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Proinflammatory Macrophage-activating Properties of the Novel Phospholipid Diacylglycerol Pyrophosphate*

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We have found that the novel phospholipid diacylglycerol pyrophosphate (DGPP), identified in bacteria, yeast, and plants, but not in mammalian cells, is able to potently activate macrophages for enhanced secretion of arachidonate metabolites, a key event in the immunoinflammatory response of leukocytes. Macrophage responses to DGPP are specific and are not mediated by its conversion into other putative lipid mediators such as phosphatidic acid, lysophosphatidic acid, or diacylglycerol. The responses to DGPP are compatible with a receptor-recognition event because they are blocked by suramin. Intracellular signaling initiated by DGPP includes phosphorylation and activation of the Group IV cytosolic phospholipase A_2 and of the extracellular-signal regulated p42 mitogen-activated protein kinase (MAPK) and p44 MAPK, and membrane translocation of the protein kinase C isoenzymes α , ϵ , δ . These results establish DGPP as a novel macrophage-activating factor and suggest a potential role for this compound in triggering homeostatic cellular responses.

Leukocytes constitute the primary line of defense against infection. Recognition of foreign material by specific membrane receptors on the leukocyte surface enables these cells to mount an immunoinflammatory response that ultimately leads to killing of the microbe. During the course of these events, leukocyte membrane remodeling generates biologically active lipids that can serve both as intra- and extracellular mediators (1).

Recently, a novel phospholipid compound, diacylglycerol pyrophosphate (DGPP),¹ has been identified (2). The biochemical routes leading to biosynthesis and degradation of DGPP in those cells which produce it have recently been elucidated (3–8). DGPP is produced by phosphorylation of phosphatidic acid (PA), a reaction catalyzed by a specific kinase that has been purified from plants (3). The amount of DGPP in resting cells is barely detectable, *e.g.* less than 0.18% of the major

phospholipids in *Saccharomyces cerevisiae* (9). However, recent studies in plants have demonstrated that the concentration of DGPP increases significantly when signaling is activated by the G-protein activating peptide mastoparan (5). Thus, DGPP has characteristics of a lipid messenger molecule, suggesting its participation in novel lipid signaling pathways (9, 10).

DGPP degradation is accounted for by a two-step dephosphorylation reaction catalyzed by DGPP phosphatase. The enzyme first removes the β -phosphate from DGPP to form PA and then removes the phosphate from PA to form diacylglycerol (DAG) (6). The reactions catalyzed by DGPP phosphatase are Mg^{2+} -independent and *N*-ethylmaleimide-insensitive (6, 7). The properties of the DGPP phosphatase reactions in yeast, bacteria, and plants are strikingly similar to the Mg^{2+} -independent PA phosphatase purified from mammalian cells (9). In fact, the Mg^{2+} -independent PA phosphatase from rat liver also displays DGPP phosphatase activity (11). The Mg^{2+} -independent phosphatase is postulated to play important roles in cellular signaling by modulating the cellular levels of several bioactive lipids including diacylglycerol, PA, lyso-PA, and sphingosine-1-phosphate (9).

DGPP production has not been documented in mammalian cells to date, and its biological role remains completely unknown (12). The above-mentioned observations, however, appear to anticipate an important role for DGPP in cellular signaling. DGPP, like DAG and lyso-PA, is produced from PA via a single enzymatic step. Because all these lipids have been shown to potently mediate cellular signaling, we have now examined the capacity of DGPP to mediate macrophage activation and the release of arachidonic acid (AA)-derived inflammatory mediators such as the eicosanoids. The results reported herein demonstrate that DGPP is a novel, potent macrophage-activating factor and suggest a role for DGPP in triggering proinflammatory cell responses.

