclase, in rapid increases in inositol 1,4,5-tris-, 1,4-bis-, and 1-monophosphates probably due to activation of phospholipase C, and in arachidonic acid release. Synthetic PAs consisting of unsaturated fatty acid diesters were as effective as PA from egg lecithin, whereas PAs with saturated fatty acids were only slightly effective and antagonized the effect of active PAs selectively, despite the fact that both types of PA analogues (sodium salts) were apparently dissolved in the incubation medium. PA-induced decreases in cAMP were not affected by omission of Ca\(^{2+}\) from incubation medium, whereas PAS with saturated fatty acids were only slightly effective and antagonized the effect of active PAs selectively. The effects of PA were cell-specific; it failed to decrease cAMP in the incubation medium. PA-induced decreases in cAMP were abolished by prior exposure of cells to islet-activating protein (pertussis toxin). This islet-activating protein treatment of cells was without effect on PA- or thrombin-induced generation of inositol phosphates. Thus, PA-induced inhibition of adenylate cyclase was (but activation of phospholipase C was not) mediated by an islet-activating protein substrate GTP-binding protein. Homologous desensitization was observed with thrombin-, bradykinin-, and PA-induced decreases in cAMP in 3T3 cells; prior exposure of the cells to any one of these agents abolished or greatly diminished the subsequent response to the same agent but did not affect the responses to others. The effects of PA were cell-specific; it failed to decrease cAMP in rabbit platelets in which labeled PA rapidly increasing in response to thrombin or A23187 was mostly outside the cells. Based on these results, it is proposed that PA interacts with its own specific membrane receptors, thereby triggering multiple effector systems in 3T3 cells.

Stimulation of Ca\(^{2+}\)-mobilizing receptors gives rise to activation of phospholipase C that is selectively involved in hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-triphosphate (1–3). These two products act as second or intracellular messengers of the receptor stimulation; diacylglycerol activates protein kinase C (4), whereas the inositol trisphosphate mobilizes Ca\(^{2+}\) from nonmitochondrial stores (5, 6). The former is rapidly phosphorylated to generate phosphatidic acid, and the latter is further hydrolyzed to inositol 1-monophosphate. Phosphatidylinositol is then resynthesized and phosphorylated, thus completing the so-called phosphatidylinositol cycle (1).

Phosphatidic acid generated as one of the intermediates of the phosphatidylinositol cycle is known to exert its own effects on cyclic nucleotide and Ca\(^{2+}\) metabolisms in certain cell types. The addition of phosphatidate or lysophosphatidate to WI-38 or VA-13 fibroblasts (7, 8) or adipocytes (9) decreased the cellular content of cAMP or inhibited prostaglandin- or methylxanthine-induced increases in cAMP. This effect of phosphatidic acid was due to inhibition of adenylate cyclase via the inhibitory guanine nucleotide-binding protein (NI) since the inhibition was effectively reversed by prior exposure of cells to IAP (pertussis toxin) (10), which causes loss of the function of NI, as a result of ADP-ribosylation of its α-subunit, the M\(_{r}\) = 41,000 protein (11). Phosphatidic acid decreased the cAMP level and inhibited membrane adenylate cyclase activity in an IAP-sensitive manner in 3T3 fibroblasts, too (12). Cyclic GMP generation was stimulated by phosphatidic acid in N1E115 neuroblastoma (13) and bovine chromaffin cells (14), probably due to increased influx of Ca\(^{2+}\) in these cells. Likewise, the effects of phosphatidic acid or lysophosphatidic acid of inducing platelet aggregation (15–17), smooth muscle contraction (18), K\(^{+}\) efflux in parotid glands (19), and neutrophil chemotaxis (20) were previously explained by their capability of mobilizing extracellular Ca\(^{2+}\), although the possibility that the Ca\(^{2+}\) ionophore properties of these phospholipids are primarily involved is still controversial (see Watson et al. (21)).

