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Specific Receptor Sites for 1-*O*-Alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (Platelet Activating Factor) on Rabbit Platelet and Guinea Pig Smooth Muscle Membranes†

San-Bao Hwang,* Ching-Shin C. Lee, Mary Jane Cheah, and T. Y. Shen

ABSTRACT: By using tritiated 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (^{3}H-PAF), we have directly identified its specific binding sites on rabbit platelet plasma membranes. The equilibrium dissociation constant for ^{3}H-PAF is 1.36 (±0.05) × 10^{-9} M at 0 °C. The number of binding sites is 1.61 (±0.34) × 10^{12}/mg of membrane, which corresponds to approximately 150–300 receptors/platelet (depending on membrane vesicle orientation). Binding of ^{3}H-PAF to rabbit platelet plasma membrane is rapid ($t_{1/2}$ < 5 min at 0 °C) and reversible. For a series of PAF analogues, their affinity for the receptor sites parallels with their relative potency to induce platelet aggregation. PAF can cause contraction of smooth muscle of heart, parenchymal strip, trachea, and ileum. Specific PAF receptor binding was demonstrated with purified plasma membrane from several smooth muscles and from polymorphonuclear leukocytes but not from presumably PAF nonresponsive cells such as erythrocytes and alveolar macrophages. It is likely that the interaction of PAF with these binding sites initiates the specific responses of platelets, polymorphonuclear leukocytes, and smooth muscles.

The platelet activating factor (PAF)[1] derived from rabbit basophils has recently been identified as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (Demopoulos et al., 1979; Benveniste et al., 1979). It is a potent mediator of the physiologic alteration of IgE anaphylaxis. It induces shape change, aggregation, and the release of granular contents of the platelet (Demopoulos et al., 1979; Benveniste et al., 1979; Hanahan et al., 1980). It also causes polymorphonuclear leukocyte (PMN) chemotaxis and enhances PMN adhesiveness, aggregation, and degranulation (Shaw et al., 1981a; O'Flaherty et al., 1981). The contraction of guinea pig ileum smooth muscle (Findlay et al., 1981) and hypotension induced by PAF (Blank et al., 1979; Muirhead et al., 1981) have also been reported. Investigations of synthetic phospholipid analogues (Tense et al., 1981; Tokumura et al., 1981; Satouchi et al., 1981; Hanahan et al., 1981) showed that the structural features necessary for biological actions are highly specific. The binding of PAF to rabbit platelets has also been reported (Shaw & Henson, 1980; Camussi et al., 1980; Brown & Thuy, 1981; Valone et al., 1982), and it was suggested that PAF action on platelets may be a receptor-mediated process. In our laboratory, we have studied the first step in the process of platelet activation and identified specific binding sites for PAF on rabbit platelet plasma membranes by using a radiolabeled PAF. Our results suggest that these binding sites are involved in the initiation of the platelet response to PAF.

In vivo administration of synthetic or natural PAF intravenously to normal rabbits induces all of the respiratory and cardiovascular alterations of IgE analphylaxis (Halonen et al., 1976). Since bronchoconstriction and thrombocytopenia can be suppressed either by infusion of prostacyclin (Vargaftig et al., 1980) or in an immune platelet depressed system (Halonon et al., 1981), platelets were considered to be the primary sites for the PAF-induced respiratory alterations. On the other hand, bradycardia, right ventricular systolic hy-

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[1] Abbreviations: PAF, platelet activating factor or 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine; DPPC, dipalmitoylphosphatidylcholine; PMN, polymorphonuclear leukocyte; IgE, immunoglobulin E; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DSC, differential scanning calorimetry.

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pertension, and the initial phase of systemic hypotension occur to the same extent in the presence or absence of circulating platelets (Halonen et al., 1980). Therefore, the cardiovascular alterations have been suggested to be independent of the circulating platelets. To clarify whether these physiological alterations are produced by PAF acting directly on the effector cells or indirectly through some other mechanisms, a determination of PAF-specific binding sites on various effector cells in combination with the measurement of smooth muscle contraction by PAF should be very informative. In our study, plasma membranes from circulating and respiratory systems have been isolated and purified for PAF-receptor binding studies, and strips or rings of smooth muscle containing tissue from respiratory and cardiovascular systems have been dissected for tension studies. The results suggest that such a broad survey of PAF-responsive cells offers a direct approach to clarify the mechanisms of action of PAF.

Materials and Methods

The synthetic ^{3}H-labeled PAF, 1-*O*-[1,2-$^3H_2$]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, was purchased from New England Nuclear (Boston, MA) with a specific activity of 45 Ci/mmol. Unlabeled PAF (L-α-lecithin, β-acetyl, γ-*O*-alkyl) was obtained from Calbiochem. *rac*-1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, and *rac*-1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine were obtained from R. Berchtold (Berne, Switzerland). The purity of these samples was confirmed by TLC. Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All reagents were either analytical or reagent grade.

Differential Scanning Calorimetric Measurements of Liposomes Prepared with PAF and PAF Analogues. Dipalmitoylphosphatidylcholine (DPPC) liposomes with various molar ratios of PAF or PAF analogues to DPPC were prepared as described previously (Hwang & Shen, 1981), and the endothermic transition curves of the liposomes were recorded on a differential scanning colorimeter (Model DSC-2, Perkin-Elmer) at a heating rate of 2.5 °C/min. Both temperature and transition energy were calibrated as described (Hwang & Shen, 1981).

