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Ca^{2+} MOBILIZATION WITH LEUKOTRIENE A_4 AND EPOXYTETRAENES IN HUMAN NEUTROPHILS

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Abstract—The biosynthesis of leukotrienes and lipoxins involves epoxide-containing intermediates which may be subject to several routes of transcellular metabolism. We have examined the capacity of leukotriene A_4 (LTA_4) and 15*S*-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid [5(6)-epoxytetraene] to stimulate the mobilization of free cytosolic calcium ($[Ca^{2+}]_i$) in human blood neutrophils. To gain insight into structure–activity relationships, a putative intermediate in lipoxin biosynthesis, 5*S*-hydroxy-14,15-oxido-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid [14(15)-epoxytetraene], was prepared by total synthesis. When added to fura-2 loaded neutrophils, each of these compounds provoked a rapid and transient increase in $[Ca^{2+}]_i$ (maximum by 8 sec) which returned to baseline within 60–90 sec. Ca^{2+} mobilization with LTA_4 was dose dependent and, at 1 μM, the efficacies of LTA_4 and LTB_4 were quantitatively similar. The 5(6)-epoxytetraene and 14(15)-epoxytetraene were less potent than LTA_4. Prior exposure of the cells to ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) (60 sec, 3 mM) did not diminish either the amplitude or the extent of $[Ca^{2+}]_i$ elicited by LTA_4. Methyl esters of LTA_4 and 5(6)-epoxytetraene were less potent than their corresponding free acids, whereas the free acid of 14(15)-epoxytetraene and its methyl ester were quantitatively similar. Results from alcohol trapping studies showed that these epoxides were intact during the initial phase of Ca_i^{2+} mobilization (t_0–10 sec) stimulated by LTA_4, 5(6)-epoxytetraene, and 14(15)-epoxytetraene. In addition, the individual mixtures of products formed upon aqueous hydrolysis of each of the epoxides did not stimulate changes in $[Ca^{2+}]_i$. In each case, the products formed were identified by physical methods including reverse phase high pressure liquid chromatography, ultraviolet spectroscopy and gas liquid chromatography–mass spectrometry. These results indicate that, when added to human neutrophils, LTA_4, 5(6)-epoxytetraene and 14(15)-epoxytetraene each stimulate a rapid mobilization of $[Ca^{2+}]_i$. Moreover, they suggest that intermediates in the biosynthesis of leukotrienes and lipoxins possess intrinsic activities that may serve to amplify cellular responses within their cell of origin or act on adjacent cells during their transcellular metabolism.

The generation of lipoxygenase-derived products of arachidonic acid is associated with the activation of a wide range of cell types and plays a central role in inflammation and the regulation of smooth muscle tone in both non-vascular and vascular tissues [1–3]. A pivotal step in the formation of these compounds is the generation of epoxide-containing intermediates [1–3]. It is now recognized that the 5-lipoxygenase which transforms arachidonic acid to 5-HPETE can also generate leukotriene A_4 (LTA_4††) [4]. The findings with this enzyme [4], as well as those obtained with both the 12-lipoxygenase and 15-lipoxygenase [5, 6], emphasize the importance of the single dioxygenation, double dioxygenation and epoxide generating abilities of lipoxygenases (i.e. multiple enzymatic activities).

Once formed *within* cells, LTA_4 can be converted to either LTB_4 or LTC_4 by enzymes which are compartmentalized within specific cells types [1]. In recent years, interest has turned to the possibility that lipoxygenase-catalyzed reactions within one cell type can be altered by interactions with a different cell type [7–15]. For example, in addition to serving as an intermediate within its cell type of origin, LTA_4 can also be released by or escape from human neutrophils [11]. LTA_4 may then be transformed via several transcellular routes [8–16]. It can be stabilized in the extracellular milieu by albumin [8] and

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†† Abbreviations: RP-HPLC, reverse phase high pressure liquid chromatography; GC, gas–liquid chromatography; MS, mass spectrometry; THF, tetrahydrofurane; DMAP, *N*,*N*-4-dimethylaminopyridine; 15-HETE, 15*S*-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; 5-HPETE, 5*S*-hydroperoxyeicosatetraenoic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; lipoxin A_4 (LXA_4), 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; lipoxin B_4 (LXB_4), 5*S*,14*R*,15*S*-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; leukotriene A_4 (LTA_4), 5*S*-*trans*-5(6)-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; leukotriene B_4 (LTB_4), 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; leukotriene C_4 (LTC_4); leukotriene D_4 (LTD_4); 5(6)-epoxytetraene, 15*S*-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; and 14(15)-epoxytetraene, 5*S*-hydroxy-14,15-oxido-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid.

transformed by transcellular metabolism to LTB_4 by either a specific plasma protein [9], human red cells [13] or renal epithelial cells [16]. Exogenous LTA_4 can also be transformed to LTC_4 by either endothelial cells [10, 14], mast cells [11], or platelets [12, 15]. Alternatively, LTA_4 may be enzymatically converted to 5*S*,6*R*-dihydroxyeicosatetraenoic acid (5*S*,6*R*-DHETE) [16] or, in the absence of appropriate cell types, it can undergo non-enzymatic hydrolysis [17, 18].

Interactions between the 5- and 15-lipoxygenases can lead to the formation of lipoxins [19]. These compounds display both a distinct pattern of biological activities and unique structures [1]. Evidence for the role of a 5(6)-epoxytetraene intermediate in the formation of LXA_4 and LXB_4 has been presented [20–22]. In addition to the 5(6)-epoxytetraene route, other biosynthetic routes have been proposed [23–25]. For example, the generation of lipoxins could involve the formation of a 14(15)-epoxytetraene intermediate [21, 24, 26]. Together the results of these studies emphasize the importance of epoxide-containing intermediates in the formation of leukotrienes and lipoxins both within their cell types of origin and in transcellular metabolism.

Despite the key role of LTA_4 in the formation of leukotrienes, few studies have examined its intrinsic activity or direct role(s) in stimulus–response coupling. When added to lung strips, trachea or human bronchus, LTA_4 provokes contractions which correlate with the extent of its tranformation to leukotrienes (i.e. LTB_4, LTC_4, LTD_4) in these tissues [27]. Administration of LTA_4 in the guinea pig (*in vivo*) causes bronchoconstriction [28]. LTA_4 also inhibits adrenocorticotrophic hormone-induced corticosterone production [29], and when added to human neutrophils, it provokes aggregation and degranulation [30, 31].

In the current study, we have utilized fluorimetric measurements of $[Ca^{2+}]_i$ as an early, sensitive index of cell activation to assess the effects of LTA_4 and two epoxide intermediates, 5(6)-epoxytetraene and 14(15)-epoxytetraene, on human neutrophils. Trapping studies were performed to assess the role of the epoxide structure during the Ca^{2+} mobilization. In addition, 5*S*-hydroxy-14,15-oxido-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid [14(15)-epoxytetraene] was prepared by total organic synthesis, and the structure–activity relationships for neutrophil Ca^{2+} mobilization by LTA_4, 5(6)-epoxytetraene and 14(15)-epoxytetraene were examined.

