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# Potential phospholipid source(s) of arachidonate used for the synthesis of leukotrienes by the human neutrophil

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The present study has employed two approaches to address the question of whether there are specific phospholipid sources of arachidonate used for leukotriene biosynthesis in the human neutrophil. Firstly, g.c.–m.s. analysis indicated that arachidonate was lost from all major arachidonate-containing phospholipid subclasses during cell activation with ionophore A23187. On a molar basis, the rank order of breakdown among the three major phospholipids was: 1-alk-1-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine > 1-alkyl-2-arachidonoyl-*sn*-3-phosphocholine > 1-acyl-2-arachidonyl-*sn*-3-phosphoinositol. Leukotrienes released into the supernatant fluid accounted for only 10–35% of the total arachidonate depletion. Phospholipid sources were also identified in labelling experiments where the specific radioactivity of arachidonate in phospholipid subclasses, as well as leukotrienes produced during cell activation, was measured. The specific radioactivity of arachidonate within 1-acyl-linked molecular species of phosphatidylcholine and phosphatidylinositol was initially high relative to the leukotrienes and decreased rapidly with stimulation. By contrast, the specific radioactivity of arachidonate in all three subclasses of phosphatidylethanolamine, 1-acyl, 1-alkyl, and 1-alk-1-enyl, was 3–5-fold below that of the leukotrienes throughout cell activation. Of the six major arachidonate-containing subclasses, only in the case of 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine did the specific radioactivity correlate well with that of leukotriene $B_4$ and 20-hydroxyleukotriene $B_4$. These data strongly suggest that 1-ether-linked phospholipids are an important source of arachidonate used for leukotriene biosynthesis.

## INTRODUCTION

Arachidonic acid and its metabolites are important inflammatory effector molecules. Yet a great deal remains unknown as to the exact phospholipid sources of endogenous arachidonate which enters into eicosanoid pathways during cell activation. It has been recognized for some time that there is a diversified group of phospholipid molecular species which make up the membranes of mammalian cells. This variety of phospholipid molecular species is especially pronounced in several inflammatory cells where there is a high content of 1-alkyl- and 1-alk-1-enyl-linked molecular species [1–7]. These ether-linked phospholipids often contain the major pools of arachidonate in these cells [1–3,5,8]. This is particularly true in the human neutrophil, where 71 and 66% of the total arachidonate in ethanolamine- and choline-linked phospholipid is contained in 1-alk-1-enyl-2-arachidonoyl-GPE and 1-alkyl-2-arachidonoyl-GPC respectively [1,8]. Moreover, these are two of the major arachidonate-containing subclasses which undergo degradation during cell activation [8–12]. The high content of arachidonate in ether-linked phospholipids, along with the capacity of enzymes (phospholipases) to mobilize this arachidonate, suggests that these phospholipids may be important sources of arachidonate for eicosanoid biosynthesis. However, other than the fact that arachidonate is released, there has been no direct evidence to indicate that these or other phospholipids are unique sources of arachidonate which enter the lipoxygenase pathway.

The phospholipid sources of arachidonic acid for eicosanoids have typically been identified by studies which measure the depletion of labelled or unlabelled arachidonic acid during cell activation. It is often assumed in these experiments that loss of arachidonate label or mass from a phospholipid correlates with that phospholipid being a source of arachidonate for eicosanoid production. However, there are at least two reasons why the interpretation of such experiments is suspect. (1) We have recently demonstrated that, on a mass basis, the two major eicosanoid products released by the neutrophil at 8 min, namely $LTB_4$ and 20-OH-$LTB_4$, accounted for only a small percentage of the loss of arachidonate from all phospholipids during neutrophil activation with ionophore A23187 [8]. Consequently, it is difficult in the human neutrophil to determine which phospholipid source(s) provides arachidonic acid that ultimately forms eicosanoids by measuring arachidonate depletion alone. The fact that only a small portion of released arachidonate forms leukotrienes raises intriguing questions as to whether there are specific phospholipids which are closely linked to leukotriene biosynthesis. (2) Another major problem which has

Abbreviations used: PE, ethanolamine-linked phospholipids; PC, choline-linked phospholipids; PI, inositol-linked phospholipids; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; GPI, *sn*-glycero-3-phosphoinositol; $LTB_4$, leukotriene $B_4$; 20-OH-$LTB_4$, 20-hydroxyleukotriene $B_4$; HBSS, Hank's Balanced Salt Solution; HSA, fatty-acid-free human serum albumin; LQ, leukotriene quotient (defined in the text).

hampered the interpretation of isotope-labelling experiments with arachidonic acid is the fact that exogenously added arachidonic acid is distributed into phospholipid molecular species differently from the endogenous arachidonate [13–16]. For example, in the neutrophil, most labelled arachidonic acid is taken up into 1,2-diacyl molecular species, whereas the largest pools of unlabelled arachidonate are found in 1-alkyl- and 1-alk-1-enyl molecular species. Resulting differences in specific radioactivity have made it very difficult to determine the contribution of arachidonate from a particular phospholipid during neutrophil activation when measuring label alone.

