Interaction of platelet-activating factor with cultured guinea pig tracheal epithelial cells

Laurie CHURCHILL, Floyd H. CHILTON and David PROUD*

Department of Medicine, Division of Clinical Immunology, The Johns Hopkins University School of Medicine, Baltimore, MD 21224, U.S.A.

The present study has examined the interaction of platelet-activating factor (PAF) with cultured guinea pig tracheal epithelial cells (GTE). PAF stimulated GTE to release endogenous arachidonic acid and metabolize it to prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$). Prostanoid production by GTE in response to PAF was dose-dependent (0.1–100 nM) and was maximal within 5 min. PGE_2 and $PGF_{2\alpha}$ levels increased by 3.3 ± 0.8 and 3.2 ± 0.6 ng/ 10^6 cells respectively over basal levels in response to 100 nM-PAF. The ability of GTE to synthesize and/or catabolize PAF was also examined. GTE readily incorporated [3H]acetate into a product which migrated on t.l.c. with PAF. However, further characterization of this product suggested that label had not been incorporated into PAF, but rather that it was incorporated into another lipid product with chromatographic characteristics similar to those of PAF. In contrast, GTE readily metabolized PAF to inactive products. When [3H]PAF was incubated with GTE, 50% of the total [3H]PAF added was catabolized in approx. 15 min. The major route of catabolism of PAF by GTE was the deacetylation–reacylation pathway, which yielded 1-*O*-[3H]alkyl-2-acyl-*sn*-glycerophosphocholine. Determination of the nature of the long-chain acyl group incorporated into the *sn*-2 position of the newly synthesized products revealed that oleic and linoleic acids were the major fatty acids present. Taken together, these results suggest that respiratory epithelial cells respond to stimulation by PAF with enhanced production of PGE_2 and $PGF_{2\alpha}$, and also have the capacity to modulate inflammatory reactions in the airways by their ability to degrade this potent inflammatory mediator.

INTRODUCTION

In recent years, a growing body of evidence has accumulated supporting a role for platelet-activating factor (PAF) in the pathogenesis of allergic and inflammatory events in the airway. Although the production of this potent biologically active phosphoglyceride was originally described in rabbit leucocytes [1], it is now known that several cell types that participate in inflammatory reactions have the capacity to synthesize and catabolize PAF [2–8]. Animal studies have shown that intravenous or aerosolized administration of PAF leads to the accumulation of inflammatory cells within the lung [9,10]. Furthermore, PAF is known to activate several types of inflammatory cells, including neutrophils, eosinophils and platelets [11–13]. Administration of PAF to the mucosal surface of the airways has also been shown to cause bronchoconstriction and subsequent hyper-reactivity both in humans [14] and in several animal models [15–17]. More recently, it has been demonstrated that both antigen-induced inflammatory cell infiltration and airway hyper-responsiveness in sheep can be inhibited upon pretreatment with a selective PAF antagonist [18].

The ability of PAF to induce biological responses when administered to the mucosal surface of the airways raises the possibility that the actions of PAF may be due, at least in part, to interactions of this mediator with the respiratory epithelial cell. Support for this hypothesis is provided by a recent demonstration that the response of the isolated guinea pig trachea to PAF is attenuated by removal of the epithelium [19]. Furthermore, it is now clear that the epithelial cell is more metabolically active than was previously appreciated. For example, respiratory epithelial cells can generate a variety of lipid mediators, including prostanoids, hydroxyeicosatetraenoic acids and

leukotrienes [20–23], and epithelial production of arachidonic acid metabolites is increased in response to inflammatory stimuli, such as bradykinin [20,23]. In addition, epithelial cells are known to actively degrade inflammatory mediators, such as neuropeptides [24]. In light of this increased appreciation of the ability of epithelial cells to generate and degrade inflammatory mediators, and of the possibility that the biological actions of PAF in the airways could be mediated, to some extent, via effects on respiratory epithelial cells, the present study used cultured guinea pig tracheal epithelial cells (GTE) to more thoroughly examine the interactions of PAF with respiratory epithelial cells.

MATERIALS AND METHODS

Materials

Cholera toxin, trypsin, essentially fatty-acid-free human serum albumin (HSA), phospholipase C, phospholipase A_2 , PAF, synthetic prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$), Pipes and bis(trimethylsilyl)trifluoroacetamide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). C_{16} -PAF (optically active isomer) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). A stock solution of C_{16} -PAF (2 mM) was prepared in ethanol and was diluted to the appropriate concentration with buffer for the PG production experiments. Phosphatidylcholine for t.l.c. standards and 1-*O*-alkyl-2-lyso-glycerophosphocholine (1-*O*-alkyl-2-lyso-GPC) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). [3H]PGE₂, [3H]PGF_{2α} and 1-*O*-[3H]hexadecyl-2-acetyl-GPC (C_{16} -PAF, containing 96% C_{16} -PAF; 40.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Ham's F12 medium, penicillin/streptomycin/fungizone solution, fetal bovine serum (FBS) and gentamicin were purchased from

Abbreviations used: PAF, platelet-activating factor; GTE, guinea pig tracheal epithelial cells; HSA, human serum albumin; PG, prostaglandin; GPC, glycerophosphocholine; FBS, fetal bovine serum; T₃, tri-iodothyronine; EGF, epidermal growth factor; ECGS, endothelial cell growth supplement; r.i.a., radioimmunoassay; f.a.b.–m.s., fast-atom bombardment–mass spectroscopy.