Preparation of Rabbit Platelets. Six volumes of blood was drawn from the central ear artery directly into 1 volume of ACD solution (1.175 g of sodium citrate, 0.685 g of citric acid, and 1.0 g of dextrose per 50 mL of H_2O). The blood was centrifuged at 270*g* for 10 min, and the top platelet-rich plasma was carefully removed. Platelets were freed from soluble plasma components by the gel-filtration technique described by Horne & Simons (1978). Fractions containing the platelets were pooled and centrifuged at 270*g* for 5 min to remove any residual red blood cells. Platelet concentrations were determined with phase-contrast microscopy.

Monitoring of Platelet Aggregation. Platelet aggregation was monitored by measuring the changes in turbidity on a Chronolog Lumi-Aggregometer, Model 400 (Havertown, PA) at 37 °C. Calcium was added to a final concentration of 1 mM before the test of platelet aggregation induced by PAF or PAF analogues.

Purification of Rabbit and Human Platelet Plasma Membranes. Fresh rabbit blood (400–600 mL) was collected from abdominal artery after peritoneal incision or from ear artery into ACD solution. Platelet-rich plasma was prepared by centrifugation at 270*g* for 10 min. The plasma was separated from platelets by centrifuging the platelet-rich plasma on ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ) according to the procedure described by Pinckard et al. (1979). The platelets banded between the plasma and ficoll layers were carefully collected and resuspended in 150 mM NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA. The platelets were sedimented to the bottom of the tube by centrifugation at 1000*g* at 4 °C for 10 min. The platelet pellets were resuspended in 150 mM NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA either with or without additional 5 mM $MgCl_2$ to a final volume of about 30 mL. The platelet suspension was divided in three portions, 10 mL each, and quickly frozen with liquid nitrogen and thawed slowly at room temperature. The freezing and thawing procedure was repeated at least 3 times. For isolation of the plasma membrane fraction, the lysed platelet suspension was layered on top of a discontinuous sucrose density gradient of 12% (w/v) and 27% (w/v) sucrose in the three tubes of the SW27 rotor of a Beckman Model L8-70 ultracentrifugation and centrifuged at 63500*g* for 5 h. Unbroken platelets, granules, and other debris sedimented to the bottom of the tubes, while the membrane fractions banding between 12% and 27% were carefully collected and stored at −80 °C. The isolated membranes were used within 2 weeks, and no changes on the PAF binding have been observed. The chemical compositions and activities of several markers are similar to those reported (Baber & Jamieson, 1970).

Freshly drawn human blood was obtained from New Jersey Blood Band (New Brunswick, NJ). The platelets and plasma membranes were prepared exactly as above for rabbit platelets.

Assays for PAF-Receptor Binding. The PAF-receptor binding was characterized by a filtration technique. The incubation was begun by the addition of the isolated membrane fraction (100 μg of membrane protein unless otherwise specified) to a final volume of 1 mL containing a known amount of both labeled and unlabeled PAF or PAF analogues in 150 mM NaCl, 10 mM Tris, pH 7.5, and 0.25% bovine serum albumin (solution A) precooled in an ice bucket. The unbound PAF was separated from bound PAF by filtration of the 0.9-mL mixture through a Whatman GF/C glass-fiber filter attached to a house vacuum line. The Whatman GF/C glass-fiber filters were presoaked with washing buffer (solution A). Each tube was successively and rapidly washed 5 times with 4 mL of ice-cold solution A. The filters were then dried and placed into vials containing 10 mL of the scintillation fluid Aquasol 2 (from New England Nuclear, Boston, MA). Radioactivity was then measured in a liquid scintillation spectrophotometer (Packard, Tri-Carb 460 CD liquid scintillation system).

Preparation of Rabbit Erythrocyte Ghosts. Rabbit erythrocyte ghosts were prepared as described by Fairbanks et al. (1971).

Preparation of Plasma Membranes of Polymorphonuclear Leukocytes from Bovine Blood and Guinea Pig Peritoneal Cavity. Bovine polymorphonuclear leukocytes (PMN) were purified from the buffy coats after 1000*g* centrifugation for 10 min. The residual red blood cells were lysed with NH_4Cl, and the PMNs were separated from platelets by low-speed centrifugation (270 *g* for 10 min). The purified PMN (>80% PMNs) were then lysed repeated by freezing and thawing for at least 3 times and then were loaded onto the top of 10% (w/w), 30% (w/w), 40% (w/w), and 50% (w/w) discontinuous sucrose density gradient and centrifuged at 63500*g* for 5 h with the SW27 rotor of a Beckman Model L8-70 centrifuge. The membrane fractions collected from the interface between the 30% and 40% sucrose solution are referred to as the "light plasma membrane fraction"; those from the interface between the 40% and 50% sucrose solution, the "heavy plasma membrane fraction" (Tsung et al., 1978). Both light and heavy

plasma membranes were reported to be free of succinate dehydrogenase and β-glucuronidase activities (Tsung et al., 1978).

Neutrophil suspensions (>85% PMNs) were also prepared from peritoneal exudates obtained 24 h after an injection of 6% (w/v) sodium caseinate into 500–1000 g of male guinea pig (Cummingham et al., 1979). Plasma membranes were prepared by the freezing and thawing procedure as described above.

Preparation of Rat Alveolar Macrophage Plasma Membranes. Alveolar macrophages were isolated by lung lavage from 175–250 g of Wistar rats (Charles River Laboratories) (Robbins et al., 1981). The resuspended macrophages were lysed by repeatedly freezing and thawing (as described above for the preparation of platelet plasma membranes), and the macrophage plasma membranes banding between 30% (w/v) and 45% (w/v) in a discontinuous sucrose density gradient were carefully collected (Gennaro et al., 1979) after centrifugation at 63500*g* for 5 h with the SW27 rotor of a Beckman Model L8-70 centrifuge and stored at −80 °C. This fraction appeared to be minimally contaminated by other subcellular organelles (Gennaro et al., 1979).

