

Stimulus-Specific Induction of Phospholipid and Arachidonic Acid Metabolism in Human Neutrophils

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Abstract. Phospholipid remodeling resulting in arachidonic acid (AA) release and metabolism in human neutrophils stimulated by calcium ionophore A23187 has been extensively studied, while data obtained using physiologically relevant stimuli is limited. Opsonized zymosan and immune complexes induced stimulus-specific alterations in lipid metabolism that were different from those induced by A23187. [^3H]AA release correlated with activation of phospholipase A_2 (PLA_2) but not with cellular activation as indicated by superoxide generation. The latter correlated more with calcium-dependent phospholipase C (PLC) activation and elevation of cellular diacylglycerol (DAG) levels. When cells that had been allowed to incorporate [^3H]AA were stimulated with A23187, large amounts of labeled AA was released, most of which was metabolized to 5-HETE and leukotriene B_4 . Stimulation with immune complexes also resulted in the release of [^3H]AA but this released radiolabeled AA was not metabolized. In contrast, stimulation with opsonized zymosan induced no detectable release of [^3H]AA.

Analysis of [^3H]AA-labeled lipids in resting cells indicated that the greatest amount of label was incorporated into the phosphatidylinositol (PI) pool, followed closely by phosphatidylcholine and phosphatidylserine, while little [^3H]AA was detected in the phosphatidylethanolamine pool. During stimulation with A23187, a significant decrease in labeled PI occurred and labeled free fatty acid in the pellet increased. With immune complexes, only a small decrease was seen in labeled PI while the free fatty acid in the pellets was unchanged. In contrast, opsonized zymosan decreased labeled PI, and increased labeled DAG. Phospholipase activity in homogenates from human neutrophils was also assayed. A23187 and immune complexes, but not zymosan, significantly enhanced PLA_2 activity in the cell homogenates. On the other hand, PLC activity was enhanced by zymosan and immune complexes. Stimulated increases in PLC activity correlated with enhanced superoxide generation induced by the stimulus.

ACTIVATION of neutrophils is intimately linked to changes in phospholipid metabolism (40, 43, 44). Released arachidonic acid (AA)¹ from membrane phospholipids (PLs) provides a substrate for the formation of bioactive lipids that can modulate cell responses (4, 33, 34). Additionally, nonmetabolized AA has been shown to activate the neutrophil NADPH oxidase (2, 15). It is also possible that phospholipid remodeling resulting in the release of AA and/or the formation of lysophospholipids may be a method of altering cell membrane fluidity during signal transduction (36). Crucial cell functions (i.e., phagocytosis, carrier-mediated transport, membrane-bound enzymes) can be altered by fatty acid enrichment (21, 22, 31, 39).

1. *Abbreviations used in this paper:* AA, arachidonic acid; DAG, diacylglycerol; LTB_4 , leukotriene B_4 ; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLA_2 , phospholipase A_2 ; PLase, phospholipase; PLC, phospholipase C; PS, phosphatidylserine.

The mechanism of AA release, as well as the characterization of the lipid pool from which it originates, has been examined in several cell types (26, 29). Previous investigators have demonstrated enhanced membrane phospholipase A_2 (PLA_2) activity in neutrophil membrane preparations stimulated with FMLP (9). AA release via PLA_2 was also demonstrated in calcium ionophore-stimulated cells (40, 43, 44) and suggested as a mechanism during activation by opsonized zymosan (43, 44). Release of AA via action of a phospholipase C (PLC) in conjunction with a diglyceride lipase (28), or by a combination of PLC-diglyceride kinase- PLA_2 , has also been suggested as possible mechanisms for AA release (5). Experiments by Takenawa et al. (40) present evidence that the latter mechanism is responsible for AA release in FMLP-stimulated neutrophils, while the ionophore enhanced PLA_2 exclusively. This is supported by other investigators who show that FMLP stimulates PLC activity in neutrophils (11). Studies with [^3H]AA-labeled neutrophils indicate that phosphatidylinositol (PI) and phosphatidylcho-

line (PC) are the PL pools containing the majority of the label and that these pools are depleted during stimulation (40, 43, 44). Alternatively, studies by Okajima and Ui (20) show loss of [³H]AA label from PC, phosphatidylethanolamine (PE), and PI during FMLP stimulation of radiolabeled guinea pig neutrophils. The mechanisms of neutrophil AA release and the pool from which it is derived is even less well established for other physiologic stimuli.

Conversion of AA by neutrophils primarily results in the formation of products via the 5-lipoxygenase pathway (8). Key products of this pathway are 5-HETE and leukotriene B₄ (LTB₄). These lipid metabolites are potent chemotactic and activation agents for polymorphonuclear leukocytes (35), as well as regulators of enzyme function (4). Generation of substantial amounts of these lipoxygenase products has been elicited from neutrophils by A23187 (12, 44). In contrast, the amount of product formed in response to physiological stimuli is exceedingly small (45).