* To whom reprint requests and correspondence should be addressed at: Johns Hopkins Asthma and Allergy Center, Clinical Immunology Unit Office 2, 301 Bayview Boulevard, Baltimore, MD 21224, U.S.A.

Gibco Laboratories (Grand Island, NY, U.S.A.). Cortisol, insulin, tri-iodothyronine (T_3), epidermal growth factor (EGF) and endothelial cell growth supplement (ECGS) were purchased from Collaborative Research (Bedford, MA, U.S.A.). Ca^{2+} ionophore A23187 and Pronase were purchased from Calbiochem (La Jolla, CA, U.S.A.). A stock solution of A23187 (1 mg/ml) was prepared in ethanol. Antisera to PGE_2 and $PGF_{2\alpha}$ were purchased from Advanced Magnetics (Cambridge, MA, U.S.A.). Bradykinin was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). L-652,731 [*trans*-2,5-bis-(3,4,5-trimethoxyphenyl)-tetrahydrofuran] was obtained from Merck Sharp and Dohme (Rahway, NJ, U.S.A.). A stock solution of L-652,731 (1 mM) was prepared in dimethyl sulphoxide. All solvents were h.p.l.c. grade from Fisher Scientific (Fair Lawn, NJ, U.S.A.). 1-*O*-Hexadecyl- $[^3H]$ acetyl-GPC was synthesized as previously described [25]. The synthetic 1-*O*-hexadecyl-2-acyl-GPC standards containing $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:4}$, $C_{20:5}$ or $C_{22:6}$ acyl chains at the *sn*-2 position were prepared by acylation of 1-*O*-hexadecyl-2-lyso-GPC (Sigma Chemical Co.) as previously described [26] using the corresponding anhydrides (Nu-Chek Prep, Elysian, MN, U.S.A.).

GTE cultures

Male Hartley guinea pigs weighing 400–500 g were killed by intraperitoneal injection of sodium pentobarbital. Epithelial cells were obtained using a modification of the method of Wu and coworkers [27]. Trachea was incubated for 18–24 h at 4 °C in Ham's F12 medium containing 0.1 % Pronase and gentamicin (50 µg/ml). At the end of the incubation period, tissue was placed in F12 medium supplemented with 20 % (v/v) FBS and the epithelial cells were dislodged from the mucosal surface with media using a 3 ml syringe. Cells were centrifuged at 350 g for 8 min, washed in a second volume of F12/20 % FBS and resuspended in Ham's F12 medium containing penicillin (100 units/ml), streptomycin (100 units/ml) and fungizone (250 ng/ml) supplemented with cortisol (0.1 µM), insulin (2 µg/ml), T_3 (3 nM), cholera toxin (10 ng/ml), EGF (12.5 ng/ml) and ECGS (3.75 µg/ml) (F12-6X). The cell pellet was dispersed by repeated pipetting using a 10 ml pipette. A sample of the cell suspension was taken for the determination of cell viability, as assessed using erythrosin B, and for cell counts. GTE were seeded at a density of 50 000 cells/cm² on 35 mm 6-well cluster plates or in 75 cm² culture flasks, and were incubated at 37 °C in CO₂/air (1:19) mixture. At 24 h after plating, the cultures were washed with F12 medium to remove non-adherent cells and were then fed with fresh F12-6X medium. GTE cultures were fed daily and reached confluence within 5–7 days.

GTE cultures were examined using a phase-contrast microscope and were further characterized by immunohistochemistry for cytokeratin, as previously described [20].

Prostanoid production of GTE

Confluent cultures of GTE were incubated in F12-6X that had been supplemented with 5 % heat-inactivated FBS for 16–20 h. Plates containing GTE were then placed in a 37 °C water bath and each well was washed 3 times with 2 ml of a Pipes buffer (pH 7.4) consisting of 25 mM-Pipes, 110 mM-NaCl, 5 mM-KCl, 5.55 mM-glucose, 1 mM-MgCl₂ and 2 mM-CaCl₂ (PGCM buffer). A 1 ml portion of buffer was then added to each well for a 10 min incubation period, at the end of which time the buffer was removed and discarded. A fresh 1 ml volume of buffer was then added for a 10 min control period. At the end of the control period supernatants were removed and a fresh 1 ml volume of buffer with or without the appropriate concentration of PAF or vehicle was added to each well for an additional 10 min period. The supernatants were collected, the cells were washed with

Ca^{2+}/Mg^{2+} -free buffer and 1 ml of 0.1 % trypsin/10 mM-EGTA in Ca^{2+}/Mg^{2+} -free Pipes buffer was added to remove cells from the plate. The cell suspensions were added to F12 medium supplemented with 30 % FBS, centrifuged and resuspended. Samples were taken to determine cell number and viability. Supernatants from control and stimulated periods were centrifuged at 1200 g for 15 min. The samples were then decanted, divided into portions and stored at –80 °C until radioimmunoassay (r.i.a.) for PGE_2 and $PGF_{2\alpha}$ was performed.

