Leukotriene B₄ Activates the NADPH Oxidase in Eosinophils by a Pertussis Toxin-Sensitive Mechanism That Is Largely Independent of Arachidonic Acid Mobilization

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Leukotriene B₄ Activates the NADPH Oxidase in Eosinophils by a Pertussis Toxin-Sensitive Mechanism That Is Largely Independent of Arachidonic Acid Mobilization

Mark A. Lindsay, Rosie S. Perkins, Peter J. Barnes, and Mark A. Giembycz

Experiments were designed to investigate whether leukotriene (LTB₄) receptors can couple directly to phospholipase A₂ (PLA₂) in guinea pig eosinophils and the role of endogenous arachidonic acid (AA) in LTB₄-induced activation of the NADPH oxidase. LTB₄ (EC₅₀ ~ 16 nM) and AA (EC₅₀ ~ 6 μM) generated hydrogen peroxide (H₂O₂) in a concentration-dependent manner and at an equivalent maximum rate (5–6 nmol/min/10⁶ cells). LTB₄ stimulated PLA₂ over a similar concentration range that activated the NADPH oxidase, although kinetic studies revealed that the release of [³H]AA (t½ < 2 s) preceded H₂O₂ generation (t½ > 30 s). Pretreatment of eosinophils with pertussis toxin abolished the increase in inositol(1,4,5)trisphosphate mass, [Ca²⁺]ₜ, [³H]AA release, and H₂O₂ generation evoked by LTB₄. Qualitatively identical results were obtained in eosinophils in which phospholipase C (PLC) was desensitized by 4β-phorbol 12,13-dibutyrate with the exception that [³H]AA release was largely unaffected. Additional studies performed with the protein kinase C inhibitor, Ro 31-8220, and under conditions in which Ca²⁺ mobilization was abolished, provided further evidence that LTB₄ released [³H]AA independently of signal molecules derived from the hydrolysis of phosphatidylinositol(4,5)bisphosphate by PLC. Pretreatment of eosinophils with the PLA₂ inhibitor, mepacrine, abolished LTB₄-induced [³H]AA release at a concentration that inhibited H₂O₂ by only 36%. Collectively, the results of this study indicate that agonism of LTB₄ receptors on guinea pig eosinophils mobilizes AA by a mechanism that does not involve the activation of PLC. In addition, although LTB₄ effectively stimulated PLA₂, a central role for AA in the activation of the NADPH oxidase was excluded. The Journal of Immunology, 1998, 160: 4526–4534.

Eosinophils have been described as both tissue-preserving and tissue-damaging cells by virtue of their cytotoxicity toward invading parasites and, under certain circumstances, normal healthy tissue. The destructive capability of eosinophils resides in their ability to undergo a massive increase in O₂ consumption in response to a diverse range of stimuli, culminating in the generation of highly toxic free radicals. This metabolic response is catalyzed by the NADPH-O₂ oxidoreductase (E.C. 1.23.45.3), a multicomponent enzyme complex that catalyzes the single electron reduction of molecular O₂ to superoxide (O₂⁻), a powerful oxidizing and reducing agent (1). In the presence of superoxide dismutase, eosinophil peroxidase, and halogen (normally bromide, 2, 3), eosinophilderived 'O₂⁻ ultimately can be converted into hypobromous acid, which is known to promote the cytolysis of pneumocytes and epithelial cells (4, 5) and to release histamine from mast cells (6).

We and others have reported previously that LTB₄ promotes a robust, receptor-mediated activation of the NADPH oxidase in guinea pig eosinophils (7, 8). Biochemical and pharmacologic studies designed to elucidate the signaling pathway(s) responsible for this effect suggest that Ca²⁺ does not play an important role. Thus, although LTB₄ evokes a rapid and transient increase in [Ca²⁺]ₜ and Ins(1,4,5)P₃ mass in guinea pig eosinophils (7, 9, 10), this is evident only at concentrations far in excess of those required to activate the NADPH oxidase (7). Moreover, pretreatment of eosinophils with EGTA or (1,2-bis(o-aminophenoxy)ethane-N, N', N'', N'''-tetraacetic acid)acyetox methyl ester, at concentrations that abolish Ca²⁺ mobilization, fail to reduce the rate or amount of H₂O₂ produced in response to a maximally effective concentration of LTB₄ (7). Similarly, SK&F 96365 and its parent compound, SC 32849, which are putative inhibitors of receptor-operated Ca²⁺ entry, block LTB₄-induced Ca²⁺ mobilization without affecting 'O₂⁻ generation (8).

