Dynamics of platelet-activating-factor release and uptake in a human neutrophil suspension

Marc CLUZEL,* Charles A. ROHDE,† Donald W. MACGLASHAN, Jr.,* Bradley J. UNDEM* and Floyd H. CHILTON*†

*Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, 301 Bayview Boulevard, Baltimore, MD 21224, and †Department of Biostatistics, Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205, U.S.A.

The present study has examined the dynamics of platelet-activating-factor (PAF) synthesis, release and uptake in order to understand better the trafficking of PAF between cells and medium. Initial experiments indicated that the amount of PAF found on the outside of the cell remained constant well after the synthesis from a precursor had apparently stopped, and in spite of a continued capacity of the cell to take up and catabolize PAF. These results suggested that PAF produced and stored within the cell is eventually released to the outside of the cell at a rate proportional to that of cellular uptake. In order to estimate the amount of PAF released from the cell, the processes of PAF release and uptake were modelled using simple mathematical functions. It was found that, under the experimental conditions used in this study, the uptake of PAF could be expressed as an exponential function tending to a non-zero baseline. Utilizing this rate constant for the uptake of PAF with the amount of PAF outside the cell, the amount of total PAF released from the cell was estimated. Data from the model suggested PAF was released in amounts 10-fold higher than could actually be measured over 30 min. In fact, the model predicted more PAF could be released from the cell than is synthesized, suggesting that a portion of the PAF which is released is taken up and then released again to the outside of the cell. The potential for PAF and/or its intermediates to be recycled was verified by demonstrating that a large proportion of exogenously provided 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine is taken up by the neutrophil, converted into PAF and then released again by the cell. These results suggest that PAF trafficking between the cell and medium is complex and involves many processes, which include synthesis, release, uptake, catabolism and recycling.

INTRODUCTION

Platelet-activating factor (PAF) is a biologically active phospholipid which is produced by neutrophils and other cell types in response to a wide variety of stimuli (for reviews, see [1–3]). In neutrophils, PAF is thought to be synthesized from a membrane precursor, 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (1-alkyl-2-acyl-GPC), through the action of phospholipase A₂ [4,5] to form 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (1-*O*-alkyl-2-lyso-GPC). This intermediate is then rapidly acetylated by acetyltransferase [6–8] to 1-alkyl-2-acetyl-GPC. PAF also is rapidly taken up and catabolized by many of the same cell types that produced it, utilizing an inverse series of reactions [9–13]. Specifically, PAF is first degraded by the removal of the acetate moiety at the *sn*-2 position by acetylhydrolase to form 1-alkyl-2-lyso-GPC. This intermediary compound of both synthesis and catabolism is then reacylated with an unsaturated fatty acid to form 1-alkyl-2-acyl-GPC. In a number of cell types, enzymes of both the synthetic and catabolic pathways (i.e., acetyltransferase and acetylhydrolase) are activated during cell stimulation [4,7,8,14,15]. Because both the synthetic and catabolic pathways are functional during cell activation, the amount of PAF recovered from a cell is the result of a balance between that which is synthesized and that which is catabolized.

Although much progress has been made on the biochemical reactions involved in the synthesis and catabolism of PAF, it is still not clear whether the PAF synthesized by neutrophils is released from the cells to act as an autacoid, or retained by the cells to act as an intracellular mediator. Several studies employing

concentrated suspensions of neutrophils have demonstrated that only a small fraction of total PAF produced by the neutrophil is present in the supernatant fluid after stimulation, suggesting that the vast majority of PAF is retained by the cells [16,17]. Alternatively, when neutrophils are studied in a superfusion system where molecules released from the cells can be rapidly partitioned away from the cell, a large proportion of PAF is actually found in the superfusate fraction, that is, released from the cells [18]. These findings have led to the hypothesis that, in a cell suspension, the majority of synthesized PAF is actually released from the suspensions of neutrophils, but then rapidly taken back up by the cells, giving the appearance that it was retained by the cell. In the present study we have tested this hypothesis by labelling the membrane precursor of PAF, 1-alkyl-2-acyl-GPC, with ³H to monitor the fate of newly synthesized PAF. Simultaneously, we quantified the uptake of exogenous PAF labelled with ¹⁴C. The goals of the study were twofold: (1) to test and evaluate the assumptions implicit in this approach; and (2) to develop a simple mathematical model based on this design that allows for an estimate of the amount of PAF that, at one time, was released from the cells. Our results suggest that large quantities of PAF are released over 30 min and that a large exchange of PAF may occur between cells and medium during PAF biosynthesis.