To examine the time course of prostanoid release from PAF-stimulated GTE, both control and stimulated periods were varied from each well (1, 2, 5, 10, 15 and 30 min). Experiments were also conducted to determine if PAF-stimulated prostanoid release could be inhibited by the specific PAF receptor antagonist L-652,731 ($K_B = 20$ nM) [28]. Control and stimulated periods of 15 min were used for these experiments. During the stimulated period, the PAF antagonist or vehicle was added at $t = 0$ and PAF (1 nM) was added at $t = 5$ min.

PG radioimmunoassay

Immunoreactive PGE_2 and $PGF_{2\alpha}$ were routinely quantified by r.i.a. Antisera and $[^3H]$ ligands were diluted in a phosphate/gelatin-buffered saline solution, pH 7.4 (0.1 % gelatin, 0.1 M-KH₂PO₄/K₂HPO₄, 0.1 % NaN₃ and 0.15 M-NaCl). Samples and standards were diluted in PGCM buffer. Aliquots (100 µl) of sample were incubated with 50 µl of antiserum and 50 µl of $[^3H]$ ligand. Standard curves were run in parallel using authentic prostanoids. The mixture was incubated for 16–20 h at 4 °C, followed by the addition of 1 ml of dextran-coated charcoal (2.5 g of Nordit A Charcoal and 0.25 g of dextran/litre). After 12 min, samples were centrifuged and the supernatants were counted for radioactivity by liquid scintillation spectrometry. The limits of detection for both the PGE_2 and $PGF_{2\alpha}$ assays were 150 pg/ml. The cross-reactivity of other prostanoids with the PGE_2 antiserum are as follows: PGE_1 , 100 %; PGA_2 , 6.0 %; $PGF_{2\alpha}$, 1.3 %; 6-oxo- $PGF_{1\alpha}$, 1 %. The cross-reactivity of other prostanoids with the $PGF_{2\alpha}$ antiserum are: $PGF_{1\alpha}$, 100 %; PGE_2 , 1.1 %; 6-oxo- $PGF_{1\alpha}$, 1.1 %; thromboxane B₂, 0.5 %; PGE_2 , 0.3 %.

PAF synthesis

The ability of GTE to synthesize PAF through the deacylation-reacylation pathway was examined. Confluent 75 cm² cultures of GTE were incubated in F12-6X supplemented with 5 % FBS for 16–20 h before initiation of the experiment. All washes and incubations for PAF synthesis experiments were carried out using PGCM containing 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mg of HSA/ml at 37 °C. Cultures were washed three times with 15 ml of buffer. The cultures were then incubated with 7 ml of buffer containing 6 µM- $[^3H]$ acetate in the presence or absence of bradykinin (1 µM) or the calcium ionophore A23187 (1 µg/ml). The cultures were shaken gently at 37 °C for 5 or 30 min time periods, at the end of which 14 ml of cold methanol was added and the cells were scraped from the bottom of the flask. Chloroform (7 ml) was then added along with 10 µg of unlabelled PAF as a carrier to increase the recovery of radioactivity. The lipids were then extracted by a modification of the technique of Bligh & Dyer [29] in which sufficient formic acid was added to lower the pH of the aqueous phase to 3.

The extracted lipid products were partially purified using a silica column (40 µm pore size; 60A; J.T. Baker Research Products) washed with chloroform and eluted with methanol/water (3:1, v/v). The eluent was dried and resuspended in chloroform/methanol (1:1, v/v). Samples and unlabelled standards were then spotted on silica gel G plates, and developed in chloroform/methanol/glacial acetic acid/water (50:25:8:4, by

vol.). Standards (1-*O*-alkyl-2-acetyl-GPC, 1-*O*-alkyl-2-lyso-GPC and phosphatidylcholine) were visualized with I_2 vapour and the distribution of label in different products was determined by scanning the plate for radioactivity using a Bioscan System 200 Imaging Scanner (Bioscan, Washington, DC, U.S.A.). The various products were then isolated and the radioactivity in the respective areas was determined by liquid scintillation counting.

Further identification of radiolabelled products migrating with PAF was carried out by scraping the PAF zone from the silica gel and extracting it three times with methanol/water (3:1; v/v). The purified products were dried and hydrolysed with phospholipase A_2 as previously described [30]. After the reaction was complete, the sample was extracted by the method of Bligh & Dyer [29]. The quantity of radioactivity in both the aqueous and organic phases was determined by liquid scintillation counting.

Products isolated from the PAF region of the t.l.c. plate were also analysed by fast atom-bombardment-mass spectroscopy (f.a.b.-m.s.). Briefly, f.a.b.-m.s. was performed on a double-focusing VG 7070E spectrometer (VG Analytical, Altrincham, Cheshire U.K.). The instrument was equipped with the commercial f.a.b. introduction system, saddle-field gun and stainless steel 5 mm $\times$ 1.4 mm targets. Xenon was used as the particle source and was accelerated to 6–8 kV. The sample, with 1–2 μ l of matrix, was applied to the target surface and 1 μ l of chloroform/methanol (1:1, v/v) containing the lipid was applied to the matrix and the volatile solvent was evaporated with a stream of air. During f.a.b.-m.s. analysis, data were recorded on a VG 2000 data system.

