

PHOTOAFFINITY LABELING OF PLATELET ACTIVATING FACTOR
BINDING SITES IN RABBIT PLATELET MEMBRANES

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SUMMARY A photoreactive, radioiodinated derivative of platelet activating factor (PAF), 1-O-(4-azido-2-hydroxy-3-iodobenzamido)undecyl-2-O-acetyl-sn-glycero-3-phosphocholine ($[^{125}\text{I}]\text{AAGP}$), was synthesized and used as a photoaffinity probe to study the PAF binding sites in rabbit platelet membranes. The nonradioactive analog, LAAGP, induced rabbit platelet aggregation with an EC_{50} value of 3.2 ± 1.9 nM as compared to 0.40 ± 0.25 nM for PAF. Specific binding of $[^{125}\text{I}]\text{AAGP}$ to rabbit platelet membranes was saturable with a dissociation constant (K_d) of 2.4 ± 0.7 nM and a receptor density (B_{max}) of 1.1 ± 0.2 pmol/mg protein. Photoaffinity labeling of platelet membranes with $[^{125}\text{I}]\text{AAGP}$ revealed several ^{125}I -labeled components by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein species with apparent molecular weight of 52,000 was consistently observed and inhibited significantly by unlabeled PAF at nanomolar concentrations. The labeling was specific since the PAF antagonists, SRI-63,675 and L-652,731, at 1 μM also blocked the appearance of this band; whereas lysoPAF was not effective at the same concentration. These results suggest that the binding sites of PAF receptor in rabbit platelets reside in the polypeptide of $\text{Mr} = 52,000$.

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INTRODUCTION Platelet activating factor (PAF), produced by a number of inflammatory cells upon chemical or immunological stimulation, is a potent lipid mediator involved in a variety of pathophysiological states, including asthma, acute inflammation, stroke, thrombosis, graft rejection, ovariectomy and immune disorders (1, 2). Based on structure-function analyses, the biological effects of PAF on target cells or tissues were believed to be receptor-mediated (3). High affinity binding sites for PAF were identified on various cells and in membrane preparations of cells and tissues (4-10). Nevertheless, little was known about the molecular nature of the putative PAF receptors. Recently, we reported the successful solubilization of a $[^3\text{H}]\text{PAF}$ -receptor complex from rabbit platelet membranes by using the non-ionic detergent digitonin (11). Hydrodynamic characterization of the radioligand-receptor complex revealed that it is a $\text{Mr} = 220,000$ macromolecule. The observation that it retained the sensitivity to sodium ion and guanine nucleotide modulation led to the speculation that the regulatory components, such as GTP binding protein, might coexist with the ligand binding subunits within the complex. In order to gain more insights into the molecular feature of the binding subunits of the PAF receptor, we prepared a photoreactive derivative of PAF which was previously synthesized by Bette-Bobillo et al (12) and used it as a photoaffinity probe to identify PAF binding sites in rabbit platelet membranes. In present report, we

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demonstrated that a membrane component with binding characteristics of the PAF receptor was affinity-labeled and may represent the binding subunit(s) of the PAF receptor in rabbit platelets.

MATERIALS AND METHODS

Materials- Carrier-free Na^{125}I was from Amersham. Prestained protein markers were from Bethesda Research Laboratories. N-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) was obtained from Pierce Chemical Co. Unlabeled 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) and 1-O-hexadecyl-sn-glycero-3-phosphocholine (lysoPAF), were from Sigma. SRI-63,675, cis-(±)-1-[2-[hydroxy[[tetrahydro-2,5-dimethyl-5-[(octadecylamino carbonyl)-oxy]methyl]furan-2-yl]-methoxy-phosphinoxy]ethyl]-quino linium hydroxide was a generous gift from Dr. Dean Handley of Sandoz. L-652,731, trans-2,5-bis(3,4,5-trimethoxyphenyl)tetrahydrofuran, was kindly provided by Dr. T.Y. Shen of University of Virginia.