EXPERIMENTAL PROCEDURES

Materials—The cell line used in this study, termed P388D₁/MAB, is a subclone of the P388D₁ cell line (TIB 63) available from the American Type Culture Collection (Manassas, VA) that was selected on the basis of high responsiveness to LPS/PAF. A description of the characteristics of this subclone will be published elsewhere.² Iscove's modified Dulbecco's medium (endotoxin < 0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from HyClone Labs. (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,1,15-³H]Arachidonic acid (specific activity 100 Ci/mmol) was obtained from NEN Life Science Products. ADP, pyrophosphate, LPS (Re595), and PAF were from Sigma. Methyl arachidonyl fluorophosphonate (MAFP) was from Cayman (Ann Arbor, MI). Suramin was from Biomol (Plymouth Meeting, PA). The MAP kinase inhibitor, PD098059, was from Calbiochem (San Diego, CA).

² H. Shinohara, M. A. Balboa, C. A. Johnson, J. Balsinde, and E. A. Dennis, manuscript in preparation.

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¹ The abbreviations used are: DGPP, diacylglycerol pyrophosphate; AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; MAFP, methyl arachidonyl fluorophosphonate; PGE₂, prostaglandin E₂; PGD₂, prostaglandin D₂; PA, phosphatidic acid; lyso-PA, lysophosphatidic acid; PD098059, (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one); DAG, diacylglycerol; LPS, lipopolysaccharide; PAF, platelet-activating factor; PKC, protein kinase C; ERK, extracellular signal-regulated kinase.

Group IV cPLA₂ antibodies were kindly provided by Dr. Ruth Kramer (Lilly Research Laboratories, Indianapolis, IN). Phospho-specific p42/p44 mitogen-activated protein kinase (MAPK) (Thr²⁰²/Tyr²⁰⁴) antibody was from New England Biolabs (Beverly, MA). Protein kinase C (PKC) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

DGPP (dioctanoyl-*sn*-glycero-3-pyrophosphate, diC₈-DGPP) was chemically synthesized from dioctanoyl-*sn*-glycero-3-phosphate (diC₈-PA) (Avanti Polar Lipids, Alabaster, AL) and *ortho*-phosphoric acid as described by Riedel *et al.* (8). DiC₈-DGPP was purified by thin-layer chromatography on potassium oxalate-treated plates using the solvent system chloroform/acetone/methanol/glacial acetic acid/water (50:15:13:12:4) (The R_f for DGPP in this system is 0.33, and the R_f for PA is 0.71) (2). The purified DiC₈-DGPP migrated precisely with enzymatically synthesized DiC₈-DGPP using three different solvent systems (2). In addition, the chemically synthesized DiC₈-DGPP was enzymatically active when employed as a substrate for pure DGPP phosphatase from *Saccharomyces cerevisiae* (6). ³²P-labeled (α-³²P) DGPP was synthesized enzymatically using purified *Catharanthus roseus* PA kinase as described by Wu *et al.* (6).

Cell Culture and Labeling Conditions—P388D₁ cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO₂ in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at 10⁶ per well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium.

Stimulation of P388D₁ Cells—Our standard regimen for activating the P388D₁ cells has been described previously (13–15). Briefly, radiolabeling of the cells with [³H]AA was achieved by including 0.5 μCi/ml [³H]AA during the overnight adherence period (20 h). The cells were placed in serum-free medium for 30–60 min before the addition of LPS (200 ng/ml) for 1 h. After the LPS incubation, the cells were exposed to PAF for the time indicated, in the presence of 0.1 mg/ml bovine serum. For DGPP, the LPS priming step was omitted. When suramin was used, it was added 30 min before the stimulants.

After stimulation, supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. More than 99% of the released radioactive material remains as unmetabolized AA under these experimental conditions.

For the measurement of PGD₂ or PGE₂ production, unlabeled cells were used, and the incubations proceeded in the absence of albumin. Prostaglandins in the supernatants were measured by using radioimmunoassays specific for either PGD₂ (Amersham Pharmacia Biotech) or PGE₂ (PerSeptive Diagnostics, Framingham, MA).

Immunoblotting Studies—Cells, serum-starved for 1 h, were stimulated as described above. Afterward, the cells were washed and lysed in a buffer consisting of 1 mM Hepes, 0.5% Triton, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 4 °C. Protein was quantified, and a 100-μg aliquot was analyzed by immunoblot with antibodies against cPLA₂, p42/p44 MAPK, or the protein kinase C isoenzymes α, δ, and ε.