In order to define better the sources of arachidonate which give rise to leukotrienes, experiments presented here have taken advantage of the differential uptake of tritiated arachidonic acid by phospholipid subclasses to label all the major phospholipid subclasses of the neutrophil to different specific radioactivities. Subsequently, the cells have been activated and the specific radioactivity of arachidonate in phospholipid subclasses as well as the eicosanoid produced during cell activation has been determined. By measuring endogenous arachidonate found in cellular phospholipids after cell activation, it has been determined whether there is selective depletion of a potential phospholipid precursor. In addition, the rate of turnover of individual phospholipid subclasses has been gauged during cell activation by observing the changes in specific activities of the various phospholipids. However, the major advantage of this technique is that it has allowed us to begin to define sources of arachidonate for leukotriene biosynthesis in the human neutrophil by comparing the specific radioactivity of arachidonate in potential phospholipid precursors with that of the leukotrienes produced during cell activation.

## EXPERIMENTAL

### Materials

[5,6,8,9,11,12,14,15-$^3$H]Arachidonic acid (83 Ci/mmol) was purchased from New England Nuclear Corp. All solvents were h.p.l.c. grade from Fisher. Lipid standards used in t.l.c. were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Essential fatty-acid-free human serum albumin (HSA) was purchased from Sigma. Hank's Balanced Salt Solution (HBSS) was purchased from GIBCO. *N*-(t-Butyldimethylsilyl)-*N*-methyltrifluoroacetamide for making t-butyldimethylsilyl derivative was purchased from Aldrich. [$^2H_8$]-Arachidonic acid was generously given by Dr. Howard Sprecher (Ohio State University, Columbus, OH, U.S.A.).

### Preparation of human neutrophils

Human neutrophils were prepared from venous blood obtained from healthy human donors immediately before the neutrophil isolation as previously described [17]. After isolation, the neutrophils were adjusted to $5.0 \times 10^7$ cells/ml of HBSS for use in incubations.

### Incorporation of [$^3$H]arachidonic acid into neutrophil glycerolipids

Neutrophil glycerolipids were labelled with [$^3$H]-arachidonic acid using a strategy which labels the major arachidonate-containing phospholipid subclasses to different specific radioactivities (see the Results section). Under standard conditions, 50 $\mu$l of an aqueous solution containing [$^3$H]arachidonic acid complexed to HSA (5.0 mg/ml) was added to 1.0 ml of cell suspension ($5.0 \times 10^7$ neutrophils), bringing the final concentration of arachidonic acid to 0.02 $\mu$M. The incubation tube was shaken gently at 37 °C for 5 min. The reaction was terminated by the addition of ice-cold HBSS (5.0 ml) containing HSA (0.25 mg/ml). The cells were removed from the supernatant fluid by centrifugation (225 ***g***, 8 min). The cells were washed twice with HBSS containing HSA (0.25 mg/ml). The reaction mixture was then incubated at 37 °C for an additional 15 min to assure that all free [$^3$H]arachidonic acid had been esterified into complex lipids.

### Cell activation

Cells ($5.0 \times 10^7$) prepared in this manner were then suspended in 10 ml of HBSS and challenged with no stimulatory agent or with A23187 (2 $\mu$M) at 37 °C for various periods of time. All incubations were terminated by lowering the pH of the reaction mixture with 9% (v/v) formic acid. This was immediately followed by centrifugation (225 ***g*** for 8 min) at 4 °C. The cell supernatant fluid was then removed and extracted twice with ethyl acetate. The lipids were extracted from the cell pellet by the method of Bligh & Dyer [19].

### Chromatography of arachidonate-containing glycerolipids in the cell pellet

Glycerolipid classes were separated by normal-phase h.p.l.c. (h.p.l.c. system I) using an Ultrasphere-Si column (4.6 mm × 250 mm; Rainin Instrument Co., Woburn, MA, U.S.A.) eluted with propan-2-ol/25 mM-phosphate buffer (pH 7.0)/hexane/ethanol/acetic acid 2450:150:1835:500:3, by vol.) at 1.0 ml/min for 5 min. After 5 min the solvent was changed to propan-2-ol/25 mM-phosphate buffer (pH 7.0)/hexane/ethanol/acetic acid (2450:250:1835:500:3, by vol.) [18] A portion of the isolated phospholipids was then prepared for g.c.–m.s. analysis. Neutral lipids were separated by t.l.c. (t.l.c. system I) on layers of silica-gel G developed in hexane/diethyl ether/formic acid (15:10:1, by vol.). Purified choline and ethanolamine phospholipids were further separated into 1-acyl, 1-alkyl and 1-alk-1-enyl subclasses as described by Nakagawa *et al.* [20]. Briefly, these phospholipids were hydrolysed to diradylacylglycerols with phospholipase C (from *Bacillus cereus*) in 100 mM-Tris/HCl buffer, pH 7.4. The diradylacylglycerols were then extracted and converted into 1,2-diradyl-3-acetylglycerols with acetic anhydride and pyridine for 3 h at 37 °C. The 1,2-diradyl-3-acetylglycerols were then separated into 1-acyl, 1-alkyl and 1-alk-1-enyl subclasses by t.l.c. (t.l.c. system II) on layers of silica-gel G (Analtech) developed in light petroleum (b.p. 35–57 °C)/diethyl ether/acetic acid (90:10:1, by vol.), followed by 100% toluene. Each of the separate subclasses was then extracted from the silica gel.