MATERIALS AND METHODS

HPLC solvents were from American Scientific Products, Burdick & Jackson (HPLC grade) (Muskegon, MI). Ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), dextran (mol. wt. > 500,000), digitonin (twice recrystallized), bovine serum albumin (Cohn fraction V), LiOH, $CaCl_2$, and methyl formate were purchased from the Sigma Chemical Co. (St Louis, MO). Sep-pak C_{18} cartridges were obtained from Waters Associates (Milford, MA) and ethyl acetate (spectroscopic grade) from Mallinckrodt, Inc., (Paris, KY). Pyridine (silylation grade), hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from the Pierce Chemical Co. (Rockford, IL). Diazomethane (CH_2N_2) was prepared from *N*-methyl-*N'*-nitro-*N*-nitroguanidine (MNNG) purchased from the Aldrich Chemical Co. (Bulletin AL-132) (Milwaukee, WI). Fura-2/AM was from Molecular Probes (Eugene, OR) and Hanks' Balanced Salt Solutions (HBSS) with and without $CaCl_2$ and $MgCl_2$ were from M.A. Bioproducts (Whittaker, MD). LTB_4, LTA_4 and other eicosanoids used for chromatographic standards were from Biomol Research Laboratories, Inc. (Philadelphia, PA). Materials and other procedures utilized in the total synthesis were essentially as in Ref. 32.

Preparation of human neutrophil suspensions. Whole blood was collected from healthy volunteers by venipuncture into buffer (1:9; 100 mM sodium citrate, 130 mM glucose, pH 6.5). Neutrophils were isolated by dextran sedimentation, Ficoll centrifugation and hypotonic lysis of contaminating red cells [33]. The final cells suspensions typically contained 96% neutrophils, ~3% eosinophils, and less than 1 platelet per 200 neutrophils as determined from Wright's stained cytospin preparations.

Measurement of cytosolic free calcium by Ca^{2+}*-sensitive fura-2 fluorescence.* Neutrophils (10^7 cells/mL) were prepared in HBSS without Ca^{2+} and Mg^{2+} and incubated with fura-2/AM (stock solution, 1 mM in dimethyl sulfoxide) at a final concentration of 1 μM for 10 min at 37°. Cells were diluted 5-fold with HBSS–0.5% BSA and incubated for an additional 15 min at 37°. Following loading of the probe, the cells were washed twice by centrifugation with cold HBSS–0.1% BSA without Ca^{2+} and Mg^{2+} and kept at 10° until assay. Prior to measurements of fluorescence, cells were rapidly pelleted in a Beckman Microfuge (model B), and resuspended to a final dilution of 10^6 cells/mL in HBSS plus Ca^{2+} and Mg^{2+} in a thermostatically controlled (37°) cuvette (1.6 mL) for 5 min before addition of agonists. The final amount of BSA was 0.01% (v/v). Fluorescence measurements were performed with a SPEX (Edison, NJ) Fluorolog II (model CM-1) spectrofluorimeter equipped with continuous stirring, a beam splitter, two excitation monochrometers, and a dual mirror chopping mechanism in a specialized optical configuration to allow rapid alternating (30 Hz) excitation of fura-2 at two wavelengths, 340 nm and 380 nm. Excitation band widths were set at 6.6 nm. The ratio of emitted fluorescence signals (505 nm, 7.2 nm band width) permits calculation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) which is independent of cell number, loading of the probe or its bleaching [34]. Fluorescence signals were calibrated using 80 μM digitonin to permit equilibration of intracellular and extracellular Ca^{2+} (maximum) followed by the addition of 1.0 M Tris, 300 mM EGTA, pH > 10.0 (minimum).

Analysis of eicosanoids and alcohol trapping products. The products formed upon addition of either LTA_4, 5(6)-epoxytetraene or 14(15)-epoxytetraene to neutrophils in suspension or with buffers alone were extracted from incubations utilizing a combination of previously described techniques [18, 19, 35]. Here, incubations were terminated at the designated intervals by addition of ethanol (2 vol.) followed by the addition of 270 ng prostaglandin

B_2 (PGB_2) as an internal standard. These suspensions were each placed at 4° for 30 min followed by centrifugation (1500–2000 rpm, 15 min). Resulting supernatant fractions were decanted and the remaining pellets were suspended in MeOH (in amounts equal to that of the incubation). This procedure was repeated twice, and the resulting EtOH and MeOH containing mixtures were combined and taken to dryness by rotoevaporation under reduced pressure. Next, the materials obtained from each incubation were separately suspended in MeOH:H_2O (1:45, v/v) by vortexing in round bottom flasks (~1–2 min). Each mixture was transferred into a glass syringe (one for each incubation condition) followed by addition of HCl in aliquots until the pH of 3.5 was obtained (<60 sec to prevent degradation of the products). The samples were next rapidly loaded into cartridges containing ODS-silica (C_{18} Sep-paks), washed with 10 mL H_2O, and eluted with hexane, methyl formate and MeOH [36]. Materials eluting in each fraction were examined for UV-absorbing materials before preparation for RP-HPLC. Next, materials eluting within the methyl formate fractions were concentrated under argon and injected into an LKB HPLC dual-pump gradient system (Bromma, Sweden) equipped with an Altex Ultrasphere-ODS (4.6 mm × 25 cm) column, injector, and solvent controller (LKB, Bromma, Sweden). The column was eluted with a gradient system similar to that described by Powell [36], modified with MeOH:H_2O:acetic acid (65:35:0.01) as phase one injection (t_0–30 min) and a linear gradient with MeOH:acetic acid (99.99:0.01) as phase two (30–50 min). This HPLC system was equippped with a photodiode array rapid spectral detector linked to an AT&T PC6300, and post-HPLC run analyses were performed utilizing a 2140-202 Wavescan program (Bromma, Sweden) and Nelson Analytical 3000 series chromatography data system (Paramus, NJ). The post-HPLC extraction and recovery of PGB_2 were 70.0 ± 12.8% (mean ± SD, N = 10) and the recovery of the alcohol trapping and hydrolysis products derived from the epoxides was 81.7 ± 10.4 (N = 9 separate determinations).

Gas chromatography–mass spectrometry was performed with a Hewlett–Packard 5988A equipped with an HP 59970A workstation and software. A fused silica capillary SE-30 (Supelco, Inc., Bellefonte, PA) column 2-4004, 30 meters, 0.25 mm i.d., 0.25 μm film thickness, was employed with a temperature program. The splitless on time was 0.90; initial temperature was 150° (1 min), followed by 230° (4 min), 240° (8 min) and 245° (12.0 min) with a 12.0 min solvent delay time. The eM volts were set at 70 relative and the resulting voltage was 2770. The maximum scans per second was 0.84 in the acquisition window with low mass 100 and high mass 600. Trimethylsilyl (Me_3Si) derivatives were prepared with each compound following treatment with diazomethane, and products were identified according to published criteria [17–22].