RESULTS

DGPP-induced Arachidonic Acid Metabolism—When mouse P388D₁ macrophages were exposed to DGPP, an appreciable formation of prostaglandins such as PGE₂ was detected in a dose- (Fig. 1A) and time-dependent (Fig. 1B) manner. Positive controls using the standard activation procedure of these cells with LPS/PAF (13–15) gave a comparable response, indicating that the DGPP-stimulated prostaglandin response is physiologically relevant.

We investigated next the critical components of the DGPP molecular structure required for stimulation of AA metabolism. Pyrophosphate-containing compounds such as pyrophosphate itself and ADP were ineffective (data not shown), suggesting that the DGPP stimulatory activity arises from its lipidic nature. DGPP was not metabolized to PA or lyso-PA by the macrophages within the time frame of the current experiments (up to 30 min). This was assessed by incubating the cells with 50 μM [α-³²P]DGPP for different periods of time (5, 10, 15, and 30 min). Analysis of the distribution of ³²P radioactivity after the incubations by thin-layer chromatography (6) revealed that all the label was recovered as DGPP. Neither ³²P-labeled PA nor ³²P-labeled lyso-PA could be detected. In addition, no up-

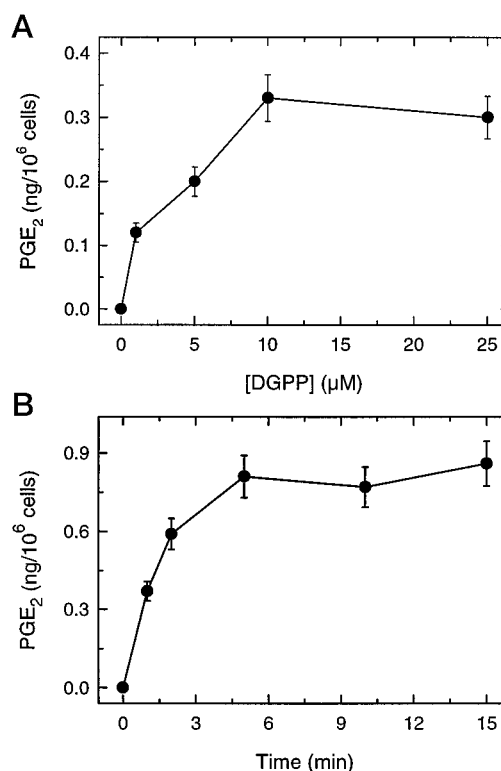


FIG. 1. DGPP induces PGE₂ production in P388D₁ macrophages. A, effect of increasing DGPP concentrations on PGE₂ production (30-min stimulation). B, time-course of PGE₂ production (50 μM DGPP).

take of DGPP by the cells could be detected, as quantitated by measuring the amount of cell-associated ³²P radioactivity after the incubations. Thus the effect of DGPP on AA mobilization appears to be specific and not mediated via its putative conversion to other bioactive lipids such as PA or lyso-PA.

AA mobilization and subsequent prostaglandin production are events typically triggered by interaction of agonists with receptors on the surface of immunoinflammatory cells such as the macrophages. The characteristics of the DGPP effect depicted in Fig. 1, along with the fact that its stimulatory potency is similar to that of the receptor agonists LPS/PAF, prompted us to investigate the possibility of whether the DGPP effect was receptor-mediated. For these experiments, we used the receptor antagonist suramin. Because of its effect of blocking receptor coupling to G-proteins, suramin is regarded as a general inhibitor of receptor-mediated processes (16). As shown in Fig. 2, prostaglandin production by DGPP was not observed if the cells were first incubated with suramin. The suramin inhibition was found to be dose-dependent, with half-maximal inhibition being observed at 30 μM. Interestingly, suramin treatment allowed us to distinguish the DGPP effects from those exerted by exogenous DAG (dioctanoyl-*sn*-glycerol), as the effects of the latter on AA release were largely insensitive to suramin (data not shown). Thus the effect of DGPP is not mediated via conversion to DAG.