Because A23187 is an ionophore, not a physiologic stimulus, it is possible that data obtained with this are not physiologically relevant and possibly confuse the issue of AA metabolism. In the present study, we have examined neutrophils to further characterize the mechanisms of AA release (using [³H]AA) and determine from which pool the AA is derived. To do this we used phagocytic stimuli for the study of both PL and AA metabolism.

Materials and Methods

Superoxide Generation (O₂⁻) from Stimulated Human Neutrophils

Superoxide anion generation was determined by means of the superoxide dismutase-inhibitable reduction of cytochrome c as described by Babior et al. (1), modified for use in a microelisa reader (model MR600; Dynatech Corp., Alexandria, VA) (18). Reduced cytochrome c was determined using an extinction coefficient for reduced-oxidized cytochrome c of 21.1 mM⁻¹ cm⁻¹ (1).

Protein Determination

Protein concentration in cell sonicates was determined by the method of Bradford (10) utilizing reagents supplied by Bio-Rad Laboratories (Richmond, CA). BSA (Miles Laboratories, Inc., Naperville, IL) was used as a standard.

Reagents and Chemicals

All solvents were of HPLC grade and were obtained from Fisher Scientific Co. (Springfield, NJ). Other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Zymosan (Sigma Chemical Co.) was opsonized in 50% human serum for 30 min at 37°C. Immune precipitates were formed by adding excess antigen (CooperBiomedical, Inc., Malvern, PA). Radiochemicals were obtained from either New England Nuclear (Boston, MA) or Amersham Corp. (Arlington Heights, IL).

Isolation and [³H]AA Labeling of Human Neutrophils

Human peripheral blood polymorphonuclear leukocytes were isolated from heparinized blood of adult volunteers by Ficoll-Hypaque density gradient centrifugation, dextran sedimentation, and hypotonic lysis as previously described (47). Neutrophils were labeled by a modification of the method of Lee et al. (24). Briefly, 120–140 million cells were suspended at a concentration of 7.5 × 10⁷ cells/ml in PBS containing 0.1% fatty acid free BSA (Miles Laboratories, Inc.). To this suspension 40–60 μCi of [³H]AA (83.3 Ci/mmol; New England Nuclear) was added and then the cells were incubated for 60 min at 37°C. We chose a radiolabel with high specific activity to minimize the cellular effects that exogenous AA can elicit (2, 15). After

the incubation, the cells were washed three times in PBS containing fatty acid free BSA (0.01%) and resuspended in PBS.

Activation of Neutrophils and Identification of AA Metabolites

Aliquots of 3–4 million [³H]AA-labeled cells (7.5–8.0 × 10⁶/ml) were placed in tubes containing either calcium ionophore A23187 (final concentration 10 μM, for 5 min), precipitated immune complexes (human serum albumin, anti-human serum albumin; 1.5 mg/ml, for 15 min), or opsonized zymosan (1.0 mg/ml, 9 × 10⁷ particles/ml, for 15 min), and incubated at 37°C. After incubation the cells were sedimented (500 g for 10 min), spun down, and assayed for the release of radiolabel. The pellets from these studies were saved for radiolabeled lipid analysis.

A 10-fold greater number of neutrophils (30–40 × 10⁶ at 7.5–8.0 × 10⁶/ml) was used for the experiments assessing the metabolism of [³H]AA. After the designated time period, the cells were pelleted (500 g for 10 min) and the supernatants (4–5 ml) extracted using C₁₈ cartridges (SEP-PAK; Waters Associates, Milford, MA). The cartridges were eluted with 4 ml of methanol and the eluent was then reduced in volume to 200 μl under a stream of nitrogen. Recovery of radiolabel from C₁₈ cartridges was ~90–95% (data not shown).

The methanol extracts were chromatographed using a 5-μm C₁₈ Nova-PAK column (Waters Associates). A flow rate of 1.5 ml/min and a two-solvent system was used. Solvent I (59:41:0.0004; water/acetonitrile/phosphoric acid) was run isocratically for 17 min. After a linear gradient of 15 min, the next portion was run isocratically for 18 min to give a final ratio of 15% solvent I and 85% solvent II (acetonitrile). Absorbance was continuously monitored at 280 and 234 nm using a variable wavelength detector (model 165; Beckman Instruments, Inc., Palo Alto, CA). Absorbance data received at 234 nm was linked to an integrator (model C-RIB; Shimadzu Scientific Instruments, Inc., Columbia, MD) while that, at 280 nm, was processed by the Ramona D radioactivity monitor (INUS, Fairfield, NJ), coupled to an Apple IIe computer.