Ultraviolet spectra which were not taken on-line with the rapid spectral detector were recorded with a Carey 118 spectrophotometer (Varian, CA).

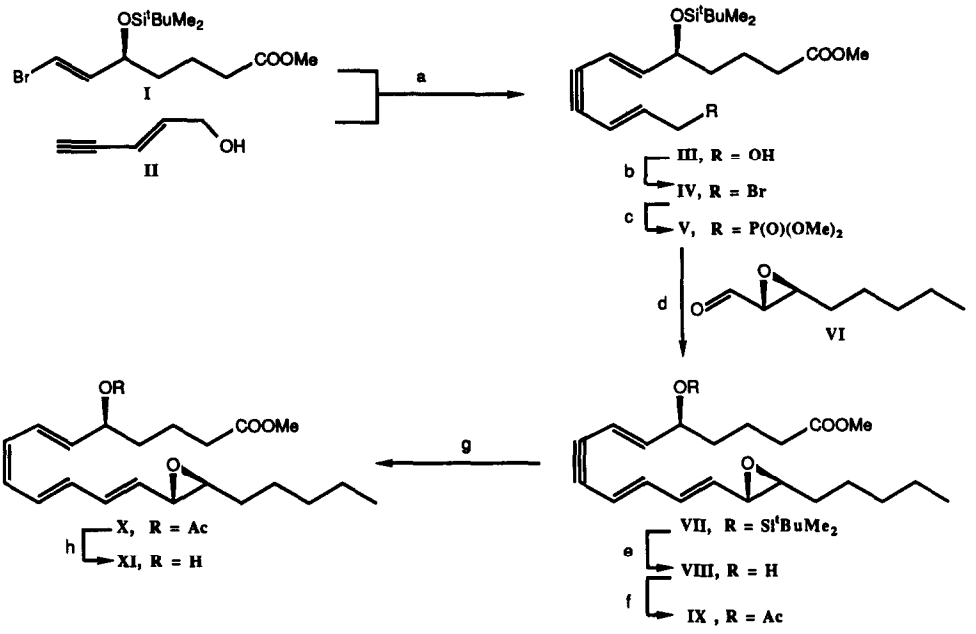

Fig. 1. Synthesis of 14,15-epoxytetraene XI. Reagents and conditions. (a) I (1.0 equiv.) [38], II (2.0 equiv.) (from Farchan Chemicals), 0.04 equiv. $(Ph_3P)_4Pd$, 0.16 equiv. CuI, Et_2NH solvent (0.3 M), 25°, 90% yield [37]; (b) 1.25 equiv. Ph_3P, 1.3 equiv. CBr_4, CH_2Cl_2 solvent (0.05 M), −10–25°, 89% yield; (c) 10 equiv. $P(OMe)_3$, CH_3CN solvent (1.0 M), 60°, 12 hr, 98% yield; (d) 0.98 equiv. LDA, THF solvent (0.1 M), −78° then add 1.5 equiv. of VI [38], −78–25°, 70% yield; (e) 1.0 equiv. nBu_4NF, THF solvent (1.0 M), 0–25°, 60% yield (plus 15% yield of corresponding δ-lactone); (f) 1.2 equiv. Ac_2O, 3.0 equiv. Et_3N, 0.005 equiv. DMAP, CH_2Cl_2 solvent (0.2 M), 0–25°, 83% yield; (g) 10% (w/w) Lindlar catalyst, H_2, hexane:EtOAc (3:1) solvent, trace quinoline, 25°, 40% yield; (h) 0.05 equiv. anhydrous K_2CO_3 in absolute MeOH:THF solvent (1:1), (0.02 M), 25°, then add anhydrous ether and filter, 90% yield.

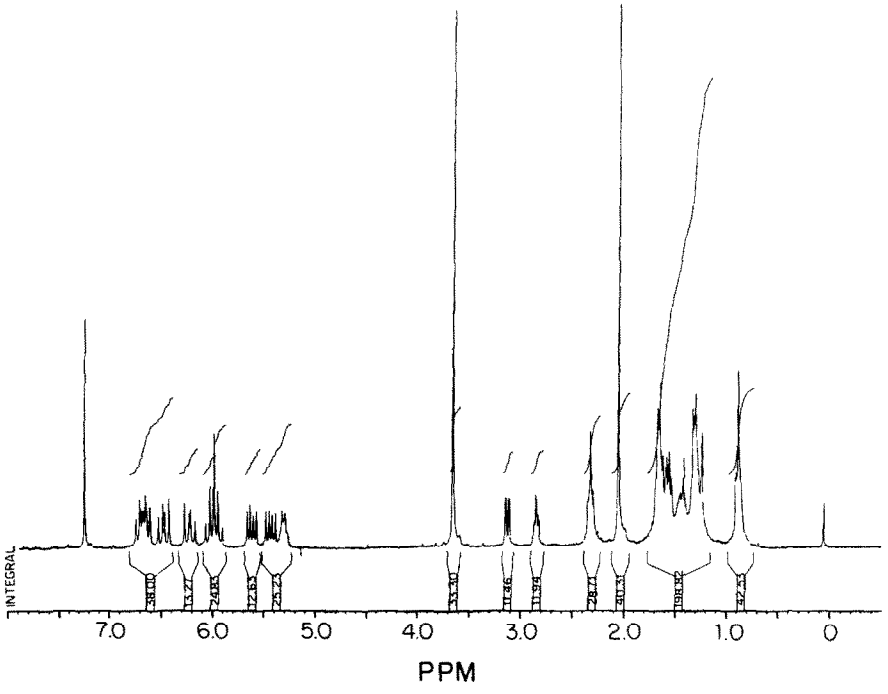

Fig. 2. ^{1}H 250 MHz ^{1}H NMR spectrum of the acetate of the 14(15)-epoxytetraene in $CDCl_3$.

Statistical analysis. A two-tailed Student's *t*-test was used for statistical analysis.

RESULTS

To investigate the actions of LTA_4, 5(6)-epoxytetraene, and 14(15)-epoxytetraene on Ca^{2+} mobilization by human neutrophils and examine the structural basis for this activity, it was first necessary to prepare both the 5(6)-epoxytetraene and 14(15)-epoxytetraene by total synthesis.

