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Isolation of a platelet membrane protein which binds the platelet-activating factor 1-0-hexadecyl-2-acetyl-SN-glycero-3-phosphorylcholine

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Summary. The phospholipid platelet-activating factor 1-0-hexadecyl-2-acetyl-SN-glycero-3-phosphorylcholine (AGEPC) initiates platelet function by interacting specifically with 1399 ± 498 (mean ± SD) high-affinity membrane receptors per platelet. In studies designated to characterize the high affinity binding site, AGEPC-human serum albumin-Sepharose was employed to isolate a 180,000 mol. wt. protein from human platelet plasma membranes. Platelet plasma membranes were isolated by adsorption of sonicated human platelets to a column of wheat germ agglutinin-Sepharose and elution with *N*-acetyl-glucosamine. The plasma membranes were solubilized in 5% sodium dodecyl sulphate and applied to a column of AGEPC-human serum albumin-Sepharose. After washing the column extensively, the specifically bound material was eluted with a five-fold molar excess of AGEPC. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the eluted material revealed a single protein with an apparent molecular weight of 180,000. This protein was not recovered from solubilized platelet membranes when chromatography was performed with a column of human serum albumin-Sepharose lacking AGEPC. The capacity of this protein to bind AGEPC suggests that it represents a constituent of the human platelet receptor for AGEPC.

Abbreviations AGEPC, 1-0-alkyl-2-acetyl-SN-glycero-3-phosphorylcholine; WGA-Sepharose, wheat germ agglutinin coupled to Sepharose beads; HPLC, high-performance liquid chromatography; HSA, human serum albumin; HSA-Sepharose, HSA coupled to AH Sepharose; AGEPC-HSA-Sepharose, AGEPC non-covalently bound to HSA-Sepharose; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; TLCK, N-α-p-tosyl-L-lysine-chloromethyl ketone; PMSF, phenylmethylsulphonylfluoride; SDS, sodium dodecyl sulphate.

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INTRODUCTION

The phospholipid platelet-activating factor, 1-0-hexadecyl/octadecyl-2-acetyl-SN-glycero-3-phosphorylcholine (AGEPC or $PAF_{acether}$) is a potent mediator of both platelet and polymorphonuclear (PMN) leucocyte function (Benveniste, Henson & Cochrane, 1972; Demopoulos, Pinckard & Hanahan, 1979; Valone *et al.*, 1979; Goetzl *et al.*, 1980; Vargaftig *et al.*, 1981; O'Flaherty *et al.*, 1981; McManus, Hanahan & Pinckard, 1981). Nanomolar quantities of AGEPC elicit human platelet aggregation and release of granular constituents (Goetzl *et al.*, 1980; McManus *et al.*, 1981; Vargaftig *et al.*, 1981) and 10- to 100-fold higher concentrations of AGEPC elicit human PMN leucocyte aggregation (O'Flaherty *et al.*, 1981) adherence (Valone & Goetzl, 1983a) enzyme

release (Goetzl *et al.*, 1980; O'Flaherty *et al.*, 1981) and chemotaxis (Goetzl *et al.*, 1980). Recent studies of the binding of AGEPC by platelets and PMN leucocytes have demonstrated the presence of specific, high-affinity binding sites and non-specific, low-affinity binding sites for AGEPC in both human platelets (Valone *et al.*, 1982) and PMN leucocytes (Valone & Goetzl, 1983b). There are 1399 ± 498 (mean $\pm$ SD) high-affinity binding sites for AGEPC per platelet and the binding sites have a $K_D = 37 \pm 13$ nM (mean $\pm$ SD). That the high-affinity binding site mediates platelet activation by AGEPC is suggested by the correlation between the K_D and the concentration of AGEPC which elicits half-maximal platelet activation (5–10 nM) and by the similar structural requirements for platelet activation and for binding of AGEPC. Although it is likely that the specific, high-affinity binding site for AGEPC is a protein receptor for AGEPC, it remains possible that it is a transport protein or a unique, saturable membrane lipid compartment. Thus, to further characterize the specific binding site for AGEPC studies were undertaken to isolate a platelet protein which binds AGEPC.

MATERIALS AND METHODS

Wheat germ agglutinin-Sepharose (WGA-Sepharose), phenylmethyl-sulphonylfluoride (PMSF), *N*-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK) and fatty acid-free human serum albumin were obtained from Sigma Chemical Co. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was obtained from Pierce Chemical Co., Rockford, IL. [^{3}H]-AGEPC (a mixture of the 1-0-hexadecyl- and 1-0-octadecyl- compounds (25 Ci/mM, New England Nuclear, Boston, MA) was purified by high-performance liquid chromatography (HPLC) on a column of 5 μm Spherisorb-NH_2 (Phase-Sep Inc., Hauppage, KY) which was developed isocratically at a flow rate of 1 ml/min with acetonitrile:water (85:15, v:v). The purified product was characterized functionally as described (Valone *et al.*, 1982; Valone & Goetzl, 1983b). AGEPC was obtained from Supelco (Bellefonte, PA). Different concentrations of this material elicited platelet aggregation which was indistinguishable from that elicited by structurally defined AGEPC. HPLC of this material revealed a predominant peak of optical absorption at 206 nm which eluted co-incident with structurally defined AGEPC, indicating that this preparation was highly purified, functionally active AGEPC.