The purpose of the present study is to show that phosphatidic acid triggers signal transduction in 3T3 fibroblasts probably as a result of its binding to particular sites or receptors in membranes. The putative phosphatidate receptors appear to be coupled to the phosphatidylinositol cycle as well as to the adenylate cyclase system.

EXPERIMENTAL PROCEDURES

Materials—IAP was purified from the 2-day culture supernatant of Bordetella pertussis (Tohama strain, phase I) according to the procedure described elsewhere (22, 23). Reagents for radioimmunoassay of cAMP were obtained from a Yamasa cAMP assay kit which was generously donated by Yamasa Shoyu Co., Ltd. (Chiba, Japan).

1 The abbreviations used are: N\(_{o}\), the guanine nucleotide regulatory protein responsible for inhibition of adenylate cyclase (this is also referred to as G); IAP, islet-activating protein (pertussis toxin); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PA, phosphatidic acid (fatty acid diester of α-glycerophosphoric acid); if necessary, the fatty acid is shown in parentheses following PA, as PA (palmitic) for dipalmitinylglycerophosphoric acid); PAF, platelet-activating factor.
...vasopressin, PA (from egg yolk lecithin), PA (oleic), PA (lauric), PA (arachidonic acid) was from Avanti Polar Lipids, Inc. All of these phosphatidic acids used were sodium salts which were apparently soluble or formed from the medium used was Dulbecco's modified Eagle's solution supplemented with 5% fetal calf serum, which was changed every 48 h until the cells were grown to confluence. These cell monolayers (3 x 10^4 cells/dish) were used immediately for cAMP studies as described below or were labeled with radioactive arachidonic acid or inositol. For the latter purposes, the confluent cells were further cultured for 44 h with [H]arachidonic acid or [H]inositol (-0.2 μCi/ml) added at 20 h before the end of the culture. Where indicated, the labeled or unlabeled cell monolayers were exposed to 100 ng/ml IAP (or its vehicle) for the final 3 h of culture to prepare IAP-treated cells. In the experiments shown in Fig. 5, IAP was replaced by thrombin, bradykinin, or PA (oleic) (or the vehicle) to prepare these agonist-pre-treated cells. The data obtained with the IAP- or agonist-treated cells were compared with those with the vehicle-treated cells.

Cyclic AMP Responses of Cell Monolayers and Cell Suspensions—The cell monolayers prepared as above were first rinsed twice with ice-cold Dulbecco's modified phosphate-buffered saline and then once with Hesper buffer A (pH 7.4). The composition of Hesper/buffer A was 15 mM Hesper, 0.4 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 2 mM CaCl₂, and 0.2% bovine serum albumin (fatty acid-free). The cAMP response of these cell monolayers was measured by a 10-min incubation at 30°C in fresh Hesper/buffer A with a further addition of 0.5 mM 3-isobutyl-1-methylxanthine and 10 μM isoproterenol. Receptor agonists or antagonists were included in the incubation medium as shown in the figure legends. Incubation was terminated by aspiration of the medium followed by acidification with HCl to 0.1 N. Cell suspensions, prepared from the monolayers as follows, were used in the experiments shown in Fig. 4. Cells were detached from dishes by being washed with Ca²⁺-/Mg²⁺-free 15 mM Hesper (pH 7.4) fortified with 0.14 M NaCl and 1 mM EGTA, collected by a 3-min centrifugation at 100 x g at 4°C, and suspended in the same medium supplemented with 5 mM MgCl₂ and 0.2% serum albumin. The cell suspensions (10⁶ cells/tube) were then incubated for 10 min at 30°C with or without 2 mM CaCl₂ in the presence of 0.5 mM 3-isobutyl-1-methylxanthine and 10 μM isoproterenol. Further additions are shown in the legend of Fig. 4. The cellular CAMP quantitatively transferred to the supernatant upon acidification was determined by a sensitive radioimmunoassay method (26). Cyclic AMP increased linearly during a 10-min incubation of cell monolayers or cell suspensions.