Synthesis of epoxytetraenes. The 14(15)-epoxytetraene was obtained by total synthesis carried out as shown in Fig. 1. The vinylbromide I and terminal acetylene II [30] were coupled using Pd(O)–Cu(I) catalysis [37, 38] leading to the dienyne alcohol III (90% yield), which was then brominated (Ph_3P–CBr_4) to give compound IV (89% yield). Exposure of bromide IV to $P(OMe)_3$ then furnished the key intermediate phosphonate V in 98% yield. Condensation of the lithioanion of V with the readily available epoxyaldehyde VI [38] gave product VII together with its *cis*-isomer at the newly generated double bond (70% yield, *ca.* 3:1 ratio *trans*:*cis*). Purification of VII was carred out by flash column chromatography using silica gel impregnated with 2% Et_3N (5% ether in petroleum ether, $R_f = 0.25$). Removal of the silyl protecting group from VII was achieved with fluoride ion leading to a mixture of the hydroxy compound VIII (60%) and the corresponding δ-lactone (15%). After chromatographic purification with silica gel–Et_3N as described above, VIII was acetylated (Ac_2O, Et_3N, DMAP) furnishing acetate IX in 83% yield, purified by HPLC (Whatman semipreparative column, EtOAc:Et_3N:hexane, 12:2:85, flow rate 11.0 mL/min, retention time 15 min, λ_{max} 301 nm.). Lindlar hydrogenation of IX gave X, purified by HPLC (same conditions as for IX, retention time 16.5 min, 40% yield) which was deacetylated with catalytic potassium carbonate in absolute ethanol affording, after dilution with ether, filtration and evaporation of the solvent at $\leq 0°$ essential pure 14(15)-epoxytetraene XI in 87% yield. The ^{1}H NMR spectrum of the acetate X is shown in Fig. 2. The 5(6)-epoxytetraene was prepared utilizing a similar synthetic strategy. Next, aliquots of methyl–LTA_4, methyl–5(6)-epoxytetraene and methyl–14(15)-epoxytetraene were each taken for saponification with LiOH in THF [19] to generate the corresponding free acids.

UV analysis of the epoxides. Immediately prior to addition of these compounds to neutrophils, their UV spectra were recorded in MeOH, and lability to HCl was determined to ensure that the epoxides were intact. LTA_4 gave a triplet of absorbance λ_{max}^{MeOH} at 280 nm with shoulders at 270 and 291 nm characteristic for its conjugated triene structure [17, 18]. The 5(6)-epoxytetraene gave λ_{max}^{MeOH} at 305.5 nm with shoulders at 291.5 and 321.0 nm, while its positional isomer 14(15)-epoxytetraene gave λ_{max}^{MeOH} at 306.5 nm with shoulders at 292.5 and 321.0. Upon addition of HCl to each cuvette, all three spectra shifted, by approximately 10 nm for LTA_4 and approximately 5 nm for the tetraene-containing epoxides (<30 sec), to shorter wavelengths, indicating the formation of the respective hydrolysis products. Since these compounds were highly susceptible to hydrolysis, the lability of each to HCl-induced hydrolysis in MeOH was tested immediately prior to their addition to cells. This procedure ensured that each compound contained an intact epoxide structure before its addition to neutrophils.

Eicosanoid-induced changes in fluorescence of fura-2 loaded human neutrophils. Next, the extent

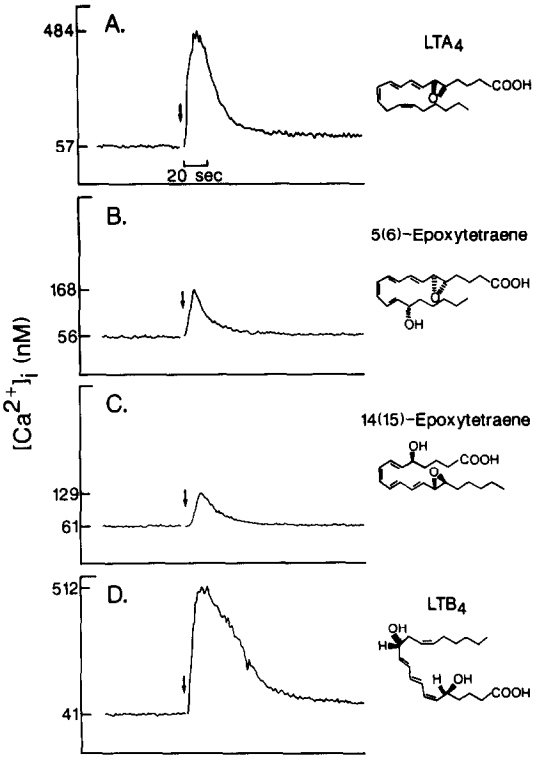

Fig. 3. LTA_4, LTB_4, 5(6)-epoxytetraene and 14(15)-epoxytetraene-induced changes of fura-2 loaded human neutrophils. Neutrophils were loaded with fura-2 as described under Material and Methods, and LTA_4 (A), 5(6)-epoxytetraene (B), 14(15)-epoxytetraene (C) or LTB_4 (D) was added at a final concentration of 1 μM. Digitonin (80 mM) and EGTA (12.5 mM) were added to determine F_{max} and F_{min}. $[Ca^{2+}]_i$ values were calculated using the 340/380 ratio as described by Grynkiewicz *et al.* [34] and are shown on the left. The tracings are representative of four separate experiments.

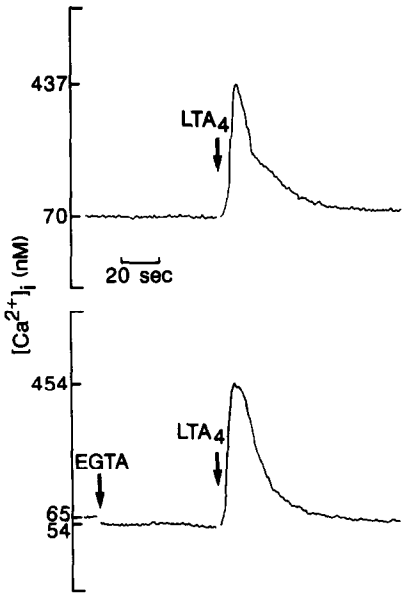

Fig. 4. Effect of the extracellular Ca^{2+} chelator EGTA on LTA_4-induced rise in $[Ca^{2+}]_i$ in neutrophils. Fura-2 loaded neutrophils were incubated in the absence (top panel) and presence (bottom panel) of 3 mM EGTA for 60 sec followed by addition of 1 μM LTA_4-free acid (arrow). Changes in $[Ca^{2+}]_i$ were measured by fura-2 fluorescence as described in Materials and Methods.

and temporal response of Ca^{2+} mobilization in fura-2 loaded neutrophils were examined for each compound. Basal $[Ca^{2+}]_i$, determined with neutrophils in suspension in the absence of additions, was 53 ± 12 nM (N = 82). Addition of LTA_4 (1 μM) to neutrophils induced a rapid, transient increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ reached a maximum level of 494 nM by 8 sec (Fig. 3). The LTA_4-induced rise in Ca^{2+} then decreased to near basal concentrations by 60–90 sec. Treatment of fura-2 loaded neutrophils with EGTA (3 mM) 60 sec before the addition of LTA_4 did not diminish either the amplitude or extent to which $[Ca^{2+}]_i$ increased when compared to LTA_4 in the absence of EGTA (Fig. 4). LTB_4 is a potent stimulus for Ca^{2+} mobilization in human neutrophils [39–41]. For purpose of comparison, the response with LTB_4 (1 μM) was examined (Fig. 3 and Table 1). At this concentration, the magnitude and extent of Ca^{2+} mobilized by LTB_4 and LTA_4 were very similar. When added at 1 μM, both the 5(6)-epoxytetraene and 14(15)-epoxytetraene elicited a rapid transient increase in $[Ca^{2+}]_i$ in neutrophils (Fig. 3). In comparison to LTA_4 and LTB_4, the amplitude of the increase of $[Ca^{2+}]_i$ induced by 5(6)-epoxytetreane and 14(15)-epoxytetraene was approximately 60% less (Table 1). All three epoxides (1 μM) induced a statistically significant increase in $[Ca^{2+}]_i$ when compared to basal levels.

