Leukotriene B_4 stimulates the release of arachidonate in human neutrophils via the action of cytosolic phospholipase A_2

James R. Burke [*], Lynda B. Davern, Kurt R. Gregor, Kenneth M. Tramposch

Dermatology Discovery Research, Bristol-Myers Squibb Pharmaceutical Research Institute, 100 Forest Avenue, Buffalo, NY 14213, USA

Abstract

Leukotriene B_4 (LTB_4) is a potent lipid mediator of inflammation and is involved in the receptor-mediated activation of a number of leukocyte responses including degranulation, superoxide formation, and chemotaxis. In the present research, stimulation of unprimed polymorphonuclear leukocytes (neutrophils) with LTB_4 results in the transient release of arachidonate as measured by mass. This release of arachidonate was maximal at an LTB_4 concentration of 50–75 nM and peaked at 45 s after stimulation with LTB_4. The transient nature of this release can be attributed, in part, to a fast (< 60 s) metabolism of the added LTB_4. Moreover, the inhibition of the reacylation of the released arachidonate with thimerosal results in greater than 4-times as much arachidonate released. Thus, a rapid reacylation of the released arachidonate also contributes to the transient nature of its measured release. Multiple additions of LTB_4, which would be expected to more closely resemble the situation in vivo where the cell may come into contact with an environment where LTB_4 is in near constant supply, yielded a more sustained release of arachidonate. No release of [^{3}H]arachidonate was observed when using [^{3}H]arachidonate-labeled cells. This indicates that the release of arachidonate as measured by mass is most probably the result of hydrolysis of arachidonate-containing phosphatidylethanolamine within the cell since the radiolabeled arachidonate is almost exclusively incorporated into phosphatidylcholine and phosphatidylinositol pools under the non-equilibrium radiolabeling conditions used. Consistent with the role of cytosolic phospholipase A_2 ($cPLA_2$) in the release of arachidonate, potent inhibition of the LTB_4-stimulated release was observed with methylarachidonylfluorophosphonate, an inhibitor of $cPLA_2$ (IC_{50} of 1 μM). The bromoenol lactone of the calcium-independent phosphospholipase A_2. failed to affect LTB_4-stimulated release of arachidonate in these cells. © 1997 Elsevier Science B.V.

Abbreviations: BSA, bovine serum albumin; $cPLA_2$, cytosolic phospholipase A_2; HBSS, Hanks buffered salt solution; HELSS, *E*-6-(Bromomethylene)tetrahydro-3-(1-napthalenyl)-2*H*-pyran-2-one; HSA, human serum albumin; LTB_4, leukotriene B_4; MAFP, methylarachidonylfluorophosphonate; $sPLA_2$, secreted phospholipase A_2

[*] Corresponding author. Fax: +1-716-8873203.

1. Introduction

The leukotrienes and prostaglandins are important lipid mediators which are derived from arachidonic acid. Polymorphonuclear leukocytes (neutrophils) have been shown to synthesize and release arachidonate and leukotriene B_4 (LTB_4) when treated with non-physiological stimuli such as the calcium ionophore A23187 or with receptor-dependent agonists such as the chemotactic peptide fMLP [1–4].

Treatment with A23187 generates substantial quantities of LTB_4 and other lipoxygenase products while the receptor-dependent agonists such as fMLP, C5a, and platelet activating factor (PAF), which activate other neutrophil functions, have been shown to produce only slight amounts of LTB_4 and metabolites [5]. However, the release of both arachidonate and its metabolites with receptor-dependent agonist treatment can be enhanced when the neutrophils are first 'primed' with agents such as granulocyte-macrophage colony-stimulating factor or tumor necrosis factor-α (TNFα) [3,6,7].

Indeed, LTB_4 itself has been shown to prime neutrophils for enhanced release of radiolabeled arachidonate and LTB_4 upon stimulation with ionophore [8]. Additionally, LTB_4 treatment of neutrophils induces adherence to the endothelium, chemotaxis, degranulation, and the generation of reactive oxygen metabolites [9–13]. These processes result, ultimately, from the binding of LTB_4 to a G-protein-coupled receptor [14].