Analysis of [³H]AA-Labeled Cellular Lipids

Cell pellets from [³H]AA-labeled cells were extracted with 100 μl of a chloroform/methanol solution (2:1) and the lipids identified by TLC. 25-μl aliquots of the organic phase were chromatographed using Silica Gel G (Analtech, Inc., Newark, DE). TLC plates were run using a solvent system previously described (23). A solvent system of chloroform/ethanol/triethylamine/water (30:34:35:4) was used for the separation of major PL classes, while neutral lipids were separated with solvent system of n-heptane/diethylether/acetic acid (75:25:4). Lipid standards were added to the sample to facilitate visualization by iodine vapors. The bands were marked and, after the iodine evaporated, the bands were scraped into vials for scintillation counting. It is possible that the 1-h [³H]AA incorporation was not sufficient to attain equilibrium within the neutrophil phospholipid pools although there is suggestive evidence to the contrary (40). Since neutrophils have a short functional lifespan in vitro, we decided against a longer incorporation time so as not to compromise their ability to respond to the stimuli.

Phospholipase Assays

Phospholipase (PLase) assays were performed as we have previously described (7, 13). Briefly, phospholipid substrates were placed in microcentrifuge tubes to which aliquots of cell homogenates were added and the two incubated together. PLA₂ assays required the use of substrates labeled at the polar head group, while the PLC assays utilized lipids labeled in the sn-2 position with radiolabeled arachidonate. When adding ³H-label in order to have a final substrate concentration of 10 μM in the reaction, it was necessary to add cold lipid (PC, PI, or PE; Sigma Chemical Co.) due to the high specific activity of these compounds. The organic solvent in which the lipids are dissolved was evaporated under nitrogen, the residue was then dissolved in sodium deoxycholate (5 μg/ml, in water) to a final substrate concentration of 50 μM. Cells were pelleted (5,000 g for 2 min), brought up in a solution of protease inhibitors (13), and immediately sonicated with 2-s-bursts maximum intensity (Branson Sonicator; power setting No. 7; Branson Sonic Power Co., Danbury, CT).

The final reaction mixture contained 10 μl of substrate, 20 μl of the cell sonicate, 10 μl 0.1 M Hepes buffer (pH 8.0 for PLC or 0.2 M Tris buffer pH 9.0 for PLA₂), and 10 μl of a 50 mM NaCl/0.4 mM CaCl₂ solution for a final PL concentration of 10 μM. The reaction mixture was begun by the addition of the sonicate and incubated for 1 h at 37°C, and then stopped by

adding 50 μ l of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (2:1), followed by 50 μ l of CHCl_3 , and then 50 μ l of 4 M KCl. The mixture was then centrifuged at 5,000 g for 1 min to separate the aqueous phase from the organic phase.

50- μ l aliquots of the organic layer were spotted on Silica Gel G plates (Analtech, Inc.) and chromatographed in either solvent system A (chloroform/methanol/acetic acid/water; 50:30:8:4) to separate the lysophospholipids from the phospholipids or solvent system B (petroleum ether/diethyl ether/acetic acid; 70:30:1) to separate the neutral lipids from the phospholipids. PLA₂ activity was determined (solvent A) by quantification of the amount of lysophospholipid produced and PLC activity was quantified by the production of labeled diacylglycerol (solvent B).

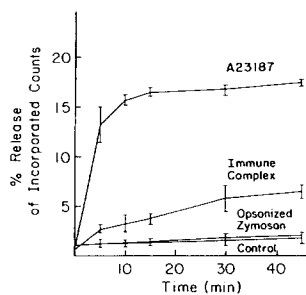
Results

Release of Radiolabel from [³H]AA-Labeled Neutrophils

Aliquots of neutrophils labeled with tritiated AA were stimulated with either calcium ionophore A23187, precipitated immune complexes, or opsonized zymosan, and the appearance of radioactivity in the supernatant was measured at various time intervals up to 45 min (Fig. 1). [³H]AA-labeled neutrophils challenged with ionophore released $15.7 \pm 0.5\%$ (mean + SEM) of incorporated radiolabel (91% of maximum release) within 10 min. Little or no additional [³H]AA was released over the next 35 min. Immune complex stimulation of labeled neutrophils also elicited [³H]AA release, however, unlike the ionophore, a gradual response was noted. Over the course of the experiment, $6.5 \pm 1.3\%$ of the incorporated counts were released but the release was still continuing at 45 min. In marked contrast, opsonized zymosan promoted no significant release of radiolabel. Additional experiments using ionophore-treated cells were performed with fatty acid free BSA in the media to facilitate "trapping" and decrease reacylation of released radiolipids. The results (expressed in %³H released) indicated no significant difference between experiments with BSA (11.8 ± 3.9 , $n = 4$) or those without BSA (11.9 ± 4.3 , $n = 8$).