PAF was also assayed using g.c.-m.s. as previously described [31]. Samples were obtained, as described above, from GTE cultures without the addition of [3 H]acetate to the incubation medium. 1-*O*-Hexadecyl-2-[2 H]₃acetyl-GPC was added at the end of the incubation as an internal standard. The incubations were terminated and the lipids were extracted and partially purified using a silica gel column as described above. The partially purified lipid fraction was treated with phospholipase C and the resulting diacylglycerols were converted to trimethylsilyl (TMS) glycerides by the addition of bis(trimethylsilyl)-trifluoroacetamide. The TMS-glycerides were analysed by selected ion-monitoring techniques to record fragment ions specific for PAF (m/z 175) and deuterated PAF (m/z 178). G.c.-m.s. was carried out on a selective mass-detection system (Hewlett-Packard 5790; Hewlett-Packard, Palo Alto, CA, U.S.A.) using a cross-linked methyl silicone capillary column. The limit of detection of this PAF assay is 500 pg.

PAF catabolism

Confluent GTE cultures grown on 75 cm² flasks were cultured for 16–20 h in F12-6X supplemented with 5% FBS, followed by three washes with PGCM containing 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mg of HSA/ml and one final wash with buffer without HSA. Buffer (7 ml) without HSA was then added to the flask. A 35 μ l portion of an aqueous solution of unlabelled PAF and 135 μ l of an aqueous solution of [3 H]PAF and unlabelled PAF, both complexed to 0.25 mg of HSA/ml, were added to give a final concentration of 0.1 μ M-PAF. The incubation was gently shaken at 37 °C for the allotted time. The reaction was then terminated and the lipids were extracted from the combined cellular and supernatant mixture. Labelled products were isolated on layers of silica gel G developed in chloroform/methanol/glacial acetic acid/water (50:25:8:4, by vol.) and the radioactivity was determined as described above.

Molecular species determination

The 1-*O*-alkyl-2-acyl-GPC metabolites formed during catabolism of [3 H]PAF by GTE after a 45 min incubation period were

purified using t.l.c. The 1-*O*-alkyl-2-acyl-GPC zone of the plate was scraped and extracted three times using methanol/water (3:1, v/v). The predominant molecular species of 1-*O*-[3 H]alkyl-2-acyl-GPC were then separated by h.p.l.c. as benzoate derivatives. The benzoate derivatives were synthesized with a slight modification of the technique of Haroldsen and coworkers [32]. 1-*O*-Alkyl-2-acylglycerols, obtained from phospholipase C hydrolysis of 1-*O*-[3 H]alkyl-2-acyl-GPC, were dissolved in 0.3 ml of benzene containing 20 mg of benzoyl chloride and 0.2 mg pyridine and incubated for 24 h at 37 °C. The benzoate derivatives produced in the reaction mixture were extracted twice with hexane/ether (1:1, v/v), and purified on a silica column (40 μ m, 60A, J. T. Baker Research Products) washed with hexane, and eluted with hexane/ether (9:1, v/v). The solvents were then removed under a stream of N₂ and resuspended in 0.5 ml of the h.p.l.c. mobile phase.

The various labelled benzoate diacylglycerol molecular species were isolated by reverse-phase h.p.l.c. using a 250 mm $\times$ 4.6 mm analytical column (Ultrasphere ODS, Beckman) eluted with acetonitrile/propan-2-ol (4:1, v/v) at a flow rate of 1 ml/min. The eluate was collected and the label in the fractions was determined by liquid scintillation counting. Radioactive peaks were identified by their elution with synthetic 1-*O*-hexadecyl-2-acyl-GPC containing specific acyl chains (C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2}, C_{20:4}, C_{20:5} and C_{22:6}) at the *sn*-2 position.

Statistical analysis

Statistical comparisons were performed using Student's paired *t* tests.

RESULTS

Epithelial cell characterization

Confluent GTE displayed a characteristic cobblestone appearance and consisted primarily of basal-type cells with few ciliated cells observed (Fig. 1). The viability of confluent cultures was routinely greater than 95%. Immunohistochemical studies of confluent cultures revealed strong cytoplasmic staining of all cells by an antibody against cytokeratin, the characteristic intermediate filament of epithelial cells.

Prostanoid production by GTE

Under basal conditions, GTE produced both PGE₂ and PGF_{2 α} (0.8 $\pm$ 0.1 ng/10⁶ cells and 0.7 $\pm$ 0.2 ng/10⁶ cells respectively), as determined by r.i.a. Although a trend toward a slight decrease in prostanoid production was observed between the first and the

Fig. 1. Phase-contrast microscopy of confluent GTE

GTE cultures demonstrated a typical cobblestone appearance and consisted primarily of non-ciliated basal-type cells. Identity of the cells as epithelial cells was further confirmed by positive immunohistochemical staining for cytokeratin.

Fig. 2. Dose-dependent increases in immunoreactive PGE_2 ($\square$) and $\text{PGF}_{2\alpha}$ ($\blacksquare$) production in response to PAF stimulation

The results are given as ng/ 10^6 cells over basal levels; means $\pm$ S.E.M. Prostanoid production was significantly elevated over buffer control levels at doses of PAF as low as 0.1 nM and above ($P < 0.05$ in each case, $n = 4$).

Fig. 3. Time course of enhanced prostanoid production by GTE in response to PAF (0.1 μM)

The amount of prostanoid released over basal levels at various incubation times (1, 2, 5, 10, 15 and 30 min) was determined (means $\pm$ S.E.M., $n = 4$). Maximal production of both PGE_2 ($\square$) and $\text{PGF}_{2\alpha}$ ($\blacksquare$) was observed within 5 min.