The enzyme responsible for the production of arachidonate is phospholipase A_2. This enzyme catalyzes the hydrolysis of phospholipids which contain an *sn*-2 arachidonoyl ester. Several different types of phospholipases A_2 have been identified; the best characterized are the 14 kDa secreted enzyme ($sPLA_2$), the 85 kDa cytosolic enzyme ($cPLA_2$), and a calcium-independent enzyme ($CaI-PLA_2$) found in myocardial tissue [15].

$cPLA_2$ is a calcium-dependent enzyme which, unlike the $sPLA_2$, has been shown to be selective for arachidonate-containing phospholipids [16,17]. The $cPLA_2$ is normally located in the cytosol, but translocates to the membrane in response to submicromolar concentrations of calcium [18,19]. Recent evidence indicates that both phosphorylation of $cPLA_2$ at serine505, which may be catalyzed by MAP kinase [20,21], and an increase in the cytosolic calcium concentration are required to activate the enzyme to produce arachidonate [20–22]. Additionally, the enzyme shows a cooperative behavior with respect to the molar fraction of arachidonate-containing phospholipids. This may account for another mechanism of regulation [23].

The involvement of $cPLA_2$ in the stimulated release of arachidonate from neutrophils is evidenced by work with permeabilized neutrophils [24], and by the observation that priming agents such as lipopolysaccharide and TNFα lead to a phosphorylation of $cPLA_2$ in neutrophils [25,26].

In this paper, we demonstrate that treatment of neutrophils with LTB_4 results in the release of arachidonate when measured by total mass, but not by radiolabel in [^{3}H]arachidonate-labeled cells. This release is shown to result from the action of $cPLA_2$ and is transient due to the quick metabolism of LTB_4 as well as the re-esterification of released arachidonate. This is the first study to show that LTB_4 alone can stimulate the mobilization of arachidonate. The results also have important implications regarding the function of neutrophils in environments in vivo where the cell may be in contact with a more continuous supply of leukotrienes.

2. Experimental

2.1. Materials

The radiolabeled arachidonic acid ([5,6,8,9,11,12,13,15-^{3}H(N)], 100 Ci mmol^{-1}) was obtained from Dupont/NEN. MK-886 was purchased from Biomol, methylarachidonylfluorophosphonate (MAFP) was from Cayman Chemicals, and HELSS was from Calbiochem. HSA (essentially fatty acid free) was purchased from Sigma.

2.2. Isolation of human neutrophils

Neutrophils were purified from whole blood (EDTA) by dextran sedimentation and centrifugation on ficoll-paque (Pharmacia LKB) for 15 min at 600 $\times g$ at 4°C. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were resuspended at a working concentration of 5×10^6 cells ml^{-1} in Hank's balanced salt solution (HBSS) containing 0.2% HSA. Purity of the cell population ($> 95\%$) was verified by Wrights stain and cell viability was obtained by trypan blue exclusion ($> 90\%$).

2.3. Measurement of released arachidonate from neutrophils

1 ml of neutrophils (5×10^6 cells ml^{-1}) were preincubated for 5 min at 37°C to equilibrate the cells, and then stimulated with LTB_4 or A23187. After various amounts of time, the reaction was

terminated on ice by the addition of 1 ml of ice cold HBSS. The samples were centrifuged at $600 \times g$ for 15 min at 4°C. Heneicosanoic acid (21:0) was added to the cells as an internal standard. The lipids were then extracted by the procedure of Bligh and Dyer [27]. After drying under a stream of nitrogen, the sample was resuspended in hexane. The free fatty acids were isolated using silica SPE columns preconditioned with hexane by eluting with 1:1 hexane:ether mixture. After removing the solvent under a stream of nitrogen, the fatty acids were derivatized to pentafluorobenzyl esters by adding 30 μl of 30% pentafluorobenzyl bromide in silylation grade acetonitrile and 30 μl of diisopropylethylamine. The contents were mixed well and incubated for 40 min at 40°C. Solvents and excess reagents were evaporated under a stream of nitrogen and the derivatives were redissolved in 200 μl of gas chromatographic grade hexane. Analysis of the fatty acids was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with an electron capture detector, an HP7673 autosampler, and a HP 486 Vectra data processor. The PFB esters were separated on a fused silica capillary column (30 m × 0.25 mm id, DB-17, J&W Scientific). The injector temperature was 250°C and the detector temperature was 290°C. The initial column temperature was 40°C for 1 min and increased to 220°C at a rate of 20°C min^{-1} and then to 280°C at a rate of 5°C min^{-1}. Helium was used as a carrier gas (0.8 ml min^{-1}) and nitrogen was used as the makeup gas. Samples in hexane were injected in the splitless mode and the derivatized arachidonate was quantitated using the heneicosanoic acid as an internal standard.