Metabolism of [³H]AA from Labeled Neutrophils

Differences between activation by physiologic stimuli or the calcium ionophore were not only apparent in the magnitude of [³H]AA release, but extended to [³H]AA metabolism as well. Stimulation of [³H]AA-labeled neutrophils with ionophore resulted in conversion of most of the released radiolabel into 5-lipoxygenase products (Fig. 2). Conversion was primarily to radiolabeled 5-HETE and LTB₄ (along with their isomers and oxidation products). Distribution of radio-



counts expressed as a percentage of the total counts incorporated. Results are expressed as the mean and SD of two experiments, with each data point performed in triplicate.

Figure 1. Time course of [³H]AA release by stimulated human neutrophils. Neutrophils labeled with [³H]AA were exposed to either calcium ionophore A23187 (10 μ M), precipitated immune complexes (1.5 mg/ml), opsonized zymosan (1.0 mg/ml), or PBS. Samples of each supernatant were collected for liquid scintillation counting and released

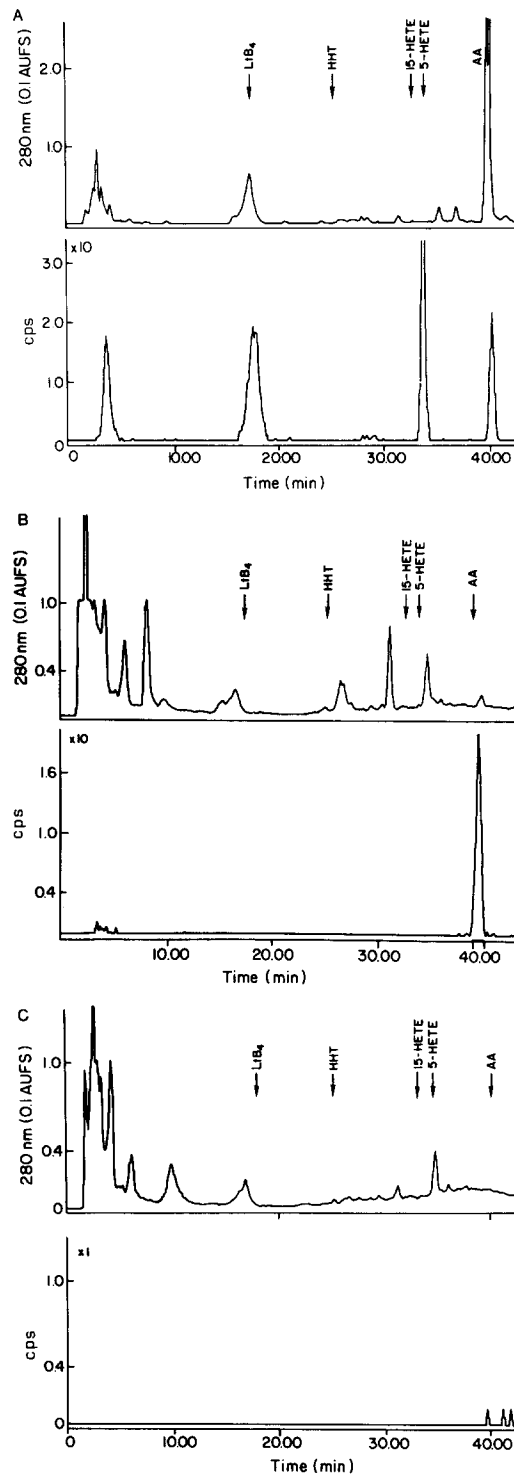


Figure 2. Reverse phase HPLC of supernatants from polymorphonuclear leukocytes activated by ionophore (A), immune complexes (B), or opsonized zymosan (C). Each chromatogram illustrates metabolite release by either UV absorbance at 280 nm (top) or by monitoring radiolabel (bottom). Retention times of synthetic reference standards are indicated by the arrows. Each chromatogram is representative of three experiments.

label was $13 \pm 7\%$ in AA, $27 \pm 5\%$ in 5-HETE, and $43 \pm 18\%$ in LTB₄ (mean \pm SD, $n = 3$). The distribution of radiolabel indicated that the method of incorporation used la-