### Specific-radioactivity analysis of arachidonate in phospholipid subclasses

Phospholipid classes and subclasses were separated by the above procedures. The amount of label in each of the glycerolipids was determined by liquid-scintillation counting (automatic external standard for quench correction). The molar quantity of unlabelled arachidonic acid was determined by hydrolysing the glycerolipids

with 2 M-KOH in methanol/water (3:1, v/v) for 30 min at 60 °C to liberate esterified arachidonic acid. [$^2H_8$]Arachidonic acid (250 ng) as an internal standard was added to the reaction mixture. After 30 min, additional water was added and the pH was adjusted to 3.0 with 6 M-HCl. The free arachidonic acid was extracted with hexane. The arachidonic acid was then converted into the t-butyldimethylsilyl ester as described previously [21]. The solvents were removed from the sample with a stream of $N_2$ and the sample suspended in hexane. The t-butyldimethylsilyl arachidonate was analysed by g.c.–m.s. using selected ion-monitoring techniques to monitor the M-57 ion at $m/z$ 361 and the t-butyldimethylsilyl [$^2H_8$]arachidonate M-57 ion at $m/z$ 369. G.c.–m.s. was carried out on an HP selective mass detection system (HP 5790). G.c. was performed using a cross-linked methylsilicone column. This column was threaded into the mass-spectrometric ion source. The initial column temperature was 80 °C and programmed at 220 °C at 30 °C/min. At that point, the temperature was increased 10 °C/min to 260 °C. The injector temperature was 250 °C, and the g.c.–m.s. interface line was at 280 °C. Helium was used as a carrier gas, and 1–2 $\mu$l of the samples was injected in the splitless mode. Specific radioactivities of each arachidonate-containing subclass were determined from the label and mass measurements and expressed as nCi/nmol.

### Specific radioactivity analysis of leukotrienes in the cell supernatant fluid

Leukotrienes were isolated from the ethyl acetate extraction mixture by reverse-phase h.p.l.c. using an Ultrasphere-ODS column (2.0 mm × 250 mm) eluted with methanol/water/$H_3PO_4$ (2750:2250:1, by vol.), pH 5.7, at 0.25 ml/min. After 5 min, the leukotrienes were eluted from the column by increasing the methanol composition from 55 to 100% over 15 min. The eluate was collected, and 10% of each 1 min fraction was counted for radioactivity in order to determine the distribution of $LTB_4$ and 20-OH-$LTB_4$. Both leukotrienes were collected and the mass quantity of each was determined by their u.v. absorbance at 270 nm [22]. The amount of label in each of the leukotrienes was determined by liquid-scintillation counting. Specific radioactivities were determined from the label and mass measurements and expressed as nCi/nmol.

## RESULTS

### Neutrophil-labelling strategies

Initial studies were done to establish a labelling strategy in which the specific radioactivities of the major arachidonate-containing subclasses were different with respect to each other. This was necessary in order to correlate the specific activities of leukotrienes produced by the neutrophil and endogenous arachidonate within the phospholipids of the neutrophil. Previous experiments have demonstrated that neutrophils differentially incorporate exogenous arachidonic acid into phospholipid subclasses. For example, whereas exogenous arachidonate was preferentially incorporated into 1,2-diacyl molecular species, the largest pools of endogenous arachidonate were contained in 1-alkyl and 1-alk-1-enyl pools. In experiments presented here, we took advantage of the differential labelling of different phospholipid subclasses (1-acyl, 1-alkyl, 1-alk-1-enyl) in order to achieve large specific-radioactivity differences among them. Neutrophils were labelled for 5 min with [$^3$H]-arachidonic acid as described in the Experimental section. An additional 15 min incubation after the wash was found to be necessary in order to bind all free [$^3$H]arachidonic acid into complex lipids. The specific radioactivity of arachidonate in the major phospholipid classes and subclasses is shown in Table 1. By using this labelling strategy, the specific radioactivities of PC and PI as classes were similar and ranged from 40 to 50 nCi/nmol. By contrast, the specific radioactivity of PE (5.0 nCi/nmol) was 10-fold less than that seen for PC or PI. In these experiments, PE, PC and PI were focused on because they contain > 95% of the arachidonate in the resting neutrophil. The incorporation of exogenous arachidonic acid into phospholipids was not a reflection of the size of arachidonate in their pools, since PE contained the largest mass quantity of arachidonate and incorporated only small quantities of exogenous arachidonic acid. The specific radioactivity was also determined in the subclasses (1-acyl, 1-alkyl and 1-alk-1-enyl) of PC and PE. The specific radioactivity in all diacyl molecular species was high relative to that of alkylacyl or alk-1-enylacyl molecular species of PC and PE. In contrast with PE and PC, PI contained only 1-acyl-linked molecular species. As indicated in Table 1, this particular labelling strategy provided differential

