PAF Binding to a Single Receptor in Corneal Epithelium Plasma Membrane

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PURPOSE. To study the binding characteristics and the expression of platelet-activating factor receptors (PAF-R) in corneal epithelium to elucidate the site of action of PAF.

METHODS. Binding of [3H]PAF was investigated in subcellular fractions of the epithelia of bovine corneas and in membranes from cultured rabbit corneal epithelial cells. Dose-response inhibition curves of [3H]PAF-specific binding were generated using increasing concentrations of several PAF-R antagonists. RNA from rabbit corneal epithelial cells was probed for PAF-R expression by reverse transcription-polymerase chain reaction (RT-PCR) with specifically designed degenerated primers.

RESULTS. Scatchard analysis showed a high-affinity binding site in bovine and rabbit corneal epithelium. The dissociation constant (Kd) and the maximum binding sites (Bmax) in a bovine membrane preparation and similar rabbit fraction were 0.77 ± 0.03 nM and 180 ± 21 femtomoles/mg protein and 4.3 nM and 1.3 picomoles/mg protein, respectively. Specific PAF-binding sites were found in bovine preparations enriched in plasma membranes with a Kd = 69.6 pM and Bmax = 80 femtomoles/mg protein; no specific binding was found in nuclei or microsomal fractions. RT-PCR of rabbit corneal epithelium generated a single product of the predicted size (478 bp). The deduced amino acid sequence of the purified PCR product was 87% homologous to human PAF-R. The hetroepines BN 50727 and BN 50730 and the PAF structural analogues CV 3988 and CV 6209 competitively inhibited [3H]PAF binding to corneal epithelium with similar potency. WEB 2086 BS was two orders of magnitude less active in antagonizing PAF binding.

CONCLUSIONS. Corneal epithelium contains a single population of receptors localized in the plasma membrane. PAF antagonists exert their actions by blocking this PAF-R. The partial sequence deduced in rabbit corneal PAF-R show a higher homology to the human PAF-R. (Invest Ophthalmol Vis Sci. 1999;40:790–795)

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-phosphocholine) is a potent mediator in a wide variety of inflammatory and immune responses in many cells and organisms. PAF is generated from membrane phosphoglycerides and exerts its biologic effects by interacting with specific receptors. PAF accumulates in the cornea after alkali burn, and in corneal epithelium, PAF promotes molecular responses, such as Ca2+ influx, rapid activation of phospholipase A2, release of arachidonic acid, and activation of the mitogen-activated protein kinase cascade. At the transcriptional level, PAF induces expression of enzymes involved in the degradation of the extracellular matrix such as collagenase type I, the 92-kDa gelatinase, and urokinase plasminogen activator. It also amplifies the inflammatory process by inducing cyclooxygenase-2 expression and increasing prostaglandin synthesis. These activities of PAF can be blocked by selective PAF receptors (PAF-R) antagonists. During eye inflammation, several species (for review see reference 9). There is 83% overall homology in amino acid residues and 90% identity in the primary structure of the transmembrane region in PAF receptors cloned from these species.

The human PAF-R comprises 342 amino acids with seven putative transmembrane domains, characteristic of the G-protein receptor superfamily. Rat PAF-R mRNA has been identified in several tissues such as spleen, small intestine, kidney, liver, and brain. Studies have shown an intracellular high-affinity binding site in microsomal membranes isolated from rat hippocampus and cerebral cortex. This binding site is potently blocked by the PAF-R antagonist BN 50730, a synthetic hetrazepine. It has been hypothesized that this binding site represents either an intracellular PAF-R or a subtype that could be involved in the induction of transcription factors. We have recently shown that corneal epithelial cells contain functionally active PAF-Rs that are coupled to Ca2+ channels and that the PAF-R antagonist BN 50730 blocks the influx of Ca2+ stimulated by PAF.