In exploring alternative activators of oxidative metabolism, we initially assessed the role of PLD, PKC, and PtdIns 3-kinase, which have been implicated in oxidant production from human neutrophils in response to a variety of G protein receptor-coupled agonists including LTB₄ (11–19). Studies with wortmannin have also suggested that PtdIns 3-kinase-dependent processes are recruited in human eosinophils for the activation of the NADPH oxidase (20). However, despite these data, PLD- and PtdIns 3-kinase-driven mechanisms do not appear to be involved in LTB₄-induced H₂O₂ generation from guinea pig eosinophils (7), and an inhibitor of PKC, Ro 31-8220, suggests that PKC plays only a relatively minor role (7).
Collectively, these results suggest that an alternative signaling pathway(s) controls the assembly and subsequent activation of the NADPH oxidase in guinea pig eosinophils. One possibility is that arachidonic acid (AA) plays a central role. Persuasive evidence for this proposal derives from several sources including the general finding that AA and other free fatty acids promote the formation of free radicals from intact and electrophoretically phagocytoses, as well as in a cell-free system (21–31). The mechanism of action of AA is still unknown, but it has been shown to exert a number of intracellular effects relevant to the activation of the NADPH oxidase. In particular, AA interacts synergistically with GTPγS in promoting the association of the small GTP-binding protein, p21ras, with a membrane fraction prepared from differentiated HL-60 cells (32).

A role for AA in the activation of the NADPH oxidase is also supported by pharmacologic experiments in which inhibitors of PLA2 have been shown to suppress O2 generation (33–35). In addition, there is now compelling evidence that PLA2 is regulated by distinct G proteins (36–43), denoted as Ga (36), suggesting that certain receptors can couple directly to PLA2 and signal the release of AA in a manner that is not dependent upon second messenger molecules derived from the hydrolysis of PtdIns(4,5)P2 by PLC (37, 42, 44).

In this article, we report the results of studies designed to investigate whether LTB4 receptors can couple directly to PLA2 in guinea pig eosinophils and the role of endogenous AA in LTB4-induced activation of the NADPH oxidase.

A preliminary account of some of these data was presented to the American Thoracic Society/American Lung Association (45).

Materials and Methods

**Drugs and analytical reagents**

The following drugs and analytical reagents were used. 4α- and 4β-PDBu were purchased from Scientific Marketing Associates (Barnet, Kent, U.K.); Percoll was from Pharmacia (Uppsala, Sweden); and HBSS was from Flow Laboratories (Rickmansworth, Hertfordshire, U.K.). Fura-2-AM (pentapotassium salt), Ro 31-8220, and ionomycin were obtained from Calbiochem (Nottingham, U.K.), and n-myo Ins(1,4,5)P3 (hexadecosumid salt) was from Semat Technical (St. Albans, Hertfordshire, U.K.). D-[inositol,1-3H(3H)]Ins(1,4,5)P3 (21 Ci/mmol) and [5,6,8,9,11,12,14,15(3H)JA (74 Ci/mmol) were from NEN/DuPont (Stevenage, Hertfordshire, U.K.). LTB4 and ZM 230,487 were generously donated by Bayer (Stoke Poges, Slough, U.K.) and Zeneca (Macclesfield, Cheshire, U.K.), respectively. Fluoroprofen, pertussis toxin (PTX), unlabelled AA, and all other drugs and reagents were purchased from Sigma Chemical (Poole, Dorset, U.K.).

**Induction, harvesting, and purification of eosinophils**

Eosinophils were elicited into the peritoneum of male Dunkin-Hartley guinea pigs (~1 kg) by weekly i.p. injection of human serum (1 ml/animal), obtained as a by-product of human granulocyte isolation. This procedure led to the production of eosinophil/macrophage-rich peritoneal exudates, essentially devoid of neutrophils and platelets, within 2 to 6 wk.

Three to six days after plasma injection, guinea pigs were anesthetized with 0.1% BSA (w/v) and incubated for 5 min with 4α-PDBu or 4β-PDBu. Hydrogen peroxide generation was measured fluorimetrically (\( \lambda_{\text{excitation}} = 350 \text{ nm} \); \( \lambda_{\text{emission}} = 460 \text{ nm} \); slit width = 5 nm) using a thermostatically controlled spectrofluorimeter fitted with a Peltier stirrer. Changes in fluorescence were monitored continuously for 5 to 20 min, and negative first derivative plots of the reduction in fluorescence were constructed to obtain the peak rates of scopoletin extinction. These values were converted to rates of H2O2 generation, which preliminary experimentation identified as being the most reproducible measure of oxidase activity, and quantified by interpolation from a standard curve constructed to known concentrations of H2O2.