Table I. Cellular Distribution of [³H]AA-Labeled Lipids in Human Neutrophils

	Total radiolabeled lipids*			
	Control	A23187	Opsonized zymosan	Immune complexes
	%	%	%	%
Triacylglycerol	22.0 ± 1.6	26.2 ± 2.2	24.1 ± 1.9	24.0 ± 1.8
DAG	2.3 ± 0.2	— ‡	4.7 ± 0.5§	3.0 ± 0.3
Monoacylglycerol	0.4 ± 0.1	— ‡	0.3 ± 0.1	0.4 ± 0.1
Free fatty acid	1.2 ± 0.3	6.9 ± 1.6§	2.5 ± 0.7	2.0 ± 0.3
PE	3.0 ± 0.5	3.1 ± 0.7	2.5 ± 0.6	3.4 ± 0.8
PI	58.1 ± 5.3	34.3 ± 6.7§	46.1 ± 6.7§	47.4 ± 6.9§
PS	11.3 ± 4.0	14.2 ± 4.3	8.0 ± 1.4	9.4 ± 2.5
PC	11.2 ± 1.8	9.0 ± 1.2	13.3 ± 1.1	10.2 ± 1.2

* Cells were treated with A23187 (10 μM, 5 min), immune complexes (1.5 mg/ml 15 min), or opsonized zymosan (1.0 mg/ml, 15 min). Results expressed as mean ± SEM of 4–5 donors. Each assay performed in triplicate.

‡ Interference by [³H]AA metabolites (8.9 ± 1.3%).

§ P vs. control <0.05, one-tailed test.

beled a pool that was readily available for hydrolysis by PLase and subsequent metabolism by 5-lipoxygenase.

Activation of [³H]AA-labeled neutrophils by physiologic stimuli presented a different pattern from that of ionophore-treated cells. Immune complex-stimulated cells released [³H]AA, however the released [³H]AA was not metabolized to any significant extent. The sensitivity of the detection method was such that metabolism of 0.004% of the radiolabeled AA released by the immune complexes could have been detected. Since opsonized zymosan failed to release [³H]AA there was, therefore, no metabolism. Small peaks detected by absorbance at 280 nm were seen in both immune complex and zymosan-stimulated cells that chromatograph just before LTB₄. It is possible that these represent an isomer(s) of LTB₄.

Distribution of [³H]AA in Cellular Lipids after Stimulation of Human Neutrophils

To determine the source of the released [³H]AA, the cell pellets were assayed for labeled PLs and neutral lipids after [³H]AA incorporation and stimulation (Table I). Incorporation of [³H]AA into neutrophil PLs was primarily into PI with lesser amounts in PC and phosphatidylserine (PS). This is consistent with other investigators who have shown that these pools are preferentially labeled with AA (40, 43, 44). Our high percentage of radiolabeled PI may be due to the short incorporation time, perhaps favoring the more metabolically active pool as has been previously suggested (32, 46). Upon activation with all three stimuli, label was decreased in the PI pool (Table I), but the decrease was greatest in ionophore-stimulated cells.

Of the neutral lipids, triglycerides contained the most radiolabel followed by diacylglycerol. The labeling in the triglyceride bands increased slightly after stimulation supporting the belief that free AA is not derived from this pool, but might enter it by reacylation. Initial TLC studies using radiolabeled standards showed that 5-HETE primarily migrated with the monoglyceride marker while exhibiting significant spillover to the diglyceride band (data not shown). HPLC analysis indicated that only ionophore-stimulated neutrophils produced significant quantities of [³H]5-HETE (Fig. 2). Therefore, due to interference by 5-HETE (or other mono-HETES) during A23187 stimulation, measurements of labeled monoglycerides and diglycerides could not be per-

formed. Stimulation with zymosan initiated a small, but significant, increase in the amount of [³H]AA-labeled DAG without a concurrent release of AA. Conversely, no significant increases in either of these lipid products were seen with immune complexes as the stimulus. It must be noted that variability among donors and lack of sensitivity of the assay did not allow for precise quantitation. Neutrophils from two out of four of the donors showed slight increases in [³H]AA-labeled DAG and [³H]AA, as well as slight decreases in [³H]AA-containing PC during immune complex stimulation. Minimal loss of [³H]AA from PC in ionophore-stimulated cells was also seen in the majority of donors. However, this was not statistically significant by Student's one-tailed *t*-test (30).

Phospholipase Activities in Human Neutrophil Homogenates

Because TLC analysis of [³H]AA-labeled lipids did not give unequivocal answers to questions about precursor pools and the mechanism of AA release from stimulated human neutrophils, the issue was therefore approached from another direction. Sonicates of resting human neutrophils were tested for PLase activity by the formation of labeled lysophospholipids or DAG from the hydrolysis of defined radiolabeled substrates. PC, PI, and PE were utilized to determine if the enzymatic activity in the homogenates exhibited a preference for a specific substrate. Table II shows that of the three substrates tested, PC was most consistently hydrolyzed by PLA₂ of all the donors tested. PLA₂ activity, using PE and PI as substrates, was minimal. Alternatively, PLC preferentially hydrolyzed PI, with an activity >15-fold that seen with PE or PC. PLC activity was present in cells from all of the donors.