Synthesis of 1-O-(11-phthalimidoundecyl)-2-O-acetyl-sn-glycero-3-phosphocholine (PAGP)- PAGP was prepared according to the published procedure (12) by using (+)isopropylidenglycerol as the starting material with modification (13). HPLC analysis of the resulting product with a nucleosil 50-5 column (4.6 mm x 25 cm) eluted with a solvent system of acetonitrile/methanol/85% phosphoric acid (130/5/1.5 V/V) (14) at flow rate of 1 ml/min indicated the presence of 5% of the regioisomer (Rt=21 min) as detected by UV absorption at 280 nm. The desired PAGP (Rt= 22 min) was freed from its regioisomer by preparative HPLC and was identical to the material synthesized by Betto-Bobillo et al (12) by comparison with the reported ^1H NMR spectrum.

Preparation of 1-O-(4-azido-2-hydroxy-3-iodobenzamido)-undecyl-2-acetyl-sn-glycero-3-phosphocholine (IAAGP)- 1-O-(11-aminoundecyl)-2-acetyl-sn-glycero-3-phosphocholine (AMAGP, Fig.1) and 1-O-(4-azido-2-hydroxy-3-benzamido)undecyl-2-acetyl-sn-glycero-3-

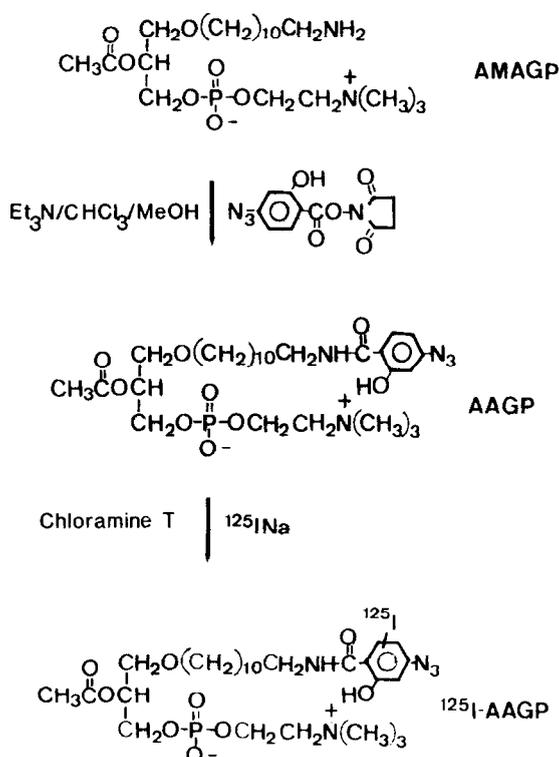


Fig.1. Synthesis of [^{125}I]AAGP, a photoreactive analog of PAF.

phosphocholine (AAGP) were prepared and purified as described (12). AAGP (2.5 mg, 7 μ mol) in 0.2 ml of methanol containing 50 μ l of concentrated ammonia solution was then treated with iodine (2.5 mg, 10 μ mol) which was dissolved in 0.2 ml of 95% alcohol. After overnight incubation at 4°C, the reaction mixture was dried under N₂ and redissolved in 50 μ l of methanol followed by injection onto a HPLC nucleosil 50-5 column (4.6 mm x 25 cm). The column was eluted with a solvent system of chloroform/methanol/H₂O (65/35/6) at a flow rate of 1 ml/min. As analyzed by UV absorption at 280 nm, the retention time for the iodinated product and AAGP were 8 min and 8.5 min respectively. The fraction at 8 min was collected in the dark and identified by FAB mass spectrometry as the monoiodinated compound with a molecular weight of 482 (M⁺).

Preparation of [¹²⁵I]AAGP- Radioiodination of AAGP was carried out by the method of Bolton and Hunter (15) with modification. Briefly, 0.25 nmol of AAGP in 5 μ l of methanol was mixed with 10 μ l of 0.5 M phosphate buffer pH 7.4. Na¹²⁵I (0.5 μ Ci, 5 μ l) was added followed by 10 μ l of chloramine T (50 μ g/ml in 0.5 M phosphate buffer). After standing at room temperature for 2 min, the reaction was terminated by the addition of 10 μ l of sodium bisulfate (30 mg/ml). Five μ l of KI (10 mg/ml) was then added and the iodinated product was extracted into chloroform by mixing 200 μ l of methanol, 200 μ l of chloroform and 100 μ l of 10% NaCl and vortexed for 2 min to affect phase separation. The chloroform layer was removed and washed once more with 10% NaCl. The isolated ¹²⁵I-product was further purified by HPLC as described above. Over 75% of radioactivity eluted at 8.0 min corresponding to the retention time of IAAGP was collected in the dark and stored at -20°C for one month without deterioration. Since the product was completely separated from the starting materials, the specific activity of the radioiodinated compound was assumed to be 2200 Ci/mmol.