To elucidate the site of action of PAF in corneal epithelial cells, we studied binding sites in subcellular membrane fractions from bovine corneal epithelium (a richer source of material than rabbit corneal epithelium) and in first-passage rabbit corneal epithelial cells. Several PAF-R antagonists were used to displace [3H]PAF binding from membranes. Specific binding sites have been reported in total membrane preparations from iris-ciliary body and retina. PAF-R mRNA expression has recently been shown in rat ocular tissues, including the corneal epithelium. However, the rabbit PAF-R has not yet been cloned. In the present study we show that the corneal epithelium expresses only one class of PAF-R, which is localized in the plasma membrane. Using reverse transcription-polymerase chain reaction (RT-PCR), we have also isolated a PCR product and deduced part of the sequence of rabbit PAF-R that shows a high homology to human PAF-R.

METHODS

Tritiated PAF, (1-O-[hexadecyl-1',2',3'H]-2-acetyl-sn-glycercyl-3-phosphocholine), specific activity 36 Ci/millimole, was purchased from NEN Life Sciences (Boston, MA). Unlabeled PAF was obtained from Cayman (Ann Arbor, MI). The PAF-R antagonists BN 50727 and BN 50730 were the gifts of Pierre Braquet, Institut Henri Beaufour, (Les Plessis Robinson, France), WEB 2086 BS was the gift of Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT) and CV 3988 and CV 6209 were from Biomol Reagents.
Rabbit Corneal Cell Cultures

Corneas from rabbit eyes (Pel-Freez Biologicals, Rogers, AR) were collected in sterile Dulbecco’s modified Eagle’s medium-Ham’s F-12 (DMEM-F12) (1:1) containing 50 μg/ml gentamicin and an antibiotic-antimycotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin). The endothelia and Descemet’s membranes were removed with a sterile scalpel and the epithelial surfaces incubated with Dispase II (1 U/ml; Boehringer Mannheim, Indianapolis, IN) in Hanks’ balanced salt solution medium for 1 hour in a humidified culture incubator at 37°C in 5% CO₂-95%O₂ air. The epithelia were isolated and, after centrifugation at 100g for 5 minutes, were suspended in DMEM-F12 (1:1) as described earlier but supplemented with 10 ng/ml EGF, 5 μg/ml insulin, and 10% fetal bovine serum (Gibco) and seeded in 25-cm² flasks. Media were changed three times weekly. Subconfluent cells were incubated with 0.05% trypsin (0.05%-EDTA for 30 minutes at 37°C, centrifuged at 100g for 10 minutes, and plated onto six-well tissue culture dishes (approximately 2 X 10⁵ cells/well). The cells were fed twice to three times weekly using them in the binding studies.

Preparation of Subcellular Fractions from Corneal Epithelium

The following procedures were performed at 0°C to 4°C. Corneal epithelia were either scraped from calf eyes (Pel-Freez) or from wells containing first-passage rabbit corneal epithelial cells. The tissues were homogenized in 50 mM Tris-HCl (pH 7.4), containing 0.25 M sucrose, 2 mM EGTA, 5 mM MgCl₂, 56 TIU aprotinin, and 0.2 mM PMSF. The homogenates were centrifuged at 1,000g for 10 minutes and the supernatant for 60 minutes at 105,000g to obtain a total membrane fraction. In two experiments, microsomes and a fraction enriched in plasma membranes were obtained after selective centrifugations at 50,000g and 105,000g for 1 hour, respectively, of supernatant that had been obtained after centrifugation at 12,000g for 20 minutes (mitochondrial free fraction). An enriched nuclear fraction was then prepared as described.

Radioligand Binding Assay

Rabbit corneal epithelial cells were lysed in 350 μl buffer containing guanidine isothiocyanate, total RNA was extracted using a kit (RNaseasy mini kit; Qiagen, Valencia, CA), and an aliquot was taken to determine the quantity and purity of RNA by spectrophotometry. Total RNA (2.5 μg) was denatured at 65°C for 10 minutes with 2.5 U RNase inhibitor and 0.5 μg oligo-dT in a total volume of 15 μl. First-strand cDNA was synthesized using 100 U M-MLV reverse transcriptase, 4 mM deoxyribonucleoside triphosphate, and 10 mM dithiothreitol. The mixture was heated at 37°C for 60 minutes in a total volume of 25 μl. PCR was performed using 1:10 dilution of the cDNA and 1.5 U Taq DNA polymerase in a 50-μl reaction mixture containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.01% Triton X-100), 2.5 mM MgCl₂, and 0.2 mM of each deoxyribonucleoside triphosphate.