Isolation of Sarcolemmal Membranes from Rabbit, Guinea Pig, and Rat Ventricles. Two methods were used to isolate and purify cardiac sarcolemmal vesicles from rat cardiac tissue. One is that described by St. Louis & Sulakhe (1976) using brief extraction of homogenate with KCl, followed by banding in two successive discontinuous sucrose density gradients of the extracted particles. The other is that described by Jones et al. (1979) where the cardiac microsomes are incubated in the presence of ATP, Ca^{2+}, and oxalate to increase the density of the sarcoplasmic reticulum vesicles to facilitate the separation. In the same preparation, both sarcolemmal and sarcoplasmic reticulum vesicles were collected for PAF-receptor binding.

Preparation of Membranes from Rabbit and Guinea Pig Ileums. Microsomal membranes from the ileum were prepared as described (Bolger et al., 1982) except that the resulting membranes were further separated by a discontinuous sucrose density gradient of 13%, 31%, and 60% (w/w), and the membranes at each sucrose interface were collected.

Membrane Preparation from Lung and Trachea. Lung and trachea from guinea pigs (Charles River) or rabbits (New Zealand white) were excised and placed in the medium containing 150 mM NaCl, 10 mM Tris, and 0.75 M KCl, pH 7.5, with or without 5 mM $MgCl_2$. The dissected tissues were then homogenized with a polytron (Kinematica GmbH, Switzerland) at a setting of 5 for 30 s 3 times. The differential purifications are essentially the same as those described by Bolger et al. (1982). The membranes, after being washed 2 times with Tris medium without KCl, were then loaded over a discontinuous sucrose density gradient as described above. The membrane bands at different sucrose density interfaces were collected.

Results

Nonspecific Interaction of PAF with DPPC Bilayers. To demonstrate that PAF action on platelets is not due to any nonspecific interaction with membrane phospholipids, effects of PAF and 1-stearoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (1-acyl-PAF analogue) on the endothermotropic transition behavior of DPPC multilamellar dispersions (or Bangham's liposomes) were compared. Figure 1 shows the differential thermal scans of various mixtures of PAF and 1-acyl-PAF analogue with DPPC. Even though PAF is much more potent than 1-acyl-PAF analogue in platelet aggregation and degranulation, both of them induced almost identical changes of the thermal behavior, in terms of the decrease of the phase transition and pretransition temperatures (Figure 2A), the half-width of the phase transition peak (Figure 2B), and the

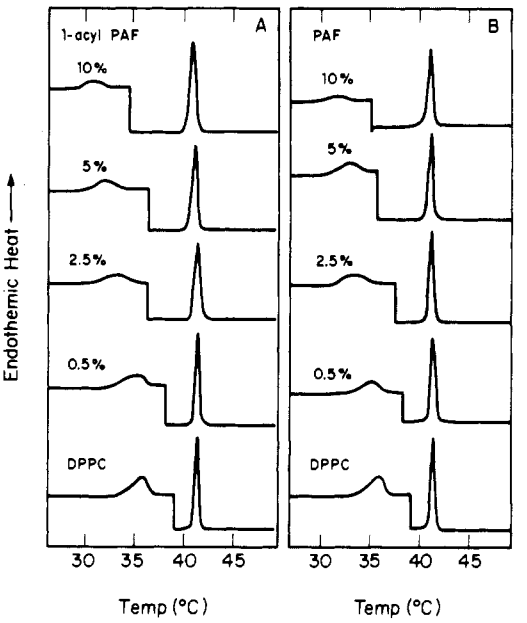

FIGURE 1: Differential thermal scans of pure DPPC liposomes and DPPC liposomes mixed with 1-acyl-PAF analogue (A) and PAF (B) at 0.5%, 2.5%, 5%, and 10% mole ratio. 50 mM phosphate buffer, pH 7.0, was used. The thermal scan curves at and near the pretransition temperature were amplified 4 times.

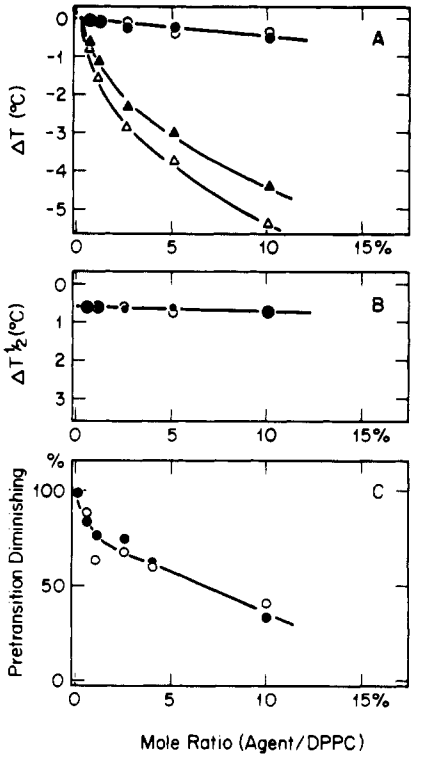

FIGURE 2: (A) Changes of temperature at phase transition and pretransition as a function of PAF and PAF analogue (1-acyl-PAF analogue). (●) Phase transition temperature changes induced by PAF; (○) phase transition temperature changes induced by 1-acyl-PAF analogue; (▲) pretransition temperature changes induced by PAF; (△) pretransition temperature changes induced by 1-acyl-PAF analogues. (B) Changes of half-width of phase transition peak as a function of PAF (●) and 1-acyl-PAF analogue (○). (C) Transition heat changes for pretransition in the presence of PAF (●) or 1-acyl-PAF analogue (○).