Fig. 4. Time course of catabolism of 1- O - ^3H alkyl-2-acetyl-GPC by GTE

The distribution of label in 1- O - ^3H alkyl-2-acetyl-GPC ($\blacksquare$) as well as in the two major metabolites, 1- O - ^3H alkyl-2-acyl-GPC ($\square$) and 1- O - ^3H alkyl-2-lyso-GPC ($\bullet$), is shown at various incubation times (5, 15, 30 and 45 min) (means $\pm$ S.E.M., $n = 4$).

second 10 min periods of buffer exposure, statistical significance was not achieved. PAF was a potent stimulus for prostanoid biosynthesis by GTE. Generation of PGE_2 and $\text{PGF}_{2\alpha}$ by GTE was significantly enhanced by 0.1 nM-PAF ($P < 0.05$) and was maximal at 0.1 μM -PAF (3.3 ± 0.8 and 3.2 ± 0.6 ng/ 10^6 cells for PGE_2 and $\text{PGF}_{2\alpha}$ respectively above basal production) (Fig. 2). Vehicle alone had no effect on prostanoid synthesis by GTE. At all doses of PAF used, the levels of immunoreactive PGE_2 produced by GTE were not significantly different from $\text{PGF}_{2\alpha}$ levels. Enhanced production of PGE_2 and $\text{PGF}_{2\alpha}$ stimulated by 1 nM-PAF was inhibited by $79 \pm 7\%$ and $84 \pm 16\%$ respectively by the PAF antagonist L-652,731 (1 μM) ($n = 3$), whereas vehicle alone had no effect on prostanoid production.

Studies were also conducted to determine the time course of PAF-induced prostanoid production. Exposure of GTE to 0.1 μM -PAF resulted in a rapid increase in both PGE_2 and $\text{PGF}_{2\alpha}$ levels. The quantities of prostanoids observed reached a maximum within 5 min. Furthermore, these levels were maintained throughout the incubation period (30 min) (Fig. 3).

PAF production by GTE

Several assays were used in an attempt to measure any PAF that may have been produced by GTE. Initially, GTE were incubated with ^3H acetate to determine if these cells have the capacity to incorporate this intermediate into PAF. GTE incorporated ^3H acetate into a product that migrated on t.l.c. with authentic PAF. The quantity of radioactivity incorporated was not increased by stimulation with either A23187 (1 $\mu\text{g}/\text{ml}$) or bradykinin (1 μM). After treatment of the partially purified radiolabelled product with phospholipase A_2 , the radioactive component of the molecule remained in the organic phase. If ^3H acetate had been incorporated into the sn -2 position of the molecule, it would have been removed by phospholipase A_2 treatment. Since acetate is a water-soluble product, a significant amount of radioactivity would have been present in the aqueous phase. However, it was still possible that the labelled molecule was PAF and that the labelled component resided in the sn -1 position alkyl chain. To test this possibility, the phospholipase A_2 -treated material in the organic phase was separated by t.l.c. This analysis indicated that the molecule still migrated with PAF and not lyso-PAF, suggesting that the labelled molecule was not PAF. Furthermore, g.c.-m.s. analysis of the combined cellular and supernatant extract failed to detect PAF.

Initial attempts to identify the ^3H -labelled product migrating with PAF on t.l.c. focused on the possibility that it may be a sphingolipid, since sphingomyelin had an R_f value similar to that of PAF in the t.l.c. system used. Analysis of the PAF region of the t.l.c. plate by f.a.b.-m.s. did, indeed, identify sphingomyelin as a major component of this fraction. No detectable levels of PAF were observed in these samples by f.a.b.-m.s.

PAF catabolism by GTE

Experiments were next performed to determine whether ^3H PAF is catabolized by GTE. Cultured GTE were incubated with 1- O - ^3H alkyl-2-acetyl-GPC for various time periods and the labelled metabolites were examined by t.l.c. Within 5 min, significant ($P < 0.05$) increases in 1- O - ^3H alkyl-2-lyso-GPC and 1- O - ^3H alkyl-2-acyl-GPC concomitant with a decrease in ^3H PAF were detected. As shown in Fig. 4, the relative amount of ^3H PAF continued to decrease throughout the 45 min incubation period, with 50% degradation occurring by approx. 15 min. 1- O - ^3H alkyl-2-acyl-GPC increased to account for nearly 50% of the total radioactivity in the reaction mixture after 30 min of incubation. 1- O - ^3H alkyl-2-lyso-GPC levels were only slightly, but significantly ($P < 0.05$), elevated at all time periods.

Fig. 5. H.p.l.c. separation of 1-*O*-[³H]alkyl-2-acylglycerobenzoate derivatives derived from newly synthesized 1-*O*-alkyl-2-acyl-GPC obtained from the incubation of [³H]PAF with GTE for 45 min

The retention times of 1-*O*-[³H]alkyl-2-acylglycerobenzoate standards containing C_{20:5}/C_{22:6} (1), C_{20:4} (2), C_{18:2} (3), C_{18:1} (4), C_{16:0} (5) or C_{18:0} (6) at the *sn*-2 position are indicated by the arrows. These data are representative of three separate experiments.

