Regulation of Phosphatidylinositol Breakdown and Leukotriene Synthesis by Endogenous Prostaglandins in Resident Mouse Peritoneal Macrophages*

Paul D. Wightman‡ and Aimee Dallob
From the Department of Biochemistry and Molecular Biology, Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065

Mouse peritoneal macrophages synthesize large amounts of prostaglandins and leukotrienes in response to certain inflammatory stimuli. Lipopolysaccharide and phorbol esters stimulate prostaglandin formation but not leukotriene synthesis. Zymosan and the calcium ionophore, A23187, stimulate the formation of both prostaglandins and leukotrienes, as well as the phospholipase C-catalyzed breakdown of phosphoinositides. We have examined the interrelationships among phosphoinositide breakdown and prostaglandin and leukotriene synthesis in resident mouse peritoneal macrophages. We demonstrate that macrophages synthesize basally prostaglandin (PG)E₂ and PGI₂ and that these products begin to accumulate from the time of initial plating of the macrophages. The presence of these prostaglandins imparts a downward modulation of zymosan-stimulated phosphoinositide breakdown and, as a result, a downward modulation on leukotriene formation. Inhibition of the basal release of prostaglandin by indomethacin resulted in enhanced zymosan-stimulated phosphoinositide breakdown and an exactly corresponding enhancement of leukotriene release. This enhancement, resulting from the inclusion of indomethacin at the time of plating, was reversed by also including PGE₂, PGI₂, or dibutyryl cAMP. Dibutyryl cAMP, when added in the presence of zymosan and in the absence of indomethacin treatment, inhibited phosphoinositide breakdown and leukotriene synthesis in a parallel fashion, with no effect on prostaglandin release. These data demonstrate that phospholipase C activation is regulated in part by prostaglandin tone and that leukotriene synthesis, unlike prostaglandin synthesis, is dependent on phosphoinositide breakdown.

Mouse resident peritoneal macrophages when treated with zymosan exhibit a multicomponent response which includes the activation of phospholipase C and the breakdown of phosphoinositides (1, 2), the generation of free arachidonic acid, and the synthesis of leukotriene C, and prostaglandins E₂ and I₂ (3-5). Macrophages can be stimulated with agents such as zymosan (4, 6) and the Ca²⁺ ionophore, A23187 (6, 7). Other inflammatory agents, such as charide and phorbol esters stimulate prostaglandin formation but not leukotriene synthesis. Zymosan and the calcium ionophore, A23187 (6,7). Other inflammatory agents, such as calf serum, 10 units/ml heparin, 100 pg/ml streptomycin, and 100 units/ml penicillin. Two ml of lavage fluid (2 × 10⁶ cells) were added to each 16-mm well in a 24-well multidish and incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO₂ in air. Each well was then washed with four volumes of phosphate-buffered saline to remove non-adhered cells, and the medium was replaced with 1 ml of fresh medium devoid of heparin. The cells were then incubated overnight as described above. This protocol for cell culture routinely produced a 2- to 4-fold increase in intracellular cAMP levels compared to unstimulated cells.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: 3M RIKER, 3M Health Care Group, 270-28-06 3M Center, St. Paul, MN 55144.


demonstrated that such elevations in cAMP can result in the inhibition of phosphoinositide breakdown in platelets (14) and neutrophils (15). We have undertaken the present study to examine the interrelationships among phosphoinositide breakdown, leukotriene C₄ and prostaglandin E₂ synthesis, and alterations in cAMP in mouse peritoneal macrophages stimulated with zymosan. We report that leukotriene C₄ synthesis is dependent on phospholipase C-mediated phosphoinositide breakdown and that these two events are down-regulated by prostaglandins. The prostaglandins which impart this down-regulation are synthesized basally and accumulate during the period between the time the macrophages are first placed in culture on day 1 and are stimulated with zymosan on day 2.

EXPERIMENTAL PROCEDURES

Materials

Female CFW mice were purchased from Charles River Breeding Laboratories, Wilmington, MA. Serum and culture media were purchased from Gibco. Tissue culture multwell plates were from Costar, Cambridge, MA. Zymosan was purchased from ICN Biochemicals, Cleveland, OH. Islet activating protein was from List Biologicals, Campbell, CA. (2-³H)Myo-inositol and ³²PO₄ were obtained from Amersham Corp. [³H]PGE₂ and [³H]LTC₄ were from Du Pont-New England Nuclear. LTC₄ and LTC₄ antisera were from our laboratories at Merck Frosst, Montreal. PGE₂ antisera was purchased from Miles Research Products, Elkhart, IN. Cytochalasin D, cycloheximide, PGE₂, PGI₂, and dibutyryl cAMP were all obtained from Sigma.