Table 1. Effects of LTA_4, 5(6)-epoxytetraene,14(15)-epoxytetraene and their corresponding methyl esters on $[Ca^{2+}]_i$ in neutrophils

Compound	Maximal $[Ca^{2+}]_i$ increase (nM)
LTA_4 (FA)	441 ± 33*†
LTA_4 (ME)	112 ± 25*†
5(6)-Epoxytetraene (FA)	203 ± 104*†
5(6)-Epoxytetraene (ME)	37 ± 53*
14(15)-Epoxytetraene (FA)	121 ± 52†
14(15)-Epoxytetraene (ME)	102 ± 34†
LTB_4	493 ± 90†

The basal $[Ca^{2+}]_i$ and peak increase in $[Ca^{2+}]_i$ after addition of a compound were calculated as described [34]. The maximum $[Ca^{2+}]_i$ increase (in nM) after addition of a 1 μM dose was determined by subtracting the basal $[Ca^{2+}]_i$ from the peak $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ was 53 ± 12 nM (N = 82). FA and ME are abbreviations for the free acid and methyl ester derivatives of these compounds respectively. Data are the means ± SD of four experiments.

* Statistical difference between FA and ME derivatives ($P < 0.05$).

† Statistically significant from basal ($P \leq 0.05$).

To determine whether a free carboxylic acid at carbon-1 was required for this response, the methyl ester derivative of each compound was tested. The methyl ester of LTA_4 was a less potent inducer of $[Ca^{2+}]_i$ mobilization when compared to its corresponding free acid (Table 1). The methyl ester of

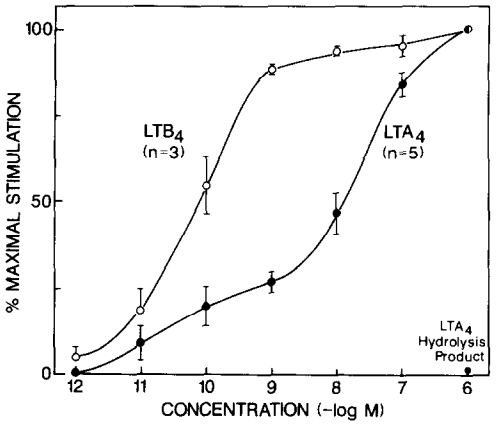

Fig. 5. Concentration dependence of LTA_4 and LTB_4 on $[Ca^{2+}]_i$ in neutrophils. Fura-2 loaded neutrophils were exposed to various concentrations of LTA_4 and LTB_4. After F_{max} and F_{min} were determined as described [34], the basal and peak $[Ca^{2+}]_i$ were calculated as detailed in Materials and Methods. Each point (mean ± SEM) represents the percent of maximum $[Ca^{2+}]_i$ increase induced by different doses of each compound using 1 μM as 100%. Basal $[Ca^{2+}]_i$ averaged 53 ± 12 nM (mean ± SEM, N = 82).

$[Ca^{2+}]_i$. That the effects of these two compounds on neutrophil $[Ca^{2+}]_i$ are stereochemically selective is illustrated by the fact that their non-enzymatic hydrolysis products did not elicit a rise in $[Ca^{2+}]_i$ in neutrophils in suspension.

Stability of epoxides during Ca^{2+} mobilization by human neutrophils. It was of interest to determine if the epoxides remained intact during the time course of Ca^{2+} mobilization by neutrophils. To this end, the stability of these epoxides during Ca^{2+} mobilization by neutrophils was examined by determining the amounts of products formed following addition of each compound at 1 μM to fura-2 loaded neutrophils. At 10 sec following additions and continuous monitoring of fura-2 fluorescence (t_0–8 sec), 2 vol. of alcohol was rapidly added to the cell suspensions. For each experiment, neutrophils from the same donor were incubated separately with the three epoxides. Here, as in previous studies [18, 22], the amounts of alcohol trapping products formed served as an index of the intact epoxide present in the incubations.

Incubations with LTA_4. Following addition of LTA_4 (1 μM) to neutrophils (1×10^6 cells/mL) in suspension, ethanol was added (10 sec), and the materials extracted were examined utilizing an RP-HPLC system equipped with a photodiode array detector (as described under Materials and Methods). When the chromatogram was recalled at 270 nm wavelength to detect conjugated triene-containing materials, two major component were observed (Fig. 6). The materials which eluted beneath these two peaks (denoted as A and B) displayed UV spectra indicative of a conjugated triene structure. The two materials gave physical properties consistent with those previously reported for the 12-*O*-ethyl derivatives of LTA_4 [18]. Therefore, these compounds were identified as the two ethanol trapping products derived from LTA_4, namely, the 5-hydroxy-12-*O*-ethyl-6,8,10,14-eicosatetraenoic acids (the carbon-12 epimers 12*R*-*O*-ethyl- and 12*S*-*O*-ethyl-derivatives of LTA_4).

The other components present in this chromatogram were identified as the non-enzymatic

Table 2. Concentration dependence of the effects of 5(6)-epoxytetraene and 14(15)-epoxytetraene on $[Ca^{2+}]_i$ in human neutrophils

Compound	Concentration (nM)	Maximal $[Ca^{2+}]_i$ increase (nM)
5(6)-Epoxytetraene	1000	203 ± 104*
	100	43 ± 24*
	10	0
Hydrolysis products	1000	16†
14(15)-Epoxytetraene	1000	121 ± 52*
	100	19 ± 9†
	10	0
Hydrolysis products	1000	9†

The basal $[Ca^{2+}]_i$ and peak increase in $[Ca^{2+}]_i$ after addition of each compound were calculated as detailed in Materials and Methods (and Ref. 34). The maximal $[Ca^{2+}]_i$ after additions was calculated as described in Table 1. Basal $[Ca^{2+}]_i$ was 53 ± 12 nM (N = 82). Data are the means ± SD of four separate experiments except for hydrolysis products, where N = 1.

* Statistically significant from basal $[Ca^{2+}]_i$ ($P \leq 0.05$).

† Not statistically significant from basal level.

14(15)-epoxytetraene stimulated $[Ca^{2+}]_i$ which was statistically significant when compared to basal levels. Unlike its free acid, the methyl ester of the 5(6)-epoxytetraene did not stimulate increases in $[Ca^{2+}]_i$.