**Measurement of \([\text{Ca}^{2+}]_{i}\)**

Eosinophils were suspended at 230 × 10^6/ml in buffer B and stored on ice until required. Assays, performed in duplicate, were conducted at 37°C in a total volume of 300 µl and were initiated by the addition of 30 µl (7 × 10^6 cells) of eosinophil suspension to 240 µl of prewarmed buffer B. The cell suspensions were incubated for 5 min, after which LTB4 (30 µM) was added. Reactions were terminated by the addition of 300 µl TCA (1 M), and Ins(1,4,5)P3 mass was subsequently extracted (49) and measured using a competitive protein-binding assay (50). The detection limit and IC50 of these assays are <0.4 and 1 pmol Ins(1,4,5)P3, respectively.

In previous studies (7, 51), we established that exposure of guinea pig eosinophils to LTB4 (1 µM) produces a transient increase in \([\text{Ins(1,4,5)P3}]_{i}\) and mass in the \([\text{Ca}^{2+}]_{i}\); for both indices of activation, peak responses occur 5 s after the stimulus and then decay rapidly to basal or near basal levels (7, 51). Thus, in the experiments performed herein, measurements were made at the 5-s time point.

**Measurement of \([\text{Ca}^{2+}]_{i}\)**

Eosinophils (10^7/ml) were suspended at 37°C in buffer C supplemented with 0.1% BSA (w/v) and incubated for 30 min with fura-2-AM (1 µM). The cells were washed three times in buffer B, and the ability of LTB4 to elevate \([\text{Ca}^{2+}]_{i}\) was determined using well-established spectrofluorimetric methods (\( \lambda_{\text{excitation}} = 340/380 \text{ nm} \); \( \lambda_{\text{emission}} = 510 \text{ nm} \); slit width = 4 nm).

**Measurement of \([\text{H}^{1}]\AA\)**

Agonist-induced release of \([\text{H}^{1}]\AA\) from eosinophils was performed using a modification of the method detailed in Cockcroft and Stuche (44).

Eosinophils (10^7/ml) were prelabeled with \([\text{H}^{1}]\AA\) (1 µCi/ml) for 240 min at 37°C and subsequently incubated at a concentration of 3 × 10^6/ml. Aliquots (100 µl) of eosinophils were transferred to Eppendorf tubes containing 80 µl buffer C (with or without Ca2+ / Mg2+ and inhibitors) and incubated for 5 min at 37°C before the addition of stimulants. Samples were incubated for a further 10 min at 37°C and the reaction subsequently terminated by the addition of 500 µl of ice-cold NaCl (0.9% w/v). Eosinophils were sedimented by centrifugation...
(12,000 × g for 5 min) and an aliquot of the supernatant counted in 2 ml ACS II (Amersham). In the text, the term [3H]AA refers to all tritiated species released from eosinophils.

Initial experiments revealed that the uptake of [3H]AA by guinea pig eosinophils occurred in a time-dependent manner (t_{1/2} = 11.5 ± 2.6 min, n = 3), reached equilibrium at approximately 15 min, and remained constant for 120 min. In a study by Debbagh and colleagues (52), analysis of radiolabeled species released from agonist-stimulated eosinophils revealed that they consisted almost entirely of free fatty acids, indicating that this method represents a satisfactory index of AA release and that it is formed following the activation of PLA2 rather than DAG lipase (44, 52).

Statistical analysis

Data in the text, tables, and figure legends refer to the mean ± SEM of “n” independent determinations taken from different cell preparations. Where appropriate, Student’s t test (two-tailed) or the one-way analysis of variance/Newman-Keuls test was used to assess significance between “control” and “treatment” groups. The null hypothesis was rejected when p < 0.05.