Methods

Cell Culture—Resident peritoneal macrophages were obtained by peritoneal lavage with 5 ml of cold medium containing 1% fetal calf serum, 10 units/ml heparin, 100 µg/ml streptomycin, and 100 units/ml penicillin. Two ml of lavage fluid (2 × 10⁶ cells) were added to each 16-mm well in a 24-well multidish and incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO₂ in air. Each well was then washed with four volumes of phosphate-buffered saline to remove non-adhered cells, and the medium was replaced with 1 ml of fresh medium devoid of heparin. The cells were then incubated overnight as described above. This protocol for cell culture routinely produced a 2- to 4-fold increase in intracellular cAMP levels compared to unstimulated cells.

The abbreviations used are: PGE₂, prostaglandin E₂; LTC₄, leukotriene C₄; PI, phosphatidylinositol; EGTA, (ethylenbis(oxyethylenenitrilo))tetraacetic acid.

9176
resulted in approximately 2.5 x 10^6 cells and 25 μg of cellular protein/16-mm well. In some experiments indomethacin, dbcAMP, PGE_2, or PGI_2 were added to the plates prior to addition of the cells and included throughout the overnight incubation.

**Phospholipid Metabolism Studies**—Following their overnight incubation as described above, the macrophages were washed with four volumes of phosphate-buffered saline, and the medium was replaced with 1 ml of Puck's saline, formulation A, containing 50 μCi/ml ^32^PO_4_. The cells were preincubated for 1 h and then challenged with 100 μg of zymosan. At the times indicated, the medium was quickly removed and replaced with 1 ml of 0.1 N HCl, the cells broken, and quantitatively removed from the well surface by mild sonication. These sonicates were then transferred to 13 x 100-mm test tubes and the lipids extracted by the method of Bligh and Dyer (16). The lipids were separated by thin layer chromatography as described (17), identified by their comigration with internal authentic standards, scraped, and then quantified by liquid scintillation counting.

**Monitoring of Phosphoinositide Breakdown and Eicosanoid Synthesis**—Macrophages prepared as described were labeled with 5 μCi/ml [^3^H]myo-inositol which was included in the culture medium during the overnight period of incubation. The following morning, the medium was removed, the cells washed with four volumes of phosphate-buffered saline, and the medium was replaced with 1 ml of Puck's saline A containing 10 mM LiCl. Following the addition of zymosan, the cells were incubated for the times indicated in individual experiments under those conditions described. The medium was then removed and saved for quantification of eicosanoids. The cells were acidified with 0.1 N HCl, and the organic and aqueous components were separated as described above. Inositol phosphates were separated and quantified in the aqueous-methanol phase by the use of Dowex/formate as described (18).

Analysis of eicosanoids by radioimmunoassay utilized specific antisera for PGE_2, PGI_2, and LTC_4 (18) as described by Humes (19).

**RESULTS**

**Effects of Zymosan on Phosphoinositide Metabolism**—Resident mouse peritoneal macrophages were prelabeled with ^32^PO_4_, incubated for various times with zymosan, and the effects on phospholipid metabolism were determined. As shown in Fig. 1, the addition of zymosan produces rapid changes in the components of the phosphoinositide cycle. At the earliest time measured, both phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate levels are increased, indicative of the sequential phosphatidylase action of PI and phosphatidylinositol 4-phosphatase. Subsequently, phosphatidylinositol 4,5-bisphosphate is decreased as it is degraded to form diacylglycerol and inositol 1,4,5-trisphosphate. This formation of diacylglycerol is indicated with the generation of its phosphorylated product, phosphatidic acid, which accumulates in a linear fashion throughout the 15-min incubation period. The subsequent conversion of phosphatidic acid to PI is demonstrated by the linear increase in PI following a brief lag period. That these early perturbations are unique to the PI cycle are shown by the lack of change in phosphatidylcholine as compared with the control cultures.