Concentration dependence of the effects of LTA_4, LTB_4, 5(6)-epoxytetraene and 14(15)-epoxytetraene on the fluorescence of fura-2 loaded human neutrophils. The amplitude of increase in $[Ca^{2+}]_i$ was dependent upon the concentrations of eicosanoids added to neutrophils (Fig. 5 and Table 2). In contrast, the aqueous hydrolysis products of LTA_4, namely 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4, were without effect at 1 μM (Fig. 5). These findings with LTB_4 and $[Ca^{2+}]_i$ are consistent with those recently reported [41]. Results in Table 2 indicate that 5(6)-epoxytetraene and 14(15)-epoxytetraene are less potent than LTA_4 and LTB_4 for mobilization of

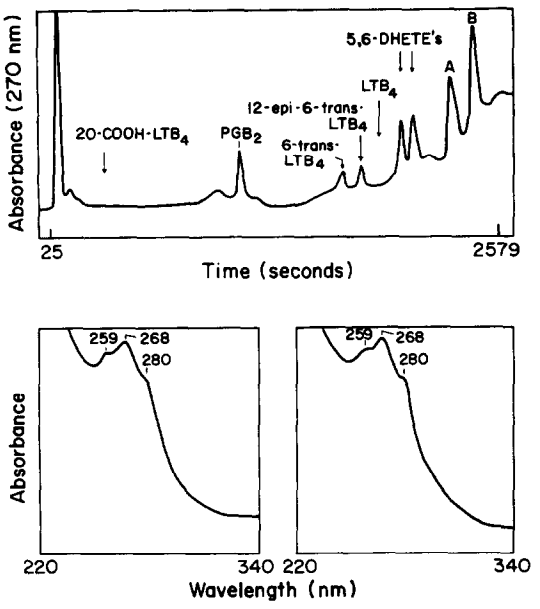

Fig. 6. RP-HPLC analysis of products formed upon addition of acidic ethanol to human neutrophil incubated with LTA_4 (1 μM, 37°, 10 sec). Upper: Products were extracted and subjected to RP-HPLC with a gradient two phase system as described in Materials and Methods and in Results. Lower: UV spectra of material beneath peaks denoted A and B. The HPLC system was equipped with a photodiode array rapid spectral detector. UV spectra were recorded on-line, and post-run analyses were performed utilizing a 2140-202 Wavescan program and a Nelson Analytical data system. Spectra were taken from retention times 37:25 and 39:31 min. Materials under the peak denoted A are shown in the left panel, and materials under peak B are shown in the right panel. The profiles and spectral data are representative of N = 3. Retention times for LTB_4 and 20-COOH-LTB_4 were 31:00 and 6:20 min respectively.

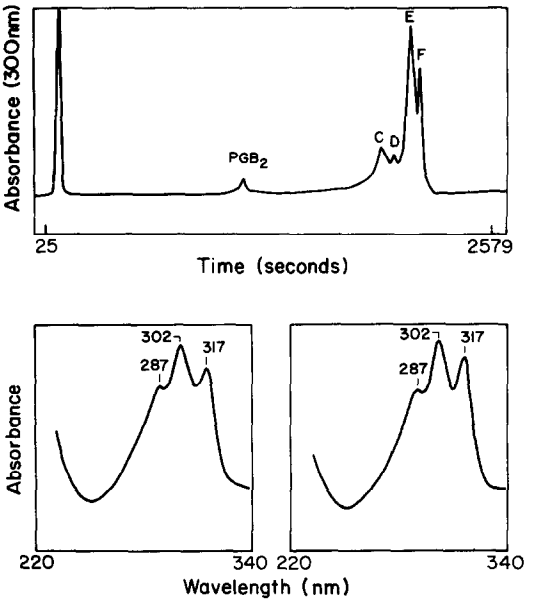

Fig. 7. RP-HPLC analysis of products formed upon addition of acidic ethanol to human neutrophils incubated with 5(6)-epoxytetraene (1 μM, 37°, 10 sec). Upper: Chromatogram recalled at 300 nm (see Materials and Methods and legend to Fig. 6) utilizing the Wavescan program. Lower: UV spectra recalled from the Wavescan program: (left panel) spectrum at 34:25 min (material beneath peak labeled E); and (right panel) spectrum at 35:09 min (material beneath peak labeled F). The profiles and spectral data are representative of N = 4. The retention times for LXA_4 and LXB_4 were 14:29 and 10:30 min respectively.

hydrolysis products of LTA_4, namely 6-*trans*-LTB_4 (27.39 min), 12-epi-6-*trans*-LTB_4 (29.19 min) and the two 5,6-DHETEs (33.01, 34.01 min). The presence of these non-enzymatic hydrolysis products indicated that a portion of the added LTA_4 underwent hydrolysis, while the majority of the compound remained intact at 10 sec and was converted to its 12-*O*-ethyl derivatives upon addition of alcohol. Here, neither LTB_4 (31.00 min) nor its ω-oxidation products [20-COOH-LTB_4 (6.20 min) and 20-OH-LTB_4 (5.03 min)] were detected in these samples (N = 3). The lower limit of detection for LTB_4 and its ω-oxidation products was ~1–2 ng, which would represent detection of a minimum of ~0.2% enzymatic conversion of LTA_4 in this system. These findings indicated that during the maximal level of $[Ca^{2+}]_i$ mobilization (<10 sec) evoked by 1 μM LTA_4 in fura-2 loaded neutrophils, neither LTB_4 nor its oxidation products were detected.

To determine if the presence of fura-2 within neutrophils inhibited or blocked the utilization of LTA_4 to generate LTB_4 by these cells, experiments were performed under the same conditions with neutrophils that were not loaded with fura-2. Following these incubations (1 μM LTA_4, 10 sec), chromatographic profiles were obtained which were essentially identical to those reported in Fig. 6. That is, neither

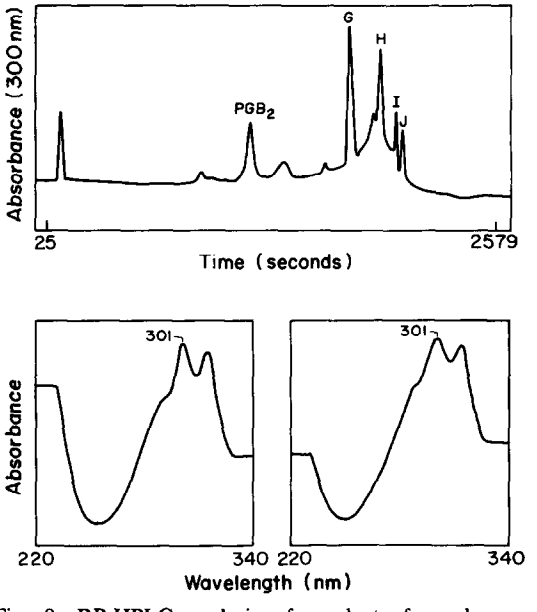

Fig. 8. RP-HPLC analysis of products formed upon addition of acidic ethanol to human neutrophils incubated with 14(15)-epoxytetraene (1 μM, 37°, 10 sec). Upper: Chromatogram recalled at 300 nm (see Materials and Methods and legends to Figs. 6 and 7) utilizing the Wavescan program. Lower: UV spectra recalled from the Wavescan program: (left panel) spectrum at 28:40 min (material beneath G); and (right panel) spectrum at 31:25 min (material beneath H).