For those experiments which measured the effect of thimerosal, an inhibitor of fatty acid acylation [28,29], cells were stimulated according to the procedure above except that the cells were incubated for 5 min with 50 μM thimerosal prior to stimulation. For those experiments which measured the effect of MK-886 or HELSS, a 5 min preincubation with the agent was used. A 20 min preincubation was used with MAFP.

2.4. [³H]Arachidonate-labeling of neutrophils

Isolated neutrophils (4.4×10^7 ml^{-1}) were labeled for 15 min at 37°C with 44 nM [³H]arachidonate.

Cells were pelleted and washed with HBSS containing 1% HSA to remove unincorporated [³H]arachidonate. These cells were then repelleted and resuspended to a concentration of 5×10^6 cells ml^{-1} as described above. The analysis of the phosphoglycerol pools into which the [³H]arachidonate was incorporated was accomplished by extracting the lipids by the method of Bligh and Dyer [27]. Extracted lipids were then separated into glycerolipid classes by TLC using Silica Gel G plates developed in chloroform/methanol/acetic acid/water (50:25:8:3, v/v). Glycerolipids were visualized against standards by exposure to iodine vapor or located using a radiochromatogram imaging system (Bioscan, Washington, DC), and the amount of radioactivity was determined by zonal scraping and liquid scintillation counting.

These [³H]arachidonate-labeled neutrophils were stimulated and quenched in the same manner as described above. Cells were removed by centrifugation and the supernatants analyzed for the release of label by liquid scintillation counting.

2.5. Leukotriene biosynthesis by neutrophils

To 1 ml of cells which had been stimulated and quenched as described above was added 200 μl of 9% formic acid and PGB_2 to a final concentration of 250 ng ml^{-1}. After the eicosanoids were extracted twice with 4 ml ethyl acetate, the combined organic layers were evaporated under nitrogen and reconstituted in ethanol. Leukotrienes were then analyzed by HPLC using an ultra-sphere ODS column (2.1 mm × 250 mm, Rainin) with spectroscopic detection at 280 nm. The mobile phase was 55:45 methanol:water pH 5.7 with phosphoric acid at a flow rate of 0.3 ml min^{-1}. After 5 min, the methanol was increased to 100% over 50 min. Spectrophotometric detection was at 270 nm. The mass of LTB_4 was quantitated by peak integration normalized by the internal standard PGB_2.

3. Results

3.1. Release of arachidonate from neutrophils treated with LTB_4

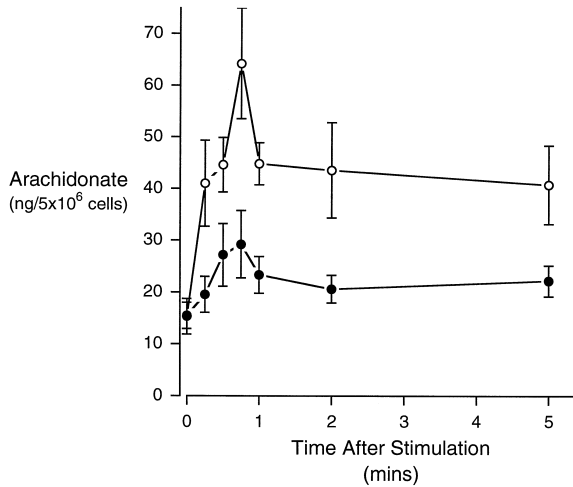Treatment of neutrophils with LTB_4 resulted in a transient production of arachidonate, as determined

Fig. 1. LTB$_4$-stimulated release of arachidonate from neutrophils. Neutrophils were stimulated with 75 nM LTB$_4$ either in the absence, closed circles, or presence, open circles, of HSA (2 mg ml^{-1}). The mass of arachidonate produced was then determined by gas chromatography as described in Section 2. The data represent the average of measurements in duplicate from three separate donors. The error is defined as the standard deviation.