depression of the pretransition enthalpy change (Figure 2C). As already pointed out, both the depression of the phase transition temperature and the degree of the broadening of the transition peak reflect the mode of interaction of the disturbant molecules with PC in the bilayer (Hwang & Shen, 1981); these results suggest that there is no specificity on the interaction of PAF and 1-acyl-PAF analogues with PC molecules, and therefore, such nonspecific interaction with membrane phospholipids is probably not involved in the action of PAF on platelets. Also, it is worth noting that neither PAF nor 1-acyl-PAF analogue significantly broadens the transition peak or depresses the phase transition temperature, and a high concentration is required to diminish the pretransition. Therefore, the induced changes of the thermotropic behavior must be due to disturbance of DPPC bilayer at the boundary region [for detailed discussion, see Hwang & Shen (1981)]. These results are probably attributable to the minor structural difference between PAF, 1-acyl-PAF analogue, and DPPC in the region near the glycerol backbone.

Binding of ^{3}H-PAF to Rabbit Platelet Plasma Membranes Prepared in the Absence of Mg^{2+} Ions during Lysis. Rabbit platelet plasma membranes were prepared in the presence or absence of Mg^{2+} ions during the freezing and thawing procedure to lyse the platelet. Without the addition of Mg^{2+} during lysis, the platelet plasma membranes show a lower dissociation constant (K_D), and the binding affinity of the PAF to receptor could be stimulated with choline and choline analogues as shown below.

The specific binding at 0 °C of ^{3}H-PAF to isolated rabbit platelet plasma membranes without Mg^{2+} during the platelet lysis is given in Figure 3A. Nonspecific binding is nonsaturable in the presence of excess unlabeled PAF (1000-fold). The specific binding is defined as the total amount of ^{3}H-PAF bound minus the nonspecific binding and is shown to be saturable. A Scatchard analysis from plotting the bound/free ratio of the labeled PAF as a function of the PAF concentration that is bound to the receptor revealed the presence of a binding site with a dissociation constant (K_D) of 1.75×10^{-8} M. At saturation, the 100 μg of plasma membrane protein present in the incubation medium is capable of binding 3×10^{-13} mol of ^{3}H-PAF. In other words, there are 1.8×10^{12} receptor sites/mg of membrane protein in the incubation medium. Specific binding was linear with platelet plasma membrane protein concentration over the range of 0–175 μg of membrane protein as tested here (data not shown).

The affinity of PAF-receptor binding could be stimulated with choline or choline analogues. When 150 mM NaCl was replaced with 150 mM choline chloride, the saturable feature of the specific binding of ^{3}H-PAF to rabbit platelet plasma membranes at 0 °C was obtained (Figure 3B). However, the affinty of the PAF-specific binding site shows a 10-fold increase with a K_D of 1.37×10^{-9} M in the Scatchard plot (not shown). The x-axis intercepts in the Scatchard plot corresponding to the total receptor sites were almost identical with either NaCl or choline chloride in the incubation medium. An increase of the affinity of the PAF-specific binding was also observed with several choline analogues, including the 2-(dimethylamino)ethanol, 2-(methylamino)ethanol, and 2-aminoethanol derivatives. The reason for such stimulation is still unknown. At least the low affinity shown by PAF-receptor sites in the NaCl incubation medium is not due to the inhibition of Na^+ ions, since identical PAF-receptor binding could be obtained in either the presence or the absence of sodium ions. Furthermore, no stimulation or inhibition of the binding of radiolabeled PAF was found with addition of Ca^{2+} (2 mM),