Results

Effect of LTβ4 and AA on H2O2 generation

LTB4 evoked a rapid, transient, and concentration-dependent generation of H2O2 from eosinophils with an EC_{50} of 16.2 ± 3.6 nM and a maximal rate of production of 5.51 ± 0.91 nmol/min/10^6 cells (Fig. 1). Kinetically, H2O2 was detected after a lag of approximately 5 s in eosinophils exposed to a maximally effective concentration of LTB4 (1 μM) and increased steadily for a minimum of 90 s; the estimated t_{1/2} was ~30 s (Fig. 2). Under identical experimental conditions, exogenous AA generated H2O2 from eosinophils in a comparable rate (6.15 ± 0.75 nmol/min/10^6 cells at 30 μM) to LTB4 but, on a molar basis, was approximately 340 times less potent (EC_{50} = 71.6 ± 15.2 nM; Fig. 1) and resulted, maximally, in a 2.7-fold increase in the amount of H2O2 released (2.46 ± 0.13% of the total radioactivity incorporated after 10 min incubation at 37°C). In contrast, the elaboration of [3H]AA from eosinophils exposed to LTB4 was significantly augmented under identical experimental conditions (Fig. 2). This effect was very rapid (t_{1/2} = 2.2 ± 1.6 s at 1 μM; Fig. 2) and concentration dependent (EC_{50} = 71.6 ± 15.2 nM; Fig. 1) and resulted, maximally, in a 2.7-fold increase in the amount of [3H]AA released (2.46 ± 0.13% of the total radioactivity incorporated). The ability of LTB4 to mobilize [3H]AA from eosinophils was a receptor-mediated phenomenon, as it was abolished by U-75,302 (55), a selective LTB4 receptor antagonist that has been shown previously to block LTB4-induced Ca^{2+} mobilization, Ins(1,4,5)P_{3} accumulation, and H2O2 generation in guinea pig eosinophils (7).

Temporally, the release of [3H]AA from LTB4-challenged eosinophils preceded the generation of H2O2 (Fig. 2). However, the
To LTB4 (1 nM) activation were also measured. Exposure of guinea pig eosinophils AA release was detected in the absence of H2O2 generation, whereas at higher concentrations of LTB4 (10 nM to 3 μM) the concentration-response curve was displaced approximately fourfold to the right of the curve that described the activation of the NADPH oxidase. Although these results are difficult to interpret, they do not exclude the possibility that endogenous AA per se is a direct effector of NADPH oxidase, as reported in other leukocytes (21–30, 33–35), and additional experiments were performed to assess this possibility.

Effect of PTX on LTB4-induced Ins(1,4,5)P3 accumulation, Ca2+ mobilization, [3H]AA release and H2O2 generation

To understand further the way in which LTB4 activates PLA2 and the role of AA in H2O2 generation, eosinophils were pretreated with PTX (500 ng/ml; 120 min; 37°C); ADP ribosylates PTX and thereby inactivates the Go and Gi families of heterotrimeric GTP-binding proteins. The indices of PLA2, PLC, and NADPH oxidase activation were also measured. Exposure of guinea pig eosinophils to LTB4 (1 μM) for 5 s resulted in an approximately sixfold increase in Ins(1,4,5)P3 mass and elevated the [Ca2+]i fivefold, from 82 to 411 nM; both of these responses were absent in cells pretreated with PTX (Figs. 4A and 5A). Similarly, PTX abolished [3H]AA release and H2O2 generation over the entire concentration range (0.1 nM to 3 μM) in which LTB4 was active (Figs. 6A and 7A). The concentration of PTX used and the time of preincubation did not affect cell viability or responsiveness, as evidenced by the robust generation of H2O2 evoked by 1 μM of 4β-PDBu (Fig. 7A).

Effect of PDBu on LTB4-induced Ins(1,4,5)P3 accumulation and Ca2+ mobilization

The finding that PTX abolished the increase in Ins(1,4,5)P3 mass, [Ca2+]i, and [3H]AA prompted us to perform additional experiments in an attempt to dissociate the activation of PLA2 from the generation of signal molecules derived from the hydrolysis of PtdIns(4, 5)P2 by PLC. To this end, eosinophils were pretreated with 4β-PDBu, which uncouples many G protein-linked receptors from PLC-β in a variety of cell types by a PKC-dependent process (56).

Pretreatment of eosinophils with 4β-PDBu (1–100 nM; 5 min; 37°C) inhibited LTB4 (1 μM)-induced Ins(1,4,5)P3 accumulation and Ca2+ mobilization in a concentration-dependent manner (IC50 ~ 35 and 70 nM, respectively) under conditions in which 4α-PDBu (100 nM; 5 min; 37°C) was inactive (Figs. 4B and 5B). Signaling through PLC was abolished in cells pretreated with 100 nM of 4β-PDBu for 5 min at 37°C and these conditions were used in all further experiments.