Phosphatidic Acids May Stimulate Membrane Receptors—The cell monolayers labeled with [H]arachidonic acid or [H]inositol were rinsed two to three times with Hesper/buffer A supplemented with 50 μM nonradioactive arachidonic acid and incubated at 37°C for 10 min in 1 ml of the same solution. This preincubation was achieved to reduce the nonspecific background of release during the subsequent release assay. The monolayers were then incubated at 37°C for 10 min in 1 ml of fresh medium with additions shown in the figure legends. Essentially all of the radioactivities released into the supernatant during incubation were identified as arachidonic acid and its metabolites (12).

Generation of [H]inositol Phosphates in Cell Monolayers—The cell monolayers labeled with [H]inositol were washed two to three times with Hesper/buffer A before incubation in the same fresh solution fortified with 5 mM LiCl and additions for various lengths of time as shown in the figure legends and Table I. Temperature was maintained at 30°C and was increased to 4°C for 4 min. The incubation was terminated by aspiration of the medium followed by the addition of ice-cold 10% trichloroacetic acid. The supernatant of the completely disrupted cells was free of trichloroacetic acid by shaking several times with diethyl ether and was applied to a column of Dowex 1-X8 (formate form, 100-200 mesh, 0.5 ml bed volume) which was eluted stepwise successively with (a) 2 x 6 ml of 5 mM inositol; (b) 2 x 6 ml of 60 mM ammonium formate, 5 mM sodium tetraborate; (c) 2 x 6 ml of 0.2 M ammonium formate, 0.1 M formic acid; and (d) 2 x 6 ml of 0.4 M ammonium formate, 0.1 M formic acid and (e) 2 x 5 ml of 1 M ammonium formate, 0.1 M formic acid (27, 28). The radioactivities in fractions c-e were determined in Aquasol (New England Nuclear) with a scintillation spectrometer as the % contents of inositol 1-monoo-, 1,4-bis-, and 1,4,5-triphosphates, respectively.

RESULTS

Some Phosphatidic Acids Induced (But Others Did Not) Cellular CAMP Increase and Arachidonic Acid Release—PA from egg lecithin as well as PAs with unsaturated fatty acids such as oleic or linoleic acid decreased cellular cAMP and increased arachidonic acid release in 3T3 fibroblasts in concentration-dependent manners (Fig. 1). In this and the following experiments in which decreases in cAMP were measured, the incubation medium was supplemented with isoproterenol to increase cAMP and thus to enhance the inhibitory agonist-induced decrements in the nucleotide, as done in our previous studies.
studies to allow an accurate estimation of the cAMP suppression in intact cells (12, 32–34). The degree of decreases in cAMP induced by PA was as great as, or even greater than, those induced by thrombin, an efficient agonist of 3T3 cell membrane receptors negatively coupled to adenylate cyclase (see Ref. 12). PA from lecithin was also as effective as thrombin in inhibiting 3T3 cell membrane adenylate cyclase (not shown) under conditions identical with those described in a previous paper (12). The incubation medium for cellular cAMP assay was always fortified with methylxanthine, a potent inhibitor of cAMP phosphodiesterase. Thus, PA is very likely to decrease cellular cAMP as a result of adenylate cyclase inhibition rather than phosphodiesterase activation.

PA with saturated fatty acid such as palmitic, lauric, or myristic acid was much less potent than any PA with unsaturated fatty acid in decreasing cAMP (Fig. 1A) and increasing arachidonic acid release (Fig. 1B). Taking advantage of the weak action of PA with saturated fatty acid, the dose-response relationship for PA (oleic) was studied in the presence and the absence of PA (lauric) (Fig. 2). PA (lauric) was antagonistic to PA (oleic) in the sense that dose-response curves for PA (oleic)-induced cAMP decrease and arachidonic acid release were both shifted to the right. Thus, PA with unsaturated fatty acids and PA with saturated fatty acid appear to share the same sites in the cells with marked cellular responses triggered by the former, but not by the latter. The results are compatible with the idea that PAs with unsaturated fatty acids are antagonists or partial agonists of a receptor.