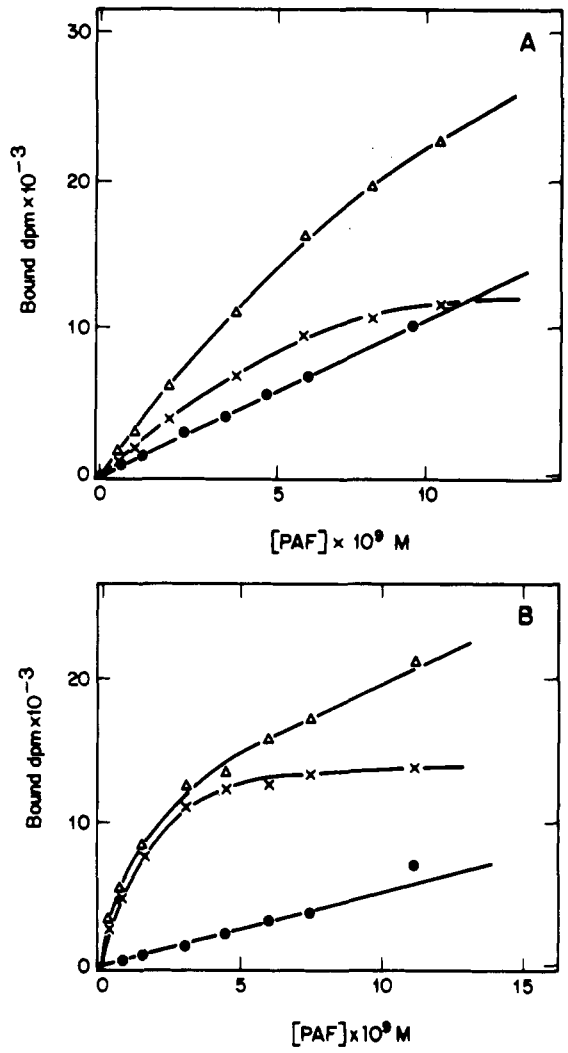

FIGURE 3: (A) Binding of ^{3}H-PAF to rabbit platelet plasma membranes prepared in the absence of Mg^{2+} and assayed in 150 mM NaCl, 10 mM Tris, and 0.25% BSA, pH 7.5, as a function of concentration of ^{3}H-PAF: (△) total binding; (●) nonspecific binding; (×) specific binding. Nonspecific binding was defined as the amount of binding not inhibited by 1000 times unlabeled PAF. Specific binding was defined as the total amount of ^{3}H-PAF bound minus the nonspecific binding. 100 μg of membrane protein was used each tube. Each point is the mean of the triplicate, and the standard derivation is less than 5% of the count. (B) Binding of ^{3}H-PAF to rabbit platelet plasma membranes prepared in the absence of Mg^{2+} and assayed in 150 mM choline chloride, 10 mM Tris, and 0.25% BSA, pH 7.5, as a function of ^{3}H-PAF concentration: (△) total binding; (●) nonspecific binding; (×) specific binding. Each point is the average of the triplicate, and the standard derivation is less than 5% of the total count.

or Mg^{2+} (5 mM), or full depletion of the sodium or more than 90% replacement of H_2O with D_2O in the incubation medium. The specific binding of PAF to its receptor is sensitive to heat and protease treatment. No specific binding could be detected after pretreatment of platelet plasma membranes for 5 min at 80 °C or overnight at room temperature. The specially bound ^{3}H-PAF also decreases with the preincubation time in the presence of pancreatic protease (data not shown).

Binding of ^{3}H-PAF to Rabbit Platelet Plasma Membranes Prepared in the Presence of Mg^{2+} during Lysis. If the platelet plasma membranes were prepared in the presence of 5 mM $MgCl_2$, a higher affinity of the PAF-receptor specific binding was demonstrated (Figure 4). The PAF-receptor specific binding remains saturable, but a Scatchard plot indicates a higher affinity of PAF-receptor specific binding ($K_D = 1.3 \times 10^{-9}$ M) as compared to that shown in Figure 3A. However, the affinity is almost identical to that of the platelet plasma

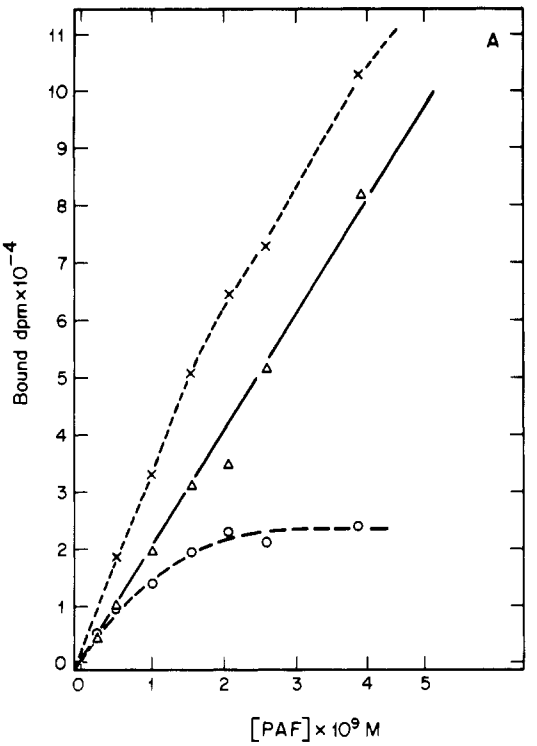

FIGURE 4: Binding of ^{3}H-PAF to rabbit platelet plasma membranes prepared in the presence of Mg^{2+} during cell lysis. The assay was done with 100 μg of membrane proteins in 150 mM NaCl, 10 mM Tris, and 0.25% BSA, pH 7.5, at 0 °C: (×) total binding; (Δ) nonspecific binding; (○) specific binding. Each point represents the average of the triplicate determination.

membranes prepared in the absence of Mg^{2+} ions during lysis and incubated with choline chloride (Figure 3B). Again the number of the receptor sites falls into the same range of 3.0 × 10^{-13} mol/100 μg of membrane protein. No further stimulation on the receptor binding in the incubation medium with choline chloride was observed (not shown).

Specificity of PAF Binding Sites. If we are indeed measuring a specific receptor responsible for mediating platelet activation, it should be possible to demonstrate a close correlation between concentration of PAF or PAF analogues required to displace 50% of the specific binding and the concentration of the phospholipid required to elicit a biological activity response. Figure 5A shows the relative potencies of PAF and several PAF analogues as platelet aggregating agents. If each of the analogues was added in several concentrations to the incubation mixture of ^{3}H-PAF with rabbit platelet plasma membranes, the inhibition of ^{3}H-PAF binding due to the presence of unlabeled analogue can also be determined (Figure 5B). The order of potencies of these analogues in competing for ^{3}H-PAF binding sites follows exactly their orders of potencies as aggregating agents. An excellent correlation between the concentration giving 50% platelet aggregation reponse (EC_{50} platelet aggregation) and the concentration of unlabeled analogue causing half-maximal inhibition of ^{3}H-PAF receptor specific binding (EC_{50} binding) indicates that the specific binding sites of ^{3}H-PAF have the specificity expected of receptor sites that mediate the platelet aggregation response to PAF analogues.