Stimulation of Phospholipase Activities in Human Neutrophils

Increased PLA₂ activity was apparent in whole cell homogenates when neutrophil suspensions were stimulated before sonication (Table III). Cells were exposed for 5 min to 10 μM ionophore, or 15 min to either 1.5 mg/ml precipitated immune complexes or 1.0 mg/ml opsonized zymosan, and phospholipase activity was assayed using PC as a substrate. Separate time periods were chosen for each individual stimulus based on the maximal rate of [³H]AA and/or O₂⁻ release

Table II. Phospholipase Activities in Human Neutrophils*

Substrate	PLA ₂	PLC
	pmol/min/mg	pmol/min/mg
PC	3.0 ± 0.7	0.6 ± 0.2
PI	0.3 ± 0.2	51.0 ± 12.4
PE	1.1 ± 0.8	2.6 ± 0.9

* Results expressed as mean ± SEM of 4-5 donors.

(Figs. 1 and 3). Therefore, we reasoned that maximal PL activity would be associated with the times chosen. The results are expressed as percent activity as compared with the unstimulated control. PLA₂ activity was enhanced by both (A23187), acting via an influx of extracellular calcium and by a phagocytic, Fc receptor-mediated stimulus (immune complexes). The increased enzymatic activity induced by both ionophore or immune complexes were not significantly different from each other, suggesting that AA may be released by both stimuli in whole cells via PLA₂. In contrast, activation of neutrophils by opsonized zymosan resulted in only a slight enhancement of PLA₂ activity.

The effects of these stimuli on PLC activation were different from those found with PLA₂ (Table IV). PLC activity in homogenates of stimulated neutrophils was assayed using PI and PE as substrates. Examination of Table IV shows that A23187 was unable to stimulate PLC activity to any great extent with either substrate. On the other hand, both physiologic stimuli were able to increase PLC activity significantly.

Superoxide (O₂⁻) Generation from Stimulated Human Neutrophils

To ensure that the stimuli being used were able to activate neutrophils, each stimulus was tested for its ability to initiate superoxide production from neutrophils. Neutrophils were exposed to ionophore, opsonized zymosan, and immune complexes in the same manner as they were in previous tests. Fig. 3 shows that opsonized zymosan and immune complexes were the stimuli capable of initiating a substantial superoxide release. These stimuli elicited significant amounts of superoxide from human neutrophils during the first 10 min of stimulation that continued to increase over a 40-min time period. In contrast, activation by A23187 resulted in fivefold less cytochrome c reduction, as compared with opsonized zymosan or immune complexes, with levels slightly over those of the control (1.3 vs. 0.7 nmol/10⁵ cells). The stimuli that initiated maximal superoxide were those that increased PLC activity, suggesting a possible correlation between the two.

Table III. Stimulation of PLA₂ Activity in Human Neutrophil Sonicates

Stimulus*	Activity (control)‡
	%
None (control)	100
A23187	250 ± 45
Immune complexes	210 ± 20
Opsonized zymosan	105 ± 12

* Cells stimulated as described in Table I.

‡ Results expressed as mean ± SEM of 4 donors, each assay performed in triplicate (n = 5, A23187).

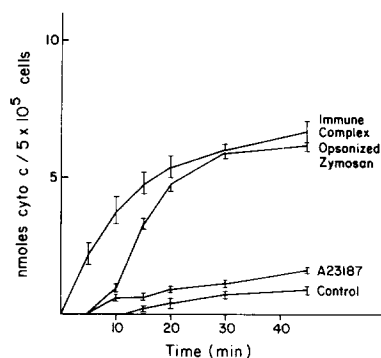


Figure 3. Time course of O₂⁻ release by stimulated human neutrophils. A comparison of the ability of various stimuli to promote superoxide anion release. The concentration of the stimuli used is described in Table I. Results are expressed as the mean of two experiments (±SEM), each data point being the mean of triplicate determinations.

Discussion

The data presented demonstrate that activation of specific PLases, as well as 5-lipoxygenase, in human neutrophils is dependent upon the stimulus encountered. This is especially important since these enzymes are ultimately responsible for the membrane remodeling that accompanies and is probably essential for cell activation (42, 46). Our studies link specific stimuli to the initiation of these specific enzymatic activities. The relationships that we observed among PLase activation and cellular function are summarized in Table V.