**Table 1. Specific activity of arachidonate in glycerolipid classes and subclasses of human neutrophil**

Neutrophils ($50 \times 10^6$) were pulse-labelled for 5 min, washed twice, and incubated for an additional 15 min as described in the text. Incubations were terminated by extracting the lipids from the cell pellet, and the amount of label and mass in each arachidonate-containing glycerolipid was determined as described in the text. Specific radioactivities of each glycerolipid were determined from these two measurements and are expressed as nCi/nmol. 1-Acyl-, 1-alkyl- and 1-alk-1-enyl- represent an acyl, alkyl or alk-1-enyl linkage respectively at the *sn*-1 position of the arachidonate-containing phospholipid. These data are the averages for four separate experiments.

| Glycero-lipid class | Specific radioactivity (nCi/nmol) | | | | |
|---|---|---|---|---|---|
| | Overall | Subclass... | 1-Acyl | 1-Alkyl | 1-Alk-1-enyl |
| PE | 5.0±0.7 | | 10.7±3.6 | 6.4±2.4 | 3.5±0.3 |
| PC | 45.5±6.0 | | 125.2±50.4 | 29.7±2.8 | – |
| PI | 51.5±5.3 | | 51.5±5.3 | – | – |

labelling of all of the major arachidonate-containing subclasses to different specific radioactivities, thereby allowing us to perform the precursor–product studies described. Moreover, previous studies [13] using this labelling strategy revealed that the individual molecular species which make up a particular phospholipid subclass all have similar specific radioactivities. This indicated that the *sn*-1 chain linkage and not the length or degree of unsaturation of the fatty acyl chain at that position was the major factor which contributed to the differential labelling. Therefore the specific radioactivity of arachidonate in each phospholipid subclass was representative of all individual molecular species of that subclass and not just an average of several molecular species with large specific-radioactivity differences. Other labelling strategies, which involved incubating the cells with arachidonate for longer periods of time, were also performed (results not shown). However, the specific radioactivities of 1-acyl-2-arachidonoyl-GPC, 1-alkyl-2-arachidonoyl-GPC and 1-acyl-2-arachidonoyl-GPI became more uniform as a function of incubation time, and therefore this technique was not used for precursor–product studies.

### Loss of endogenous arachidonate from phospholipid subclasses upon challenge with ionophore A23187

Experiments were initially done to determine which arachidonate-containing phospholipid subclasses were broken down most rapidly during cell activation. The mass quantity of arachidonate in each phospholipid subclass was determined in cells which had received no stimulus or ionophore A23187 for 4 min (Fig. 1). A 4 min period was chosen here because most of the $LTB_4$ and 20-OH-$LTB_4$ had been produced by this time point. In the resting neutrophil, the predominant pools of endogenous arachidonate were found in 1-alk-1-enyl-2-arachidonoyl-GPE (45%), 1-alkyl-2-arachidonoyl-GPC (15.0%) and 1-acyl-2-arachidonoyl-GPI (12.0%). All major arachidonate-containing phospholipid subclasses were broken down during cell activation with ionophore. This reflects the non-specific nature by which ionophore stimulates the neutrophil. On a molar basis, the rank order of breakdown was: 1-alk-1-enyl-2-arachidonoyl-GPE > 1-alkyl-2-arachidonoyl-GPC > 1-acyl-2-arachidonoyl-GPI. Leukotrienes released into the supernatant fluid accounted for 10–35% of the loss of arachidonate from phosphoacylglycerol subclasses. We have recently reported a more complete analysis of the kinetic of release and the profile of products [8].