Cell and Tissue Specificity of PAF-Receptor Binding. In addition to rabbit platelet plasma membranes, membranes from isolated cells or dissected tissues were also examined for the presence of specific PAF-receptor sites. The equilibrium dissociation constant (K_D) and the number of receptor sites from a variety of cells and tissues were summarized in Table

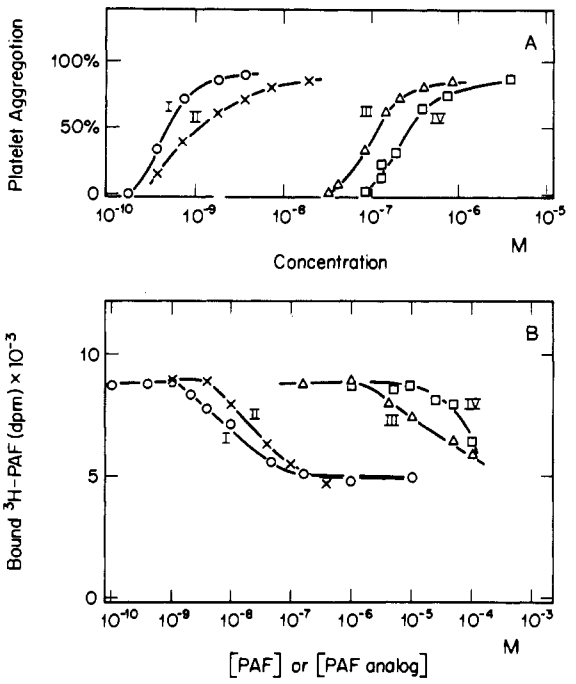

FIGURE 5: (A) Extent of platelet aggregation induced by PAF (I) and PAF analogues *rac*-1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (II), *rac*-1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (III), and 1-palmitoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (IV). (B) Effect of PAF (I) and PAF analogues (II–IV) on ^{3}H-PAF binding to rabbit platelet plasma membranes. Rabbit platelet plasma membranes were prepared in the presence of Mg^{2+} during cell lysis. 1 nM ^{3}H-PAF was incubated with 100 μg of membrane protein in the presence of the indicated concentration of PAF and PAF analogues. Each value represents the mean of triplicate determination.

Table I: Dissociation Constant and Number of Receptor Sites for PAF

source of membranes	dissociation constant, K_D (M)[a]	no. of receptor sites/mg of membrane protein[a]
rabbit platelet	1.36 (±0.05) × 10^{-9}	1.61 (±0.34) × 10^{12}
human platelet	4.5 × 10^{-9}	1.83 × 10^{11}
bovine blood PMN	4.93 × 10^{-9}	1.08 × 10^{12}
guinea pig peritoneal PMN	7.58 × 10^{-9}	1.13 × 10^{11}
rabbit ileum	4.87 × 10^{-9}	1.59 × 10^{11}

[a] $n = 3$.

I. Human platelet plasma membranes, plasma membranes, both heavy and light fractions, from bovine blood PMN and guinea pig peritoneal PMN, and membranes from rabbit ileum, guinea pig ileum and lung, and rat trachea also show PAF-specific binding. Because of the high nonspecific binding and low number of receptor sites per unit membrane prepared, the dissociation constant and the number of receptor sites on membranes from guinea pig lung and rat trachea were not determined. So far, no specific PAF receptor binding has been found with rabbit erythrocytes and rat alveolar macrophages, which are presumably PAF nonresponsive cells. Also, no specific PAF receptor binding was found with membrane preparations from several other smooth muscle tissues including ventricles from guinea pigs, rats, and rabbits, trachea tissues from guinea pigs and rabbits, and lung tissues from rats and rabbits.

Rate of Association and Dissociation of ^{3}H-PAF from Rabbit Platelet Plasma Membranes. The specific binding of ^{3}H-PAF (1 nM) to purified rabbit platelet plasma membranes

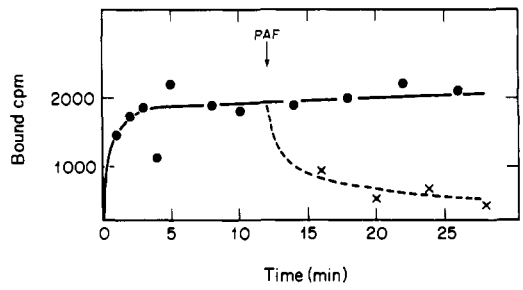

FIGURE 6: Time course of ^{3}H-PAF bound to rabbit platelet plasma membranes. ^{3}H-PAF (1 nM) was incubated with rabbit platelet plasma membrane at 0 °C. In the same figure, a time course of inhibition of bound ^{3}H-PAF with excess unlabeled PAF (1 μM) added at 12 min is also shown.

was rapid, with a half-time of less than 1 min at 0 °C (Figure 6). Equilibrium between bound and unbound ^{3}H-PAF was reached within 5 min and was maintained until 30 min after addition as tested here. There was no evidence of spontaneous dissociation or additional binding of PAF during this time. Also, no ^{3}H-PAF degradation was found from the filter-bound materials, extracted by the Bligh and Dyer's technique (Bligh & Dyer, 1959), separated on thin-layer chromatography with a solvent system of $CHCl_3$/MeOH/CH_3COOH/H_2O (100:50:14:6 v/v/v/v), and detected on a Bioscan Bid System 100/800 (Bioscan, Inc., Washington, DC). The reversibility of ^{3}H-PAF binding was tested by adding a large excess (1 μM) of unlabeled PAF to an equilibrated mixture of ^{3}H-PAF and platelet plasma membranes. As also demonstrated in Figure 6, ^{3}H-PAF binding to platelet plasma membranes is readily reversible, and the reversibility is completed within 5–10 min at 0 °C.