EXPERIMENTAL

Materials

1-[³H]Alkyl-2-lyso-GPC (52 Ci/mmol) obtained from ox heart

Abbreviations used: PAF, platelet-activating factor; GPC, *sn*-glycero-3-phosphocholine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; HSA, human serum albumin; DMSO, dimethyl sulphoxide; HBSS, Hanks Balanced Salt Solution; TMS, trimethylsilyl.

† To whom correspondence should be sent, at the following address: Section on Pulmonary Medicine, The Bowman Gray School of Medicine, 300 S. Hawthorne Road, Winston-Salem, NC 27103, U.S.A.

and containing predominantly $C_{16:0}$ alkyl chains and 1- O - $[^3H]$ alkyl-2-acetyl-GPC (56 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). 1-Alkyl-2-acetyl-glycero-3-phospho[*methyl*- ^{14}C]choline (56 mCi/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). All labelled compounds were > 98% radiochemically pure. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and the Ca^{2+} ionophore A23187 was from Calbiochem Corporation (La Jolla, CA, U.S.A.). Lipid standards used in t.l.c. were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 1-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-hexadecyl-2-acetyl-GPC) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Essentially-fatty-acid-free human serum albumin (HSA), phospholipase C from *Bacillus cereus* (type XIII), dilinolein (dilinoeoylglycerol), 1-monolinoleoyl-*rac*-glycerol and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Silica columns were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.), and t.l.c. plates were from Analtech (Newark, DE, U.S.A.). All solvents were h.p.l.c. grade from Fisher Scientific (Pittsburgh, PA, U.S.A.). Hanks Balanced Salt Solution (HBSS) was purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). 1- O -Hexadecyl- $[^3H_3]$ acetyl-*sn*-glycero-3-phosphocholine (1- O -alkyl-2- $[^3H_3]$ acetyl-GPC) was synthesized as previously described [19].

Incorporation of 1- $[^3H]$ alkyl-2-lyso-GPC into 1- $[^3H]$ alkyl-2-acyl-GPC of the human neutrophil

Human neutrophils were prepared from venous blood obtained from healthy donors. Neutrophils were isolated by dextran sedimentation, followed by Ficoll/Paque density centrifugation as previously described [11]. Isolated neutrophils were suspended in HBSS with Ca^{2+} (1.2 mM) and HSA (1 mg/ml) at a concentration of 10 million cells/ml and incubated for 30 min at 37 °C in presence of 1- O - $[^3H]$ alkyl-2-lyso-GPC (1 nM). The neutrophils were then washed twice with HBSS with Ca^{2+} (1.2 mM) and HSA (1 mg/ml) (225 g, 8 min, 4 °C) and resuspended at a concentration of 10 million cells/950 μ l in HBSS with Ca^{2+} (1.2 mM) without HSA. Neutrophils were then transferred to 1.5 ml Eppendorf tubes and prewarmed for 5 min. At this point, two aliquots were removed to determine the total radioactivity of the solution and the distribution of the label before stimulation.

Stimulation of the human neutrophils

Neutrophils labelled with 3H as described above were challenged with no stimulatory agent or with the Ca^{2+} ionophore A23187 (1.0 μ M) at 37 °C for various periods of time. Under standard conditions, 1- O -alkyl-2-acetyl- $[^{14}C]$ GPC was suspended in an aqueous solution containing HSA (20 mg/ml), then added to the cell suspension along with the stimulatory agent, bringing the final concentration of 1- O -alkyl-2-acetyl- $[^{14}C]$ GPC to 110 nM. Cell suspensions were incubated at 37 °C for various periods of time, and reactions were terminated by centrifugally removing the cells from the supernatant fluid (10000 g, 1 min). The cell pellets were then resuspended in 1 ml of methanol. Unlabelled PAF (1 μ g) and 1-alkyl-2-acyl-GPC (1 μ g) were added to the cell pellet or supernatant fluid in order to optimize recovery of these labelled lipids in subsequent chromatography steps.