Primers used to amplify the PAF-R were specifically designed according to the sequence of the human PAF-R using highly conserved areas when compared with the PAF-R from guinea pig and rat. The sequence of the PAF-R1 primer was 5' AAG-ATC-TTC-ATG-GTG-AAT-CTC-AC 3' and of the PAF-R2 primer was 5' GTG-AGC-AGG-GTG-CGC-ATG-AC 3'. Amplification. The reaction was started at 95°C for 10 minutes, followed by 36 cycles of 95°C, 30 seconds; 60°C, 30 seconds; 72°C, 45 seconds; and a final cycle of 72°C, 7 minutes. Amplification. The reaction was started at 95°C for 10 minutes, followed by 36 cycles of 95°C, 30 seconds; 60°C, 30 seconds; 72°C, 45 seconds; and a final cycle of 72°C, 7 minutes. Amplification.
RESULTS

Binding of PAF to Bovine and Rabbit Corneal Epithelial Membranes

When membrane preparations (100 μg protein) from bovine and rabbit corneal epithelium were incubated with [3H]PAF for 45 minutes, PAF-specific binding increased with the concentration of labeled ligand and reached saturation (Figs. 1A). Scatchard analysis of total membranes from bovine and rabbit corneal epithelium (105,000g pellet) indicated a single population of PAF binding sites (Figs. 1A, 1B).

The equilibrium $K_d$ for bovine corneal epithelium was 0.17 ± 0.03 nM and represents the affinity of the PAF binding sites. The $B_{\text{max}}$, indicating the total number of specific binding sites present, was 180 ± 21 femtomoles/mg protein. Data were processed from the Scatchard plot and represent the average ± SEM of three experiments. For first-passage rabbit corneal epithelial cells the $K_d$ was 4.3 nM and the $B_{\text{max}}$ 1.3 picomoles/mg protein. Data were obtained from two experiments. Bovine plasma membranes had a higher binding affinity for PAF than the total membrane preparation containing plasma and microsomal membranes, as shown in Figure 1C. The $K_d$ was 69.6 pM and the $B_{\text{max}}$ was 80 femtomoles/mg protein. An enriched microsomal fraction prepared from the same tissues that excluded plasma membranes did not specifically bind PAF. Also, no specific binding was found in enriched nuclear membrane preparations (data not shown). A second experiment produced similar results.

Displacement of [3H]PAF Binding by PAF Antagonists

Competitive studies with unlabeled PAF showed that the binding was reversible with a 50% inhibitory concentration (IC50) of 0.1 nM (Fig. 2A). The PAF-R antagonists produced a dose-dependent inhibition of specific [3H]PAF binding to the membranes of bovine corneal epithelium. The curves generated from the displacement of labeled PAF parallel those of unlabeled PAF. BN 50730 and BN 50727 (PAF-R antagonists containing hetrazepine rings) were specific inhibitors with IC50 values of 15.6 nM and 12.5 nM, respectively. PAF-R antagonists structurally related to PAF such as CV 3988 and CV 6209 were also very potent (Fig. 2B). WEB 2086 BS was two orders of magnitude less active than the other compounds.
conserved PAF-R sequences in rabbit corneal epithelium. Our experiments and by using a strategy based on amplification of because they could be reversed by PAF-R antagonists. In the corneal epithelium, which affect the inflammatory and wound shown that PAF exerts both fast and slow responses in the by PAF, the characteristics of the PAF-R in corneal epithelium

DISCUSSION

Expression of PAF-R in Rabbit Corneal Epithelial Cells

Rabbit corneal epithelial cells yielded a single RT-PCR product of the expected fragment size (478 bp; Fig. 3A). The signal was strongly expressed in rabbit corneal epithelium. Negative control cells (without RNA or without reverse transcriptase) showed no bands. The rabbit corneal epithelial PCR product was purified and subjected to DNA sequence analysis. Comparison of the deduced amino acid sequence of the 478-bp product with those of other cloned PAF-R from different species (Fig. 3B) showed that it is most closely related to the human PAF-R (87% identity), followed by guinea pig PAF-R (85%); the homology of the rabbit PCR product to mouse and rat was 79%.