Fig. 2. A23187-stimulated release of arachidonate from neutrophils. Neutrophils were stimulated with 2.5 μM A23187 in the presence of HSA (2 mg ml^{-1}). The mass of arachidonate produced was then determined by gas chromatography as described in Section 2. The data represent the average of measurements in duplicate from three separate donors. The error bars represent the standard deviation.

by gas chromatography–electron capture to measure the total mass, which peaked at 45 s (see Fig. 1). This is in contrast to the release observed when stimulated with the calcium ionophore A23187 (Fig. 2). The A23187-induced release is not transient and peaks at 5 min. Both A23187 and LTB$_4$ failed to stimulate the release of oleic or linoleic acids (data not shown). Arachidonate produced upon stimulation with LTB$_4$ was not cell-associated but was released extracellularly (results not shown). The presence of HSA increased the amounts of arachidonate by about 3-fold, presumably by sequestering the arachidonate once outside the cell.

As shown in Fig. 3, the release of arachidonate was dependent on the concentration of LTB$_4$ up to approximately 50 nM. Above this concentration, no further increase in arachidonate production was observed which is consistent with a saturation effect. While 50 nM LTB$_4$ is a substantially higher concentration than the value of 48–270 pM reported for the dissociation constant of LTB$_4$ from its receptor [30], the binding of LTB$_4$ to the albumin present in the assay greatly reduces its effective concentration [31].

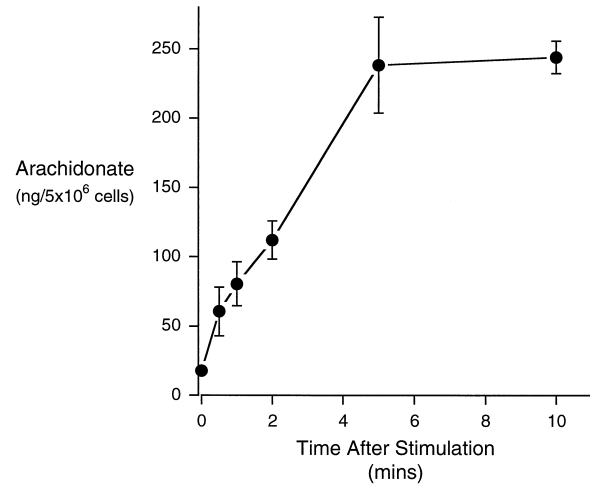Using neutrophils which had been radiolabeled with [3H]arachidonate, TLC analysis of the phospho-

lipid classes showed most of the [3H]arachidonate was incorporated into phosphatidylcholine and phosphatidylinositol pools (23.3 ± 1.5% and 30.8 ± 1.9%,
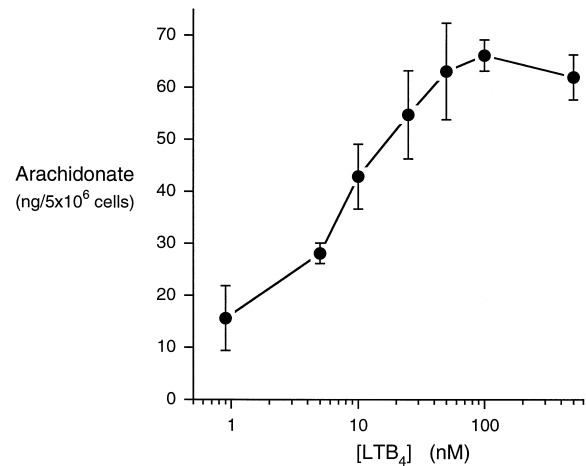