LTB_4 nor its ω-oxidation products were detected following a 10-sec exposure of neutrophils to LTA_4. These results from two separate experiments suggest that the presence of fura-2 within neutrophils did not alter their response to LTA_4.

Incubations with the 5(6)*-epoxytetraene.* When the 5(6)-epoxytetraene (1 μM) was added to neutrophils (1×10^6 cells/mL) in suspension, and acidic ethanol (2 vol.) was injected to terminate these incubations (10 sec), several strongly absorbing materials were observed at 300 nm following extraction and RP-HPLC (Fig. 7, upper panel). The two major components, which eluted beneath the peaks labeled E and F, and less prominent components, which eluted beneath the peaks labeled C and D, each displayed UV spectra indicative of conjugated tetraene structures. The UV spectra of each of these materials recorded on-line (Fig. 7, lower panels) showed three main bands of intense absorbance at 287, 302 and 317 nm. These findings (chromatographic behavior and UV analysis) suggested that the four compounds originated from the 5(6)-epoxytetraene and that they were positional isomers [22, 42]. The materials that eluted beneath these peaks (C, D, E, F) were treated with diazomethane, converted in to Me_3Si derivatives, and subjected to GC–MS analysis. Material from peak E gave A C value of 23.5, and the mass spectrum showed ions of high intensity at *m/e* 173 (base peak; $Me_3SiO^+{=}CH{-}(CH_2)_4{-}CH_3$), 203 ($Me_3SiO^+{=}CH{-}(CH_2)_3{-}COOCH_3$) and 171 (elimination of CH_3OH from *m/e* 203). Ions of lower intensity were observed at *m/e* 231 ($CH_3{-}CH_2{-}O{-}(Me_3SiO^+){=}CH{-}(CH_2)_4{-}CH_3$), 307 (M − 231), and 438 (M − 100). The material from peak F gave a C value of 23.7, and its mass spectrum was virtually identical to that of the material from E, namely ions at 173 (base peak), 203, 171, 231, 307 and 438. The presence of these ions and high intensity at *m/e* 173 (base peak) in the mass spectra of the Me_3Si derivatives of these compounds was characteristic of those recently documented for the parent compound 5,15-dihydroxy-14-*O*-ethyl-6,8,10,12-eicosatetraenoic acid [22].

The materials from peaks C and D were also treated with diazomethane, converted to Me_3Si-derivatives, and analyzed by GC–MS. This material gave a broad peak on GC and C value of 23.0–23.1. The mass spectrum showed prominent ions at *m/e* 203 (base peak, $Me_3SiO^+{=}CH{-}(CH_2)_3{-}COOCH_3$), 171 (203 − 32; elimination of CH_3OH) and 173 ($Me_3SiO^+{=}CH(CH_2)_4{-}CH_3$). Ions of lower intensities were observed at 365 (M − 173), 335 (M − 203), 262 and 438 (M − 100). The presence of these ions suggests that the parent compound is a 5,15-dihydroxy-6-*O*-ethyl-7,9,11,13-eicosatetraenoic acid. Taken together, the results from HPLC, UV and GC–MS analysis indicate that the four compounds that eluted within peaks C–F were ethanol trapping products derived from 5(6)-epoxytetraene. The two major products were the 5,15-dihydroxy-14-*O*-ethyl-6,8,10,12-eicosatetraenoic acids (the 14-*O*-ethyl derivatives of the 5(6)-epoxytetraene) and the minor products were the 5,15-dihydroxy-6-*O*-ethyl-7,9,11,13-eicosatetraenoic acids (the 6-*O*-ethyl derivatives). Although the complete stereochemistry of these four ethanol trapping products was not determined in the present study, it is likely, by analogy with the trapping products of LTA_4 [18, 43], that the two major products (from E and F) are the carbon-14 epimers (i.e. 14*R*-*O*-ethyl- and 14*S*-*O*-ethyl-derivatives of the 5(6)-epoxytetraene), both of which would contain an all-*trans*(6,8,10,12; E,E,E,E) geometry. The products from C and D most likely represent the carbon-6 epimers (i.e. 6*S*-*O*-ethyl- and 6*R*-*O*-ethyl-derivatives of the 5(6)-epoxytetraene) containing a *cis* double bond at carbon-11 which remained intact from the 5(6)-epoxytetraene. The presence of these four trapping products following incubations with 5(6)-epoxytetraene (1 μM) and addition of alcohol at 10 sec suggests that the epoxide remained intact during the maximal level of Ca^{2+} mobilization (N = 4). Moreover, within 10 sec the epoxide was not converted to appreciable amounts of either LXA_4, LXB_4 or their isomers (<10% hydrolysis products). Incubations performed with the 5(6)-epoxytetraene added to buffer containing 0.5% albumin in the absence of neutrophils (10 sec, 37°) and stopped with the addition of acidic ethanol gave virtually identical profiles. In three other experiments, trapping studies with methyl-5(6)-epoxytetraene also showed that the 14-*O*-ethyl- and 6-*O*-ethyl-derivatives were the major products formed following its addition to neutrophils (10 sec, 37°).

Incubations with the 14(15)*-epoxytetraene.* Similar experiments were performed with the 14(15)-epoxytetraene. Following addition of the epoxide (1 μM) to either neutrophils in suspension or buffer containing 0.5% albumin and termination with acid ethanol (10 sec), four products were resolved by RP-HPLC (Fig. 8, upper panel). The materials that eluted under peaks labeled G–J each displayed UV spectra (Fig. 8, lower panels) indicative of a conjugated tetraene structure, namely strong absorbance at 289, 301, and 316 nm. Materials from G–J were treated with diazomethane, converted to Me_3Si-derivatives, and subjected to further analysis by GC–MS. The two major products from G and H gave virtually identical ions in these spectra, namely ions of high intensity at *m/e* 203 (base peak; $Me_3SiO^+{=}CH{-}(CH_2)_3{-}COOCH_3$) and 173 ($Me_3SiO{=}CH(CH_2)_4{-}CH_3$). Ions of lower intensity were at *m/e* 335, 438 (M − 100; rearrangement followed by loss of $O{=}CH{-}(CH_2)_4{-}CH_3$) and 448 (M − 90; or loss of Me_3SiOH). These two products gave C values of 23.8 and 23.6 respectively. The materials from I and J also gave similar results. However, the base peaks of their methyl-Me_3Si-derivatives were at *m/e* 173, which is consistent with that reported for the parent compound 5,15-dihydroxy-14-*O*-alkyl-eicosatetraenoic acid.