Our investigations into the molecular mechanisms of PAF receptor-mediated AA mobilization have highlighted the requirement for a rise in intracellular Ca²⁺ levels, an event that occurs within seconds after PAF addition (17). Given the above results suggesting that DGPP could signal as well in a receptor-mediated manner, we examined the possibility that DGPP induces intracellular Ca²⁺ mobilization in P388D₁ cells. To this end, a protocol identical to that previously used for PAF was used, *i.e.* using fura2-loaded cells (17). The results of these

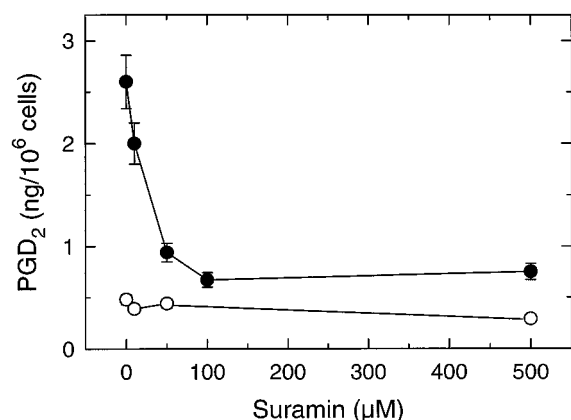


FIG. 2. Effect of suramin on DGPP-induced PGD₂ production (50 μM, 30-min stimulation) (closed circles) compared with unstimulated control cells (open circles).

experiments revealed that, unlike PAF, DGPP failed to induce any detectable change in the intracellular Ca²⁺ concentration (not shown). In turn, these data indicate that DGPP does not act as a Ca²⁺ ionophore.

DGPP Activates the MAP Kinase Cascade and Cytosolic Phospholipase A₂—By what mechanism does DGPP stimulate prostaglandin production? A direct approach to determining the identity of the PLA₂(s) responsible for AA mobilization and subsequent prostaglandin synthesis is to use selective inhibitors for the PLA₂ forms present in these cells (13). AA mobilization induced by DGPP was completely blocked by MAFP (13), an inhibitor of the cytosolic group IV phospholipase A₂, or cPLA₂ (Fig. 3A), suggesting involvement of the latter enzyme in DGPP-induced signaling. Further support to this notion was given by the finding that DGPP induced a retardation of the electrophoretic mobility of the small but measurable amount of unphosphorylated cPLA₂ in the resting cells, indicating phosphorylation of that fraction of enzyme (Fig. 3B). Positive controls using the standard activation procedure of the P388D₁ cells with LPS/PAF (13–15) gave a similar result.

The kinases responsible for phosphorylation of the cPLA₂ have all been identified as members of the MAPK family. In the vast majority of cell types, including P388D₁ macrophages,³ the kinases responsible for phosphorylating the cPLA₂ have been identified as the extracellular signal-regulated kinases (ERK) p42 MAPK and p44 MAPK (18–21). Fig. 4A shows that DGPP stimulation of the cells led to a robust activation of both p42 MAPK and p44 MAPK, as judged by increased phosphorylation of these kinases. The time-course of activation of the ERKs by DGPP is shown in Fig. 4B. Direct proof that p42/p44 MAPK activation is involved in prostaglandin synthesis in DGPP-stimulated cells was established by using the MAP kinase inhibitor PD098059, a compound that inhibited p42/p44 MAPK phosphorylation (Fig. 5A), and PGE₂ release (Fig. 5C). PD098059 also slightly inhibited the phosphorylation of the small fraction of cPLA₂ that was not phosphorylated under basal conditions (Fig. 5B). It is important to note that phosphorylation of the cPLA₂ at Ser⁵⁰⁵ (*i.e.* the one causing the gel shift) has been demonstrated not to be required for AA mobilization and prostaglandin release under certain conditions (21, 22). Hence, cPLA₂ phosphorylation does not necessarily have to correlate with enhanced AA release, as other factors may also be involved (21–24). As a matter of fact, no correlation between DGPP-induced cPLA₂ gel shift (Fig. 5B) and PGE₂ release (Fig. 5C) is evident from our data. Therefore DGPP-induced cPLA₂