Fig. 3. Concentration dependence of LTB$_4$-stimulated release of arachidonate. Neutrophils were stimulated with various concentrations of LTB$_4$ in the presence of HSA (2 mg ml^{-1}). The reactions were quenched 45 s after stimulation and the mass of arachidonate produced was then determined as described in Section 2. The data represent the average of measurements in duplicate from three separate donors. The error bars represent the standard deviation.

respectively) with only $8.2 \pm 1.4\%$ in phosphatidylethanolamine pools (the remainder was in neutral lipids). Treatment of these [^{3}H]arachidonate-labeled neutrophils with LTB$_4$ failed to stimulate a measurable release of [^{3}H]arachidonate as shown in Fig. 4. These radiolabeled neutrophils were able to release sizable amounts of [^{3}H]arachidonate, however, when stimulated with the calcium ionophore A23187.

3.2. Effect of a reacylation inhibitor on the release of arachidonate induced by LTB$_4$

While the presence of HSA would be expected to aid in the sequestration of any hydrolyzed arachidonate once outside the cell, the transient production of arachidonate shown in Fig. 1, demonstrates that either the arachidonate is re-incorporated into cellular phospholipid pools, or is metabolized by enzymes such as cyclooxygenase or 5-lipoxygenase. In order to address the possibility of reacylation, neutrophils were treated with 50 μM thimerosal which blocks the acylation of fatty acids [29] without inducing the

Fig. 5. Effect of thimerosal on LTB$_4$-stimulated release of arachidonate. Neutrophils were stimulated with 75 nM LTB$_4$ either in the absence, open circles, or presence, closed circles, of thimerosal (50 μM). The mass of arachidonate produced was then determined as described in Section 2. The data represent the average of three determinations from a single blood donor, although the same trends were observed in three other experiments.
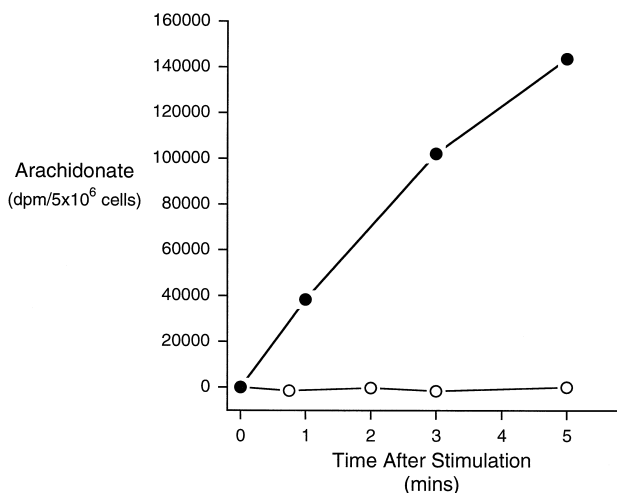
Fig. 4. LTB$_4$- and A23187-stimulated release of [^{3}H]arachidonate from [^{3}H]arachidonate-labeled neutrophils. Neutrophils were stimulated with either 75 nM LTB$_4$, open circles, or 2.5 μM A23187, closed circles. At the indicated times, samples were quenched with equal volumes of ice-cold buffer and centrifuged. The released [3H]arachidonate in the supernatant was quantitated by scintillation counting. The data represent values from which non-stimulated controls were subtracted and are the average of three determinations from a single donor, although the same trends were observed in three other experiments.
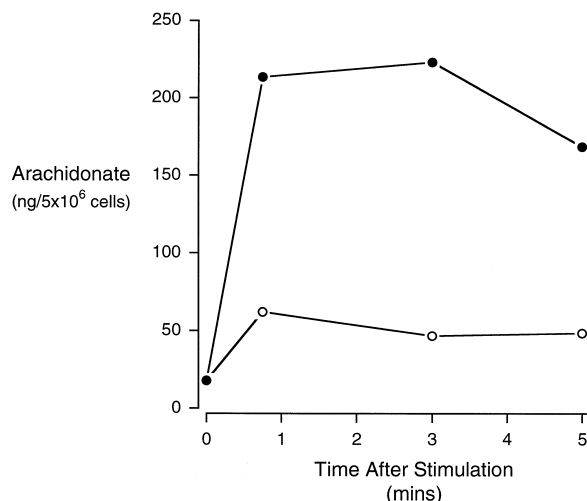
release of arachidonate itself [28]. As shown in Fig. 5, treatment with thimerosal resulted in more than 4-times as much release of arachidonate. In contrast, treatment with 10 μM MK886, an inhibitor of the 5-lipoxygenase activating protein inhibitor [32,33], did not substantially affect the measured arachidonate in LTB$_4$-stimulated neutrophils (results not shown). Thus, metabolism toward 5-lipoxygenase products was not considered to be significant (vide infra).

