Identification of Functional Platelet-Activating Factor Receptors on Human Keratinocytes

Jeffrey B. Travers, J. Clark Huff, Marek Rola-Pleszczynski,† Erwin W. Gelfand,* Joseph G. Morelli, and Robert C. Murphy*

Department of Dermatology, University of Colorado Health Sciences Center, and *Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado, U.S.A.; and †Immunology Division, Department of Pediatrics, Universite de Sherbrooke, Sherbrooke, Quebec, Canada

Platelet-activating factor (PAF) is a potent inflammatory mediator that has been shown to be produced by human keratinocytes and is thought to play a role in cutaneous inflammation. Immunofluorescence and radioligand binding studies were used to characterize PAF receptors (PAF-R) on human keratinocytes and the human epidermoid cell lines A-431 and HaCaT. Indirect immunofluorescence studies demonstrated anti–PAF-R staining of primary cultures of human keratinocytes, A-431 cells, and HaCaT cells. Primary cultures of human fibroblasts and the melanoma cell line SK-30 failed to show immunostaining above that seen with control antiserum. With indirect immunofluorescence studies of sections of normal human skin, a granular anti–PAF-R staining pattern was noted on the keratinocyte cell membranes. A-431 cells readily metabolized PAF by deacetylation-reacylation at 37°C, but not at 4°C. Binding studies on crude membrane preparations of A-431 cells conducted at 4°C demonstrated specific binding that reached saturation by 120 min. Scatchard analysis of PAF binding data revealed a single class of high-affinity (K_D = 6.3 ± 0.3 nM) PAF binding sites. The immunofluorescence and radioligand binding sites were shown to be functional PAF-Rs, as 10 pM to 1 μM PAF increased intracellular calcium in primary cultures of human keratinocytes, A-431 cells, and HaCaT cells, whereas PAF treatment of primary cultures of human fibroblasts or the melanoma cell line SK-30 did not result in changes in the intracellular calcium concentration. The structurally dissimilar PAF-R antagonists CV-6209, Ro19-3704, and alprazolam all inhibited the PAF-induced calcium changes in A-431 cells. The CV-6209 inhibition was seen at doses that competed with the PAF binding to these cells. These studies provide the first evidence for the presence of a functional PAF-R expressed on human keratinocytes, suggesting that this lipid mediator may play an important role in normal keratinocytes or in inflammatory dermatology. *J Invest Dermatol 105:816–823, 1995*

Platelet-activating factor (1-alkyl-2-acetyl-glycero-3-phosphocholine; PAF) is a potent activator of many cell types including platelets, vascular endothelium, neutrophils, mast cells, and monocytes [1,2]. By acting as both a chemoattractant to recruit and as a stimulus to activate granulocytic cells, PAF has profound proinflammatory effects. In addition to the proinflammatory effects found with the stimulation of the above cell types, PAF has mitogenic effects on cultured smooth muscle and lymphoblastic cell lines [3–5]. The majority of PAF actions are thought to be exerted through interaction with a specific receptor, because radioligand binding studies using [^{3}H]PAF have demonstrated high-affinity binding sites on PAF-responsive cells, and various structurally dissimilar PAF receptor antagonists can inhibit PAF effects [6]. The PAF receptor (PAF-R) has been cloned from guinea pig lung [7] and human leukocytes [8]. The elucidated structure of the PAF-R places it in the G-protein–coupled rhodopsin class receptor family, with seven putative transmembrane domains.

The PAF-R is functionally linked to phosphoinositol hydrolysis, and activation results in the production of inositol-1,4,5-trisphosphate and diacylglycerol [9]. Inositol-1,4,5-trisphosphate mobilizes intracellular calcium, and diacylglycerol activates protein kinase C. PAF also affects cyclic nucleotides by inhibiting cyclic adenosine monophosphate accumulation [10]. PAF activates microtubule-associated protein-2–kinase [11] and induces expression of the early response gene *c-fos* [12].

Several lines of evidence suggest that PAF may play a role in epidermal pathophysiology. First, PAF has been found in psoriatic scale [13] and in blister fluid from both traumatic [14] and bullous pemphigoid [15] blisters. Primary cultures of human keratinocytes have been shown to synthesize PAF when stimulated with the calcium ionophore A23187 [16]. Second, intradermal injections of PAF result in wheal and flare reactions [17]. Third, PAF-R antagonists administered either systemically or topically have anti-inflammatory effects in murine model systems of contact dermatitis [18,19].

Although there is much evidence suggesting a role for PAF in

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Reprint requests to: Dr. Jeffrey B. Travers, Departments of Dermatology and Pharmacology, Indiana University School of Medicine, 550 North University Boulevard, Suite 3240, Indianapolis, IN 46202.

Abbreviations: GPC, glycerophosphocholine; KLH, keyhole limpet hemocyanin; PAF, platelet-activating factor; PAF-R, PAF receptor.

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epidermal function, a keratinocyte PAF-R has not yet been demonstrated. In this study, we used immunofluorescence and radioligand binding studies to identify PAF-R immunoreactivity and specific binding sites in human keratinocytes. Characterization of PAF-induced changes in keratinocyte intracellular free calcium using the calcium-sensitive fluorescent dye indo-1 indicated that these binding sites were functional receptors. The demonstration of a functional keratinocyte PAF-R suggests that PAF may have direct effects on keratinocyte function.

MATERIALS AND METHODS

Reagents Labeled PAF (1-[^{3}H]octadecyl-2-acetyl-glycerophosphocholine [GPC], 161 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Unlabeled PAF (1-hexadecyl-2-(R)acetyl-GPC) and other phospholipids used for chromatography were purchased from Biomol Research Labs (Plymouth, PA). LysoPAF (1-hexadecyl-2-(R)lyso-GPC), S-PAF (3-hexadecyl-2-(S)acetyl-1-GPC), fatty acid-free bovine serum albumin (BSA), HEPES, phenanthroline, phenylmethylsulfonyl fluoride, leupeptin, and aprotinin A were purchased from Sigma Chemical Co. (St. Louis, MO). CV-6209 was a kind gift of Dr. Hajime Toguchi (Takeda Chemical Industries, Japan). Ro 19-3704 was kindly provided by Dr. Peter Sorter (Hoffman-La Roche, Inc., Nutley, NJ). Alprazolam was a generous gift from the UpJohn Co. (Kalamazoo, MI).