### Specific radioactivities of phospholipid subclasses and eicosanoids in neutrophils stimulated with ionophore A23187

Neutrophils prelabelled with arachidonic acid as described above were next incubated at 37 °C with ionophore A23187 or no stimulus for up to 8 min. The specific radioactivities of [$^3$H]arachidonate was determined in the diacyl, alkylacyl and alk-1-enylacyl fractions of PC and PE and compared with the specific radioactivities of $LTB_4$ and 20-OH-$LTB_4$ at 2, 4 and 8 min after cell activation. Fig. 2 shows the specific radioactivities of cell-associated PC subclasses and the leukotrienes released into the supernatant fluid during cell activation. As previously discussed (Table 1), the

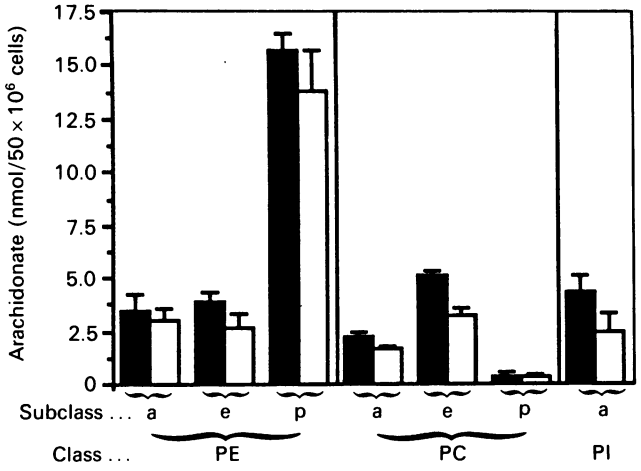


**Fig. 1. Loss of endogenous arachidonate from phospholipid subclasses upon challenge with ionophore A23187**

Neutrophils were incubated for 4 min with ionophore A23187 (2 $\mu$M) or no stimulus. Lipids were extracted from the cell pellet, and glycerolipid classes were separated by normal-phase h.p.l.c. PE and PC were further separated as diacylglycerol acetates into 1-acyl, 1-alkyl and 1-alk-1-enyl subclasses by t.l.c. The nanomolar quantities of arachidonate in 1-acyl (a), 1-alkyl (e) and 1-alk-1-enyl (p) subclasses were determined by g.c.–m.s. ■, Quantity of arachidonate in an individual subclass after a 4 min incubation with no stimulus; □, quantity of arachidonate in an individual subclass after incubation with ionophore A23187 for 4 min. These data are averages for four experiments (error bars represent ± S.E.M.)

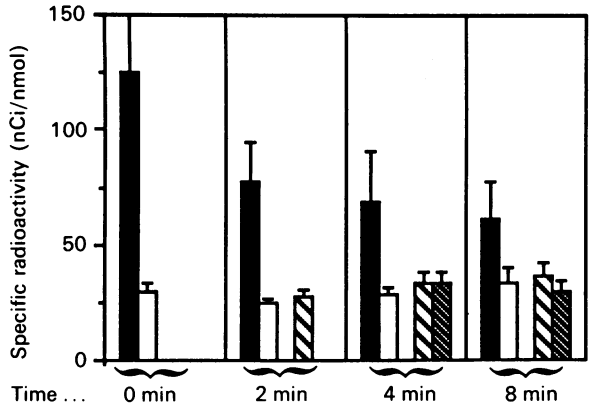


**Fig. 2. Specific radioactivities of cell-associated PC subclasses and the leukotrienes released into the supernatant during cell activation**

Neutrophils were labelled and activated with A23187 as described in the Experimental section. The specific radioactivities of arachidonate in 1-acyl- and 1-alkyl-2-arachidonoyl-GPC as well as $LTB_4$ and 20-OH-$LTB_4$ were determined at various periods of time after ionophore stimulation as described in the Experimental section. ■ and □ represent the specific radioactivities of arachidonate in 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC respectively. ▨ and ▨ represent the specific activity of $LTB_4$ and 20-OH-L respectively. There is no value for the specific radioactivity of 20-OH-$LTB_4$ at 2 min owing to the fact that only small quantities of this leukotriene were produced at this time point.

specific radioactivities of [$^3$H]arachidonate within diacyl molecular species were higher than those of 1-alkylacyl or 1-alk-1-enylacyl molecular species of PC before cell activation (0 min). After stimulation, the specific radioactivity of [$^3$H]arachidonate in 1-acyl-2-arachidonoyl-GPC decreased rapidly from 125 nCi to 61 nCi/nmol after 8 min of A23187 stimulation. The specific radioactivity of [$^3$H]arachidonate in 1-alkyl-2-arachidonoyl-GPC was 30 nCi/nmol in the resting neutrophil and remained constant between 26 and 30 nCi/nmol during cell activation. 1-Alk-1-enyl molecular species of PC contain very small quantities of arachidonate (< 4% of arachidonate in PC) relative to the large pools of arachidonate found in 1-acyl and 1-alkyl pools. Consequently, the specific radioactivity of this PC subclass has not been included in Fig. 2. After stimulation with ionophore A23187, both $LTB_4$ and 20-OH-$LTB_4$ were produced within 2 min. The specific radioactivity of $LTB_4$ and 20-OH-$LTB_4$ increased slightly from 28 and 36 nCi/nmol during cell activation. This correlated closely with the specific radioactivity of 1-alkyl-2-arachidonoyl-GPC at all time points which were tested.