3.3. Production and metabolism of LTB$_4$ in LTB$_4$-stimulated neutrophils

Since it has been reported that treatment of neutrophils with LTB$_4$ greatly enhances the ability of the cells to synthesize LTB$_4$ from exogenously added arachidonate [34], it was of interest to determine whether treatment with LTB$_4$ led to the further accumulation of LTB$_4$. Fig. 6 shows that while A23187 treatment resulted in the formation of large amounts of LTB$_4$, LTB$_4$ treatment alone did not lead to the formation of LTB$_4$ above the levels added. In fact, the added LTB$_4$ was completely metabolized in less than a minute as determined by the absence of the
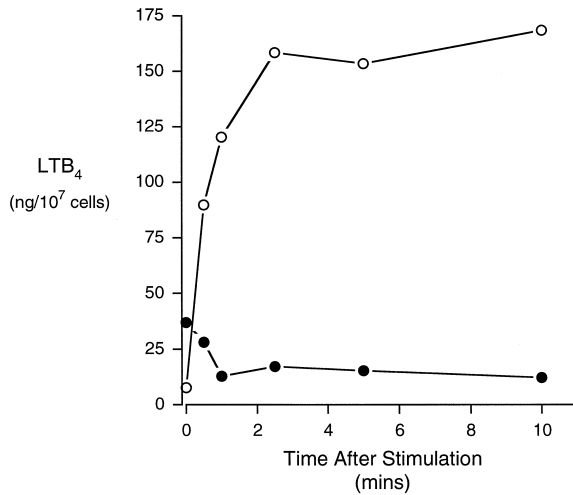
Fig. 6. LTB_4 levels in neutrophils treated with A23187 or LTB_4. Neutrophils were stimulated with either 75 nM LTB_4, closed circles, or 2.5 μM A23187, open circles. At the indicated times, samples were quenched and the LTB_4 was measured as described in Section 2. The data represent the average of two determinations from a single donor.
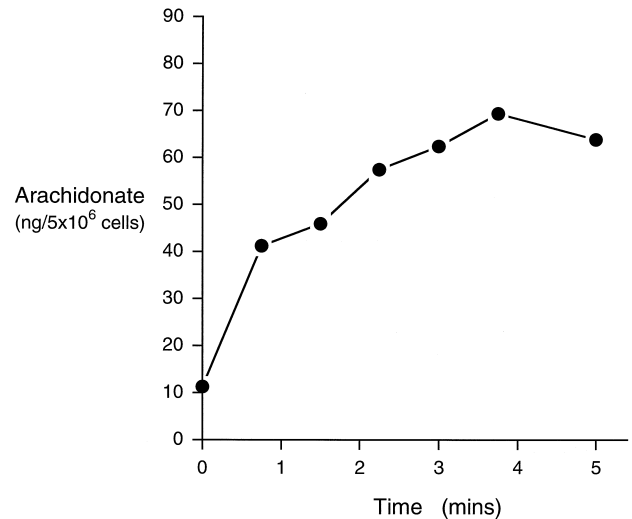
Fig. 7. Effect of multiple additions of LTB_4 on arachidonate production in neutrophils. Neutrophils were stimulated with 75 nM LTB_4 every 45 s in the presence of HSA (2 mg ml^{-1}). The mass of arachidonate produced was then determined as described in Section 2. The data represent the average of three determinations from a single donor.

exogenously added LTB_4. The production of metabolites of LTB_4 were not determined.