Discussion

We have demonstrated that ^{3}H-PAF binding to platelet plasma membranes is a saturable, heat-sensitive, and time-dependent process. At 0 °C and 1 nM ^{3}H-PAF concentration, at least 35% of the binding appears to be specific, i.e., displaceable by unlabeled PAF but unaffected by the same concentration of its biologically less potent analogues. There is a close correlation between the abilities of PAF analogues to displace ^{3}H-PAF from its specific binding sites and their abilities to cause platelet aggregation, despite the fact that different conditions were used in the binding and the aggregation assays. These data suggest that we have identified a binding site that has the characteristics expected of a specific receptor for PAF and that functions biologically in the recognition of PAF analogues by platelets leading to aggregation.

The events subsequent to ligand-receptor binding during platelet activation are still poorly understood. One pathway of platelet activation involves the release of arachidonic acid from membrane phospholipids and its subsequent transformation into endoperoxides and thromboxane A_2 (Samuelsson, 1976, 1983; Smith, 1976). Both slow-reacting substance and thromboxane A_2 productions have been observed in PAF-stimulated platelets (Mencia-Huerta et al., 1981; Shaw et al., 1981b). However, no significant inhibition of PAF-induced platelet aggregation and/or ATP release was observed with several nonsteroid antiinflammatory drugs (NSAIDS), including aspirin, diflunisal, sulindac sulfide, etc., which are cyclooxygenase inhibitors and which do not interfere with PAF-receptor binding (our published observations). It seems that thromboxane A_2 production does not play a significant role in PAF-induced rabbit platelet degranulation and aggregation. Both phospholipase A_2 and diglyceride lipase activities are predominantly associated with the intracellular vesicles (LaGarde et al., 1981). Phospholipase C has been described as a cytosolic enzyme (Mauco et al., 1979; Billah et al., 1980). It would seem unlikely that PAF-receptor interaction would result in a direct stimulation of phospholipase A_2, phospholipase C, and/or diglyceride lipase. If a second messenger is involved before activation of these enzymes, its relationship with PAF-receptor recognition remains to be clarified.

The PAF receptor in platelet plasma membrane is heat labile and protease sensitive. The lack of correlation between the effects of PAF analogues on platelet aggregation and on several physical properties of pure DPPC bilayer as detected by DSC here suggests that PAF-receptor sites are possibly a membrane protein but not phospholipids. The specific binding of PAF to platelet plasma membranes is independent of Ca^{2+}, Mg^{2+}, and monovalent ions even though PAF-induced platelet aggregation requires Ca^{2+} and Na^+. Replacement of H_2O by D_2O in the incubation medium has no effect on the PAF-receptor binding, yet no platelet aggregation could be induced by PAP when platelets was suspended in 100% D_2O/Tyrode's solution (our published observation). Clearly, the failure of platelets in D_2O suspension to aggregate is not related to PAF-receptor binding but due to some unidentified subsequent events.

Assuming an equimolecular ligand–receptor complex, it is possible to calculate the mean number of receptors per platelet from maximal binding and the yield of platelet plasma membranes. From the method described above, the yield of platelet plasma membranes is roughly 8.12 ($\pm$1.03) $\times$ 10^{-14} g of plasma membrane protein/platelet. From the maximal binding sites of 3 $\times$ 10^{-13} mol/100 μg of membrane protein, the density of receptor sites is estimated to be about 150 receptors/platelet. If the isolated membrane has formed randomly oriented vesicles with only half of the extracellular membrane surface exposed to the suspending medium, each cell than contains about 300 receptors. This number of receptors (150–300) is about an order of magnitude smaller than those for several hormones (Williams et al., 1976; Klee & Nirenberg et al., 1974) or for the chemotactic peptide *N*-formylmethionylleucylphenylalanine on rabbit or human neutrophils (Aswanikumar et al., 1977; Williams et al., 1977).

The K_D values calculated from binding data are about 10–100-fold higher than the concentrations of PAF that give half-maximal aggregation response as reported here and in the earlier literature. Thus, full occupancy of the receptors may not be required for a maximal aggregation response. Speculatively, PAF-bound receptors might stimulate a membrane enzyme, an ionophore, or a structural protein. Such process of message amplication (Yavin, 1982) may require only a small percentage of receptor occupancy by PAF.

The binding affinity of PAF to plasma membranes is dependent upon the methods of plasma membrane preparation and/or the incubation medium for the PAF-receptor binding assay. For example, choline chloride, ethanolamine chloride, etc. will increase the affinity of the PAF receptor from membranes in the absence of Mg^{2+} during cell lysis to PAF. Conversely, we cannot rule out a possible decrease of the receptor affinity during the preparation of platelet plasma membranes. Rudnick & Nelson (1978) have reported the protective effect of Mg^{2+} ions during the lysis of glycerol-loaded platelets to yield consistent preparation of platelet membrane vesicles capable of accumulating high concentrations of serotonin from the external medium. It seems likely that Mg^{2+} ions are required to improve the integrity of the isolated plasma membranes during the platelet lysis.