Platelet aggregation- Aspirin-treated rabbit platelets were prepared accordingly (16) and finally resuspended in Tyrode's buffer pH 7.2 containing 1 mM CaCl₂ and the ADP scavenger complex, creatine phosphate (1 mM) and creatine phosphokinase (10 U/ml) at a concentration of 5 x 10⁸ platelets/ml. Two hundred μ l of platelet suspension were transferred into cuvettes of the aggregameter and stimulated with 10 μ l of various concentrations of PAF and PAF analog which were dissolved in 0.15 M NaCl solution containing 0.25% fatty acid free bovine serum albumin (BSA). Changes in light transmittance were monitored by a 2-channel NKK Hema Tracer model PAC-2S (NIKO BIOSCIENCE INC. Tokyo, Japan).

Preparation of rabbit platelet membranes- Rabbit platelet membranes were prepared as described previously (11) and finally resuspended in 25 mM Tris-HCl pH 7.4 containing 5 mM EDTA, 0.1 mM PMSF and 1 μ M leupeptin with a protein concentration of 10 mg/ml and stored at -70°C until use. The protein was determined by a Bio-Rad protein assay with BSA as a standard.

Radioligand binding assay- Rabbit platelet membranes were incubated at 4°C in 0.5 ml of 25 mM Tris-HCl pH 7.4 containing 10 mM MgCl₂ and 0.1 M KCl and 0.1% BSA and various concentrations of [¹²⁵I]AAGP. After 1 h of incubation, bound and free ligands were separated by filtration under vacuum on Whatman GF/C glass fiber filters. Each filter was rapidly washed with 10 ml of ice cold buffer and the radioactivity bound to the membrane fraction was determined in a gamma counter.

Photoaffinity labeling - Platelet membrane proteins (1 mg) were incubated with 1 x 10⁶ cpm of [¹²⁵I]AAGP in 2 ml of binding assay buffer described above in the absence or presence of competing ligand at 4°C for 1 h. At the end of incubation, membranes were sedimented at 30,000 xg for 30 min at 4°C, resuspended in 1 ml of Tris-HCl buffer without BSA, and exposed, on ice, to a 254 nm wavelength ultraviolet lamp (mineralight R, UVGL-25, 4 watts UVP Inc., San Gabriel, CA) at a distance of 1.0 cm for 5 min. One ml of Tris-HCl buffer containing 0.2% BSA, 2 mM EDTA, 0.2 mM PMSF, 2 μ M leupeptin and 2 μ M unlabeled PAF was added. After incubation at 4°C for 20 min, the membranes were centrifuged at 30,000 xg for 30 min at 4°C and washed once more with Tris-HCl buffer without BSA followed by centrifugation, again at 30,000 xg. The membrane proteins were then solubilized in 0.1 ml of 0.1 M Tris-HCl pH 6.8 containing 8 M urea, 0.1 M dithiothreitol (DTT) and 3% SDS at room temperature for 30 min. The samples were analyzed by SDS-gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel according to the Laemmli method (17). At the end of electrophoresis, gel were dried and exposed to Kodak X-Omat films with intensifying screens at -70°C for two weeks.

RESULTS AND DISCUSSION

[125 I]AAGP, synthesized previously by Bette-Bobillo et al (12), was suggested to be a potential probe for photoaffinity labeling of PAF receptor. However, no further data was provided in support of its utility. In attempt to identify the binding sites of the PAF receptor, we synthesized the same compound for further characterization. The nonradioactive analog was first prepared and its effect on platelet function was examined. As shown in Fig. 2, IAAGP exhibited the agonist activity to induce platelet aggregation with a potency which is one log of magnitude less than that of synthetic PAF. The mean EC_{50} values for IAAGP and PAF were 3.2 ± 1.9 nM and 0.4 ± 0.25 nM respectively for three experiments. When rabbit platelet membranes were incubated with incremental inputs of the radioligand, [125 I]AAGP, at 4°C for an hour, the specific binding of [125 I]AAGP to the membranes reach a plateau indicating a saturation at 5 nM (Fig. 3A). Scatchard analysis of the binding data (Fig. 3B) revealed a single high affinity binding site with a dissociation constant (K_d) of 3.3 nM and a B_{max} of 1.2 pmol/mg protein in the depicted experiment, the mean K_d and B_{max} were 2.4 ± 0.7 nM and 1.1 ± 0.2 pmol/mg protein respectively for three experiments. The binding affinity of the analog was apparently in good correlation to its biological activity on rabbit platelets.