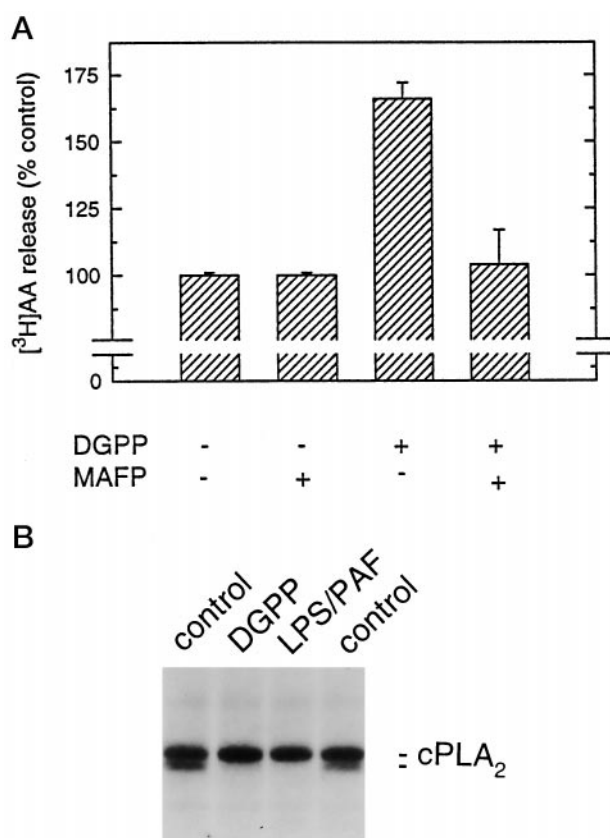


FIG. 3. cPLA₂ involvement on DGPP signaling. A, effect of MAFP on DGPP-induced AA release. The cells were incubated for 20 min without (–) or with (+) 25 μM MAFP as indicated. Afterward, the cells were either untreated (–) or treated with (+) 50 μM DGPP as indicated, and AA release was determined after 30 min. B, phosphorylation of cPLA₂ by different stimuli in P388D₁ macrophages. Cells were incubated with DGPP (50 μM, 30 min), LPS/PAF (1 h with 200 ng/ml LPS followed by 10 min with 100 nM PAF), or neither (control). Afterward, cells were lysed, and cPLA₂ mobility shift was analyzed by immunoblot.

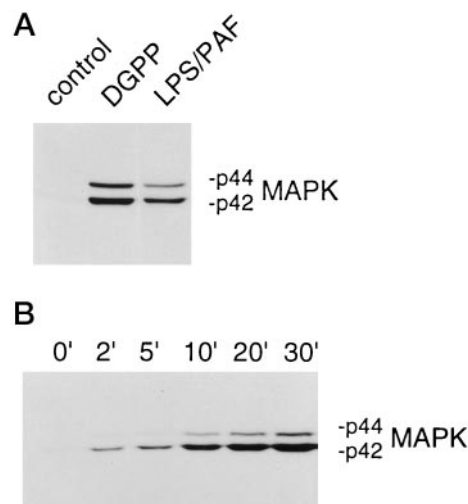


FIG. 4. Involvement of the MAPK cascade in DGPP signaling. A, phosphorylation of p42/p44 MAPK by different stimuli in P388D₁ macrophages. Cells were incubated with DGPP (50 μM for 30 min), LPS/PAF (1 h with 200 ng/ml LPS followed by 10 min with 100 nM PAF), or neither. Afterward, cells were lysed, and p42/p44 MAPK phosphorylation was analyzed by immunoblot. B, time-course of the effect of DGPP (50 μM) on p42/p44 MAPK phosphorylation.

phosphorylation leading to a gel shift is just interpreted as a consequence of DGPP-induced MAP kinase activation, without necessarily having a role in AA release.