The PAF-induced contraction of guinea pig ileum was not antagonized by muscarinic antagonist, diphenhydramine, and slow-reacting substance (SRS) antagonist FPL-55712 (Findlay et al., 1981). Also, PAF, when added to a spiral-cut guinea pig trachea suspended in a tissue bath containing Krebs–Henseleit buffer, produced a dose-dependent loss of active tissue tension, which could not be modified by aspirin or propranolol pretreatment (Prancan et al., 1982). In our laboratory, contractions of guinea pig lung parenchymal strip and the cross-cut ventricle ring induced by PAF were also observed (our unpublished results). However, in our experiments no PAF specific receptor sites were found on membranes from guinea pig trachea and heart. From the results shown in Table I, the number of the receptor sites in guinea pig ileum with the same amount of membrane protein is only one-tenth of that on rabbit platelet plasma membranes. The specific receptor binding to lung membrane preparation is too low to calculate the receptor number accurately. We can only tentatively assume that our failure to detect the receptor sites on those tissues summarized in Table I was due to the small number of PAF-specific receptor sites that could result from the small percentage of smooth muscle present in the tissue and/or the impure membrane preparations.

PAF could induce platelets to release thromboxane A_2 (Shaw et al., 1981b) and slow-reacting substance (SRS) (Mencia-Huerta et al., 1981) and stimulate the lipoxygenase pathway in polymorphonuclear leukocytes (Chilton et al., 1982). It was also found that PAF-induced respiratory alterations may depend on the circulating platelets (Halonen et al., 1981). Therefore, PAF-induced bronchoconstriction may also mediate through leukotrienes and/or thromboxane A_2 released by circulating platelets and/or PMN leukocytes. The physiological roles of PAF through direct and/or indirect actions on respiratory, circulatory, and cardiovascular systems in vivo remain an important question pending clarification. Toward this goal, the search for specific inhibitors of the PAF-receptor sites is currently in progress.

Registry No. I, 65154-06-5; II, 77286-68-1; III, 85405-05-6; IV, 79512-78-0; 1-acyl-PAF analogue, 79549-26-1; Mg, 7439-95-4.

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Effects of Physical States of Phospholipids on the Incorporation and Cytochalasin B Binding Activity of Human Erythrocyte Membrane Proteins in Reconstituted Vesicles†

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ABSTRACT: Proteoliposomes were reconstituted from a Triton extract of human erythrocyte membrane proteins and a mixture of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of varying ratios. With mixtures of egg PC and soybean PE, the protein/lipid ratio of the reconstituted vesicles was maximal at 25% PC and 75% PE, the composition which is known to have a maximum bilayer disruption (highest occurrence of lipidic particles seen by freeze–fracture electron microscopy). With mixtures of 1-palmitoyl-2-oleoyl-PC and dilinoleoyl-PE, which give vesicles with few isolated lipidic particles at room temperature, the effect was less pronounced. The specific activity of the cytochalasin B (CB) binding protein in the reconstituted vesicles, on the other hand, was increased monotonically up to severalfold as the PC content was increased in the egg PC/soybean PE mixture. A similar increase was observed when soybean PE was partially substituted by dimyristoyl-PC, cholesterol, or transphosphatidylated PE from egg PC. These findings indicate that preexisting defects in the lipid bilayer promote protein incorporation into the bilayer during reconstitution whereas reduction of the bilayer fluidity facilitates the CB binding activity in the reconstituted vesicles.

An ample body of evidence indicates that the compositions and the physical states of the lipids used in membrane reconstitution affect the incorporation of proteins and other molecules. The insertion of M-13 protein into model membranes with proper orientation depends on the phase transition of the lipids used (Wickner, 1977). Complex formation between serum apolipoproteins and lecithins (Pownall et al., 1979) and between glucagon and lecithins (Epand et al., 1981) was also found to depend on the phase states of the lipid. It has been proposed that at the phase transition many structural defects exist in the lipid bilayer, and these defects could affect the incorporation of proteins (Marsh et al., 1976). The composition and the physical state of lipids also affect the functions of reconstituted proteins. The mitochondrial ATPase incorporated into liposomes gives a well-defined maximum at a certain composition of the liposome (Kagawa et al., 1973), and this lipid composition coincides with the lipid compositions which give maximal bilayer instability (Hui et al., 1981a). A recent experiment by Woldegiorgis et al. (1982) confirmed this lipid dependency. When erythrocyte membrane proteins were reconstituted into liposomes, it was found that the composition of the exogenous lipid used in reconstitution had certain effects on the efficiency of the anion transport (Gerritsen et al., 1979; Wolosin, 1980). It is possible that the maximum amount of defects exists in the bilayer at the range of instability, and they either facilitate protein incorporation and/or affect the functions of the protein incorporated. Perhaps by the same token, this effect may also exist in natural membranes.

Glucose transport across the human erythrocyte membrane is known to be mediated by a specific protein, and cytochalasin B (CB)[1] inhibits this transport function by binding to this protein (Jung & Rampal, 1977). Successful effort has been made to isolate this transport protein, namely, band 4.5, and to reconstitute it into liposomes (Kasahara & Hinkle, 1977; Phutrakul & Jones, 1979; Sogin & Hinkle, 1980; Froman et al., 1980, 1981; Lukacovic et al., 1981). It has been shown that cytochalasin B binds specifically to this protein (Carter-Su

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DLPE, 1,2-dilinoleoylphosphatidylethanolamine; IMP, intramembranous particle; LIP, lipidic particle; TPE, transphosphatidylated product of egg PC in the presence of ethanolamine; DMPC, 1,2-dimyristoylphosphatidylcholine; SDS, sodium dodecyl sulfate; CB, cytochalasin B; Tris, tris(hydroxymethyl)aminomethane.

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