Platelet membranes preincubated with [125 I]AAGP were exposed to UV light followed by solubilization in the presence of DTT, SDS and urea at room temperature for 30 min. Analysis of the

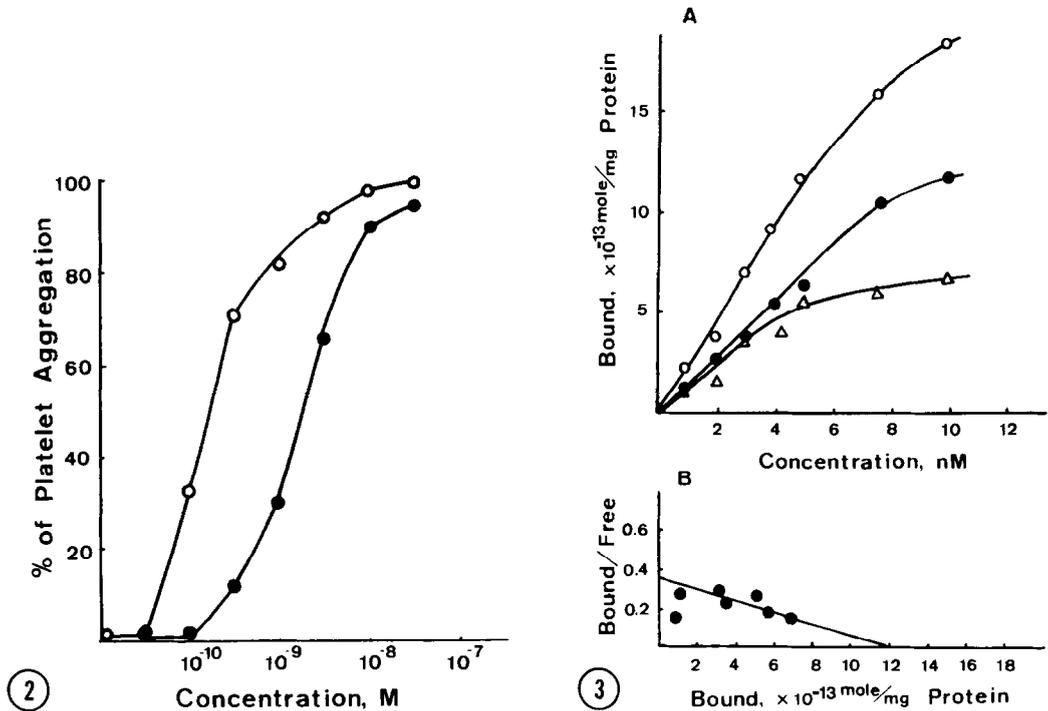


Fig. 2. Platelet aggregation induced by various concentrations of PAF (○) or IAAGP (●). The aggregation obtained in response to 10^{-6} M PAF was referred to 100%.

Fig. 3. (A) Saturation binding of [125 I]AAGP to 100 μ g of rabbit platelet membranes. The specific activity of [125 I]AAGP was adjusted to 50 Ci/mmol by the addition of unlabeled IAAGP prior to experiments. Each point represents the mean of duplicate determinations for total binding (○), specific binding (△) and non-specific binding (●). (B) Scatchard plot of specific binding of [125 I]AAGP to platelet membranes.

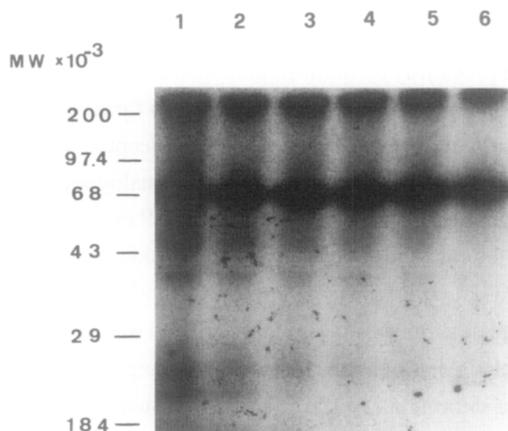


Fig. 4. Photoaffinity crosslinking of [125 I]AAGP to rabbit platelet membranes. The platelet membranes were incubated with [125 I]AAGP alone (lane 1), and separately in the presence of unlabeled PAF at 10^{-10} (lane 2), 10^{-9} (lane 3), 10^{-8} (lane 4), 10^{-7} (lane 5) and 10^{-6} M as described under "Materials and Methods". After irradiation, samples were solubilized and subjected to SDS-PAGE on a 10% acrylamide gel.