The specific radioactivities of [$^3$H]arachidonic acid were also measured in the three major subclasses of cell-associated PE during cell activation (Fig. 3). All major PE subclasses, 1-acyl, 1-alkyl and 1-alk-1-enyl, were initially low (11, 6 and 3 nCi/nmol respectively) in comparison with PC, and remained low throughout the 8 min incubation. As observed with the 1-acyl-linked molecular series of PC, the specific radioactivity of [$^3$H]-arachidonate in 1-acyl-2-arachidonoyl-GPE was high relative to 1-alkyl- and 1-alk-1-enyl-linked species. The specific radioactivity of [$^3$H]arachidonate in 1-alkyl- and 1-alk-1-enyl molecular species of PE was similar, and both remained constant between 4 nCi and 7 nCi/nmol throughout the 8 min cell activation. All the major subclasses of PE contained [$^3$H]arachidonate at a specific radioactivity that was initially low relative to the leukotrienes and remained low throughout the 8 min stimulation.

Unlike PC and PE, PI contains no 1-alkyl- or 1-alk-1-enyl-linked molecular species [13]. In fact, the major store of arachidonate in PI is found in one phospholipid, namely 1-stearoyl-2-arachidonoyl-GPI, in the human neutrophil. Consequently, the specific radioactivity of PI shown in Fig. 4 represents only [$^3$H]-arachidonate in 1-acyl-linked PI molecular species. By comparison with the leukotrienes, the specific radioactivity of 1-acyl-2-arachidonoyl-GPI was initially higher and decreased to the level of the leukotrienes only after 8 min of stimulation.

### Leukotriene quotient (LQ) for each phospholipid subclass

Previous studies have demonstrated that more than 60 and 85% of leukotrienes are produced within the first 2 min and 4 min respectively after neutrophil activation. Only a small portion of the leukotriene is produced between 4 and 8 min. Therefore the predominant pools of arachidonate which give rise to leukotriene production are mobilized within the first 4 min of cell activation. Consequently, the specific radioactivities of the phospholipids within the first 4 min are likely to provide the most important formation to precursor–product studies, since arachidonic acid which forms eicosanoids is mobilized during these early time points. In order to introduce this factor into the present precursor–product studies, an LQ was calculated for $LTB_4$ and phospholipid

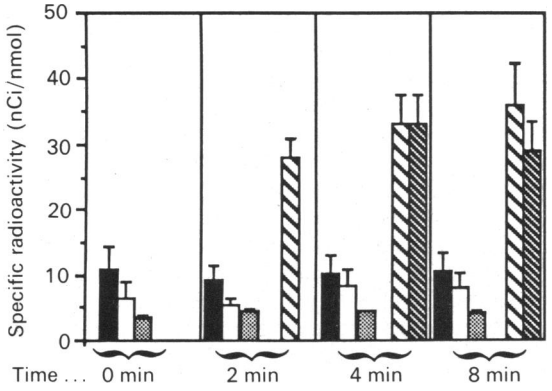


**Fig. 3. Specific radioactivities of [$^3$H]arachidonic acid in the three major subclasses of cell-associated PE during cell activation**

Neutrophils were labelled and activated with ionophore A23187 as described in the Experimental section. The specific radioactivities of arachidonate in 1-acyl, 1-alkyl and 1-alk-1-enyl-2-arachidonoyl-GPE as well as $LTB_4$ and 20-OH-$LTB_4$ were determined at various periods of time after ionophore stimulation. ■, □ and ▨ represent the specific radioactivity of arachidonate in 1-acyl-, 1-alkyl- and 1-alk-1-enyl-2-arachidonoyl-GPE respectively. ▧ and ▧ represent the specific radioactivity of $LTB_4$ and 20-OH-$LTB_4$ respectively. There is no value for the specific radioactivity of 20-OH-$LTB_4$ at 2 min owing to the fact that only small quantities of this leukotriene were produced at this time point.