The present study also we observed the partial cloning and expression of the rabbit PAF-R in corneal epithelial cells. By in situ hybridization techniques, rat cornea seems to express the PAF-R only in epithelium.13 Using our primers, we detected a PCR product in the corneal endothelium that requires further investigation (Xiang Ma, H.E.P. Bazan, unpublished results, 1998). The sequence analysis of the PCR product from rabbit corneal epithelium shows an 87% homology to the binding data suggest that there is a single PAF binding site in the corneal epithelium, that it is located in the plasma membrane and that, unlike brain tissue, it does not possess intracellular binding sites. PAF did not specifically bind to the membranes of subcellular fractions enriched in either microsomes or nuclei, despite using chelated Ca2+ and adding Mg2+ to the preparation, conditions that produce PAF binding in brain tissue.10 Moreover, the PAF competition curve (Fig. 2) was steep rather than shallow (i.e., significant changes in ligand binding were achieved over a relatively narrow range of ligand concentrations), which implies that there is a homogenous population of PAF-R.

The Kd and the Bmax values obtained for the single PAF receptor in cultured rabbit corneal epithelium were similar to those reported for the high-affinity binding sites in rabbit iris, rabbit ciliary body, and rat retinal PAF receptor.11,12 However, the Kd and Bmax values for the PAF receptor in bovine corneal epithelial membranes were lower that in the rabbit, indicating a sparser population of PAF receptors, with greater binding affinity for the agonist. The increased affinity for PAF by the bovine corneal epithelium preparations enriched in plasma membrane, compared with that in the preparations that included microsomes, represents enhanced specific binding, because the microsomal fraction did not bind to PAF.

Because our results suggested that there was only one binding site located in the plasma membrane, it was important to define the action of a variety of PAF-R antagonists. We found that two hetrazepine-derived antagonists, BN 50730 and BN 50727, had similar potency. This differs from the rat cerebral cortex10 where BN 50730 acts as a specific antagonist of the high- and low-affinity PAF intracellular (microsomal) binding sites, with an IC50 of 585 pM for each site, and BN 50730 did not antagonize binding to the synaptosomal membrane. Our previous experiments have shown that PAF induces an increase in intracellular Ca2+ in cultured rabbit corneal epithelial cells3 and that the increase is predominantly caused by influx of extracellular Ca2+. It is interesting to note that BN 50730 blocked the influx of Ca2+ into the cells, which suggests that this antagonist is acting at the plasma membrane level.

The ability of BN 50730 to displace [3H]PAF from the corneal epithelium plasma membrane-binding site may point to the characteristics of the binding sites themselves in different tissues. Several studies suggest the existence of more than one binding site in brain.9,10,25 However, other tissues contain a single class of PAF-R. Thus, PAF exerts multiple functions according to the cell signal system that is activated. The brain, because of its complexity, could require more steps to regulate the response to agonists such as PAF. For example, PAF-R has been shown to be highly expressed in microglias and less abundant in neuronal cells.9 Furthermore, there is a different distribution of PAF-R in various regions of the brain and, in addition, results in several studies suggest that PAF modulates neural functions.9

To elucidate the intracellular signaling mechanisms triggered by PAF, the characteristics of the PAF-R in corneal epithelium were investigated. Previous studies from our laboratory have shown that PAF exerts both fast and slow responses in the corneal epithelium, which affect the inflammatory and wound healing processes.3–9 These activities are receptor mediated, because they could be reversed by PAF-R antagonists. In the present study, the PAF-R was characterized by ligand binding experiments and by using a strategy based on amplification of conserved PAF-R sequences in rabbit corneal epithelium. Our