Some of the requirements for the zymosan induction of the phosphoinositide breakdown in mouse macrophages were studied. Incorporation of ^32^PO_4_ into phosphatidic acid was used as a measure of PI breakdown. These studies (data not shown) demonstrate that extracellular Ca^{2+} is not required for phosphoinositide breakdown since the replacement of Ca^{2+} with 1 mM EGTA in the medium did not significantly inhibit phosphatidic acid formation. This finding is consistent with that reported by Moscat et al. (20). These authors have also reported (20) that a guanine nucleotide-binding protein may be involved in zymosan-induced phosphoinositide breakdown. Our data suggests that this G-protein is not sensitive to pertussis toxin since a 4-h preincubation of macrophages with 200 ng/ml atlet activating protein did not inhibit zymosan-induced phosphoinositide breakdown. Rouzer et al. (21) have demonstrated that zymosan-induced release of eicosanoids from macrophages is not dependent on phagocytosis of the zymosan granule. We demonstrate that the same is true for zymosan-induced phosphoinositide breakdown since inhibition of phagocytosis with 2 μg/ml cytochalasin D did not inhibit this response. Bonney et al. (8) have reported that zymosan-induced eicosanoid release in macrophages requires new protein synthesis and is inhibited by preincubation of the cells with cycloheximide. Our data show that new protein synthesis is not required for phosphoinositide breakdown since preincubation of the cells with 2 μg/ml cycloheximide for 2 h had no effect on this response. Clearly, the requirement for new protein synthesis in zymosan-induced eicosanoid release is distal to phospholipase C activation.

**Coordinate Measurement of Zymosan-induced Phosphoinositide Breakdown and Eicosanoid Synthesis**—The breakdown of phosphoinositides and the synthesis of LTB_4 and PGE_2 by macrophages stimulated with zymosan were each measured in the same culture. The cellular phosphoinositides were labeled overnight with [^3^H]myo-inositol, and their breakdown was monitored in the cells by measuring inositol phosphates. The extracellular release of the eicosanoids was measured in the medium by radioimmunoassay as described under "Experimental Procedures."

The concentration dependence for zymosan cell stimulation was studied in the experiment depicted in Fig. 2. As shown, phospholipase C-mediated phosphoinositide breakdown and the synthesis of PGE_2 and LTC_4 are each induced at similar zymosan concentrations. The time course for these events are shown in Fig. 3. PGE_2 release was maximal by 1 h while LTC_4 release paralleled the breakdown of phosphoinositides and
Regulation of Phosphatidylinositol Breakdown in Mouse Macrophages

9178 Regulation of Phosphatidylinositol Breakdown in Mouse Macrophages

56. 20. 48' YO-

FIG. 2. The concentration dependence for zymosan-stimulated eicosanoid release and phosphoinositide breakdown. [3H]myo-inositol-labeled macrophages were incubated for 1 h in the presence of increasing concentrations of zymosan, as indicated. Total inositol phosphates (O), LTC₄ (●), and PGE₂ (▲) were quantified as stated under "Experimental Procedures," and the data depicted are as described in the legend to Fig. 1.

FIG. 3. The time dependence for zymosan-stimulated eicosanoid release and phosphoinositide breakdown. [3H]myo-inositol-labeled macrophages were incubated with 50 μg/ml zymosan for the times indicated. Total inositol phosphates (O), LTC₄ (●), and PGE₂ (▲). The data depicted are as described in the legend to Fig. 1.

Effects of dbcAMP on PI Breakdown and LTC₄ Synthesis—It has been demonstrated in a number of cell types that classes of prostaglandins can increase cellular cAMP (11–13). Furthermore, it has been demonstrated that elevations in cAMP can result in the inhibition of phosphoinositide breakdown (14, 15). That this may be a mechanism by which the basal release of PGE₂ and PGJ₂ by macrophages imparts an inhibitory tone to zymosan-stimulated phosphoinositide breakdown and, as a result, LTC₄ synthesis was investigated. Dibutylryl cAMP (50 μM) was added in the presence and absence of 0.1 μM indomethacin to macrophage cultures at the time of plating and incubated overnight at 37 °C. As shown in Fig. 7, dbcAMP completely reverses the indomethacin effect on both phosphoinositide breakdown and LTC₄ synthesis. Furthermore, dbcAMP shows significant inhibition of these two parameters in the absence of indomethacin. In a related experiment, dbcAMP was added to macrophage cultures at the same time as the addition of zymosan on the second day of culture. As shown in Fig. 8, dbcAMP partially inhibited both phosphoinositide breakdown and LTC₄ synthesis in a concentration-dependent fashion while the synthesis of PGE₂ was unaffected. Furthermore, the concentrations

Basal Release of PGE₂ and PGJ₂ by Mouse Macrophages—Mouse peritoneal macrophages are normally obtained by lavage of the peritoneal cavity and then placed in tissue culture wells to adhere the cells for a period of 2 h. The normal protocol used by most investigators (1–3, 19) includes an overnight incubation for stabilizing the cells before being used for experiments the following morning. We have measured the accumulation of prostaglandins and leukotrienes during this period of incubation. LTC₄ is not released basally (data not shown) but PGE₂ and PGJ₂ accumulate to significant quantities which increase in a linear fashion for at least 8 h (Fig. 4). Resident peritoneal macrophages when stimulated with zymosan on the second day of culture synthesize, primarily, PGE₂ and lesser amounts of PGJ₂. However, PGJ₂ is synthesized earlier and in larger quantity under basal conditions on the first day of culture than is PGE₂.