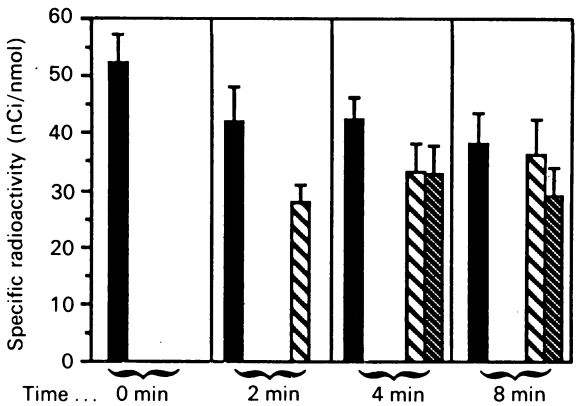


**Fig. 4. Specific radioactivities of arachidonate in 1-acyl-2-arachidonoyl-GPI, $LTB_4$ and 20-OH-$LTB_4$ during cell activation**

Neutrophils were labelled and activated with ionophore A23187 as described in the Experimental section. The specific activities of arachidonate in 1-acyl-2-arachidonoyl-GPI as well as of $LTB_4$ and 20-OH-$LTB_4$ were determined at various times after ionophore stimulation. ■ represents the specific radioactivity in 1-acyl-2-arachidonoyl-GPI. ▧ and ▧ represent the specific radioactivity of $LTB_4$ and 20-OH-$LTB_4$ respectively. There is no value for the specific radioactivity of 20-OH-$LTB_4$ at 2 min owing to the fact that only small quantities of this leukotriene were produced at this time point.

**Table 2. LQ for the various phospholipid subclasses**

Neutrophils were labelled and stimulated with A23187 as described in the Experimental section. The specific radioactivities of each phospholipid subclass as well as that of $LTB_4$ was determined at various time points after ionophore stimulation. The leukotriene quotient (LQ) for each phospholipid subclass at 2 min and 4 min was determined by using the following equations:

$$LQ_{2\,min} = \frac{(PI_{0\,min} + PI_{2\,min})/2}{LTB_{4,2\,min}}$$

and

$$LQ_{4\,min} = \frac{(PI_{0\,min} + PI_{2\,min} + PI_{4\,min})/3}{LTB_{4,4\,min}}$$

The average specific radioactivities of phospholipid subclasses and leukotrienes at the various time points used to derive the LQs are shown in Figs. 2, 3 and 4. These data are the averages ± S.E.M. for four separate experiments.

| | LQ | |
|---|---|---|
| Phospholipid subclass | 2 min | 4 min |
| 1-Acyl-2-arachidonoyl-GPE | 0.40±0.06 | 0.33±0.04 |
| 1-Alkyl-2-arachidonoyl-GPE | 0.22±0.03 | 0.20±0.01 |
| 1-Alk-1-enyl-2-arachidonoyl-GPE | 0.15±0.03 | 0.15±0.03 |
| 1-Acyl-2-arachidonoyl-GPI | 1.6±0.18 | 1.5±0.17 |
| 1-Acyl-2-arachidonoyl-GPC | 4.0±1.3 | 3.1±1.1 |
| 1-Alkyl-2-arachidonoyl-GPC | 1.1±0.19 | 0.98±0.12 |

subclasses at 2 min and 4 min after cell activation (Table 2). For example, this quotient for 2 min is the average specific radioactivity of a phospholipid subclass at 0 and 2 min divided by the specific radioactivity of $LTB_4$ at 2 min. The formulas for these quotients are given in Table 2. An $LTB_4$ quotient of 1.0 would indicate identity between the average specific radioactivity of a given phospholipid subclass and that of $LTB_4$. An additional advantage to calculating this quotient is that it takes into account the variation in the initial labelling of neutrophils from experiment to experiment. For example, the specific radioactivity of $LTB_4$ in an individual experiment was always used as the denominator for the specific radioactivity of the phospholipid subclasses from that same experiment.

The $LTB_4$ quotient for the six major arachidonate-containing subclasses at 2 min and 4 min is shown in Table 2. The ratio of the specific radioactivity of [$^3$H]arachidonate in 1-acyl-2-arachidonoyl-GPC and 1-acyl-2-arachidonoyl-GPI, 3.6 and 1.7 respectively, is greater than 1 at both 2 min and 4 min. In contrast, LQ value for all PE molecular species was 3–6-fold below 1.0 at both time points. Finally, 1-*O*-alkyl-2-arachidonoyl-GPC had an LQ value of 1.1 and 1.0. Only in the case of 1-*O*-alkyl-2-arachidonoyl-GPC did the specific radioactivity of the phospholipid subclass match that of the produced $LTB_4$. LQs were also calculated using 20-OH-$LTB_4$ as the denominator. These values were similar to those observed for $LTB_4$ (results not shown).