![Figure 2](image-url)
human PAF-R. The transmembrane domain of the PAF-R is more conserved in different species, suggesting that it is important in binding PAF. By transfecting minigenes that encode the third intracellular loop of the PAF-R, it has been recently shown that this third domain is fundamental for binding to G proteins. It was this segment that we chose for designing the downstream primer, and in the sequence analysis of the PCR product, we found complete homology of this loop to the human PAF-R (Fig. 3B).

In summary, in our study corneal epithelium expressed only one PAF-R, indicated by results in binding studies in bovine and rabbit corneal epithelial cells and by the expression of a PCR product, the predicted size and sequence of which agree with reported PAF-R cloned from other species. We also showed that species and/or tissue differences may account for variance in the activity of PAF-R antagonists.

Acknowledgments

The authors thank Laurie Varner for excellent technical assistance.

References


**Brightness Alters Heidelberg Retinal Flowmeter Measurements in an In Vitro Model**

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**PURPOSE.** The Heidelberg Retinal Flowmeter (HRF), a laser Doppler flowmetry device, has captured interest as a research and clinical tool for measurement of ocular blood flow. Concerns remain about the range and accuracy of the values that it reports.

**METHODS.** An in vitro blood-flow model was constructed to provide well-controlled laminar flow through a glass capillary for assessment by HRF. A change in material behind the glass capillary was used to simulate changing brightness conditions between eyes.

**RESULTS.** Velocities reported by the HRF correlated linearly to true velocities below 8.8 mm/sec. Beyond 8.8 mm/sec, HRF readings fluctuated randomly. True velocity and HRF-reported velocities were highly correlated, with \( r = 0.967 \) \((P < 0.001)\) from 0.0 mm/sec to 2.7 mm/sec mean velocity using a light background, and \( r = 0.900 \) \((P < 0.001)\) from 2.7 mm/sec to 8.8 mm/sec using a darker background. However, a large change in the \( y \)-intercept occurred in the calibration curve with the background change.

**CONCLUSIONS.** The HRF may report velocities inaccurately because of varying brightness in the fundus. In the present experiment, a darker background produced an overreporting of velocities. An offset, possibly introduced by a noise correction routine, apparently contributed to the inaccuracies of the HRF measurements. Such offsets vary with local and global brightness. Therefore, HRF measurements may be error prone when comparing eyes. When used to track perfusion in a single eye over time, meaningful comparison may be possible if meticulous care is taken to align vessels and intensity controls to achieve a similar level of noise correction between measurements. (Invest Ophthalmol Vis. Sci. 1999;40:795-799)

The Heidelberg Retinal Flowmeter (HRF, Heidelberg Engineering, Germany) is a noninvasive confocal scanning laser imaging device marketed for the mapping of flow magnitudes in the human fundus. It has several unique characteristics. Quantification of retinal blood flow is accomplished through a series of point measurements, each with a measurement resolution of approximately \( 10 \times 10 \mu m \) on the retinal plane, and a field depth of 400 \( \mu m \). An average clinical measurement takes approximately 5 minutes, including postmeasurement processing. Users of this technology have published various studies measuring the effects of disease and medications on blood flow in the fundus; however, many questions on the validity of HRF still remain. One concerns the arbitrary unit in the HRF report. Despite previous experiments, physical units have not been successfully correlated to the arbitrary units. Furthermore, the functional range for these units, and the normal values, are yet to be determined. Can the HRF reliably differentiate glaucomatous disease processes from healthy ones based on these values? Are these values dependent on flow or other optical factors such as pallor? Our laboratory has used HRF to measure changes in blood flow induced by hypercapnia and hyperoxia. Were these changes representative of true changes in volumetric retinal blood flow? Based on these questions, this study had two specific purposes: to determine the range of linear response, or func-