solubilized membranes by SDS-PAGE and autoradiography resolved several 125 I-labeled components (Fig. 4). The labeling to a protein band at $M_r = 68,000$ was predominant; however it was not protected in the presence of an excess of unlabeled PAF, representative of a non-specific binding protein. Since the $M_r = 68,000$ species happened to have the same molecular weight as BSA, it was very likely the contaminant of BSA which was routinely included in the binding buffer to help the solubility of [125 I]AAGP in the aqueous solution. However, we speculated that the BSA-like protein is of platelet origin (20) since the photolabeled membranes had been subjected to washing by BSA-free buffer before SDS-PAGE analysis. A diffuse band migrating with an average apparent molecular weight of 52,000 was also visualized and prevented by PAF in a dose-dependent manner with significant inhibition observed at a concentration as low as 10^{-10} M. In the depicted experiment, three additional minor species at $M_r = 40,000$, 24,000 and 21,000 were also specifically labeled. Although the protease inhibitors, PMSF, EDTA and leupeptin were employed during the preparation of the platelet membranes, it did not exclude the possibility that these smaller molecular weight species were the proteolytic products of the relatively abundant $M_r = 52,000$ species. In fact, the photolabeled membranes were solubilized at room temperature instead of by boiling, which might also increase the chance of proteolysis occurring in SDS/DTT-solubilized polypeptides as reported by others (22,23). Unfortunately, we found that boiling of samples usually resulted in poor appearance of the band in the autoradiograph, indicating that the covalent binding of [125 I]AAGP to the receptor proteins might be somewhat destabilized by heating. Prolonged exposure to UV light did not seem to improve the situation. The specificity of the labeling to $M_r = 52,000$ was further demonstrated in Fig. 5. The addition of 1 μ M lysoPAF, the inactive deacylated metabolite of PAF, to the incubation mixture, did not influence the appearance of the labeled proteins. On the contrary, the specific PAF antagonists, SRI-63,675 (18) and L-651,731 (19), completely blocked the labeling to $M_r = 52,000$ at the same concentration without affecting the $M_r = 68,000$ band. Taken together, these data clearly illustrated that the $M_r = 52,000$ protein displays binding characteristics similar to the PAF receptor and therefore may represent the specific binding component for PAF receptor in rabbit platelets. The observation that the $M_r = 52,000$ protein migrated as a broad, diffuse band suggests that it is a

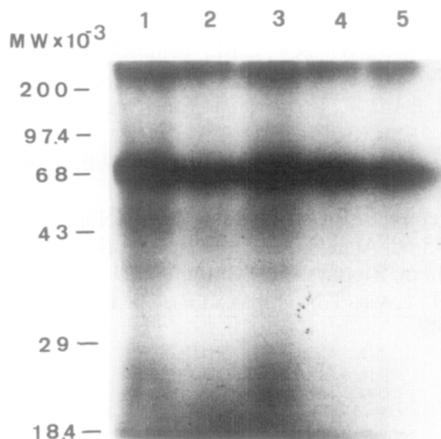


Fig.5. Specificity of the cross-linking of [125 I]AAGP to rabbit platelet membranes. Platelet membranes were incubated with [125 I]AAGP alone (lane 1), and separately in the presence of 1 μ M of unlabeled PAF (lane 2), lysoPAF (lane 3), SRI-63,675 (lane 4) and L-652,731 (lane 5) as described under "Materials and Methods".

glycoprotein which is a characteristic of many membrane receptor proteins (21). Since our previous work demonstrated that under the nondenaturing condition the PAF receptor was solubilized from rabbit platelet membranes as a large complex ($M_r=220,000$), it is suggested that the association of the $M_r=52,000$ polypeptide with other regulatory components is essential for preserving the functional activity of the solubilized PAF-receptor complex.

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