**PA Actions Independent of Ca**

A23187 was also a potent releaser of arachidonic acid from 3T3 cells (Fig. 3). Ca**2+** taken up by the cells in response to the Ca**2+** ionophore may activate such phospholipases involved in arachidonic acid release as phospholipase A2 and diacylglycerol lipase (in combination with monoacylglycerol lipase). The A23187-induced release of arachidonic acid displayed a sharp contrast with the PA-induced release in that it was not at all inhibited by PA with saturated fatty acid (Fig. 3). Thus, the result in Fig. 3 confirms that PA with saturated fatty acids antagonizes the action of PA with unsaturated fatty acids selectively, probably competing for the same receptor sites on the cells. PA (oleic) inhibited cAMP accumulation in either the presence or the absence of extracellular Ca**2+** (Fig. 4A), whereas A23187 was effective in this regard only when the incubation medium was fortified with Ca**2+** (Fig. 4B). A23187 formed a sharp contrast with PA in that it did not inhibit the adenylate cyclase activity of membrane preparations (12), which was susceptible to PA from lecithin (see above). Thus, PA may interact with the receptors negatively coupled to adenylate cyclase without mediation of Ca**2+**, which is capable of decreasing the cellular cAMP content in this cell line (12).

**Selective Refractoriness of 3T3 Cell cAMP Responses to Repeated Additions of Agonists** — Prolonged exposure of cells to a receptor agonist diminishes the response of the cells to the second addition of the same agonist. The refractoriness of the response thus provoked is referred to as homologous desensitization of the receptor. Homologous desensitization of receptors for thrombin and bradykinin is shown in Fig. 5; these agonists failed to decrease cAMP in cells that had been exposed to the same agonists for 3 h, despite normal responses of the cells to other agonists or PA (oleic). Likewise, the exposure of cells to PA (oleic) did not alter the concentration-
response relationship for thrombin- and bradykinin-induced cAMP decreases. These PA-treated cells, however, exhibited fewer responses to the subsequent challenge with PA (oleic) than did the cells that had been exposed to the vehicle or other agonists or than the same cell response to thrombin or bradykinin (Fig. 5C).

Thus, PA seems to stimulate a specific receptor which undergoes homologous desensitization during prolonged exposure to the agonist.

**Blockade by IAP of Phosphatidic Acid-induced Decreases in cAMP and Arachidonic Acid Release**—The cAMP content in 3T3 cells that had been treated with IAP for 3 h was not diminished by PA (oleic) at concentrations fully effective in the nontreated cells (Fig. 6A). The IAP treatment of cells also abolished arachidonate releasing response of cells to PA (oleic); PA caused a much smaller release of arachidonic acid from IAP-treated cells than from nontreated cells at any concentration (10 nM to 10 μM) employed (Fig. 6B). Since the Mr = 41,000 protein, the α-subunit of N, is completely ADP-ribosylated during the IAP treatment of this cell line (35), these dual responses to PA are very likely to be mediated by N, or other GTP-binding proteins, the function of which to couple receptors to effectors is lost upon IAP-catalyzed ADP-ribosylation (11, 25, 36-41).

**Activation of Phospholipase C by Phosphatidic Acids in an IAP-insensitive Manner**—We have recently shown that phosphatidic acid, like thrombin, increased the release of radioactive inositol from [3H]inositol-labeled 3T3 cell monolayers into culture media (12). The inositol thus released may have arisen from the breakdown of phosphatidylinositols in cells. The generation of inositol phosphates, direct products of phosphatidylinositol breakdown via phospholipase C, was therefore studied in Figs. 7 and 8 and Table I. Fig. 7 shows that inositol 1,4,5-tris-, 1,4-bis-, and 1-monophosphates rapidly accumulated in the incubation medium of 3T3 cell monolayers which was fortified with PA (oleic) and LiCl, an inhibitor of further dephosphorylation of inositol monophosphate. The rate of accumulation was: inositol trisphosphate > inositol bisphtpate > inositol monophosphate; the order of the rate was in agreement with the sequential dephosphorylation of inositol trisphosphate, the direct product of the phospholipase C reaction with the substrate of phosphatidylinositol 4,5-bisphosphate.