Cell Culture Primary cultures of human neonatal keratinocytes and fibroblasts were isolated from foreskins as described previously [20]. All tissue culture media and supplements were obtained from Gibco (Grand Island, NY). Human neonatal keratinocytes were grown in keratinocyte–serum free medium supplemented with antimicrobials (penicillin 10,000 U/mL, streptomycin 10 mg/mL, fungizone 25 μg/mL) and were used after the second passage (days 18–22). Human neonatal foreskin fibroblasts were grown in M-199 media with antimicrobials and were used within the first month of culture. The epidermoid cell line A-431 was obtained from American Type Culture Collection (Rockville, MD), and HaCaT cells [21] were a kind gift from Professor Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany). The keratinocyte cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 5 mM HEPES, L-glutamine, and antimicrobials. For use in the metabolism, binding, and calcium studies, adherent cells were harvested while 80% to 90% confluent by treatment with 3 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) at 37°C for 30 min, with gentle scraping. The cells were washed at least twice with Hanks' balanced salt solution (HBSS) without phenol red, which contained (in mM): 138 NaCl, 5 KCl, 0.3 KH_2PO_4, 0.3 Na_2HPO_4, 0.5 $MgCL_2$-$6H_2O$, 0.4 $MgSO_4$-$7H_2O$, 4 $NaHCO_3$, 1.3 $CaCl_2$, 5.6 glucose, and 25 HEPES at pH 7.4.

Measurement of PAF-R Immunofluorescence Immunofluorescence studies to characterize the keratinocyte PAF-R used a rabbit polyclonal antibody directed against a decapeptide expressed on the extracellular portion of the human PAF-R. This antibody (anti–hPAF-R$^{164-173}$) has been shown to be specific for the PAF-R by both fluorescence-activated cell sorter analysis and Western blotting [22]. Immunofluorescence studies used either anti–hPAF-R$^{164-173}$ or a control antibody from rabbits immunized against the carrier keyhole limpet hemocyanin (anti-KLH) alone, purified as described [22], diluted 1:100 with PBS. Keratinocytes were cultured in Vitrogen 100 (Celtrix, Santa Clara, CA) pretreated Lab-tek Chamber slides (Nunc, Naperville, IL). Subconfluent cultures were washed twice with cold PBS and incubated with either anti–hPAF-R$^{164-173}$ or anti-KLH for 2 h at 4°C. The primary antibodies were then removed, and the slides were washed with cold PBS. The cells were then incubated with a 1:100 dilution of affinity-isolated fluorescein isothiocyanate (FITC)-labeled goat F(ab')$_2$ anti-rabbit IgG (Biosource Int., Carrillo, CA) for 2 h at 4°C, then washed twice with cold PBS and dried briefly before acetone fixation. Skin specimens were taken from extra tissue from young adults undergoing cosmetic procedures. The tissues were embedded in OCT (Miles Inc., Elkhart, IN), frozen in liquid nitrogen, and stored at −70°C until used. The skin was sectioned at 5 μm on a cryostat. Sections were placed on gelatin-coated slides and incubated with anti-hPAF-R$^{164-173}$ or anti-KLH antibody for 2 h at room temperature, rinsed with PBS, then incubated with the FITC-labeled secondary antibody for 1 h at room temperature. Coverslips were mounted and the slides were examined on an Olympus fluorescence microscope.

Measurement of [^{3}H]PAF Metabolism A-431 cells (2.5×10^6) were washed and resuspended to a final volume of 0.95 ml of HBSS. To the above cell suspension, we added 50 μl of aqueous solution containing [^{3}H]PAF complexed to BSA (2.5 mg/ml), bringing the final concentration to 1×10^{-9} M. The incubation mixture was shaken gently at either 37°C or 4°C for 120 min. The reaction was terminated by addition of 3.75 ml of chloroform/methanol (1:2 v/v) to the cell suspension, and the lipids were extracted using the technique of Bligh and Dyer [23]. The recovered products were those present in both the cells and medium, and the recovery of tritium label in the lipid fraction was greater than 85%. Solvents were removed by a stream of nitrogen, and the lipids were resuspended in chloroform/methanol (1:2 v/v). The phospholipids and phospholipid standards were separated on silica gel G thin-layer chromatography (TLC) plates (Analtech, Newark, DE) using an acidic solvent system containing chloroform/methanol/glacial acetic acid/water (65:35:8:1, v/v). The distribution of radiolabel was determined using an automatic TLC linear analyzer (Berthold, Germany).

Measurement of [^{3}H]PAF Binding Subconfluent cultures of A-431 cells ($150–200 \times 10^6$ cells) were washed and resuspended to a final volume of 20 ml with PBS containing the protease inhibitors phenanthroline (1 mM), phenylmethylsulfonyl fluoride (100 μM), aprotinin A (10 μg/mL), and leupeptin (10 μg/mL). The cellular suspension was sonicated on ice, then centrifuged at $100 \times g$ to remove whole cells. The supernatant was removed and centrifuged at $100,000 \times g$ for 60 min at 4°C. The resulting pellet was resuspended in binding buffer, which contained (in mM): 10 NaCl, 5 $MgCl_2$-$6H_2O$, 5 $MgSO_4$-$7H_2O$, 10 $CaCl_2$, 5.6 glucose, and 10 Tris-HCl at pH 7.0. Aliquots of crude membrane (50 μg protein; measured by protein assay [24]) were placed in 15×85-mm glass tubes pretreated with Sigmacote (Sigma), with a final volume of 0.4 ml. Suspensions were then simultaneously exposed to 50 μl of aqueous solution containing [^{3}H]PAF complexed to BSA (10 mg/ml), plus either 50 μl unlabeled PAF, lysoPAF, or CV-6209 complexed to BSA, or BSA alone. The mixture of crude membranes, [^{3}H]PAF, and inhibitors was shaken gently at 4°C for the appropriate times. Each sample was run in triplicate. Suspensions were harvested by suction through GF/C filters (Whatman) premoistened with 10 mg/ml BSA using a Hoeffer Filtration apparatus (Hoeffer Scientific, San Francisco, CA). The tubes were then washed three times with 5 ml of cold binding buffer. The filters were air dried and placed in 10-ml scintillation vials, to which 7 ml of Scintiverse (Fisher, Fairlawn, NJ) was added. The vials were counted in a Beckman LS6800 scintillation counter that was programmed to measure each sample's quench and to determine dpm from cpm using tritium standards.