The effects of this basal release of prostaglandins on the zymosan-stimulated breakdown of phosphoinositides and synthesis of LTC₄ was studied by measuring these two latter events in macrophages whose basal release of prostaglandins was inhibited with indomethacin. As shown in Fig. 5, the dose-dependent inhibition of basal PGE₂ results in a marked increase in the zymosan-stimulated breakdown of phosphoinositides and release of LTC₄. The enhancement of phosphoinositide breakdown and LTC₄ synthesis in indomethacin-treated macrophages can be completely reversed with the addition of either PGE₂ or PGJ₂ (10⁻⁷ M) to the overnight incubation (Fig. 6).

Effects of dbcAMP on PI Breakdown and LTC₄ Synthesis—It has been demonstrated in a number of cell types that classes of prostaglandins can increase cellular cAMP (11–13). Furthermore, it has been demonstrated that elevations in cAMP can result in the inhibition of phosphoinositide breakdown (14, 15). That this may be a mechanism by which the basal release of PGE₂ and PGJ₂ by macrophages imparts an inhibitory tone to zymosan-stimulated phosphoinositide breakdown and, as a result, LTC₄ synthesis was investigated. Dibutylryl cAMP (50 μM) was added in the presence and absence of 0.1 μM indomethacin to macrophage cultures at the time of plating and incubated overnight at 37 °C. As shown in Fig. 7, dbcAMP completely reverses the indomethacin effect on both phosphoinositide breakdown and LTC₄ synthesis. Furthermore, dbcAMP shows significant inhibition of these two parameters in the absence of indomethacin. In a related experiment, dbcAMP was added to macrophage cultures at the same time as the addition of zymosan on the second day of culture. As shown in Fig. 8, dbcAMP partially inhibited both phosphoinositide breakdown and LTC₄ synthesis in a concentration-dependent fashion while the synthesis of PGE₂ was unaffected. Furthermore, the concentrations...
Regulation of Phosphatidylinositol Breakdown in Mouse Macrophages

The effects of basal prostaglandin release on zymosan-stimulated phosphoinositide breakdown and LTC₄ synthesis. Macrophages were obtained by lavage, adhered for 2 h, and then incubated overnight in the presence of [³H]myo-inositol and indomethacin at the concentrations indicated. The following morning, the media were removed and PGE₂ (●) was quantified therein by radioimmunoassay. The macrophages were washed, the medium replaced, and the cells were then incubated with 50 µg/ml zymosan for 1 h. Zymosan-stimulated LTC₄ (●) and total inositol phosphates (△) were quantified. The data depicted are as described in the legend to Fig. 1.

FIG. 5. The effects of basal prostaglandin release on zymosan-stimulated phosphoinositide breakdown and LTC₄ synthesis.

Reversal of indomethacin effects on LTC₄ synthesis and phosphoinositide breakdown by prostaglandin. Macrophages were prepared as described in the legend to Fig. 6 and incubated overnight in the presence (right panel) and absence (left panel) of 10⁻⁶ M indomethacin and PGE₂ or PGI₂ (10⁻⁷ M) as indicated. The following morning the media were removed, the macrophages were washed, the medium replaced, and the cells were incubated with 50 µg/ml zymosan for 1 h. Zymosan-stimulated LTC₄ (●) and total inositol phosphates (△) were quantified. The data depicted are as described in the legend to Fig. 1.

FIG. 6. Reversal of indomethacin effects on LTC₄ synthesis and phosphoinositide breakdown by prostaglandin.

FIG. 7. Reversal of indomethacin effects on phosphoinositide breakdown and LTC₄ synthesis by dibutyryl cyclic AMP.

The protocol for this experiment was the same as described in the legend for Fig. 7 except that dbcAMP was used at 5 x 10⁻⁴ M instead of prostaglandins. Zymosan-stimulated LTC₄ (●) and total inositol phosphates (△). The data depicted are as described in the legend to Fig. 1.