Analysis of the radiolabelled products

Lipids were extracted from the supernatant fraction and the cell pellet by the method of Bligh & Dyer [20]. After extraction, a fraction of the extract was removed and the radioactivity in 3H - and ^{14}C -labelled lipids was determined in order to calculate

recoveries. Radioactive products were then separated on layers of silica-gel H developed in chloroform/methanol/acetic acid/water (50:25:8:4, by vol.). The distribution of labelled products was determined by scanning the plate for radioactivity using a Bioscan system 200 imaging scanner (Bioscan, Washington, DC, U.S.A.). Standard phospholipids were then revealed with I_2 vapour, and the bands corresponding to 1-alkyl-2-lyso-GPC, PAF and 1-alkyl-2-acyl-GPC standards were identified. The different t.l.c. zones were scraped off the plate, and the quantity of radioactivity was determined on a Beckman liquid-scintillation counter after producing standardization curves for the two different radioisotopes. Radioactivity was counted in d.p.m. and the results were usually expressed as a percentage of total radioactivity (supernatant fluid + cells) for a given isotope. All samples were corrected for recovery by determining the radioactivity in samples before t.l.c.

M.s. of PAF

In some experiments, analysis of PAF molecular species was performed by g.l.c.-m.s. as previously described [19]. In these experiments, 1-hexadecyl-2- $[^3H_3]$ acetyl-GPC was added at the end of the experiment as an internal standard, and lipids were extracted by the method of Bligh & Dyer [20]. A PAF-enriched fraction was then obtained from the extract by silica-column purification as previously described [18]. 1-Alkyl-2-acetyl-GPC was enzymically converted into diacylglycerols by phospholipase C. The diacylglycerols were then converted into the trimethylsilyl (TMS) derivatives by the addition of BSTFA. The TMS derivatives were then analysed by selected-ion-monitoring techniques to record fragment ions specific for PAF (m/z 175) and $[^3H]$ PAF (m/z 178). G.l.c.-m.s. was carried out on a selective-mass-detection system (Hewlett-Packard 5790) employing a cross-linked methylsilicone capillary column. This column was then inserted into an m.s. column source. The initial column temperature was 100 °C and programmed to 230 °C at 30 °C/min. At this point, the temperature was increased to 300 °C at 10 °C/min. The injector temperature was 250 °C and the g.l.c.-m.s. interface line was 280 °C. Helium was used as a carrier gas, and 1 μ l of the sample was injected in the splitless mode. The limit of detection of this PAF assay is 500 pg.

Calculations

Calculations of constants were performed with the help of a pharmacokinetic computerized program PCNONLIN (Statistical Consultants, Edgewood, KY, U.S.A.) [21]. In the case of the simple bidirectional binding model used in the study

the differential equations were:

$$dz(1) = K_2 \times PAF.S - K_1 \times PAF.C$$

and

$$dz(2) = K_1 \times PAF.C - K_2 \times PAF.S$$

where $PAF.S$ and $PAF.C$ represents PAF concentrations in supernatant fluid and cell respectively, K_1 is the uptake constant and K_2 the reverse constant. Simulation curve fits with experimental data were statistically determined by Chi-square test. Integration of

$$\int_{t_0}^{t_n} A dt$$

where A represents the concentration of $[^3H]$ PAF formed in the cell and released in the supernatant, was performed according to the equation:

$$Area = (t_n - t_{n-1}) \times (At_n + A_{n-1})/2$$

where t is the time.

RESULTS

PAF release from the human neutrophil

Initial experiments were designed to obtain a cellular system in which the synthesis of PAF from a precursor and the subsequent release of this PAF could be monitored. Previously, we have demonstrated that 1-[³H]alkyl-2-lyso-GPC incubated with human neutrophils for 30 min is metabolized to 1-alkyl-2-acyl-GPC containing greater than 80 % arachidonate at the *sn*-2 position. Subsequently, it was demonstrated that cells containing 1-[³H]alkyl-2-arachidonoyl-GPC converted a portion of this phospholipid into a number of products, including [³H]PAF and

Fig. 1. Time course of the conversion of 1-O-[³H]alkyl-2-acyl-GPC to products during cell activation

Human neutrophils (1×10^7 cells/ml) containing 1-O-[³H]alkyl-2-acyl-GPC were challenged with A23187 (1 μ M) at 37 °C in 0.1 % HSA. At the indicated time points, the incubation was terminated and the labelled products from both the medium and the cells were extracted and identified by t.l.c. Labelled products were then isolated by zonal scraping, and the amount of radioactivity was quantified using liquid-scintillation counting. The results are expressed as the percentage of the initial radioactivity and are means $\pm$ S.E.M. for four different experiments. $\blacktriangle$, [³H]PAF in cells; $\circ$, [³H]PAF in medium; $\bullet$, 1-[³H]alkyl-2-acyl-GPC.