Intracellular Calcium Measurements Adherent cells were harvested while 80% to 90% confluent by treatment with 3 mM ethylenediaminetetraacetic acid in PBS at 37°C for 30 min, with gentle scraping. The cells were washed at least twice with HBSS without phenol red. Cells ($0.5–1.0 \times 10^6$/ml) were then loaded with the Ca^{++}-sensitive indicator, indo-1 AM (1 μM; Calbiochem, San Diego, CA), in HBSS at 37°C for 45 min. After loading, the cells were spun down, resuspended in HBSS, and maintained at room temperature. Before each measurement, an aliquot of the cell suspension ($1.0–1.5 \times 10^6$) was pelleted rapidly by centrifugation and resuspended in 3 ml of HBSS. PAF and PAF-R antagonists were added in 2.5–5 μl ethanol. Endothelin-1 was added in PBS buffer. Indo-1 fluorescence was monitored in a Hitachi F-4010 spectrophotometer. Measurements were performed at 37°C with constant stirring. The excitation and emission wavelengths of the fluorescent dye were 331 and 410 nm, respectively, and $[Ca^{++}]_i$ was calculated as described [25].

RESULTS

PAF-R Expression at the Cell Surface of Cultured Keratinocytes and Skin Slices To characterize the expression of PAF-R at the protein level, we used a rabbit polyclonal antibody against a decapeptide (hPAF-R$^{164-173}$), corresponding to a segment in the second extracellular loop of the putative structure of the human PAF-R, in immunofluorescence studies as described in *Materials and Methods*. The anti–hPAF-R$^{164-173}$ antibody bound to the surface of cultured human keratinocytes and to the keratinocyte cell lines A-431 and HaCaT, as shown in **Fig 1*A-D***. The granular pattern of fluorescence outlining the cells was consistent with that of a membrane receptor. Indirect immunofluorescence with control antibody (anti-KLH) did not produce similar staining **(Fig 1*F*)**. Incubation of anti–hPAF-R$^{164-173}$ antibody with primary cultures of human fibroblasts **(Fig 1*E*)** or the melanoma cell line SK-30 (data not shown) did not demonstrate differences in fluorescence above the background found with anti-KLH. Incubation of skin slices with anti–PAF-R$^{164-173}$ antibody resulted in selective binding to the epidermis **(Fig 2)** in a granular pattern on keratinocyte membranes, most intense on the suprabasal cells.