³ J. Balsinde, M. A. Balboa, and E. A. Dennis, unpublished observation.

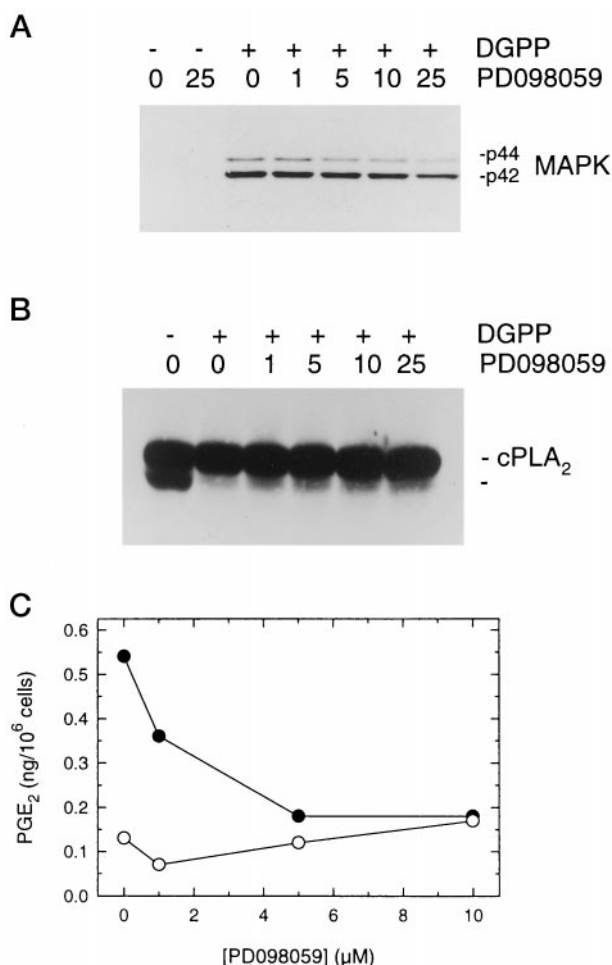


FIG. 5. **Effects of PD098059, a MAP kinase kinase inhibitor.** *A*, dose-dependent inhibition by PD098059 of DGPP-induced phosphorylation of p42/p44 MAPK. *B*, effect of PD098059 on cPLA₂ phosphorylation induced by DGPP. *C*, effect of PD098059 on DGPP-induced PGE₂ production (close circles) and controls lacking DGPP (open circles). PD098059 concentrations are expressed as micromolar in all panels.

DGPP Activates Protein Kinase C—Having thus established that DGPP behaves as an activator of the “classical” MAPK cascade module and its downstream effector cPLA₂, we sought to investigate next the effect of DGPP on signaling elements that are known to be placed upstream of the MAPK cascade. PKC has often been documented as an upstream activator of the classical MAPK kinase pathway and the cPLA₂ (23, 24). When activated, protein kinase C translocates to membranes as this is its cellular site of action. Therefore, activation of PKC by DGPP could be easily followed by monitoring its increased appearance in membranes. Translocation of the PKC isoenzymes α , ϵ , and δ was readily observed within 1 min of cell treatment with DGPP, *i.e.* earlier than the activation of p42/p44 MAPK (Fig. 6). The one other PKC isoenzyme present in these cells, PKC ζ , (25) did not translocate to membranes in response to DGPP (not shown).

DISCUSSION

Phosphatidic acid and the compounds that derive from it in a single enzymatic step, *i.e.* lyso-PA and DAG, are recognized to play key roles in cell physiology, not only as intermediate lipid metabolites, but as potent signaling mediators (26, 27). In this paper, we have identified a signaling pathway, initiated by another direct metabolite of PA, DGPP, through which leukocytes may be activated to generate immunoinflammatory lipid mediators. Such a signaling pathway includes activation of

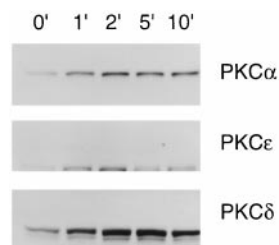


FIG. 6. **Translocation of three PKC isoenzymes to the membrane fraction of cells stimulated with DGPP (50 μ M) for the times indicated.**

several PKC isoenzymes, p42/p44 MAPK, and finally the cPLA₂. Thus DGPP adds to the number of biological structures capable of triggering a proinflammatory response by phagocytic cells.