Table 1. Time course of 1-O-[³H]alkyl-2-acetyl-GPC specific radioactivity

Human neutrophils (1×10^7 cells/ml) containing 1-O-[³H]alkyl-2-acyl-GPC were challenged with A23187 (1 μ M) at 37 °C in 0.1 % HSA/Ca²⁺ (1.2 mM) as described in the Experimental section. At the indicated time points, the incubation was terminated and the mol quantity of 1-O-hexadecyl-2-acetyl-GPC from both the cells and the medium was determined by g.l.c.-m.s. In the same samples, labelled products were extracted and separated by t.l.c. 1-O-[³H]alkyl-2-acetyl-GPC was isolated, and the amount of radioactivity was quantified by zonal scraping and liquid-scintillation counting. No internal standards were present in labelled samples, but recoveries in these samples were higher than 85 %. The specific radioactivity for PAF is expressed as the d.p.m. of 1-O-[³H]alkyl-2-acetyl-GPC/pmol of 1-O-hexadecyl-2-acetyl-GPC. Results are means $\pm$ S.E.M. for four different donors.

Time (min)...	Specific radioactivity			
	5	10	15	30
Medium	74.8 $\pm$ 10.9	71.2 $\pm$ 13.8	52.9 $\pm$ 1.13	69.8 $\pm$ 13.1
Cell	64.7 $\pm$ 10.6	66.6 $\pm$ 13.7	53.1 $\pm$ 5.08	68.0 $\pm$ 13.2

Fig. 2. Time course of the uptake of exogenous 1-O-alkyl-2-acetyl-[¹⁴C]GPC

Human neutrophils were challenged with A23187 (1 μ M). Exogenous [¹⁴C]PAF (110 nM) was added in the medium with A23187 and the incubation was terminated at the indicated time points. Labelled products in medium and cells were extracted and separated by t.l.c. The amount of radioactivity in isolated products was quantified by liquid-scintillation counting. The data are means $\pm$ S.E.M. for four different experiments. $\circ$, PAF in supernatant; $\blacktriangle$, PAF in cells; $\square$, lyso-PAF in cells; $\blacksquare$, 1-alkyl-2-acyl-GPC in cells.

1-[³H]alkyl-2-lyso-GPC during cell activation. In the present experiments we have utilized this strategy to label 1-alkyl-2-acyl-GPC. Cells were then stimulated and the amount of labelled [³H]PAF in both the cell and the supernatant fluids was determined. As shown in Fig. 1, only a small percentage of the total PAF synthesized was found in the supernatant fluid. However, the amount of PAF in supernatant fluids increased for 20 min after cell activation and remained relatively constant to 60 min. Cellular PAF reached a maximum of 27 % of the total radioactivity at 20 min and decreased to 22 % by 60 min. The amount of radioactivity in the labelled precursor of PAF, 1-[³H]alkyl-2-acyl-GPC, decreased concomitantly with the production of labelled PAF and then increased after 20 min. These data revealed that the amount of labelled PAF found in the supernatant fluids does not decrease after PAF biosynthesis from a phospholipid precursor has slowed significantly.

A major assumption of this labelling strategy is that [³H]PAF synthesized from the 1-[³H]alkyl-2-arachidonoyl-GPC represents PAF synthesized from unlabelled endogenous pools of 1-alkyl-2-acyl-GPC. In order to address this question, experiments were performed to determine if the ratio of 1-[³H]alkyl-2-acetyl-GPC/unlabelled 1-hexadecyl-2-acetyl-GPC remained constant at different times during cell activation. Cells prelabelled as described above were incubated for various times from 5 to 30 min with ionophore A23187. The radioactivity and mol quantity of PAF were determined in cells and medium by liquid-scintillation counting and m.s. respectively, as described in the Experimental section. As Table 1 shows, the ratio of 1-[³H]alkyl-2-acetyl-GPC/1-hexadecyl-2-acetyl-GPC in cells and supernatant fluids remained relatively constant throughout the time course in three separate experiments. This experiment provided evidence that [³H]PAF produced and released by the cell from 1-[³H]alkyl-2-acyl-GPC is representative of unlabelled PAF produced and released by the cell.

Fig. 3. Effect of different concentrations of exogenous 1-O-alkyl-2-acetyl-GPC on the uptake of 1-O-alkyl-2-acetyl-GPC

Human neutrophils were challenged with A23187 (1 μ M). 1-O-[3 H]alkyl-2-acetyl-GPC (0.5 nM final concn., $\square$) or 1-O-alkyl-2-acetyl-[14 C]GPC (110 nM, $\blacktriangle$) was added with A23187 and the incubation was terminated at the indicated time points. Labelled products in the medium and cells were extracted and separated by t.l.c., then radioassayed after t.l.c. zonal scraping. The data are expressed as the means $\pm$ S.E.M. for three (3 H)PAF) and four (14 C)PAF) different experiments.