## DISCUSSION

The present data, along with recent work [8], indicate that the human neutrophil has the potential to mobilize arachidonate from several major phospholipid subclasses during cell activation (1-alk-1-enyl-2-arachidonoyl-GPE > 1-alkyl-2-arachidonoyl-GPC > 1-acyl-2-arachidonoyl-GPI). However, released $LTB_4$ and 20-OH-$LTB_4$ account for only 10–35% of the arachidonate mobilized from phospholipids. Because the released leukotrienes represent, by mass, such a small proportion of the total arachidonate lost from all phospholipids, this type of mass data does not allow one to determine which, if any, of the potential phospholipid subclasses is playing a dominant role in providing arachidonate for leukotriene biosynthesis. In order to get a better idea of which phospholipids contribute arachidonate to the leukotrienes, the present study has examined the specific radioactivity of [$^3$H]arachidonate in cellular phospholipids and released leukotrienes at different times after cell activation. Initial studies demonstrated that the specific radioactivity of phospholipid classes such as PC and PE resulted from a wide range of specific radioactivities representing the respective subclasses of each class. Therefore the specific radioactivity of arachidonate in the six major arachidonate-containing subclasses of the human neutrophil was compared with that of the leukotrienes obtained during neutrophil activation. Of the six subclasses, the specific radioactivity of 1-alkyl-2-arachidonoyl-GPC matched that of the produced $LTB_4$ (i.e. LQs of about 1.0). However, it is important to point out that this phospholipid subclass has a specific radioactivity which is intermediate between those of the other major phospholipid subclasses. Consequently, it is possible that there are multiple phosphoacylglycerols which contribute arachidonate to leukotriene production and the average specific radioactivity of these phospholipids is reflected in the specific radioactivity of $LTB_4$ and 20-OH-$LTB_4$. In any case, the specific radioactivities of $LTB_4$ and 20-OH-$LTB_4$ are low relative to 1-acyl-2-arachidonoyl-GPC and -GPI. This indicates that the 1-ether-linked phosphoacylglycerols must contribute a significant amount of the arachidonate that gives rise to the leukotrienes, since the specific radioactivities of leukotrienes are well below those of these 1-acyl-linked phospholipids. It would be interesting to determine if a more physiological stimulus released arachidonate selectively from a given phospholipid and if the specific radioactivities of leukotrienes produced by these neutrophils correlated with an individual phospholipid precursor. However, relatively large quantities of leukotrienes are needed to get accurate specific-radioactivity measurements in these experiments. Consequently, it has been difficult to find such a stimulus in the human neutrophil. In any event, the present experiments reveal that the human neutrophil has the capacity to use the large stores of arachidonate in 1-ether-linked phospholipids for leukotriene biosynthesis. Whether this arachidonate comes from only one 1-ether-linked subclass or a mixture of 1-acyl- and 1-ether-linked subclasses is a question which requires further exploration with a variety of inflammatory cells.

In addition to these precursor–product findings, these studies reveal potentially important information regarding arachidonate–phospholipid turnover during cell activation. For example, the specific radioactivity of arachidonate in all 1-acyl-linked phospholipids (particularly 1-acyl-2-arachidonoyl-GPC) decreased as a function of time after cell activation with ionophore A23187. This decrease in the specific radioactivity of 1-

acyl-linked phospholipids is most likely the result of uptake of free arachidonic acid which has been liberated from a low-specific-activity pool during cell activation. Previous studies have demonstrated that free arachidonic acid is preferentially incorporated into 1-acyl-linked phospholipids during cell activation [23]. A strong candidate for the source of free arachidonic acid, which might dilute the specific radioactivities of [$^3$H]-arachidonate in these 1-acyl-linked phospholipids, is 1-alk-1-enyl-2-arachidonoyl-GPE. This PE subclass contains the largest endogenous pool of arachidonate and the lowest-specific-radioactivity pool of [$^3$H]-arachidonate in the human neutrophil. Moreover, this phospholipid subclass, on a molar basis, loses more arachidonate than does any other phospholipid subclass during cell activation. There are a number of potential explanations for this increased acylation during cell activation. For example, this rapid reacylation of 1-acyl-linked phospholipid may inhibit free arachidonic acid from entering eicosanoid biosynthesis, thereby serving as a control point for the formation of inflammatory mediators. Alternatively, increased acylation of 1-acyl-linked phospholipids with arachidonate from large pools may serve to replenish 1-acyl-linked phospholipids with arachidonate during cell activation.

Results from the present study further suggest a direct biochemical relationship in which platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) and $LTB_4$ share a common biochemical pathway in the human neutrophil [9,24,25]. In the biosynthetic pathway for both mediators, 1-*O*-alkyl-2-lyso-GPC and free arachidonic acid may be mobilized from 1-*O*-alkyl-2-arachidonoyl-GPC during activation by $Ca^{2+}$. Previous studies have demonstrated that 1-*O*-alkyl-2-lyso-GPC derived from 1-alkyl-2-arachidonoyl-GPC is acted upon by an acetyltransferase to form platelet-activating factor. The present study provides evidence that arachidonic acid which is liberated from 1-*O*-alkyl-2-arachidonoyl-GPC may be utilized by the 5-lipoxygenase for leukotriene biosynthesis. Because of the complexity of arachidonate-containing phospholipids, further studies will be necessary to test how tightly the production of platelet-activating factor and eicosanoids are coupled.

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