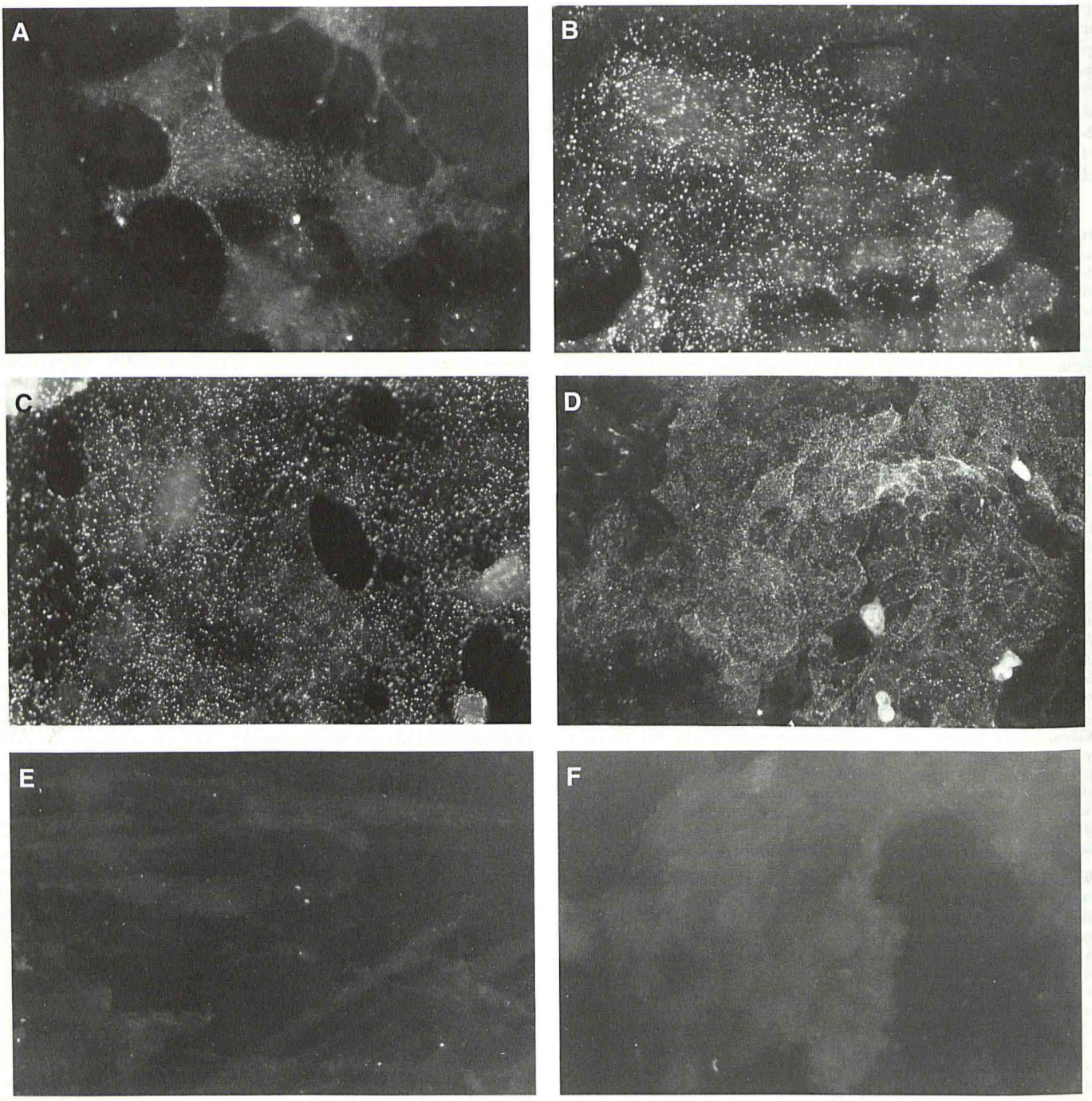

Figure 1. Indirect immunofluorescence labeling of human keratinocytes. Cultured keratinocytes were incubated with either anti–hPAF-$R^{164-173}$ or anti-KLH, washed, and incubated with FITC-conjugated goat anti-rabbit IgG, as described in *Materials and Methods*. The anti–hPAF-$R^{164-173}$ antibody gave granular staining of the cell membranes on (*A*) human neonatal foreskin keratinocytes (600×), (*B*) A-431 cells (400×), (*C*) HaCaT cells (600×), and (*D*) HaCaT cells (200×), but did not stain (*E*) human neonatal foreskin fibroblasts (400×). The anti-KLH control antibody failed to stain primary cultures of human neonatal keratinocytes, A-431 cells, HaCaT cells, or primary cultures of human neonatal foreskin fibroblasts. *F*, an example of the anti-KLH antibody staining in A-431 cells (400×).

Metabolism of PAF by A-431 Cells Because it was not known whether keratinocytes metabolize PAF, [^{3}H]PAF was incubated with A-431 cells and the products were examined by TLC, as described in *Materials and Methods*. **Figure 3** shows the distribution of label in PAF, lysoPAF, 1-alkyl-2-acyl-GPC, and neutral lipids after incubation with A-431 cells for 2 h at 37°C and 4°C. A-431 cells readily metabolized PAF at 37°C, with 1-alkyl-2-acyl-GPC the major product, indicating that these cells metabolize PAF by the deacetylation-reacylation pathway found in other tissues [26,27]. This metabolism was temperature dependent, with minimal PAF degradation at 4°C. The products shown in **Fig 3** were extracted from the complete incubation mixture and thus represent products derived from both cells and medium. Incubation of [^{3}H]PAF under standard conditions at 37°C in the absence of cells resulted in negligible metabolism of [^{3}H]PAF (results not shown).

Binding of PAF to A-431 Membranes Because ligand metabolism can result in spurious binding results, PAF binding studies