Fig. 4. Effect of cell activation on the uptake of exogenous 1-O-alkyl-2-acetyl-[14 C]GPC

Human neutrophils were challenged with A23187 (1 μ M). Exogenous [14 C]PAF (110 nM) was added in medium simultaneously with A23187 (●) or 30 min later (○). The reaction was terminated at the indicated time points. Labelled products in the medium and in the cells were extracted and separated by t.l.c., and then radioassayed after t.l.c. zonal scraping. These data are expressed as the percentage of 1-O-alkyl-2-acetyl-[14 C]GPC remaining in the supernatant fluid from 0 to 30 min (upper scale on the abscissa) or from 30 to 60 min (lower scale on the abscissa) and are the means $\pm$ S.E.M. for two different experiments.

Uptake of exogenous PAF by the human neutrophil

A second component which must be considered when trying to determine the amount of PAF released from a cell is the rate of cellular uptake and catabolism of the PAF. Certainly, the constancy of PAF on the outside of the cell, in spite of a decrease

of synthesis by the cell, could be the result of inhibited catabolism during cell activation. In order to estimate the amount of PAF which has been taken up by the cells during activation, 1-alkyl-2-acetyl-[14 C]GPC was provided to the cell suspension along with A23187. As previously described, exogenously provided [14 C]PAF was rapidly taken up by the stimulated neutrophil and catabolized to 1-alkyl-2-lyso-GPC and 1-alkyl-2-acyl-GPC (Fig. 2). It is, however, noteworthy that intact PAF was found bound to the cell at a much higher rate than it was catabolized to products. During cell activation there are likely to be varying concentrations of PAF in the supernatant fluids at different time points after cell activation, owing to the endogenous synthesis of PAF by the neutrophil. Therefore it was important to examine PAF uptake in the range of PAF concentrations likely produced and released into the supernatant fluids. The uptake of exogenous PAF at two concentrations (0.5 and 110 nM) were compared in ionophore-stimulated neutrophils. One corresponded to the highest PAF concentration (110 nM) present in the medium of our experiment, and the second (0.5 nM) was below the lowest concentration of endogenously produced PAF in the medium observed in our experiments. As Fig. 3 shows, the rates of uptake of PAF under these two conditions were similar.

Experiments were next designed to determine whether the uptake process was constant over time after A23187 activation. In these experiments, neutrophils were stimulated with A23187 for 30 min and exogenous PAF was added either simultaneously with A23187 or 30 min after cell activation. As Fig. 4 shows, the PAF uptake from 30 to 60 min was not different from the uptake observed from 0 to 30 min. These data suggested that PAF uptake had not slowed significantly after 30 min of cell activation.

The synthesis and uptake experiments suggested that PAF remained constant in the cell supernatant fluids at later time points, despite a decrease in synthesis of new PAF and a maintained capacity of the cell to take up PAF. In order to understand better this apparent discrepancy, cells containing 1-[3 H]alkyl-2-acyl-GPC incorporated into cellular lipids were stimulated with A23187 in the presence of [14 C]PAF, and the [3 H]PAF/[14 C]PAF ratios found in the supernatant fluids were measured. Logic holds that, as long as [3 H]PAF is synthesized from 1-[3 H]alkyl-2-acyl-GPC and a portion of [3 H]PAF is released, the ratio of [3 H]PAF/[14 C]PAF on the outside of the cell should continue to increase. However, if synthesis of [3 H]PAF from labelled endogenous precursor stops, the 3 H/ 14 C ratio should stabilize. As Fig. 5 shows, [3 H]PAF was rapidly produced upon cell activation with ionophore A23187. This production of [3 H]PAF occurred with the loss of [14 C]PAF, resulting in an increasing ratio of [3 H]PAF/[14 C]PAF at the earlier time points. However, this ratio reached a plateau after 30 min. That the [3 H]PAF/[14 C]PAF ratio reached a stable point indicated that, at some time after cell activation, the newly synthesized [3 H]PAF and exogenously provided [14 C]PAF were in equilibrium in the medium. This stable ratio may have been obtained because synthesis of endogenous PAF has stopped at some point after cell activation. Alternatively, exogenously provided [14 C]PAF may have been incorporated into 1-alkyl-2-acyl-2-[14 C]GPC through the deacetylation-reacylation pathway, and the stable [3 H]PAF/[14 C]PAF ratio reflects the fact that equilibrium has been reached between intracellular PAF precursor pools.