The products formed following incubation of the 14(15)-epoxytetraene with neutrophils and termination by addition of alcohol were two 6-*O*-ethyl-derivatives (under G and H) and two minor products, 14-*O*-ethyl-derivatives (under I and J). Utilizing similar considerations (*vide supra*), it is likely that the two major products represent the carbon-6 epimers (6*R*-*O*-ethyl- and 6*S*-*O*-ethyl-derivatives) that contain all-*trans* geometries, while the minor products are the carbon-14 epimers. In this case, the two C-14 epimers (14*R*-*O*-ethyl- and 14*S*-*O*-ethyl-derivatives) derived from the 14(15)-epoxytetraene are likely to contain an 8-*cis* double bond which remained intact from the 14(15)-epoxytetraene.

Thus, following a 10-sec exposure to neutrophils, the epoxide remained intact until the injection of acidic ethanol, at which time it was converted to trapping products (Fig. 8, N = 4).

DISCUSSION

In the present study we have measured the mobilization of $[Ca^{2+}]_i$, which has been implicated as an early and sensitive signal involved in neutrophil activation [44–46], in response to epoxide intermediates of leukotriene and lipoxin biosynthesis. Here, we report that these epoxides, in particular LTA_4, can rapidly stimulate a transient increase in $[Ca^{2+}]_i$. The 5(6)-epoxytetraene and 14(15)-epoxytetraene (a putative precusor in the biosynthesis of lipoxins [26]) were also effective but less potent than LTA_4 in this system (Table 1).

LTB_4 has been shown previously to be a potent stimulus for these cells utilizing intracellular Ca^{2+} as one of the important signals in its response coupling [39–41, 45, 46]. Here, LTB_4 proved to be more potent than its precursor LTA_4 in mobilizing $[Ca^{2+}]_i$ (Fig. 5 and Table 1). Studies reported by Goldman and Goetzl [47] provide evidence for the presence of both high- and low-affinity receptors for LTB_4 on human neutrophils. Whether LTA_4 is recognized by specific surface receptors, or shares the site(s) accepted by LTB_4 in these cells remains to be determined. Nevertheless, the action of LTA_4 appears to be highly specific since its aqueous hydrolysis products (6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4) did not elicit changes in $[Ca^{2+}]_i$ in neutrophils (Fig. 5). Results from Ca^{2+} chelation studies using EGTA (Fig. 4) show that the LTA_4-induced rise in $[Ca^{2+}]_i$ was exclusively dependent upon intracellular Ca^{2+} stores, and independent of extracellular Ca^{2+}. Together, these results suggest that LTA_4 mediates Ca_i^{2+} mobilization in human neutrophils via a mechanism which is independent of Ca^{2+} influx.

Since previous studies have shown that biological responses to LTA_4 are correlated with its degree of transformation to leukotrienes [27, 28], it was necessary to determine whether intact epoxides were present during the mobilization of Ca^{2+} [t_0–10 sec] by neutrophils. To this end, alcohol trapping studies were performed (Figs 6–8). In these experiments, each epoxide was incubated with neutrophils at a maximally effective concentration for Ca^{2+} mobilization (i.e. 1 μM from Fig. 3), and changes in fura-2 fluorescence were monitored followed by addition of acidic ethanol at t = 10 sec. Results from these analyses indicated that the epoxide structure in each compound was intact both before its addition to neutrophils and during the course of Ca^{2+} mobilization. Following these incubations, the epoxides were recovered as their respective alkyl trapping products (Figs 6–8). The trapping products were identified by physical methods including GC–MS (see Results). Therefore, we conclude that an intact epoxide structure is required for mobilization of $[Ca^{2+}]_i$ by these three compounds with neutrophils.

Activated neutrophils can release LTA_4, which can undergo transformation by transcellular routes [8–16]. The results of such studies imply that, during this process, LTA_4 must first "exit" the neutrophil, second, come in contact with the plasma membrane of the "acceptor cell," and then traverse or gain access to intracellular compartments prior to its enzymatic transformation to leukotrienes. This scheme of events is consistent with the reported transcellular metabolism observed with LTA_4, namely its production in one cell type and transformation by another [8–16]. In view of these results, the present observation has several implications. First, that LTA_4 added to the outside of neutrophils stimulates the mobilization of $[Ca^{2+}]_i$ suggests an "autocrine" activity. In this context LTA_4 may act in the cell of origin as an intrinsic amplifier via augmentation of Ca^{2+}-dependent enzymatic steps and activation of other Ca^{2+}-sensitive pathways, or it may exit the cell and act back upon the cell of origin to further amplify cell activation. Second, LTA_4 may possess "paracrine"-like activity. Once generated in the cell of origin, LTA_4 may exit this neutrophil, come in contact with an acceptor cell, and thus influence the activation state of this adjacent cell.

The responses to exogeneous LTA_4, 5(6)-epoxytetraene, and 14(15)-epoxytetraene at equimolar amounts (1 μM) were rapid in onset, reaching maximal within 8 sec, and qualitatively similar to the response evoked by native LTB_4 (1 μM), albeit to a lesser extent. At lower concentrations a rank order was established which indicated that LTA_4 was more potent than either the 5(6)-epoxytetraene or the 14(15)-epoxytetraene (Fig. 5 and Table 1). It is not possible to conclude from the present results, however, whether the epoxytetraenes represent less potent full agonists or if they serve as partial agonists. Nevertheless, additional evidence for the structural requirements for Ca^{2+} mobilization by these epoxides is afforded by a comparison of the compounds studied (Fig. 3). LTA_4 proved to be the most potent of the three epoxides tested. Here, the —OH group at carbon-15 and loss of the *cis*-pentadiene unit at carbon-11 through carbon-15 reduced the activity (LTA_4 vs 5(6)-epoxytetraene). The free carboxylic acid appears to be of more importance in the case of LTA_4, since its methyl derivative was significantly less potent (Table 1).

In addition to routes involving a 5(6)-epoxytetraene generated from 15-HETE, biosynthesis of LXA_4 and LXB_4 from 15-HPETE may involve the formation of a 14(15)-epoxytetraene [21, 26]. In the present study, 5(6)-epoxytetraene was of approximately equal potency to the 14(15)-epoxytetraene in stimulating Ca^{2+} mobilization. This finding indicates that both of these compounds display intrinsic biological activity. LXA_4 promotes both chemotaxis and chemokinesis with neutrophils [48–50]. It is of interest then that its precursor can stimulate Ca^{2+} mobilization in these cells.

Previous reports documented that LTA_4 added to suspensions of neutrophils induced aggregation [30] and degranulation [31] responses, but failed to elicit significant superoxide generation [31]. Our findings indicate that LTA_4 can also stimulate a rapid and transient increase in $[Ca^{2+}]_i$ in neutrophils. In summary, the present results indicate that LTA_4 and the epoxytetraenes are stimulants for the mobilization of intracellular Ca^{2+} in human neutrophils. They also suggest that pharmacological intervention at the

level of synthetic inhibitors of LTB_4 synthetase may accentuate the Ca^{2+}_i mobilization activity of LTA_4 as a result of substrate accumulation. In view of transcellular studies with eicosanoid epoxides [8–16], it is likely that upon formation these intermediates can mobilize Ca^{2+} which may contribute to cell activation.

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