Determination of the molecular species of 1-*O*-[³H]alkyl-2-acyl-GPC formed

The nature of the acyl chain at the *sn*-2 position of the 1-*O*-[³H]alkyl-2-acyl-GPC that was formed when GTE were incubated with [³H]PAF was determined after conversion of these molecules to their respective benzoate derivatives and analysis by h.p.l.c. The h.p.l.c. profile of the diradylglycerobenzoate derivatives is shown in Fig. 5. The major acylation products were 1-*O*-hexadecyl-2-oleoyl-GPC (C_{18:1}) and 1-*O*-hexadecyl-2-linoleoyl-GPC (C_{18:2}) which accounted for 35 % and 28 % of the radiolabel respectively in the 1-*O*-[³H]hexadecyl-2-acyl-GPC. Smaller percentages of radioactivity were eluted with 1-*O*-hexadecyl-2-arachidonoyl-GPC (C_{20:4}) and 1-*O*-hexadecyl-2-palmitoyl-GPC (C_{16:0}) (12 % and 8 % of the total radioactivity in the 1-*O*-[³H]hexadecyl-2-acyl-GPC fraction respectively). Less than 3 % of the radioactivity was eluted with 1-*O*-hexadecyl-2-(C_{20:5}, C_{22:6} or C_{18:0})-GPC.

DISCUSSION

PAF is known to induce the release of arachidonic acid and its subsequent metabolism to bioactive metabolites in a number of cells and tissues [33–36]. Furthermore, several studies have suggested that the actions of PAF may be mediated, at least in part, by the secondary production of these arachidonic acid metabolites. For example, Brunelleschi and colleagues reported that PAF-induced relaxation of histamine-contracted guinea pig trachea was directly correlated with enhanced production of PGE₂ [19]. Both PAF-induced relaxation and PGE₂ production were significantly attenuated by cyclo-oxygenase inhibitors and by the removal of the epithelium, suggesting that epithelial-derived prostanoids contribute to the observed effects of PAF on this tissue. The present study clearly demonstrates that PAF is, indeed, a potent stimulus for enhanced biosynthesis of both PGE₂ and PGF_{2α} from cultured GTE. Enhanced production of these prostanoids by airway epithelial cells may be important in view of the reported actions of PGE₂ and PGF_{2α} on airway smooth muscle tone and responsiveness. For example, PGF_{2α} has been reported to produce severe bronchospasm in asthmatics [37]. In contrast, although PGE₂ has a direct bronchodilator action, Walters and colleagues have demonstrated that it pro-

duces hyper-reactivity to subsequent histamine and methacholine challenges in normal individuals [38]. Thus PAF-induced synthesis of PGE₂ and PGF_{2α} from respiratory epithelial cells may contribute to the changes in airway resistance that are observed after intratracheal instillation of PAF in humans [14].

A number of inflammatory cells, including neutrophils, mast cells and macrophages as well as endothelial cells, synthesize PAF via the deacylation–reacylation pathway in response to stimulation with inflammatory mediators or A23187 [2,3,39,40]. In the deacylation–reacylation pathway, 1-*O*-alkyl-2-acyl-GPC is deacylated by the action of phospholipase A₂ to form 1-*O*-alkyl-2-lyso-GPC, which is subsequently acetylated by an acetyltransferase enzyme to form PAF. In the present study we have examined the ability of cultured GTE to synthesize PAF by incubation of these cells with [³H]acetate in both the presence and the absence of bradykinin or A23187. Subsequent analysis of the combined cellular and supernatant lipid extract indicated that GTE did not incorporate [³H]acetate into PAF. These results suggest that GTE do not synthesize significant amounts of PAF via the deacylation–reacylation pathway. Furthermore, PAF was not detected by g.c.–m.s. or f.a.b.–m.s., suggesting that, if PAF is synthesized by the deacylation–reacylation pathway or by other pathways (i.e. the *de novo* pathway), the amount produced is below the limit of detection in these systems.

It is of interest to note that GTE did incorporate [³H]acetate into a product that migrated with PAF but was not identified as PAF upon further analysis. Preliminary results suggest that the radiolabelled product may be sphingomyelin, since sphingomyelin has an *R_f* value similar to that of the radiolabelled product and was identified as one of the major components in this region of the t.l.c. plate by f.a.b.–m.s. Furthermore, Holtzman and colleagues have previously shown that sphingomyelin is one of the major phospholipids present in pulmonary epithelial cell membranes [41]. These results clearly indicate that extreme caution should be taken when using [³H]acetate incorporation to quantify PAF production in cells.

The present study also suggests that tracheal epithelial cells may play an important role in the regulation of local concentrations of PAF through their ability to catabolize this potent inflammatory mediator. GTE appear to catabolize PAF by the same deacylation–reacylation pathway that has been described for several cells, including neutrophils, macrophages and mast cells [42–44]. Significant quantities of 1-*O*-[³H]alkyl-2-lyso-GPC are formed upon incubation of [³H]PAF with tracheal epithelial cells, indicating that acetylhydrolase, present within the cell, first removes the acetyl moiety from PAF. This intermediate product then appears to be rapidly reacylated to form 1-*O*-alkyl-2-acyl-GPC.