To determine whether neutrophils encountering a more constant concentration of LTB_4 have the capacity to release more arachidonate, multiple additions of LTB_4 were made at 45 s intervals. As shown in Fig. 7, this procedure indeed resulted in a more sustained release of arachidonate. This release of arachidonate is likely underestimated since the reacylation of arachidonate represents the major route of metabolism of the fatty acid (see Fig. 5).

3.4. Role of $cPLA_2$ in the generation of arachidonate in LTB_4-stimulated neutrophils

To determine which of the three phospholipases A_2 is responsible for the production of arachidonate in differentiated cells, pharmacological agents were employed to determine effects on arachidonate release in the cells. The methylarachidonylfluorophosphonate (MAFP) is a potent inhibitor of $cPLA_2$ which does not inhibit the $sPLA_2$ [35]. It has also been shown to inhibit the mobilization of arachidonate in PAF-stimulated P388D$_1$ [36]. Treatment of neutrophils with MAFP inhibited the release of arachidonate with IC$_{50}$ values of 1 and 0.4 μM when

stimulated with LTB_4 and A23187, respectively (Fig. 8). Since MAFP also inhibits the CaI–PLA_2 [37], it is unclear whether the $cPLA_2$ or the CaI–PLA_2 is
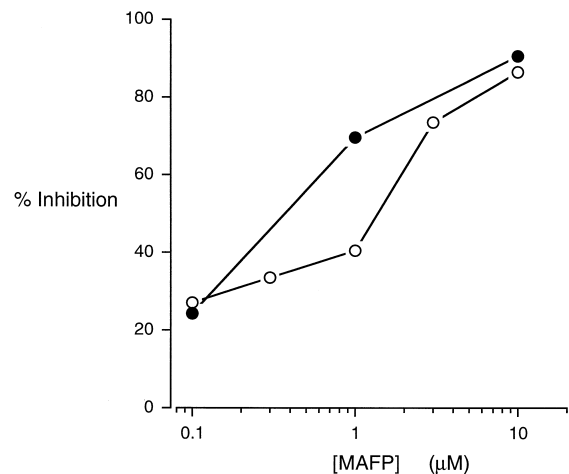
Fig. 8. Inhibition of LTB_4- and A23187-stimulated arachidonate production by MAFP. Neutrophils were preincubated with the indicated amount of MAFP for 20 min prior to a 45 s stimulation with either 75 nM LTB_4, open circles, or 2.5 μM A23187, closed circles. The mass of arachidonate produced was then determined as described in Section 2. The data represent the percent inhibition relative to a control without inhibitor and are the average of three determinations from a single donor.

responsible for the production of arachidonate in these cells. To address this question, the cells were treated with the bromoenol lactone (HELSS) inhibitor of CaI–PLA_2. This compound is a mechanism-based inactivator of the CaI–PLA_2 which does not inhibit $sPLA_2$ or $cPLA_2$ [38], and has been shown to block enzyme activity in A-10 smooth muscle cells and $P388D_1$ cells at concentrations as low as 1–5 μM [39,40]. Following HELSS treatment of neutrophils, the arachidonate release induced by LTB_4 was inhibited less than 10% at HELSS concentrations as high as 100 μM (results not shown). These results are consistent with the involvement of $cPLA_2$, rather than $sPLA_2$ or CaI–PLA_2, in the LTB_4-stimulated production of arachidonate in neutrophils. Indeed, LTB_4 has been shown to act through the LTB_4 receptor to modulate $cPLA_2$ and arachidonate release [4].

The role of $cPLA_2$ in the production of arachidonate is consistent with the observation that LTB_4 treatment of neutrophils leads to the complete conversion of the enzyme into the phosphorylated state as determined by SDS–PAGE and immunoblot analysis [4]. This is consistent with LTB_4 acting through a receptor-mediated phosphorylation cascade. We have determined that this phosphorylation occurred by 45 s (results not shown) which demonstrates that the time course of phosphorylation of $cPLA_2$ correlates with the time required for maximal release of arachidonate.