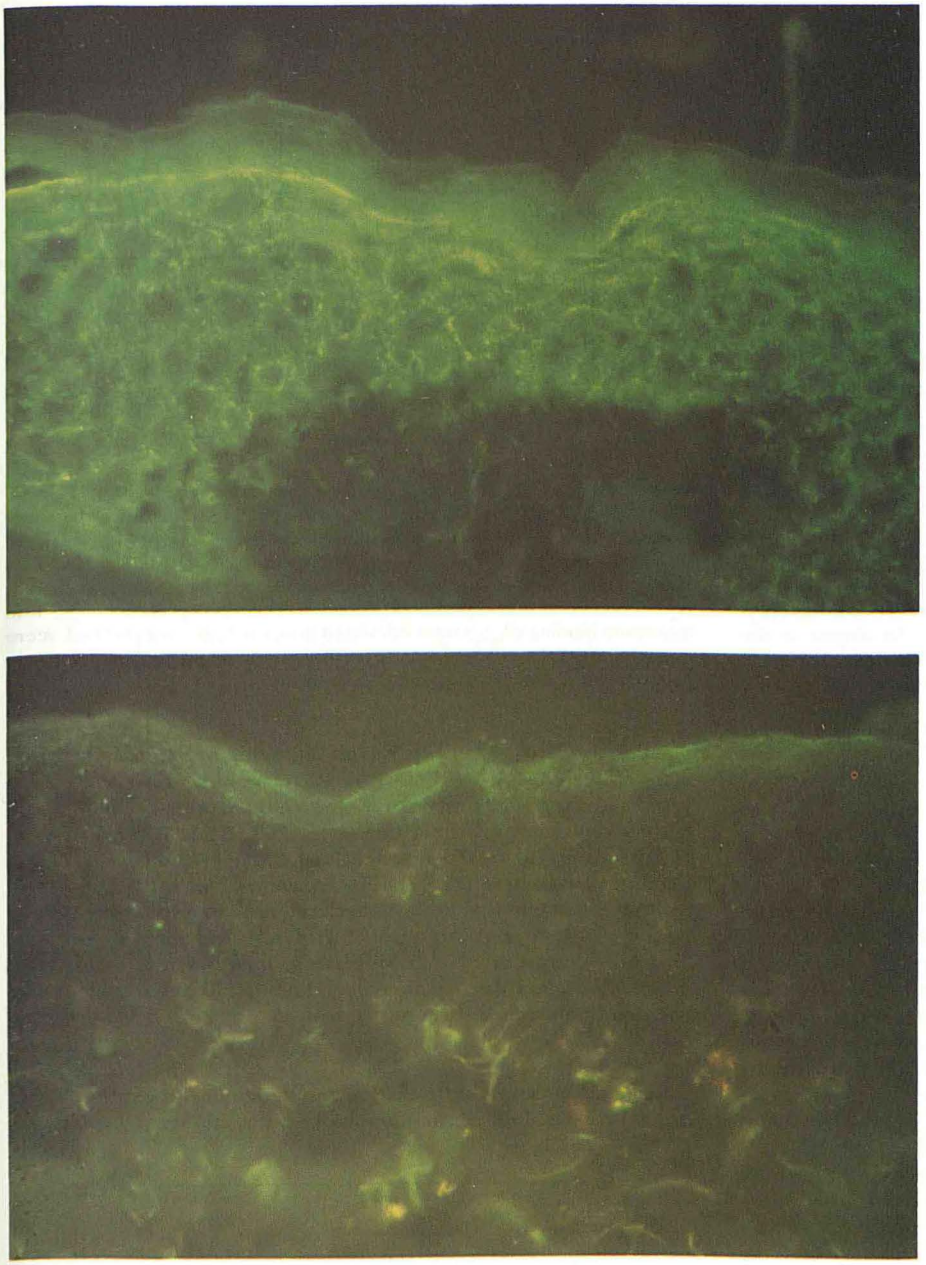

Figure 2. Indirect immunofluorescence labeling of sections of normal human skin. Five-micron–thick cryostat sections were incubated with either anti–hPAF-$R^{164-173}$ or anti-KLH, washed, and incubated with FITC-conjugated goat anti-rabbit IgG as described in *Materials and Methods*. Granular staining of the keratinocyte cell membranes was seen with the anti–hPAF-$R^{164-173}$ antibody (*A*), but not with the anti-KLH antibody (*B*) (400×).

were conducted at 4°C. The kinetics of PAF binding to A-431 membranes were examined. Crude membrane preparations of A-431 cells (approximately 50 μg protein) were incubated in triplicate with 1 nM of [^{3}H]PAF, with or without 1 μM of unlabeled PAF, and the binding was quantified as described in *Materials and Methods*. As indicated in **Fig 4**, specific binding reached saturation by 120 min. The affinity and number of PAF binding sites in A-431 cells were determined by constructing saturation binding isotherms. As demonstrated in **Fig 5**, PAF specific binding increased with the concentration of labeled ligand and reached saturation. The data were then subjected to Scatchard analysis [28] to quantitate the number of binding sites present and their apparent affinity. Scatchard analysis of four separate experiments gave a linear plot (representative experiment shown in **Fig 5**), indicating a single population of PAF binding sites. The equilibrium dissociation constant (K_D), representing the affinity of the PAF binding site, was calculated to be 6.3 ± 0.3 nM. The B_{max}, representing the total number of specific binding sites present, was calculated to be 1.0 ± 0.1 pmol/mg protein. Assuming an equimolar ligand-receptor complex and 100×10^6 cells yielding 2.1 mg of crude membrane protein under our experimental conditions, the above B_{max} corresponds to 120 ± 12 binding sites per A-431 cell. The specificity of PAF binding was examined by conducting competition binding studies with unlabeled PAF, the PAF-R antagonist CV-6209, and lysoPAF **(Fig 6)**. Both unlabeled PAF and CV-6209 (50% inhibitory concentration = 4.9 and 10.5 nM, respectively) competed with [^{3}H]PAF for binding to A-431 membranes, whereas lysoPAF was inactive.

PAF-Induced Intracellular Calcium Mobilization To determine whether the binding sites on human keratinocytes were functionally active, we measured changes in $[Ca^{++}]_i$ induced by PAF using the fluorescent probe indo-1. PAF caused a transient increase in intracellular free calcium in a dose-dependent manner. As illustrated in **Fig 7**, $[Ca^{++}]_i$ began to increase 5 to 10 seconds after the addition of PAF and returned to baseline within several minutes. Repeat stimulation with PAF resulted in little change in $[Ca^{++}]_i$, suggesting homologous desensitization (results not shown). Dose-response curves in **Fig 8** characterize the relation between maximal changes in $[Ca^{++}]_i$ and PAF concentration for

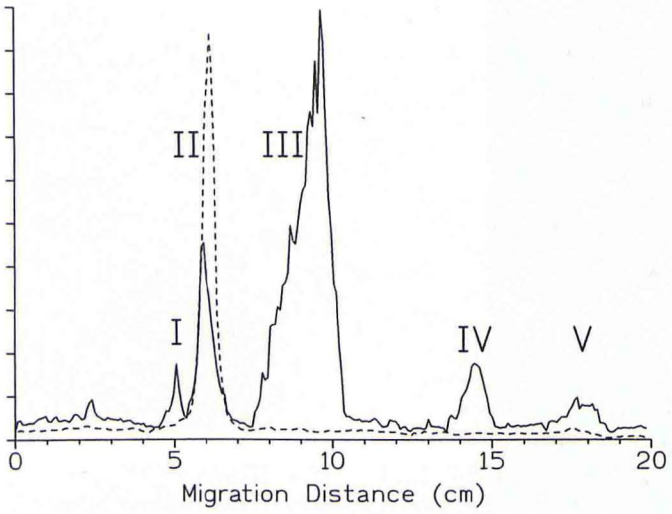

Figure 3. Distribution of label in products after incubation of 1-[^{3}H]octadecyl-2-acetyl-GPC with A-431 cells. The cells were incubated for 120 min at 4°C (*dashed line*) and 37°C (*solid line*), and the lipids were extracted as described in *Materials and Methods*. An aliquot of the labeled products was separated on TLC and radioassayed. The peak numbers identify the following lipids: 1-[^{3}H]octadecyl-2-lyso-GPC (peak I, RF = 0.26), 1-[^{3}H]octadecyl-2-acetyl-GPC (peak II, RF = 0.31), 1-[^{3}H]octadecyl-2-acetyl-GPC (peak III, RF = 0.51), unknown component (peak IV, RF = 0.77), and neutral lipid comigrating with 1-[^{3}H]octadecyl-2-acetyl glycerol (peak V).

primary cultures of human keratinocytes and A-431 cells. PAF treatment of indo-1–loaded HaCaT cells resulted in calcium responses similar to those in A-431 cells (data not shown). The change in $[Ca^{++}]_i$ was evident at a PAF concentration as low as 10 pM. The median effective concentration for the curves generated were 780 pM and 1.5 nM for primary cultures of human keratinocytes and A-431 cells, respectively. S-PAF, the biologically less active enantiomer of PAF, was a much less potent stimulus for A-431 calcium mobilization than was native PAF. The structurally dissimilar PAF-R antagonists CV-6209, Ro19-3704, and alprazolam all inhibited PAF-induced calcium flux in a dose-dependent