Isolation of human platelet membranes

Platelets from healthy adult volunteers who had not taken aspirin or other platelet-active drugs for at least 7 days before venipuncture were collected into citrate anticoagulant (Valone *et al.*, 1979; Valone *et al.*, 1982). The platelets were washed three times by centrifugation at 1600 *g* for 15 min onto cushions of autologous erythrocytes after which the contaminating erythrocytes were removed by centrifugation at 200 *g* for 10 min. The platelets in the supernate were then sedimented by centrifugation at 1600 *g* for 15 min and resuspended at a concentration of 1×10^9/ml in 'membrane buffer' (0·25 M sucrose, 5 mM Tris-HCl pH 7·4 0·1 mM TLCK and 0·3 mM PMSF). The final platelet preparations routinely contained less than one erythrocyte and one leucocyte per 500 platelets as assessed microscopically. The platelets were lysed by sonication on ice for two 30 sec intervals at setting 5 on a Branson sonicator. In two experiments the sonicate was centrifuged at 1600 *g* for 15 min and no pellet was obtained. Therefore, the sonicate was applied directly to a column of 5 ml of WGA-Sepharose for isolation of platelet membranes (Rotman & Linder, 1981). Fractions of 4·6 ml were collected at a flow rate of 1 ml/min and the elution of proteins from the column was monitored by optical absorption at 280 nm. Non-specifically bound material was removed by washing the column sequentially with 40 ml of membrane buffer, 100 ml of 1·0 M NaCl in membrane buffer and 50 ml of membrane buffer. The last wash was continued until no protein had eluted from the column for at least 25 ml after which the platelet membranes were recovered by developing the column with 40 ml of 0·2 M *N*-acetyl-glucosamine.

Affinity chromatography

Platelet membrane proteins which bind AGEPC were isolated by affinity chromatography on a column of AGEPC bound non-covalently to human serum albumin (HSA) which was coupled to AH Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). HSA was coupled to AH Sepharose (HSA-Sepharose) by mixing 500 mg HSA with 50 mg of EDC and 3 g of swollen, washed AH Sepharose. The pH of the mixture was adjusted to 4·5 and the mixture was incubated for 18 hr at 20° on a rocker-arm apparatus. Twenty milligrams of EDC were then added to the mixture and after further incubation for 3 hr at 20° the HSA-Sepharose was recovered by centrifugation at 200 *g* for 5 min. The HSA-Sepharose was washed five times with Hanks's buffer after which the packed gel

was resuspended in an equal volume of Hanks's buffer. For two preparations of HSA-Sepharose 4·8 mg and 2·5 mg of HSA were bound per ml of packed gel. AGEPC was then bound non-covalently to HSA-Sepharose (AGEPC-HSA-Sepharose) by mixing 6 ml of the packed gel with 100 μl of methanol containing 500 μg of AGEPC and 3800 c.p.m. of [^{3}H]-AGEPC. Control columns were prepared by mixing 6 ml of HSA-Sepharose with 100 μl of methanol alone. After incubating the mixture for 30 min at 20° the gel was recovered by centrifugation at 200 ***g*** for 5 min and washed three times with Hanks's buffer. The washed gel was packed into a column and washed further with 100 ml of 'affinity buffer' (5 mM Tris-HCl pH 7·4, 0·2% sodium dodecyl sulphate (SDS), 0·1 mM TLCK and 0·3 mM PMSF) which removed less than 5% of the bound [^{3}H]-AGEPC. For four sequential columns, 20–65% of the radiolabeled [^{3}H]-AGEPC was bound to the gel indicating that 100–325 μg of AGEPC was bound per 6 ml of packed gel.

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli utilizing a Bio-Rad apparatus (Richmond, CA). One microgram of protein was placed in a glass tube, dried with a centrifugal evaporator, resuspended in 120 μl of tracking buffer (20% glycerol, 2% mercaptoethanol, 4% SDS, 0·13 M Tris-HCl pH 6·8), incubated for 1 hr at 37° and applied to the top of a 5% slab gel. After subjecting the gels to 120 V for 4–5 hr, the gels were fixed and stained utilizing a silver strain kit (Bio-Rad) which reliably detects 0·1 μg of protein. The quantity of protein in different samples was determined using a colorimetric assay (Bio-Rad) and HSA as the protein standard.