4. Discussion

In the present study, the unexpected finding that LTB_4 stimulates the release of arachidonate was observed. This release was not observed with cells which had been radiolabeled with [^{3}H]arachidonate. This, along with the fact that the release was transient, explains why other researchers measuring the effect at 5 min of treatment with LTB_4 on arachidonate release have not made this observation [4,41]. It is interesting to note that the time course for arachidonate production observed in the present research correlates well with the 30–60 s required for neutrophil aggregation stimulated by LTB_4 [42].

Potent inhibition by MAFP, but not by HELSS, of the release of arachidonate demonstrates that $cPLA_2$

is responsible for this release. That the release of arachidonate is concomitant with the phosphorylation of the $cPLA_2$ induced by LTB_4 supports the idea that phosphorylation of the enzyme is important in the regulation of its function [15].

The phospholipid source for this arachidonate release is most probably phosphatidylethanolamine since two-thirds of the endogenous arachidonate in neutrophils has been shown to be in this phospholipid class [43]. This is also consistent with the lack of LTB_4-induced release of [^{3}H]arachidonate in neutrophils radiolabeled with [^{3}H]arachidonate since it was shown that the [^{3}H]arachidonate was mainly localized into phosphatidylcholine and phosphatidylinositol pools with very little associated with phosphatidylethanolamine in these non-equilibrium labeled cells. That phosphatidylethanolamine is the major source of released arachidonate has also been demonstrated in A23187-stimulated neutrophils and mast cells [43–46].

While stimulation of neutrophils with LTB_4 leads to the release of arachidonate, it is not clear what purpose this serves. It may be that the production of arachidonate, which is then rapidly reacylated, is simply an alternate form of phospholipid remodeling usually thought to be catalyzed by acyltranferases. This putative remodeling may then prime the cell for optimal production of arachidonate and eicosanoids when treated, either subsequently or simultaneously, with some other agent. Indeed, LTB_4 has been shown to prime neutrophils for enhanced release of arachidonate when stimulated with fMLP [4]. Moreover, priming of neutrophils with LTB_4 leads to the increased synthesis of leukotrienes when stimulated with A23187 [34,47]. A particularly intriguing idea is that the LTB_4-stimulated release and reacylation reported here may represent a remodeling of arachidonate from phosphatidylethanolamine to phosphatidylcholine. Indeed, phosphatidylcholine has been shown to be the source of arachidonate used for leukotriene biosynthesis in neutrophils [44] and is the phospholipid into which free arachidonate is incorporated (vide supra).

Another important consideration is that the present work uses isolated neutrophils. Therefore, the important signaling between different cells in vivo has been removed. Thus, while the arachidonate resulting from the stimulation of neutrophils by LTB_4 is shown not

to be used in producing additional leukotrienes, it may be that this arachidonate is utilized by other cells in vivo to produce eicosanoid products. This would be similar to the example of hepatocyte biosynthesis of LTB_4 which requires LTA_4 produced by a neighboring leukocyte, for instance, since hepatocytes lack the 5-lipoxygenase needed for LTA_4 generation [48].

Moreover, the transient release of arachidonate observed when stimulated with LTB_4 also underestimates the importance of this process in vivo. As shown in Fig. 7, if the neutrophils are treated with multiple additions of LTB_4, a more sustained release of arachidonate is observed. This may more closely resemble an environment in vivo where it can be envisioned that neutrophils may be found in an inflamed tissue, for instance, where LTB_4 is constantly produced from neighboring cells. This in vivo environment, therefore, would be expected to yield a more sustained and prolonged production of arachidonate from neutrophils.

In conclusion, the present research demonstrates that treatment of neutrophils with LTB_4 results in the production of arachidonate. The results also provide mechanistic information on the role of leukotrienes in the priming of neutrophil function. Moreover, these findings have important ramifications on the role of neutrophils in vivo where the cells may encounter environments of constant leukotriene levels [49,50].

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