Studies examining the catabolism of PAF by other cell types have indicated that the *sn*-2 position of 1-*O*-alkyl-2-lyso-GPC can be reacylated with a variety of fatty acyl chains. In rabbit macrophages, murine mast cells, human and rabbit platelets and unstimulated human and monkey neutrophils, the predominant fatty acid chain incorporated into the *sn*-2 position is arachidonic acid (C_{20:4}) [42–47]. In contrast, rabbit and rat neutrophils acylate 1-*O*-alkyl-2-lyso-GPC with C_{18:2}, suggesting that species variation occurs [4,48]. The present study has demonstrated that in cultured GTE the major fatty acyl chains which are incorporated into the *sn*-2 position of 1-*O*-alkyl-2-lyso-GPC are C_{18:1} and C_{18:2}, with smaller amounts of C_{20:4} and C_{16:0}. Several factors that may influence the acylation profile must be taken into consideration when interpreting these data. The GTE culture medium is supplemented with 5 % FBS for the last 16–20 h of culture. Prior to this time the cells are grown in serum-free F12-6X medium. Linoleic acid (C_{18:2}; 0.3 μM) is the only fatty acid present in F12 medium. It is possible, therefore, that the linoleic

acid content of these cells has been enhanced and may account for the incorporation of primarily $C_{18:1}$ and $C_{18:2}$ into the *sn*-2 position. In support of this hypothesis, a recent study has shown a shift in the acylation profile of 1-*O*-alkyl-2-acyl-GPC from $C_{20:4}$ to $C_{22:6}$ when the culture medium for murine mast cells is supplemented with $C_{22:6}$ [44]. PAF-induced prostanoid biosynthesis may also have a significant effect on the observed acylation profile. It is clear that the concentration of PAF used in the catabolism studies ($0.1 \mu\text{M}$) is sufficient to stimulate maximal prostanoid production. Arachidonic acid may be shunted to the cyclo-oxygenase pathway, resulting in lower levels of $C_{20:4}$ being available for incorporation into the *sn*-2 position of 1-*O*-alkyl-2-lyso-GPC. A similar hypothesis has been suggested in a previous study in which the acylation profile of neutrophils is shifted from $C_{20:4}$ to $C_{18:2}$ and $C_{18:1}$ when cells are stimulated with A23187 [49].

In summary, the present study demonstrates that respiratory epithelial cells release both PGE_2 and $\text{PGF}_{2\alpha}$ in response to stimulation by PAF. Furthermore, GTE have the capacity to degrade PAF through a deacetylation–reacylation pathway. These results suggest that respiratory epithelial cells may modulate the effects of PAF within the airways through the release of secondary inflammatory mediators as well as by the regulation of local concentrations of this potent inflammatory mediator.

We gratefully acknowledge the excellent assistance of Theresa Connel and Dana Schmidt. The authors also express their appreciation to Dr. Massimo Triggiani for his helpful comments throughout this project. This work was supported in part by National Institute of Health Grants AI 24985 and AI 26771 and by an Institutional Award from The Johns Hopkins University. L. C. is a fellow of the American Lung Association. This is manuscript no. 52 from the Johns Hopkins Asthma and Allergy Center, Baltimore, MD, U.S.A.