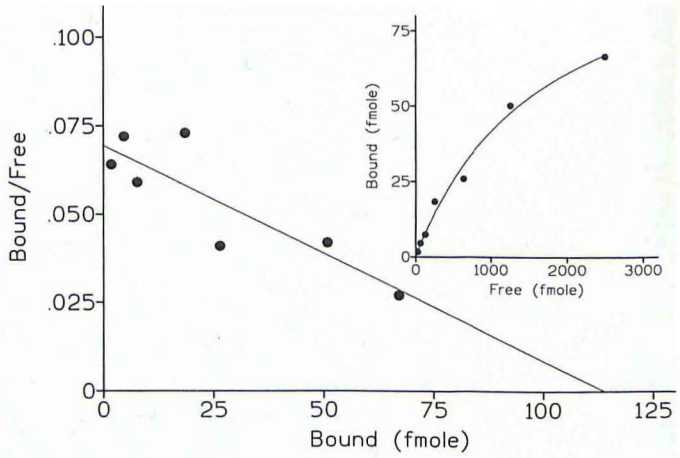

Figure 5. Saturation kinetics and Scatchard analysis of [^{3}H]PAF binding to A-431 membranes. Saturation binding isotherms were constructed (*inset*) by equilibrium (120 min) binding of 0.05 to 10 nM [^{3}H]PAF to A-431 membranes. The data were subjected to Scatchard analysis. The data pictured are from a single experiment using triplicate samples and is representative of four separate experiments. The binding affinity (K_D) and maximum binding (B_{max}) were calculated from the Scatchard plot and were found to be 6.3 ± 0.3 nM and 1.0 ± 0.1 pmol/mg protein, respectively (mean ± SEM from four separate experiments).

manner, as illustrated in **Fig 9**. This inhibition of the PAF-induced increase in $[Ca^{++}]_i$ by these PAF-R antagonists was specific for PAF, because pretreatment of A-431 cells or primary cultures of human keratinocytes with 10 nM CV-6209, 1 μM Ro19-3704, or 100 μM alprazolam did not affect 10 nM endothelin-1–induced calcium mobilization **(Fig 7)**. The concentrations of PAF, S-PAF, or PAF-R antagonists did not affect cellular integrity as measured by trypan blue dye exclusion. PAF (100 nM) treatment of indo-1–loaded primary cultures of fibroblasts, primary cultures of human melanocytes, and the melanoma cell line SK-30 did not affect the baseline fluorescence (data not shown).

DISCUSSION

These studies demonstrate that human keratinocytes express functional PAF-Rs. Indirect immunofluorescence studies using a poly-

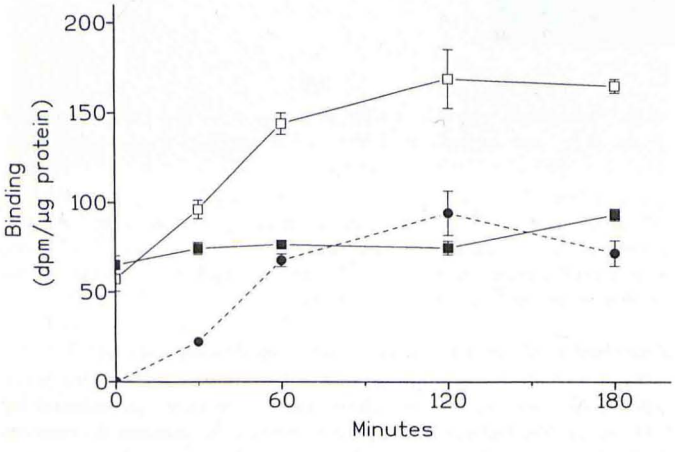

Figure 4. Kinetics of [^{3}H]PAF binding to A-431 cell membranes at 4°C. Crude membrane protein (50 μg) was incubated with 1 nM [^{3}H]PAF for the indicated time and harvested as described in *Materials and Methods*. Specific binding (*closed circles*) was the difference between PAF binding in the absence (*open squares*) and presence (*closed squares*) of excess unlabeled PAF (1 μM). Each point is the mean ± SEM PAF binding of two separate experiments using triplicate samples.

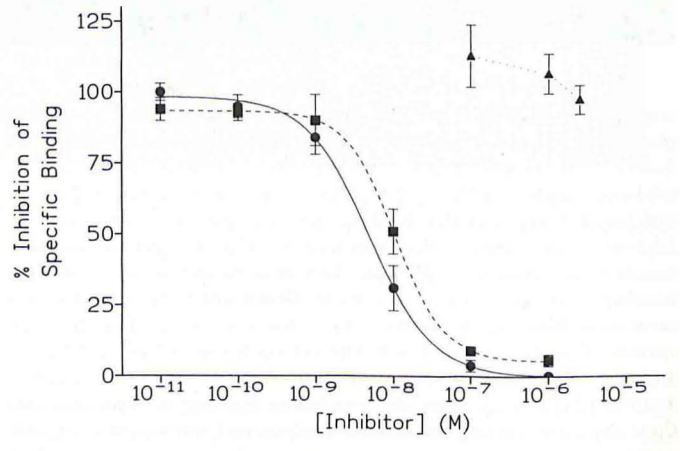

Figure 6. Competition of [^{3}H]PAF binding to A-431 membranes by PAF and CV-6209. A-431 membranes were incubated with 1 nM [^{3}H]PAF and different concentrations of unlabeled 1-hexadecyl-2-acetyl-GPC (*circles*), CV-6209 (*squares*), lysoPAF (*triangles*), or BSA alone for 120 min and harvested as described in *Materials and Methods*. The data are expressed as the mean ± SEM percentage of specific binding observed at each concentration of analogue from three or four separate experiments.