Effect of PDBu on LTB4-induced [3H]AA release

Eosinophils in which PLC was desensitized demonstrated a significant (3.7-fold) increase in the basal elaboration of [3H]AA (2.9 ± 0.17% of total radiolabel incorporated) over the amount released spontaneously (0.78 ± 0.18%; Fig. 6B). Also, the addition of LTB4 to 4β-PDBu-treated cells further increased the release of [3H]AA (to 3.94 ± 0.21% of the total incorporated radioactivity at the maximally effective concentration) indicating that LTB4 receptors can stimulate PLA2 by a PLC-independent mechanism (Fig. 6B). This effect was concentration dependent with an EC50 (72.3 ± 13.2 nM) not significantly different from that obtained in eosinophils treated with vehicle (see above; Fig. 6B). The maximum change in [3H]AA release from “control” cells (1.6%) was routinely greater (~30%) than in eosinophils in which PLC was desensitized (1.1%). This difference was not due to depletion of “releasable” radiolabel, since a combination of 4β-PDBu and ionomycin (100 nM each) liberated 8.9 ± 1.3% (n = 3) of the total [3H]AA incorporated into membrane lipids.

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Table I. Lack of effect of flurbiprofen and ZM 230,487 on LTB4- and AA-induced H2O2 generation from guinea pig eosinophils

<table>
<thead>
<tr>
<th></th>
<th>Rate H2O2 Generation (nmol/min/10^6 cells)</th>
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<tbody>
<tr>
<td></td>
<td>LTB4 (1 μM)</td>
</tr>
<tr>
<td></td>
<td>Control (nmol/min/10^6 cells)</td>
</tr>
<tr>
<td></td>
<td>Control (nmol/min/10^6 cells)</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Flurbiprofen (3 μM)</td>
<td>4.16 ± 0.26</td>
</tr>
<tr>
<td>ZM 230,487 (50 μM)</td>
<td>3.99 ± 0.37</td>
</tr>
</tbody>
</table>

*Eosinophils were pretreated with flurbiprofen or ZM 230,487 for 10 min before being exposed to LTB4 (1 μM) or AA (10 μM). The generation of H2O2 by the cells was then measured spectrophotometrically by monitoring the reduction in fluorescence of scopoletin from which peak rates of H2O2 generation were calculated. Data represent the mean ± SEM of four independent determinations performed with different cell preparations.

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FIGURE 4. Effect of PTX and PDBu on LTB4-induced Ins(1,4,5)P3 accumulation. Eosinophils (10^6/ml) were treated with or without PTX (500 ng/ml; 120 min; 37°C; A), 4β-PDBu (1 to 100 nM; 5 min; 37°C; B), or 4α-PDBu (100 nM; 5 min; 37°C; B), and the ability of a maximally effective concentration (1 μM) of LTB4 to increase Ins(1,4,5)P3 mass was determined. Data points and bar charts represent the mean ± SEM of four experiments performed with different cell preparations. See Materials and Methods for further details. * p < 0.001; significant inhibition of LTB4-induced Ins(1,4,5)P3 accumulation.
Effect of PDBu on LTβ-r-induced H2O2 generation

Experiments were performed in eosinophils in which PLC was desensitized to determine the extent to which activation of NADPH oxidase was dependent upon signaling through PLA2. As shown in Figure 7B, LTβ-r failed to augment the rate of H2O2 production in eosinophils pretreated with 4β-PDBu (100 nM; 5 min; 37°C) at any concentration examined despite the fact that these cells expressed a modest basal rate (1.3 ± 0.32 nmol/min/106 cells) of oxidative metabolism. This low level of H2O2 production was presumably due to the fact that phorbol diesters evoke a robust and prolonged activation of the NADPH oxidase in guinea pig eosinophils (7).

Role of PKC and Ca2+ in LTβ-r-induced [3H]AA release

To provide further evidence that the activation of PLA2 by LTβ-r occurs independently of PLC, the role of DAG-induced PKC activation and Ins(1,4,5)P3-induced Ca2+ mobilization were examined. Pretreatment (5 min) of eosinophils with the PKC inhibitor Ro-31,8220 (1 to 20 μM) had no significant effect on basal or LTβ-r (1 μM)-induced [3H]AA release from guinea pig eosinophils (Fig. 8). Thus, PtdIns(4, 5)P2-derived DAG is not required for LTβ-r-induced [3H]AA release.