Model for determining PAF release from the cell

We next developed a model which takes into account the rate of synthesis of PAF from its precursor and the subsequent release of PAF, as well as its uptake and catabolism. In this model we have considered that, at a given time, the amount of PAF that had at one time been released from the cells is equal to the amount of PAF synthesized by the cell and found in the

Fig. 5. Time course of the endogenous-PAF/exogenous-PAF ratio in supernatant fluids

Human neutrophils containing 1-*O*-[³H]alkyl-2-acyl-GPC in cellular phospholipids were challenged with A23187 (1 μ M) in the presence of 1-*O*-alkyl-2-acyl-[¹⁴C]GPC (110 nM). At the indicated time points, the incubation was terminated, and labelled products in the supernatant fluid were extracted and separated by t.l.c. The amounts of radioactivity in 1-*O*-[³H]alkyl-2-acyl-GPC and 1-*O*-alkyl-2-acyl-[¹⁴C]GPC were quantified in the same samples by liquid-scintillation counting and using the standardization curves for the two isotopes. The data are expressed as the ratio of d.p.m. of 1-*O*-[³H]alkyl-2-acyl-GPC and 1-*O*-alkyl-2-acyl-[¹⁴C]GPC respectively in supernatant fluids. Results are means $\pm$ S.E.M. for four different experiments.

supernatant fluids along with the amount of PAF which has been taken up during the period of cell activation. Therefore, in order to extrapolate the amount of PAF released from cell from the amount of PAF observed in medium, an uptake constant for PAF had to be obtained. A convenient way to accomplish this is to describe PAF uptake as a mathematical function which does not change over the time of the reaction and is independent of the concentration of the substrate provided to the cells. Both of these assumptions were tested in the previous sets of experiments (Figs. 3 and 4). We had shown that, under our experimental conditions, the amount of PAF taken up/min was a fixed fraction of the PAF present in medium from 0 to 30 min. These elements, associated with the shape of the PAF-uptake curve, suggested that the loss of PAF in the supernatant could be expressed by an exponential function tending to a non-zero baseline. This may represent the point at which the release of PAF from inside the cell is proportional to the amount of PAF taken up by the cell. Because there are binding sites (receptor, hydrophobic etc.) for PAF on the cells and sites (such as albumin) for PAF in the supernatant fluid, we hypothesized that PAF in the medium is in a dynamic process which can be represented by:

where PAF.S is PAF on extracellular binding sites and PAF.C are cellular binding sites. K_1 is the rate constant for cellular uptake of PAF, and K_2 represents the rate constant for the movement of cellular PAF back to binding sites in the supernatant fluids. Therefore PAF concentrations in supernatant fluids may be expressed as

$$d \text{ PAF.S} / dt = K_1 \text{ PAF.S} + K_2 \text{ PAF.C} \quad (2)$$

After integration, eqn. (2) may be written as:

$$\text{PAF.S}(t) =$$

$$\{ \text{PAF}_0 [1 - K_2 / (K_1 + K_2)] \} e^{-(K_1 + K_2)t} + K_2 \text{ PAF}_0 / (K_1 + K_2) \quad (3)$$

where PAF_0 represents the initial amount of PAF added in

Fig. 6. A model for PAF uptake

Neutrophils were prepared and stimulated as described in the Experimental section. The amount of [¹⁴C]PAF remaining in the supernatant fluids was determined, and these data were fitted by computerized analysis to the model described in the Results section to obtain an uptake and reverse constant. The uptake constant and reverse constant was $0.34 \pm 0.03 \text{ min}^{-1}$ and $0.03 \pm 0.003 \text{ min}^{-1}$ respectively. The P value obtained by a chi-square test between the experimental and simulated curve was 0.0005. $\diamond$, Experimental uptake; —, theoretical uptake.

supernatant fluids. As a result, PAF uptake corresponds to a monoexponential process defined by $-(K_1 + K_2)$ and reaching a baseline defined by $[(K_2 \text{ PAF}_0) / (K_1 + K_2)]$. After baseline determination, K_1 may be calculated graphically. To accomplish this we employed a computer program which utilizes the experimental data obtained from our uptake experiments with [¹⁴C]PAF to obtain an uptake constant K_1 using eqn. (2) described above. Fig. 6 illustrates the actual experimental data from an uptake experiment and the theoretical uptake curve extrapolated from those data using the simple bidirectional mode.