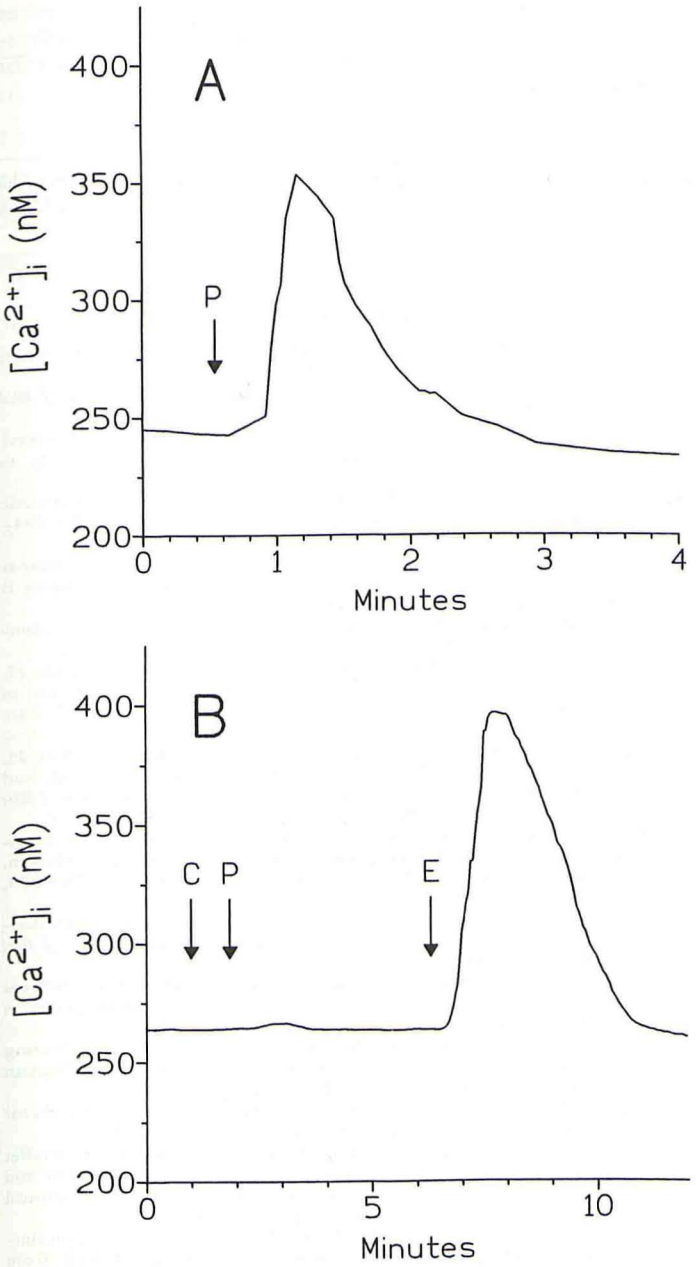

Figure 7. PAF-induced intracellular Ca^{++} mobilization in human neonatal foreskin keratinocytes. Indo-1–loaded keratinocytes were incubated in HBSS containing 1.4 mM $CaCl_2$ and challenged with (*A*) 1 nM PAF or (*B*) 10 nM CV-6209 before PAF treatment. As shown (*B*), pretreatment of cells with CV-6209 did not affect the calcium mobilization induced by 10 nM endothelin-1. These are typical results from at least four or five separate experiments.

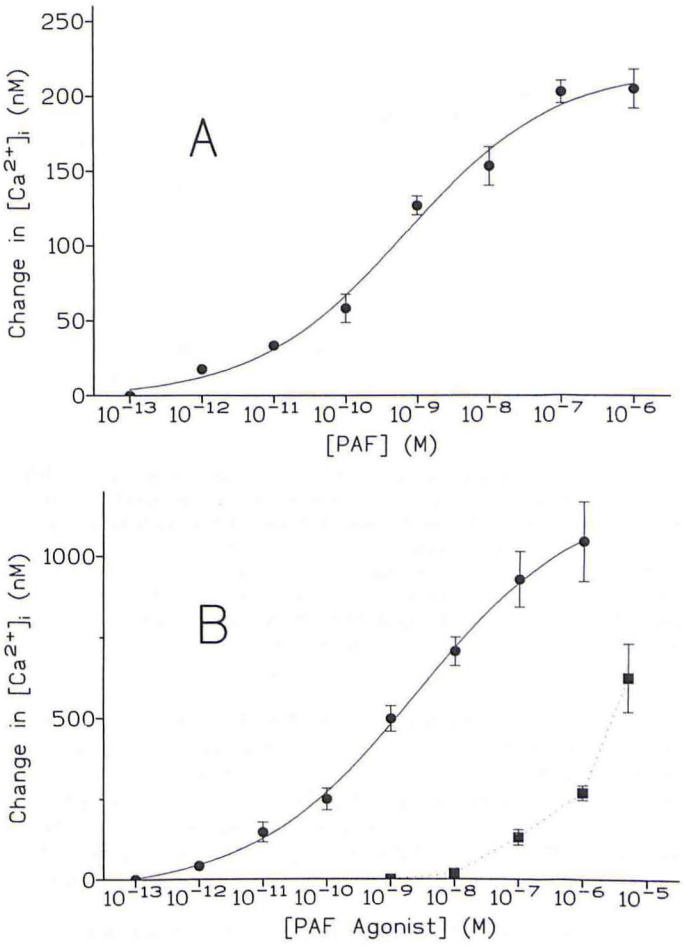

Figure 8. Concentration dependence of intracellular Ca^{++} mobilization. Indo-1–loaded (*A*) human neonatal foreskin keratinocytes or (*B*) A-431 cells were treated with 100 fM to 1 μM PAF (*circles*) or 1 nM to 5 μM S-PAF (*squares*), and the peak change in $[Ca^{++}]_i$ was calculated. The values are mean ± SEM from three to eight separate experiments.

clonal anti-peptide antibody (anti–hPAF-$R^{164-173}$), which has been shown to be specific for the PAF-R in monocytic, neutrophilic, and B-lymphoblastic cell lines [22], showed binding of anti–hPAF-$R^{164-173}$ antibody to primary cultures of human keratinocytes and the keratinocyte cell lines A-431 and HaCaT. Microtome sections of normal human skin also demonstrated a granular, cellular membrane pattern of anti-$hPAF^{164-173}$ antibody binding localized to the epidermis **(Fig 2)**. Anti-$hPAF^{164-173}$ immunoreactivity was slightly more prominent in the squamous and granular layers, with less in the basal layer. Anti–hPAF-$R^{164-173}$ staining spared the horny layer (stratum corneum), suggesting that cellular viability is necessary for PAF-R expression.