Table II and Figure 9 show the results of experiments in which the Ca2+ sensitivity of LTβ-r-induced [3H]AA release was explored. In fura-2-AM-treated eosinophils incubated in Ca2+-free medium, EGTA (100 μM) abolishes the ability of LTβ-r (0.1 nM to 1 μM) to increase the [Ca2+]c. Under these conditions, the release of [3H]AA effected by a maximally effective concentration (1 μM) of LTβ-r to increase the [Ca2+]c in fura-2-AM-loaded eosinophils was determined. Data points and bar charts represent the peak change in [Ca2+]c, and are the mean ± SEM of four experiments performed with different cell preparations. See Materials and Methods for further details.* p < 0.001; significant inhibition of LTβ-r-induced Ca2+ mobilization.

Discussion

Previous studies have established that LTβ-r activates the NADPH oxidase in guinea pig eosinophils by a mechanism that does not involve Ca2+ or the activation of PLD or PtdIns 3-kinase (7, 8). A major role for PKC has also been excluded based on inhibitor studies with the staurosporine analogue, Ro 31-8220 (7). A review of the cellular signaling pathways that regulate oxidative metabolism in neutrophils and macrophages led us to examine the possible second messenger role of AA. This was an attractive target because exogenous AA activates the NADPH oxidase in a variety of cell types, including eosinophils.

FIGURE 5. Effect of PTX and PDBu on LTβ-r-induced Ca2+ mobilization. Eosinophils (107/ml) were treated with or without PTX (500 ng/ml; 120 min; 37°C; A), 4β-PDBu (1 to 100 nM; 5 min; 37°C; B), or 4α-PDBu (100 nM; 5 min; 37°C; B), and the ability of a maximally effective concentration (1 μM) of LTβ-r to increase the [Ca2+]c in fura-2-AM-loaded eosinophils was determined. Data points and bar charts represent the peak change in [Ca2+]c, and are the mean ± SEM of four experiments performed with different cell preparations. See Materials and Methods for further details.* p < 0.001; significant inhibition of LTβ-r-induced Ca2+ mobilization.

FIGURE 6. Effect of PTX and 4β-PDBu on LTβ-r-induced [3H]AA release. Eosinophils (107/ml) were labeled with [3H]AA (1 μCi/ml; 2 h), washed thoroughly, and treated with or without PTX (500 ng/ml; 120 min; 37°C) or 4β-PDBu (1 μM; 5 min; 37°C). The ability LTβ-r to release [3H]AA was then determined. Data points represent the mean ± SEM of four experiments performed with different cell preparations. See Materials and Methods for further details.* p < 0.05; significant stimulation of [3H]AA release in 4β-PDBu-treated eosinophils. ** p < 0.001; significant stimulation of [3H]AA release by 4β-PDBu. NS, no significant difference in [3H]AA release compared with vehicle-treated eosinophils (C).
of phagocytes (21–31), and inhibitors of PLA₂ attenuate agonist-stimulated oxidative metabolism (33–35).

In guinea pig eosinophils, AA affected a robust generation of H₂O₂ of a magnitude equivalent to that produced by LTB₄, and the ability of LTB₄ to generate H₂O₂ was subsequently determined. Data points represent the mean ± SEM of four experiments performed with different cell preparations. See legend to Figure 1 and Materials and Methods for further details. *, p < 0.01; significant inhibition of H₂O₂ generation in 4β-PDBu-treated cells. NS, no significant increase in H₂O₂ generation from control rate.

The reason for the shallow [³H]AA concentration-response curves is currently unclear, this observation nevertheless prompted the question of whether a causal relationship exists between these two parameters. This was particularly relevant given that the activation of the NADPH oxidase by LTB₄ was not mediated by a prostanoid, thromboxane A₂, or leukotrienes (see above). Circumstantial evidence to support this hypothesis was the critical observation that the kinetics of [³H]AA release were very rapid and preceded H₂O₂ generation. Another important consideration was whether the level of AA achieved in LTB₄-stimulated eosinophils was sufficient to activate the NADPH oxidase. Although not formally investigated in this study, Wolf et al. (58) have reported that following stimulation of pancreatic islets with glucose, the intracellular concentration of AA, measured by mass spectrometry, reached 50 to 100 μM, which if reproduced in agonist-stimulated eosinophils, is far in excess of the concentration required to promote maximally oxidative metabolism.