Determining PAF release using a PAF-uptake constant

As has been previously discussed, the total PAF that has at one time been released from the cell represents that which is in the supernatant fluid along with that which was taken up by the cell during that same time. Therefore, the total amount of PAF that has been released at any given time can be expressed [22] as:

$$\text{PAF.R}(t_n) = \text{PAF.S}(t_n) + K_1 \int_{t_0}^{t_n} \text{PAF.S} dt \quad (4)$$

where PAF.R represents the total amount of PAF released from t_0 to t_n . Fig. 7 illustrates the measured amount of [³H]PAF released from the neutrophil at several time points during cell activation. By summing the amount of PAF at a given time with the amount of PAF that was taken up by the cell up to that time, the theoretical amount of PAF which was released from the cell can be estimated. As can be seen from the left vertical axis of Fig. 7, the actual amount of [³H]PAF measured in the supernatant fluid of the neutrophil represents under these experimental conditions only a small percentage of the PAF that had one time been released from the cell. In addition, the amount of PAF released to the outside of the cell continues to increase throughout the 30 min incubation.

A concern with the model was the fact that exogenous PAF was added to the system. Exogenous PAF at concentrations used in our model did not affect the amount of endogenous [³H]PAF

Fig. 7. Experimental and theoretical time-course measurement of PAF release

Left-hand ordinate: by using the amount of endogenously produced 1-*O*-[³H]alkyl-2-acetyl-GPC found in the supernatant fluid and the PAF uptake constant calculated from Fig. 6, we estimated the amount of 1-*O*-[³H]alkyl-2-acetyl-GPC that had been released from neutrophils from zero time to a given point, according to the equation described in the Results section. The results are expressed in d.p.m. and represent means $\pm$ S.E.M. for four experiments. Right-hand ordinate: by measuring by g.l.c.-m.s. the mol quantity of endogenously produced PAF present in medium and by determining the uptake constant with 1-*O*-[³H]alkyl-2-acetyl-GPC (0.5 nM) added in the same sample, an estimate of the mole quantity of PAF that had been released from human neutrophils was obtained as described above. These results are expressed in pmol and are means $\pm$ S.E.M. for three experiments.

observed in the medium (results not shown). To further eliminate a potential role of exogenous PAF on the release of endogenous PAF, we performed experiments where endogenous PAF production and release was measured by g.l.c.-m.s. and where PAF uptake was quantified by using exogenous labelled [³H]PAF. Because [³H]PAF had a much higher specific radioactivity than [¹⁴C]PAF, the [³H]PAF could be added at much lower concentrations (0.5 pmol/10⁷ neutrophils). At this concentration, [³H]PAF was not detectable by our g.l.c.-m.s. measurements and therefore did not interfere with endogenous PAF measurements. This concentration also represents 1/20 of the molar quantity of PAF detected in supernatant fluid of stimulated neutrophils. Under these conditions, a potential effect of exogenous PAF on endogenous PAF was unlikely. As observed in Fig. 7 (right vertical axis), extrapolated PAF release obtained using low concentrations of exogenous [³H]PAF was similar to that estimated using higher concentrations of [¹⁴C]PAF. These data suggest that exogenous PAF at the concentrations chosen using [¹⁴C]PAF did not modify the amount of PAF released.

An important observation in this experiment was the fact that release continued well after synthesis had slowed. In fact, the estimated amount of PAF released at later time points was more than could have been derived from the intracellular pools observed in Fig. 1. Therefore these data suggest that the sustained PAF found on the outside of the cell was not totally due to the shuttling of intracellular PAF to the outside of the cell. It is important to remember that, in this model, PAF that is taken up and released several times will be counted more than once. It is difficult to design an experiment to determine the extent to which recycling of PAF might occur. However, labelled lyso-PAF can be provided to the cell during cell activation, and the distribution of labelled PAF (cell as against supernatant fluid) can be determined. Any PAF found on the outside of the cell must have been derived from labelled lyso-PAF that was taken up, converted into PAF and released again. In the experiment illustrated in Fig. 8, 1-*O*-[³H]alkyl-2-lyso-GPC was provided to the cell 10 min after cell

Fig. 8. Localization of PAF in cells and medium when produced from exogenously provided 1-[³H]alkyl-2-lyso-GPC

Human neutrophils were challenged with A23187 (1 μ M) as described in the Experimental section. 1-*O*-[³H]alkyl-2-lyso-GPC (2.5 nM final concn.) was then added 10 min after the addition of A23187. Incubations were terminated at indicated time points and labelled products in medium and cells were extracted, separated by t.l.c., and then radioassayed by liquid-scintillation counting. The data are expressed as percentages of total newly synthesized PAF which was cell-associated or present in the medium. Results are means $\pm$ S.E.M. for two separate experiments. □, [³H]PAF in cells; ■, [³H]PAF in medium.