Because of the large number of cells needed and the advantages inherent in using a homogeneous source of cells instead of primary cultures (which can contain small amounts of other nonkeratinocyte skin cells), we used the cell line A-431 in both the metabolism and binding experiments. A-431 cells metabolized PAF at 37°C by transacylation, similar to that found in other cells such as neutrophils [26], lymphocytes [27], and platelets [29]. To avoid binding artifacts due to ligand metabolism, we conducted binding assays on crude membrane preparations of A-431 cells at 4°C, a temperature at which these cells do not appreciably metabolize PAF. [^{3}H]PAF specific binding increased over time and reached equilibrium by 120 min. Scatchard analysis of saturation binding isotherms revealed a single class of high-affinity binding sites, which had a K_D of 6.3 ± 0.3 nM **(Fig 5)**. This K_D found for A-431 cells is similar to reported values for human platelets [30] and Raji lymphoblasts [31], and is somewhat higher than reported values for human neutrophils [32]. The number of binding sites was estimated to be 120 per cell. PAF and CV-6209, but not the biologically inactive PAF precursor lysoPAF, competed with [^{3}H]PAF for binding to A-431 membranes.

The anti–PAF-$R^{164-173}$ immunoreactivity and [^{3}H]PAF binding sites found on human keratinocytes were shown to be functional PAF-Rs because PAF treatment resulted in intracellular calcium mobilization. The median effective concentration of the PAF-induced calcium mobilization was similar to the K_D derived from the binding studies. The pattern of PAF-induced increase in keratinocyte $[Ca^{++}]_i$ was similar to that described in other cell

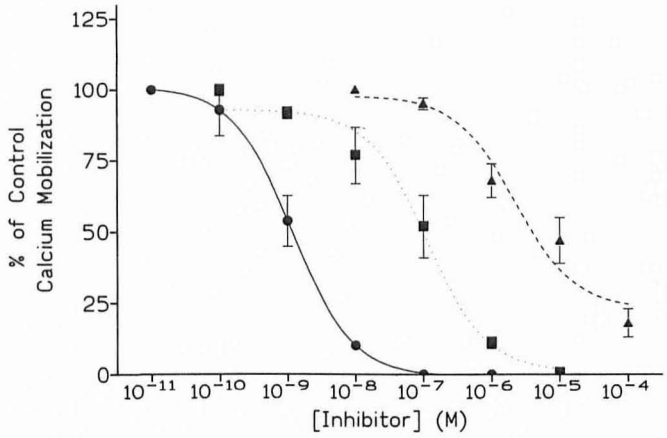

Figure 9. Concentration dependence of the inhibition of PAF-induced intracellular calcium mobilization in A-431 cells by PAF-R antagonists CV-6209, Ro19-3704, and alprazolam. Indo-1–loaded A-431 cells were pretreated with various doses of CV-6209 (*circles*), Ro19-3704 (*squares*), or alprazolam (*triangles*) for 1 min, followed by 500 pM PAF. The peak change in $[Ca^{++}]_i$ was calculated. Each point is the mean ± SEM of the percentage of peak change of $[Ca^{++}]_i$ induced by PAF alone from three to five separate experiments.

types [31]. The structurally dissimilar PAF-R antagonists CV-6209, Ro19-3704, and alprazolam all inhibited this PAF-induced response **(Fig 9)**, yet had no effect on endothelin-1–induced intracellular calcium mobilization **(Fig 7)**. Primary cultures of human fibroblasts or the melanoma cell line SK-30 did not display anti–hPAF-$R^{164-173}$ immunoreactivity, and PAF treatment of these cells did not result in intracellular calcium mobilization (results not shown).

In platelets, the PAF-R is functionally linked to inositol-specific phospholipase C, with resultant production of inositol-1,4,5 trisphosphate and diacylglycerol [9]. The intracellular calcium mobilization that is seen with PAF-R activation is due at least in part to inositol-1,4,5 trisphosphate-stimulated release of intracellular (nonmitochondrial) stores of calcium [33]. Our finding that PAF-R activation stimulates an increase in keratinocyte $[Ca^{++}]_i$ is consistent with a previous report that PAF treatment of cultured human keratinocytes (but not fibroblasts) resulted in phosphoinositol hydrolysis [34]. The neutral lipid diacylglycerol formed by phosphoinositol hydrolysis is known to activate protein kinase C by facilitating translocation of this enzyme from the cytosol to the extracellular membrane [35].

We detected 1-alkyl neutral lipids when A-431 cells were incubated with PAF **(Fig 3)**. The 1-alkyl-2-acetylglycerols, which are neutral lipid products of PAF degradation by phospholipase C, also activate protein kinase C [3]. Alternatively, 1-alkyl-2-acylglycerols, which can also be produced by PAF degradation (transacylation followed by phospholipase C degradation), can inhibit protein kinase C activation [36]. These 1-alkyl neutral lipids are thought to exert their effects independent of the PAF-R and thus would not be blocked by PAF-R antagonists. Inasmuch as PAF acting through its receptor and PAF degradation products could theoretically have either similar or opposite biochemical effects, demonstration of a functional keratinocyte PAF-R (inhibited by PAF-R antagonists) is an important first step in the future evaluation of PAF effects on this cell type.

The role of the PAF-R in keratinocyte function is not clear. However, the finding by Pignol *et al* [37] that PAF potentiates interleukin-1 production by lipopolysaccharide-stimulated guinea pig keratinocytes suggests that activation of the keratinocyte PAF-R may augment inflammation. PAF-R antagonists have been shown to have antiinflammatory effects in animal models of cutaneous inflammation, including allergic and irritant contact dermatitis in the mouse [17,18] and burn edema formation in the rabbit [38]. The availability of PAF-R antagonists may allow further characterization of this potent autacoid in keratinocyte pathophysiology, information that could lead to better treatment strategies for inflammatory dermatoses.

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