Although identified in 1993 (2), the cellular roles of DGPP and the enzymes involved in its metabolism, namely PA kinase and DGPP phosphatase, have remained obscure. It has been speculated that DGPP might simply represent a product of signal attenuation of PA-mediated signaling (2, 3). Thus plasma membrane increases in PA as a consequence of activation of the phosphoinositide turnover could be attenuated by its conversion to DGPP via PA kinase, which has been demonstrated to reside in the plasma membrane (2, 3). It has been speculated as well that DGPP might represent a precursor species for the PA to be utilized either for specific signaling functions or phospholipid synthesis (6). Studies in plants have shown DGPP to be a minor polar lipid that dramatically increases and then decreases in concentration when cells are activated (5, 10). This behavior, typical of lipid messenger formation and attenuation, suggests that DGPP is itself a signaling molecule and not just a by-product of PA metabolism.

PA and lyso-PA have been long known to possess stimulatory activity on cells when applied as an extracellular stimulus. lyso-PA, acting via a specific surface receptor, appears to mediate a myriad of biological effects under physiological settings (26). On the other hand, it seems logical to rationalize that the extracellular signaling ability of PA is more relevant under pathophysiological settings, where relatively large amounts of PA arising from damaged tissue membranes can be made accessible for stimulation of cells in the vicinity. Given the obvious structural similarities among DGPP, PA, and lyso-PA, we wondered whether DGPP would have extracellular activating capacity as well. Our results have provided strong evidence to document that DGPP is capable on its own of activating immunoinflammatory signaling in macrophages. This is a very remarkable discovery because DGPP has not been identified in mammalian cells (10). Thus it is possible that immunocompetent cells may have evolved to recognize a distinctive lipid on the membranes of foreign organisms, thus adding to the specificity of the immunoinflammatory reaction. Two other pieces of evidence herein reported are consistent with the aforementioned possibility. First, the DGPP effect arises from its lipidic nature and is specific (*i.e.* is not mediated by its conversion to either PA or lyso-PA); second, the DGPP effects are strongly inhibited by suramin, which suggests the involvement of a proteinaceous component of the plasma membrane in DGPP recognition. We cannot rule out, however, that suramin may have acted in our experiments in a receptor-independent manner.

The current results raise an intriguing question as to the identity of the membrane receptor to which DGPP binds to activate macrophage AA metabolism. Although it is possible that DGPP interacts with a specific surface receptor, the structural similarities between DGPP and lyso-PA raise, as well, the possibility that DGPP is acting as a surrogate for lyso-PA and

hence interacting with a lyso-PA receptor. A receptor for lyso-PA, *vzg-1/lpA1/edg-2*, has recently been identified (28, 29). Northern blot analysis of mRNA from P388D₁ macrophages failed to detect expression of the *vzg-1/lpA1/edg-2* lyso-PA receptor (27, 28) on these cells.⁴ Moreover, this receptor does not bind DGPP when assayed by a specific receptor binding assay (28, 29).⁴ Therefore, the DGPP effects reported herein are not mediated by this lyso-PA receptor. Recently, two other lyso-PA-like receptors have been identified (30, 31). It will be interesting to investigate their relationship, if any, to DGPP signaling. It is interesting to note in this regard that the effects of DGPP herein reported do not involve intracellular Ca²⁺ movements, whereas signaling through the lyso-PA receptor is usually accompanied by this response (26).

In summary, the current results demonstrate that DGPP is a potent and specific extracellular mediator and provide keys to understanding the biological significance of this novel phospholipid compound. The studies reported here provide the foundation for future molecular studies directed toward understanding the intriguing biological role of DGPP.

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⁴ J. Chun, personal communication.

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