Not only PA but also thrombin provoked rapid generation of inositol phosphates when added to the 3T3 cell monolayer. The yield of inositol trisphosphate was strictly dependent on the concentrations of thrombin (Fig. 8A) and oleic PA (Fig. 8B). The effective concentration ranges (0.03-3 unit/mi for thrombin and 0.1–10 μM for PA (oleic)) were essentially the same between the inositol phosphate generation (Fig. 8) and the cAMP decrease (Fig. 5) or the arachidonate release (Fig. 6 and Ref. 12). The disparity between these responses was, however, found for their differential susceptibility to IAP treatment; the generation of inositol phosphates in response to PA (oleic), thrombin, or vasopressin was not affected by prior exposure of cells to IAP (Table I), the procedure which effectively abolished the cAMP-decreasing and arachidonate-releasing responses of the cells to the same agonists. No inositol phosphates increased upon the addition of
cell monolayers prelabeled with \([3H]\)inositol were incubated with increasing concentrations of thrombin for 3 min to determine the accumulation of inositol 1,4,5-trisphosphate (Ins-P) and was 10 min for inositol 1-monophosphate (Ins-P) and inositol 1,4-bisphosphate (Ins-P). The data are percentages of the values obtained without additions. These “none” values (dpm/dish) are shown in parentheses.

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<th>Additions</th>
<th>IAP treatment</th>
<th>Accumulation of</th>
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<tr>
<td></td>
<td></td>
<td>Ins-P</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5397 ± 183)</td>
</tr>
<tr>
<td>PA (oleic) (10 µM)</td>
<td>236 ± 3</td>
<td>212 ± 13</td>
</tr>
<tr>
<td>Thrombin</td>
<td>220 ± 6</td>
<td>171 ± 13</td>
</tr>
<tr>
<td>(1 unit/ml)</td>
<td>217 ± 7</td>
<td>208 ± 10</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>230 ± 14</td>
<td>160 ± 7</td>
</tr>
<tr>
<td>(100 nM)</td>
<td>225 ± 14</td>
<td>163 ± 3</td>
</tr>
<tr>
<td>A23187 (10 µM)</td>
<td>98 ± 5</td>
<td>88 ± 10</td>
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<td></td>
<td>101 ± 4</td>
<td>98 ± 5</td>
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![Fig. 7. Time courses of generation of inositol tris-, bis-, and monophosphates in 3T3 cells in response to PA (oleic). The cell monolayers prelabeled with \([3H]\)inositol were incubated with (●) or without (○) 1 µM PA (oleic) for various length of time as described under “Experimental Procedures.” The data (means of duplicate experiments) are the increments during incubation in radioactivities in the inositol 1,4,5-tris-(A), 1,4-bis-(B), and 1 mono-(C)-phosphate fractions.

![Fig. 8. Comparison between thrombin and PA (oleic) in their ability to increase inositol 1,4,5-trisphosphate in 3T3 cells. The cell monolayers prelabeled with \([3H]\)inositol were incubated for 3 min with increasing concentrations of thrombin (A) or PA (oleic) (B) as described under “Experimental Procedures.” The thrombin- and PA-induced increments in radioactivities in the inositol 1,4,5-trisphosphate fraction were plotted. Each point represents the mean of two experiments.