[³H]AA Release

[³H]AA release from cell membranes has often been used as a model system for studies of receptor-mediated PLase activation, particularly PLA₂ (43, 44). To directly relate [³H]AA release with PLase activation, we measured these functions in neutrophils activated by a panel of unrelated stimuli. We have shown that neutrophils release [³H]AA when stimulated with either A23187 or immune complexes but not when opsonized zymosan, which binds to cells via the C3b receptor, is used. Release of labeled AA from neutrophils correlated with enhanced PLA₂ activity, as measured in sonicates of stimulated cells but not with enhanced PLC activity (see Tables III and IV). Furthermore, activation of 5-lipoxygenase only occurred with A23187 and did not correlate with activation of PLA₂ and consequent [³H]AA release.

Table IV. Stimulation of PLC Activity in Human Neutrophil Sonicates

Stimulus*	Activity (Control)‡	
	PI	PE
	%	%
Control	100	100
A23187	97.8 ± 6.3	91.0 ± 52.7
Opsonized zymosan	152.3 ± 33.6§	331.5 ± 162.2
Immune complexes	135.3 ± 29.3§	500.3 ± 188.8

* Cells stimulated as described in Table I.

‡ Results expressed as mean ± SEM, n = 4 donors.

§ P vs. control <0.05.

Table V. Relationships Between PL Metabolism and Superoxide Release

Function	Stimuli		
	A23187	Immune complex	Oposonized zymosan
PLA ₂	+	+	-
[³ H]AA release	+	+	-
[³ H]AA metabolism	+	-	-
PLC	-	+	+
DAG	-	±	+
O ₂ ⁻	±	+	+

PLA₂ Activity

PLA₂ activity can be detected in neutrophil sonicates by the use of defined radiolabeled substrates (7). We used this method to measure PLA₂ activity after cell stimulation. The technique was able to detect increases in activity in the stimulated cells, however it was not possible to use it to accurately measure small differences in activity. The results we obtained with this *in vitro* PLA₂ assay correlated with the results obtained by observing [³H]AA release, that is activity was increased in cells stimulated with ionophore and immune complexes before sonication but not when zymosan was used as a stimulus. The results are consistent with several other studies reporting activation of PLA₂ by the ionophore (40, 43, 44). Although there are reports in the literature of receptor-mediated PLA₂-activation by FMLP (29), there are no reports concerning immune complex activation of PLA₂ in neutrophils. However, studies using peritoneal macrophages show that in this cell PLA₂ enzyme may be linked to the Fc2b receptor, thus providing a potential link between immune complex stimulation and PLA₂ action (27, 38).

PLC Activity

We also examined PLC activity in the same fashion. Studies of PLC activity in inflammatory cells have centered around the inositol response with particular attention to the inositol phosphates as second messengers (3). For example, FMLP-stimulated neutrophils show increases in the inositol phosphates, particularly IP₃, during the first 10 s post-stimulation (41). We demonstrated enhanced PLC activity vs. PI with the neutrophil sonicates upon prior stimulation with immune complexes and opsonized zymosan. This technique utilizes PI as a substrate and the enzyme activity has been shown to be calcium dependent (7). Calcium-dependent PLC activity towards PI has also been seen in preparations of ram seminal vesicles (19) and in FMLP-stimulated guinea pig neutrophils (25), and is characterized by its extended response upon activation. We also observed enhanced activity using PE as a substrate, although this activity was one-tenth that obtained when using PI as the substrate. However, the concentration of PE in the cell is much greater than PI, and this small activity may be enough to provide the appropriate lipid molecules (i.e., DAG) from sources other than PI. Ionophore stimulation by itself was unable to increase PLC activity, thus supporting data presented by a number of investigators (40, 44). Since the activity that we observed is calcium dependent, it is possible that additional signals are required for PLC activation.

Distribution of Cellular [³H]AA

We also examined the changes in distribution of [³H]AA label in neutrophils exposed to these same stimuli. Analysis of [³H]AA-labeled cellular lipids was performed at time points that correlated with the maximal rate of [³H]AA release. The technique used for labeling cells primarily labeled PI and to a lesser extent PC and PS, however the only significant loss of radiolabel during stimulation came from PI. Treatment with ionophore depleted this pool considerably. This probably occurred via PLA₂ since the ionophore has not been shown to activate a neutrophil PLC, but has been shown to induce a PLA₂ capable of using PI as a substrate (40). The decreases in PI were not as marked when opsonized zymosan or immune complexes were used as the stimulus (Table I).