Having established that the release of [³H]AA by LTB₄ had the required concentration and time dependence to activate the NADPH oxidase, experiments were performed with PTX to determine the extent to which signaling through PLA₂ and PLC were implicated in the release of [³H]AA and H₂O₂ by LTB₄. Unfortunately, this approach was unsuccessful, since PTX abolished all effects (H₂O₂ generation, [³H]AA release, Ins(1,4,5)P₃ accumulation, and Ca²⁺ mobilization) evoked by LTB₄ indicating the involvement of one or more members of the Gi or Go family of heterotrimeric GTP-binding proteins. Although we cannot state with certainty which of the G proteins were involved in mediating the aforementioned effects of LTB₄, recent studies have established that guinea pig peritoneal eosinophils express a number of G proteins, including Gαi₃ and Gao (but not Gαi₁), that are PTX substrates and are known to couple to PLC-β in a number of cells and tissues (59). Another possible candidate is Gαz₂, although it has not been formally identified in guinea pig eosinophils.

Table II. Effect of depletion of extracellular Ca²⁺ on LTB₄-induced AA generation from guinea-pig eosinophils

<table>
<thead>
<tr>
<th></th>
<th>Without Ca²⁺/Mg²⁺</th>
<th>+ EGTA</th>
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<tbody>
<tr>
<td>LTB₄ (1 μM)</td>
<td>1.29 ± 0.25</td>
<td>0.96 ± 0.20</td>
</tr>
<tr>
<td>4β-PDBu (1 μM)</td>
<td>2.70 ± 0.42*</td>
<td>1.87 ± 0.35*</td>
</tr>
</tbody>
</table>

*Eosinophils (10⁷/ml) were labeled with [³H]AA, and the amount of radiolabel released by LTB₄ (1 μM) or vehicle in the absence (+100 μM EGTA) and presence of Ca²⁺ (1 mM) and Mg²⁺ (1 mM) was measured at 60 s. Data represent the mean ± SEM of three independent determinations performed with different cell preparations.
LTB₄ promotes the hydrolysis of PtdIns(4, 5)P₂ in guinea pig eosinophils with the formation of Ins(1,4,5)P₃ and, by definition, a stoichiometrically equivalent amount of diglyceride (7). Potentially, these second messengers could act upstream of PLÁ₂ through their ability to release Ca²⁺ from intracellular stores and activate PKC, thereby indirectly regulating the availability of free AA. Given that PTX abolished all effects of LTB₄, additional experiments were deemed necessary to establish how LTB₄ generates H₂O₂ and the role of PLC-derived signal molecules in [³H]AA release. To this end, two studies were performed. Initially, eosinophils were incubated with 4β-PDBu, which is known to uncouple (desensitize) certain serpentine receptors from PLC-β (but not PLÁ₂) (44, 56), at a concentration that abolished LTB₄-induced Ins(1,4,5)P₃ accumulation and Ca²⁺ mobilization. It was reasoned that if LTB₄ receptors can couple directly to PLÁ₂, then [³H]AA release should be unaffected. Treatment of cells with 4β-PDBu significantly enhanced the basal elaboration of [³H]AA. This was somewhat unexpected given that phorbol diesters are inactive in platelets (60), HL60 cells, and neutrophils under roughly comparable conditions (44, 61). However, a survey of the literature revealed that the ability of phorbol diesters per se to release AA varies depending on cell type (see Ref. 62), which might be dictated by the complement and/or activation requirements (e.g., Ca²⁺ dependence) of PLÁ₂ isoenzymes present in the cell of interest. Of significance to this study was the finding that LTB₄ increased the release of [³H]AA from eosinophils in which PLC was desensitized above that produced by 4β-PDBu per se, with an EC₅₀ identical to eosinophils in which PLC was not desensitized. An interpretation of these results is that LTB₄ releases [³H]AA by a PLC-independent mechanism. Unfortunately, the observation that the desensitization produced by 4β-PDBu also was associated with [³H]AA and H₂O₂ release did not permit unequivocal conclusions to be drawn, and a second approach was adopted to corroborate or refute this interpretation. Logic dictates that if the formation of DAG by LTB₄ is necessary for the activation of PLÁ₂, then an inhibitor of PKC should inhibit [³H]AA release. Similarly, a requirement for Ins(1,4,5)P₃ in this response implies that the activation of PLÁ₂ is Ca²⁺ dependent and that the accumulation of Ins(1,4,5)P₃, can account, at least in part, for Ca²⁺ mobilization. Experiments designed to test these assumptions failed to implicate DAG and Ins(1,4,5)P₃ in LTB₄-induced [³H]AA release. Thus, pretreatment of eosinophils with the PKC inhibitor Ro 31-8220 did not inhibit LTB₄-induced [³H]AA release at concentrations (1 to 20 μM) known to inhibit PKC in leukocytes (63, 64). Since LTB₄ does not activate PLD (an alternative source of diglyceride) in guinea pig eosinophils (7), these data indicate that the activation of PKC by PtdIns(4, 5)P₂-derived DAG cannot be involved in the activation of PLÁ₂. Moreover, LTB₄ mobilized appreciable [³H]AA from eosinophils in which Ca²⁺ mobilization was abolished. Therefore, by definition, this Ca²⁺-independent PLÁ₂ cannot require Ins(1,4,5)P₃-evoked Ca²⁺ release for activity. The regulation of the Ca²⁺-dependent PLÁ₂(s) was also dissociated from the activation of PLC as evinced by the finding that the Ca²⁺ required for [³H]AA release were mobilized by LTB₄ over a concentration range that did not detectably increase Ins(1,4,5)P₃.