A23187 to 3T3 cells (Table I), despite the foregoing results that the Ca\textsuperscript{2+} ionophore was as effective as thrombin or PA in increasing arachidonic acid release (Fig. 3) and decreasing cellular cAMP (Fig. 4) in this cell line. Likewise, the membrane adenylate cyclase activity was lowered by thrombin (12) or PA (see above), but not by A23187 (12). The cell responses to A23187 could be accounted for by Ca\textsuperscript{2+} mobilized by its Ca\textsuperscript{2+} ionophore action. Therefore, it is very likely that receptors for thrombin or PA are coupled to adenylate cyclase inhibition and phospholipase C activation rather directly without mediation of intracellular Ca\textsuperscript{2+}. Inositol 1,4,5-trisphosphate, a product of the phospholipase C reaction, then mobilizes Ca\textsuperscript{2+} from intracellular stores (5, 6), which is in turn responsible for arachidonic acid release via activation of Ca\textsuperscript{2+}-dependent phospholipase A\textsubscript{2} and/or diacylglycerol lipase in 3T3 cells. IAP seems to ADP-ribosylate a GTP-binding protein that is involved in this action of inositol triphosphate leading to arachidonic acid release since it did block receptor-coupled arachidonic acid release, but did not prevent the A23187-induced release (12) as well as the receptor-mediated production of inositol phosphates (Table I).

**Failure of IAP to inhibit agonist-induced accumulation of inositol mono-, bis-, and triphosphates in 3T3 cells**

The 3T3 cell monolayers exposed to 100 ng/ml IAP (+) or vehicle (−) in the presence of \([3H]\)inositol were incubated with additions indicated as described under “Experimental Procedures.” The incubation time was 3 min to determine the accumulation of inositol 1,4,5-trisphosphate (Ins-P) and was 10 min for inositol 1-monophosphate (Ins-P) and inositol 1,4-bisphosphate (Ins-P). The data percentages of the values obtained without additions. These “none” values (dpm/dish) are shown in parentheses.
Release of PA from rabbit platelets in response to thrombin and A23187

Rabbit platelets were labeled with [3H]arachidonic acid and incubated without (control) or with thrombin or A23187 for 10 min. These cells (intracellular) and the medium (extracellular) were then analyzed separately for the radioactivity contents of the various lipid fractions by means of thin-layer chromatography. See "Experimental Procedures" for details. The radioactivities (dpm/dish, from duplicate experiments) of the lipid fractions in control cells are shown in parentheses.

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<th>Lipid Fraction</th>
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<tr>
<td>Intracellular</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>18,200, 15,710</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>4,110, 3,850</td>
</tr>
<tr>
<td>Dacylglycerol</td>
<td>3,290, 2,730</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>395, 280</td>
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<tr>
<td>Thrombin A23187</td>
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Fig. 9. Failure of PA (oleic) to inhibit cAMP accumulation in platelet cells and adenylyl cyclase activity of platelet membranes. Platelet cell suspensions (A) or platelet membranes (B) were incubated with increasing concentrations of PA (oleic), epinephrine (C), or thrombin (D) as described under "Experimental Procedures." The cAMP accumulation (A) and adenylyl cyclase activity (B) are plotted as percentages of the control values obtained without these additions which were 504 pmol/106 cells and 918 pmol/mg of protein/min, respectively. Each point is the mean of duplicate experiments.

clase activity was affected by PA (oleic) in platelets under the same conditions (Fig. 9). No arachidonic acid was released from platelets in response to PA, either (not shown). Thus, the actions of PA appear to be cell-specific, most likely dependent on whether the specific receptor sites are occurring or not on the cell surface.


discussion

The following findings presented in this study are in accordance with the idea that the effects of phosphatidic acids on 3T3 cells result from their binding to particular sites or specific receptors located on the outer surface of the cells.