activation, and the conversion of this precursor into cell-associated and released PAF was monitored. A period of 10 min was chosen here because that appears to be the time when PAF synthesis from endogenous precursors has slowed and yet the amount of PAF released from the cell remains constant. 1-*O*-[³H]alkyl-2-lyso-GPC is rapidly converted into [³H]PAF and 1-*O*-[³H]alkyl-2-acyl-GPC after it is provided to the neutrophil. A significant portion of this PAF is then released from the cell. These data verify that there is a potential for PAF and/or its metabolic intermediates to be shuttled back and forth from the medium to the cell.

DISCUSSION

The principal observation of the present study was that the amount of PAF found on the outside of the cell remained constant well after the synthesis from a precursor had apparently stopped and in spite of a continued capacity of the cell to take up and catabolize PAF. These results support the hypothesis that PAF produced and stored within the cell is eventually released to the outside of the cell at a rate proportional to that of cellular uptake.

An alternative explanation to the result of a constant amount of PAF in the supernatant fluids was that synthesis of PAF from a precursor had not stopped. Two sets of findings rendered this alternative unlikely. (1) Previous studies which monitored the mass of 1-alkyl-2-arachidonoyl-GPC, a major precursor of PAF [23], during cell activation indicate that the bulk of this phospholipid is depleted during the first 10 min of neutrophil activation with ionophore A23187. Similar results here (Fig. 1) suggest that most of this precursor is converted into products within the first 10 min, and there is an actual increase in this molecule from 20 to 60 min (Fig. 1). (2) In experiments where cells containing 1-*O*-[³H]alkyl-2-acyl-GPC incorporated into cellular lipids were stimulated with A23187 in the presence of

exogenous [^{14}C]PAF, the ratio of [^3H]PAF produced from 1- [^3H]alkyl-2-acyl-GPC/[^{14}C]PAF reached a steady state in the supernatant fluids. This suggests that, at this point, no more [^3H]PAF is added to the [^{14}C]PAF pool and newly synthesized [^3H]PAF and exogenously provided ^{14}C are in equilibrium. Another explanation for the constant release of PAF in spite of its decreased synthesis was that the uptake of PAF slows significantly after cell activation. This hypothesis was found untenable by the finding that the cellular uptake of PAF remained constant throughout the course of the experiment (30 min).

If indeed PAF synthesis stops after 10–20 min, and cellular uptake remains rapid and constant, one can only estimate the actual amount of PAF released by a cell by considering the amount of PAF found in the extracellular medium and the uptake kinetics for PAF. In order to appreciate how much PAF is released from the neutrophil, the processes of PAF release and uptake were modelled by using simple mathematical functions. It was found that, under the experimental conditions used in the present study, the uptake of PAF followed a simple exponential function tending to a non-zero baseline. From these data, the first-order rate constant of uptake of PAF was determined. Utilizing this rate constant along with the amount of PAF found outside of the cell, we could estimate the total amount of PAF that had actually been released from the cell. Data from the model suggested that much larger quantities of PAF had been released from the cell than are measured in the supernatant fluids. In fact, this model predicts that more PAF could be released from the cell than is synthesized. This suggests a portion of the PAF which is outside the cell is taken up by the cell and then cycled back to the outside of the cell. Because PAF itself may be recycling or the molecule may be catabolized to 1-alkyl-2-lyso-GPC or 1-alkyl-2-acyl-GPC and then re-formed by the inverse series of reactions and released, it is difficult to design experiments which measure the extent of recycling in the system. However, experiments where labelled lyso-PAF is given at 10 min after cell activation indicate that a relatively large portion of lyso-PAF is converted into PAF and released from the cell. These data reveal that there is a potential for PAF and/or its intermediates to be shuttled from the outside of the cell into the cell and back out during cell activation.

Our findings point out many important aspects of PAF turnover which must be considered when trying to measure PAF release. Clearly, the amount of PAF which can be detected outside the cells depends not only on factors such as the rate of synthesis and release of the molecule, but also the rates of uptake and catabolism. In fact, a previous study from our laboratory revealed that relatively large quantities of PAF can be released from the cell if that PAF can be partitioned from the uptake system [18]. In addition, this study points out other factors, such

as PAF recycling, which appear to be critical in determining the amount of PAF found outside of the cell. Taken together these results suggest that PAF trafficking between the cell and medium is complex and involves many processes, which include synthesis, release, uptake, catabolism and possible recycling.

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