FIG. 8. Selective inhibition of phosphoinositide breakdown and LTC₄ synthesis by dibutyryl cyclic AMP.

of dbcAMP at which half-maximal inhibition occurs is virtually identical for both phosphoinositide breakdown and LTC₄ synthesis. These data suggest that LTC₄ synthesis, unlike PGE₂ synthesis, is dependent on phospholipase C-mediated phosphoinositide breakdown. Indeed, this is consistent with earlier reports that substances such as phorbol myristate acetate and lipopolysaccharide, which do not stimulate PI breakdown in macrophages, induce the synthesis of PGE₂ but not LTC₄ (6). Efforts to measure immediate elevation of cAMP in macrophages treated with PGE₂ or PGI₂ have not been successful. Similarly, we have not been able to demonstrate inhibition of phosphoinositide breakdown and LTC₄ synthesis with the short term addition of these prostaglandins. Clearly, however, the long term incubation of macrophages in the presence of prostaglandins, whether secreted basally or added exogenously, is inhibitory to these two processes. That the prostaglandin effect is in fact mediated by cAMP remains to be established.

DISCUSSION

Much attention has been given to the respective roles of phospholipase C and phospholipase A₂ in the response of macrophages to inflammatory stimuli. Moscat et al. (22) have measured the release of arachidonic acid and the generation of diacylglycerol or inositol phosphates in zymosan-stimulated mouse peritoneal macrophages. These authors conclude that phospholipase C and diacylglycerol lipase act in concert to release free arachidonic acid. This conclusion is based in part on their inability to detect phospholipase A₂ in cell-free assays. Emilsson et al. (1, 10, 23) have monitored the release...
of free arachidonic acid, the phospholipase A₂-catalyzed generation of lysophosphatidylinositol and the phospholipase C-catalyzed production of inositol phosphates in mouse peritoneal macrophages stimulated with a variety of agents. These agents include zymosan, phorbol esters, Ca²⁺ ionophores, and diacylglycerols. These authors conclude that the major pathway for arachidonic acid release in macrophages is via deacylation of phospholipids by phospholipase A₂. However, neither of these investigators have distinguished between the extracellular release of free arachidonic acid and either of the major oxygenated products of arachidonic acid, PGE₂ and LTC₄, in the macrophage. The relevance of that pool of arachidonic acid released extracellularly to that converted intracellularly to its oxygenated products has not been addressed. Indeed, we have observed in resident mouse peritoneal macrophages and the macrophage cell lines P388D₁ and RAW264.7 that the inclusion of albumin in the culture media can increase the stimulus-dependent extracellular accumulation of arachidonic acid as much as 10-fold with no effect on the amounts of prostanooids released.²

In the present study we have made simultaneous measurements of phospholipase C-catalyzed phosphoinositide breakdown and synthesis of LTC₄ and PGE₂ from intracellular arachidonic acid in macrophages stimulated with zymosan. We show that modulation of phosphoinositide breakdown results in a coordinate modulation of LTC₄ release. As zymosan-stimulated phosphoinositide breakdown is enhanced by inhibition of basal prostaglandin release, so is zymosan-stimulated LTC₄ synthesis (Figs. 5–7). In addition, the inhibition of zymosan-stimulated phosphoinositide breakdown by dbcAMP results in the inhibition of LTC₄ synthesis but not PGE₂ synthesis (Fig. 8). These data demonstrate that LTC₄ synthesis, unlike PGE₂ synthesis, is dependent on phospholipase C-catalyzed PI breakdown and that the independent regulation of these biosynthetic pathways (6) may be attributed to this fact. Phosphoinositide breakdown may regulate LTC₄ synthesis by either providing substrate from arachidonic acid-rich diacylglycerol or by the formation of two important second messengers, inositol 1,4,5-triphosphate, and diacylglycerol. Inositol 1,4,5 triphosphate can mobilize intracellular Ca²⁺ needed for the activation of either phospholipase A₂ or 5-lipoxygenase, and diacylglycerol can activate protein kinase C which may in turn regulate other phospholipases.

While it remains difficult to attribute the relative roles of phospholipases C and phospholipases A₂ to the synthesis of eicosanoids by macrophages, it is clear that both lipolytic pathways are operative in the macrophage. Prostaglandins can be synthesized independent of phospholipase C activation while leukotriene synthesis is wholly dependent on the activation of this enzyme.

REFERENCES


* P. D. Wightman and A. Dallob, unpublished data.
Regulation of phosphatidylinositol breakdown and leukotriene synthesis by endogenous prostaglandins in resident mouse peritoneal macrophages.

P D Wightman and A Dallob


Access the most updated version of this article at [http://www.jbc.org/content/265/16/9176](http://www.jbc.org/content/265/16/9176)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/265/16/9176.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/16/9176.full.html#ref-list-1](http://www.jbc.org/content/265/16/9176.full.html#ref-list-1)