First, slight alterations of the structure of phosphatidic acids caused striking changes in their activities. The acids with low activities acted as antagonists (or competitive inhibitors) of the active acids or agonists. For instance, PAs consisting of unsaturated fatty acids were full agonists, whereas PAs of saturated acids were partial agonists and antagonists (Figs. 1 and 2). Lauric PA failed to inhibit the effect of A23187 (Fig. 3), thrombin, and bradykinin (not shown), suggesting that the inhibition was due to an antagonism selectively directed to the effect of active PA. The critical micelle concentration of PA is probably so low that PA apparently dissolved must be present as invisible tiny vesicles in incubation or culture media under the experimental conditions in the present study. A possibility could not be excluded, therefore, that PA (lauric) interacted with the vesicles of PA (oleic) in Fig. 2 to form aggregates which were still invisible, thereby making PA (oleic) inaccessible to cell membranes. PA (lauric) was only inhibitory to arachidonate release from cells that had been previously exposed to PA (oleic) for a short while, making it likely that the interaction occurs at cell membranes.

Second, desensitization (or refractoriness to the repeated challenge with agonists) was observed with PA as well as other typical receptor agonists such as thrombin and bradykinin (Fig. 5); the potency of PA (oleic) to decrease cAMP was markedly, but the potency of thrombin or bradykinin was not at all, lowered by prior exposure of cells to the same PA. The refractoriness of cells to the second challenge with the same agents may have arisen from receptor desensitization since the cells once exposed to a low concentration (2 μM) of A23187 were as responsive to the second addition of the Ca2+-ionophore as were the cells exposed to the vehicle (data not shown).

Third, PA-induced decrease in cellular cAMP or PA-induced release of arachidonic acid was reversed by prior exposure of 3T3 cells to IAP for 2 h (Fig. 6). This IAP treatment procedure gave rise to ADP-ribosylation of the membrane M, = 41,000 protein, an α-subunit of Ni, in 3T3 cells (35). The ADP-ribosylated Ni is not capable of being coupled to receptors any longer (42, 43), despite its unaltered functions by itself to bind GTP, hydrolyze GTP, and interact with the adenylyl cyclase catalyst (44, 45). Thus, susceptibility to IAP is strongly suggestive of an involvement of N2-coupled receptors.

Fourth, incubation of 3T3 cells with PA provoked rapid generation of inositol 4,5-trisphosphate accompanied by increases in inositol bios- and monophosphates (Fig. 7), as did incubation with other receptor agonists, thrombin and vasopressin (Table I). A23187 was without effect in this regard. Stimulation of Ca2+-mobilizing receptors is known to trigger the activation of phospholipase C, the specific substrate of which is phosphatidylinositol 4,5-bisphosphate, leading to the generation of inositol 1,4,5-trisphosphate followed by its dephosphorylation to inositol bis- and monophosphates (2, 3, 5, 6). Receptor-coupled activation of phospholipase C is apparently independent of intra- and extracellular Ca2+ but is probably dependent on locally sequestered Ca2+ (2, 3, 5, 6). Thus, the inositol phosphate generation in response to PA may arise from PA receptor-linked activation of phospholipase C in 3T3 cells.

Fifth, a possibility has been ruled out that the Ca2+ ionophore action of PA (see the "Introduction") is responsible for PA-induced decreases in cAMP since PA (oleic) decreased cAMP in the same dose-dependent manner in either the
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Presence or the absence of extracellular Ca\(^{2+}\) (Fig. 4A). PA (oleic) was still effective even after the sonication of the PA solution in the presence of ECTA, the procedure which may render the PA vesicles completely depleted of Ca\(^{2+}\). Cellular cAMP was decreased by A23187 as well, suggesting an involvement of Ca\(^{2+}\). A23187 did not decrease cAMP in a Ca\(^{2+}\)-free medium (Fig. 4B) nor inhibit membrane adenylate cyclase (12). A Ca\(^{2+}\) ionophore action is involved in A23187-induced (but not in PA-induced) decreases in cellular cAMP. In fact, the A23187-induced decrease in cAMP was distinct from the decrease induced by PA in its insusceptibility to IAP treatment of cells (12).