The most likely explanation for the fact that the PI pool appears to be the only pool affected may be linked to the method of incorporation. The fatty acid carrier and duration of labeling play a large role in determining which PLS contain radiolabel (40, 44). In the present study, substantial amounts of [³H]AA were in a highly active, cellular pool (PI) (32, 46), while low-turnover pools of high cellular concentration (i.e., PC) contained much less. Therefore, a small or moderate increase in PLA₂ activity using the PC pool may not appear to release significant amounts due to the very low specific activity within the labeled PC pool. Alternatively, liberation of [³H]AA by immune complex stimulation may occur by the pathway of PLC coupled with a DAG kinase and a PLA₂ acting on phosphatidic acid as seen by Takenawa et al. (40). Examination of [³H]AA-labeled neutral lipids shows that significant changes were observed during zymosan activation. Stimulation with opsonized zymosan resulted in increased levels of [³H]AA-DAG. Immune complex stimulation resulted in no significant increases in labeled AA in cell pellets, nor was the labeled DAG increase as prominent as in the zymosan-treated cells. FMLP stimulation of rabbit neutrophils has been shown to generate an increase in [³H]AA-DAG production, the maximum level occurring at 30 s followed by a steep decline (42). In the present study, cellular lipids were extracted and analyzed at times corresponding to the maximal rate of [³H]AA and/or O₂-release (Figs. 1 and 3). At this time it is not clear what mechanism is responsible for the [³H]AA-DAG accumulation during zymosan stimulation. Due to the transient existence of [³H]AA-DAG formed by the breakdown of the polyphosphoinositides, it is unlikely that the majority of [³H]AA-DAG is derived from this source during zymosan stimulation. The most probable explanation is that the labeled DAG is generated via a calcium-dependent PLC-activated downstream of an initial PLC-polyphosphoinositide event.

[³H]AA Metabolism

Previous investigators have suggested that the rate limiting step in 5-lipoxygenase activity is the release of AA (44). In contrast, the results we report would suggest that 5-lipoxygenase must be separately activated. Challenge of [³H]AA-labeled neutrophils with ionophore resulted in the metabolism of [³H]-AA primarily into 5-lipoxygenase products. These results are similar to those obtained by a number of investigators examining ionophore-activated neutrophils and confirm that this method of labeling neutrophils labels a pool

of AA that is accessible to 5-lipoxygenase (8, 12, 24, 35). Surprisingly, in the case of activation by the immune complexes, released AA is not metabolized.

The separation of stimulus-induced PLA₂ activity from 5-lipoxygenase action has been demonstrated in both neutrophils (12) and macrophages (20). Clancy et al. (12) have shown that FMLP- and C5a-stimulated neutrophils could only produce 5-HETE and LTB₄ when exogenous AA was added, suggesting that 5-lipoxygenase but not PLA₂ was activated. In contrast to the results obtained by these investigators with FMLP, stimulation of [³H]AA-labeled neutrophils with immune complexes resulted in activation of PLA₂ and release of [³H]AA but not its metabolism via 5-lipoxygenase. It is obvious from the data we present that not all stimuli induce release of [³H]AA and that conversion of AA to 5-lipoxygenase metabolites by activated cells is not automatic. An explanation for this phenomenon is that conditions used for [³H]AA incorporation may not label the proper phospholipid pool from which the AA is released. This possibility has been proposed for other cell types that metabolize AA (20). However, the fact that ionophore-stimulated cells metabolize the labeled precursor indicates that the labeled pool is susceptible to 5-lipoxygenase action. Moreover, it is improbable that unlabeled AA released by immune complex stimulation would be preferentially metabolized over that of the labeled precursor. On the other hand, studies have shown that 5-lipoxygenase products can be reacylated by ionophore-stimulated neutrophils (37). It is possible that immune complexes or zymosan activate 5-lipoxygenase but the products are reacylated rapidly.

Evidence in the literature for physiologic stimuli initiating synthesis of 5-lipoxygenase products is contradictory (6, 12). The most effective method of stimulating 5-lipoxygenase product formation is by using a calcium ionophore, whereby extracellular calcium is allowed into the cell. This stimulus strongly activates both PLA₂ and 5-lipoxygenase. It is interesting to note that unregulated influxes of calcium are a characteristic of dying or damaged cells (16). A mechanism of release such as this would fit the proinflammatory nature of these mediators as well as the suicidal nature of the neutrophil itself.

Superoxide (O₂⁻) vs. PLase Activity

Those stimuli that gave the best superoxide response were those that enhanced PLC activity (DAG formation), but not PLA₂ activity (AA release). It is interesting to note that these products have been shown to directly activate neutrophil NADPH oxidase in whole cells and cell fractions (2, 14, 15, 17, 41). If these lipids are involved in mediating the superoxide response, then stimuli initiating a strong response may be characterized by the ability to form DAG via PLC activation.

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