The reason for the reduction in the magnitude of LTB₄-induced [³H]AA release in eosinophils in which PLC was desensitized was not formally addressed, but it is likely that 4β-PDBu, by abolishing Ca²⁺ influx, reduced the pool of Ca²⁺-dependent PLÁ₂ that LTB₄ would normally mobilize. This proposal is consistent with the fact that the magnitude of LTB₄-induced [³H]AA release in EGTA-treated eosinophils and in cells in which PLC was desensitized were almost identical.

Collectively, these data provide persuasive evidence that LTB₄ can liberate [³H]AA in eosinophils independently of signal molecules derived from the activation of PLC. Furthermore, the demonstration that PTX abolished LTB₄-induced [³H]AA release from guinea pig eosinophils, and the evidence that PLÁ₂ is regulated by distinct G proteins in many cells (36–43), suggests that LTB₄ releases AA from membrane phospholipids by acting at receptors that couple directly to PLÁ₂ via a member of the Gi or Go family of heterotrimeric GTP-binding proteins.

The role of AA in LTB₄-induced activation of the NADPH oxidase was also assessed in eosinophils in which PLC had been desensitized. A prediction, based on previous evidence that PLC-driven processes are not involved (7), was that the capacity of
eosinophils to produce H$_2$O$_2$ should be preserved. However, contrary to expectation, LTB$_4$ failed to generate H$_2$O$_2$ in 4β-PDBu-treated eosinophils under conditions in which the release of [³H]AA was essentially unchanged. Thus, despite circumstantial data to the contrary (see Introduction), these results indicate that LTB$_4$ activates the NADPH oxidase by a mechanism divorced from its ability to couple to PLA$_2$ and generate AA. This interpretation is corroborated, in part, by the results obtained with the PLA$_2$ inhibitor mepacrine, which abolished [³H]AA release at a concentration that exerted a relatively modest effect on H$_2$O$_2$ generation. However, if it is assumed that mepacrine (50 μM) inhibits H$_2$O$_2$ generation by acting solely at the level of PLA$_2$, then these data suggest that the liberation of AA by LTB$_4$ does play a relatively minor role (~36%) in the activation of the NADPH oxidase. It is striking that in other cells, in particular in human neutrophils, mepacrine effectively suppresses the magnitude of the respiratory burst evoked by FMLP and serum-opsonized zymosan (33, 34) at concentrations significantly lower than those used in this study (34). Moreover, in FMLP-stimulated human eosinophils, mepacrine inhibited O$_2^-$ generation in a concentration-dependent manner (35), although in this study the release of [³H]AA was not measured. The reason(s) for this discrepancy is unknown, but possibilities include differences in species or cell type (eosinophil vs neutrophil) and/or, more fundamentally, in the signaling pathways recruited for the activation of the NADPH between agonists (51). The possibility also remains that eosinophils “elicited” into the peritoneal cavity might not generate H$_2$O$_2$ in the same way as their unstimulated circulating counterparts.

In conclusion, the results of this study prove persuasive evidence that agonism of LTB$_4$ receptors on guinea pig eosinophils mobilizes AA by a PTX-sensitive mechanism that does not involve the generation of signal molecules derived from the hydrolysis of PtdIns(4, 5)P$_2$ by PLC. Moreover, despite the ability of LTB$_4$ to stimulate PLA$_2$, a central role for AA in the activation of the NADPH oxidase was largely excluded using both a pharmacologic and a biochemical approach. Thus, the primary cellular signaling pathways recruited by LTB$_4$ that control the assembly and subsequent activation of the NADPH oxidase complex in guinea pig eosinophils remain to be elucidated.

References