Sixth, PA did not decrease cAMP or inhibit adenylate cyclase in rabbit platelets, despite the fact that thrombin and epinephrine were strongly effective in this regard in the same cells (Fig. 9). Epinephrine-induced inhibition of platelet membrane adenylate cyclase was reversed by IAP treatment of the membranes (46), indicating that epinephrine (\(\alpha\)-adrenergic) receptors are negatively coupled to adenylate cyclase via \(\mathrm{G}\) in this cell type. Thus, the failure of PA to inhibit adenylate cyclase in platelets makes it very likely that PA interacts with \(\mathrm{N}\)-coupled receptors which are present in 3T3 cells but absent in platelets.

Taken together, we would like to propose that the initial step of the interaction of phosphatidic acid with 3T3 cells is its binding to a particular receptor site on cell membranes, triggering multiple effector systems including inhibition of adenylate cyclase and activation of phospholipase C and other phospholipases involved in arachidonic acid release and cellular calcium mobilization. The effects of phosphatidic acid on 3T3 cells were not mimicked by other phospholipids such as phosphatidylcholine and phosphatidylserine or arachidonic acid. Since inclusion of mepacrine, an inhibitor of phospholipase \(A_2\), in incubation media did not exert any influence on the effects of phosphatidic acid added (data not shown), it is unlikely that lysophosphatidic acid is a real agonist of the putative phosphatidic acid receptor.

Characteristics of this putative receptor for PA deserve brief discussion as follows. First, the receptor is negatively coupled to adenylate cyclase via an IAP substrate (or \(\mathrm{N}\)) and positively coupled to phospholipase C without being mediated by any IAP substrate. It is likely, therefore, that there are two subtypes of PA receptors: the one coupled to an IAP substrate and the other not, in 3T3 cells. The decrease in cellular cAMP might trigger Ca\(^{2+}\) influx which is responsible for the activation of phospholipase \(A_2\) (diacylglycerol lipase) releasing arachidonic acid from membrane phospholipids since PA-induced arachidonic acid release and \({\text{\(^{45}\)Ca}^{2+}}\) influx (12) were abolished by IAP treatment of cells. Alternatively, GTP-binding (IAP substrate) proteins might play an additional role in the activation of phospholipase \(A_2\).

Second, platelet-activating factor (PAF), one of the receptor agonists, is a phospholipid bearing some similarities in structure to PA. A possibility that PA shares the same receptor with PAF is, however, unlikely since not all of the actions of PA on 3T3 cells were mimicked by PAF, e.g. there was no significant generation of inositol phosphates during incubation of the cells with PAF (data not shown). Moreover, the maximal decrease in cellular cAMP was much smaller when 3T3 cells were incubated with PAF than when the cells were incubated with PA (data not shown).

Finally, PA is one of the important metabolites rapidly produced upon stimulation of Ca\(^{2+}\)-mobilizing receptors in a variety of cell types. In the case of 3T3 cells, stimulation of membrane receptors by thrombin, bradykinin, or vasopressin triggered the same effector systems as those initiated by PA. It is tempting to speculate, therefore, that PA generated following stimulation of these receptors is responsible for the effects observed. In fact, most of the PAs generated in stimulated platelets were outside the cells (Table II); PA is capable of binding to its extracellular receptors under these conditions. The receptor desensitization experiments in Fig. 5, however, did not support this speculation because 3T3 cells that did not respond to thrombin or bradykinin after preexposure to these agonists responded to PA in just the same degree as did the nonexposed control cells. Hence, PA receptors that are desensitized by prior addition of PA itself must not have been stimulated during exposure of the cells to thrombin or bradykinin. A possibility is not ruled out as yet that cellular responses to agonists of Ca\(^{2+}\)-mobilizing receptors could be amplified by endogenously generated PA which stimulates its own receptors to an extent too weak to cause desensitization.

REFERENCES
Phosphatidic Acids May Stimulate Membrane Receptors