REFERENCES

- Benveniste, J., Henson, P. M. & Cochrane, C. G. (1972) *J. Exp. Med.* **136**, 1356–1377
- Mueller, H. W., O'Flaherty, J. T. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 6213–6218
- Musch, M. W., Billah, M. M. & Siegel, M. I. (1987) *Biochem. Biophys. Res. Commun.* **144**, 1243–1250
- Chilton, F. H., O'Flaherty, J. T., Ellis, J. M., Swendsen, C. L. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 6357–6361
- Camussi, G., Aglietta, M., Coda, R., Bussolino, F., Piacibello, W. & Tetta, C. (1981) *Immunology* **42**, 191–199
- Arnoux, B., Duval, D. & Benveniste, J. (1980) *Eur. J. Clin. Invest.* **10**, 437–441
- Lee, T., Lenihan, D. J., Malone, B., Roddy, L. L. & Wasserman, S. I. (1984) *J. Biol. Chem.* **259**, 5526–5530
- Robinson, M. & Snyder, F. (1985) *Biochim. Biophys. Acta* **837**, 52–56
- Lellouch-Tubiani, A., Lefort, J., Simon, M.-T., Pfister, A. & Vargaftig, B. B. (1988) *Am. Rev. Respir. Dis.* **137**, 948–954
- Arnoux, B., Denjean, A., Page, C. P., Nolibé, D., Morley, J. & Benveniste, J. (1988) *Am. Rev. Respir. Dis.* **137**, 855–860
- Shaw, J. O., Pinckard, N., Ferrigni, K. S., McManus, L. M. & Hanahan, D. J. (1981) *J. Immunol.* **127**, 1250–1255
- McManus, L. M., Hanahan, D. J. & Pinckard, R. N. (1981) *J. Clin. Invest.* **67**, 903–906
- Kroegel, C., Yukawa, T., Dent, G., Venge, P., Chung, K. F. & Barnes, P. J. (1989) *J. Immunol.* **142**, 3518–3526
- Cuss, F. M., Dixon, C. M. S. & Barnes, P. J. (1986) *Lancet* **ii**, 189–192
- Chung, K. F., Aizawa, H., Leikauf, G. D., Ueki, I. F., Evans, T. W. & Nadel, J. A. (1986) *J. Pharmacol. Exp. Ther.* **236**, 580–584
- Coyle, A. J., Urwin, S. C., Page, C. P., Touvy, C., Villain, B. & Braquet, P. (1988) *Eur. J. Pharmacol.* **148**, 51–58
- Patterson, R., Bernstein, P. R., Harris, K. E. & Krell, R. D. (1984) *J. Lab. Clin. Med.* **104**, 340–345
- Soler, M., Szelczak, M. W. & Abraham, W. M. (1989) *J. Appl. Physiol.* **67**, 406–413
- Brunelleschi, S., Haye-Legrand, I., Labat, C., Norel, X., Benveniste, J. & Brink, C. (1987) *J. Pharmacol. Exp. Ther.* **243**, 356–363
- Churchill, L., Chilton, F. H., Resau, J. H., Bascom, R., Hubbard, W. C. & Proud, D. (1989) *Am. Rev. Respir. Dis.* **140**, 449–459
- Eling, T. E., Danilowicz, R. M., Henke, D. C., Sivarajah, K., Yankaskas, J. R. & Boucher, R. C. (1986) *J. Biol. Chem.* **261**, 12841–12849
- Holtzman, M. J., Aizawa, H., Nadel, J. A. & Goetzl, E. J. (1983) *Biochem. Biophys. Res. Commun.* **114**, 1071–1076
- Salari, H. & Chan-Yeung, M. (1989) *Am. J. Respir. Cell. Mol. Biol.* **1**, 245–250
- Fossard, N., Rhoden, K. J. & Barnes, P. J. (1989) *J. Pharmacol. Exp. Ther.* **248**, 292–298
- Clay, K. L., Murphy, R. C., Andres, J. L., Lynch, J. & Henson, P. M. (1984) *Biochem. Biophys. Res. Commun.* **121**, 815–825
- Gupta, C. M., Radhakrishnan, R. & Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4315–4319
- Wu, R., Yankaskas, J. R., Chen, E., Knowles, M. R. & Boucher, R. C. (1985) *Am. Rev. Respir. Dis.* **132**, 311–320
- Hwang, S., Lam, M., Biftu, T., Beattie, T. R. & Shen, T. (1985) *J. Biol. Chem.* **260**, 15639–15645
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Waku, K., Ito, H., Bito, T. & Nakazawa, Y. (1974) *J. Biochem. (Tokyo)* **75**, 1307–1312
- Cluzel, M., Undem, B. J. & Chilton, F. H. (1989) *J. Immunol.* **143**, 3659–3665
- Haroldsen, P. E., Voelkel, N. F., Henson, J. E., Henson, P. M. & Murphy, R. C. (1987) *J. Clin. Invest.* **79**, 1860–1867
- Chilton, F. H., O'Flaherty, J. T., Walsh, C. E., Thomas, M. J., Wykle, R. L., DeChatelet, L. R. & Waite, B. M. (1982) *J. Biol. Chem.* **257**, 5402–5407
- Kawaguchi, H. & Yasuda, H. (1986) *Biochim. Biophys. Acta* **875**, 525–534
- Lefort, J., Rotilio, D. & Vargaftig, B. B. (1984) *Br. J. Pharmacol.* **82**, 565–575
- Lin, A., Morton, D. R. & Gorman, R. R. (1982) *J. Clin. Invest.* **70**, 1058–1065
- Cuthbert, M. F. (1973) in *The Prostaglandins: Pharmacology and Therapeutic Advances* (Cuthbert, M. F., ed.), pp. 253–286, J. B. Lipincott, Philadelphia
- Walters, E. H., Bevan, C., Parish, R. W., Davis, B. H. & Smith, A. P. (1982) *Thorax* **37**, 438–442
- Albert, D. H. & Snyder, F. (1983) *J. Biol. Chem.* **258**, 97–102
- McIntyre, T. M., Zimmerman, G. A., Satoh, K. & Prescott, S. M. (1985) *J. Clin. Invest.* **76**, 271–280
- Holtzman, M. J., Grunberger, D. & Hunter, J. A. (1986) *Biochim. Biophys. Acta* **877**, 459–464
- Robinson, B. W. S., McLemore, T. L. & Crystal, R. G. (1985) *J. Clin. Invest.* **75**, 1488–1495
- Chilton, F. H., O'Flaherty, J. T., Ellis, J. M., Swendsen, C. L. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 7268–7271
- Triggiani, M. & Chilton, F. H. (1989) *Biochim. Biophys. Biophys. Acta* **1006**, 41–51
- Chabot, M. C., Schmitt, J. D., Bullock, B. C. & Wykle, R. L. (1987) *Biochim. Biophys. Acta* **922**, 214–220
- Kramer, R. M., Patton, G. M., Pritzker, C. R. & Deykin, D. (1984) *J. Biol. Chem.* **259**, 13316–13320
- Malone, B., Lee, T. & Snyder, F. (1985) *J. Biol. Chem.* **260**, 1531–1534
- Ramesha, C. S. & Pickett, W. C. (1986) *J. Biol. Chem.* **261**, 15519–15523
- Chilton, F. H., Ellis, J. M., Olson, S. C. & Wykle, R. L. (1984) *J. Biol. Chem.* **259**, 12014–12019