RESULTS

The initial step in the isolation of a platelet binding protein for AGEPC was to isolate platelet membranes. Washed human platelets were disrupted by sonication on ice and then applied at 4° to a column of 5 ml of WGA-Sepharose (Fig. 1). The column was washed until no additional protein eluted as determined by optical absorption at 280 nm after which the platelet membranes were recovered by developing the column with 0·2 M *N*-acetyl-glucosamine. In four experiments, 21·6 ± 3·5 mg of membrane protein (mean ± SD) per 10^{10} platelets was recovered from the WGA-Sepharose column. To obtain solubilized membrane proteins, the column fractions containing the platelet membranes were pooled, mixed with SDS at a final concentration of 5% SDS and incubated for 2 hr at 37°. The solubilized proteins were then applied at 20° to a column of 3 ml of AGEPC-HSA-Sepharose which was

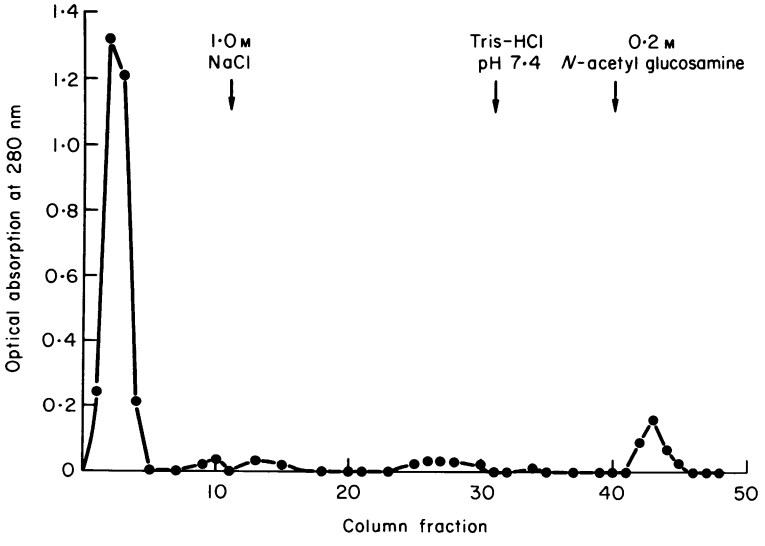

Figure 1. Isolation of human platelet membranes by affinity chromatography on wheat germ agglutinin-Sepharose. Eight × 10^5 platelets were disrupted by sonication and applied to a column of 5 ml of WGA-Sepharose which was developed as described in the methods.

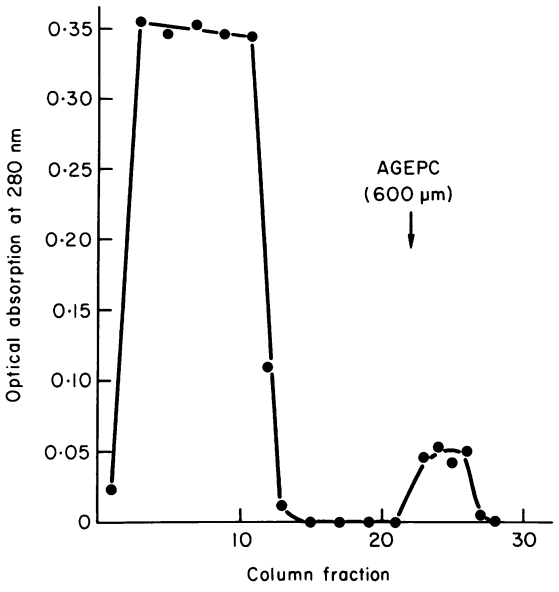

Figure 2. Isolation of a human platelet membrane protein which binds AGEPC by affinity chromatography on AGEPC-HSA-Sepharose. The platelet membrane preparation shown in Figure 1 was dissolved with 5% SDS, and one-half of the material was applied at room temperature to a column of 3 ml of AGEPC-HSA-Sepharose which was washed with 100 ml of affinity buffer. Fractions of 4·6 ml were collected at a flow of 1 ml/min. The column was developed with 600 μm AGEPC as described in the text.

washed until no protein eluted as assessed by optical absorption at 280 nm (Fig. 2). Membrane proteins which remained bound to AGEPC-HSA-Sepharose were then eluted specifically with a five- to 10-fold molar excess of AGEPC by mixing 500 μg of AGEPC in 50 μl of methanol with a suspension of the gel and 1 ml of the affinity buffer. After incubating the mixture for 30 min at 20° the membrane proteins eluted by AGEPC were recovered by washing the column with 10 ml of buffer containing 50 μg of AGEPC per ml. In six experiments, $11{\cdot}0 \pm 4{\cdot}6$ μg of protein (mean ± SD) per 10^{10} platelets were eluted by AGEPC from the AGEPC-HSA-Sepharose column. To assess the possibility that the recovered proteins were eluted non-specifically by the small quantity of methanol added with the AGEPC, control columns were developed in an identical manner with methanol alone. For two columns no protein was eluted by methanol alone.

To determine the number of proteins which were eluted from the AGEPC-HSA-Sepharose column, 1 μg of the pooled protein peak was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3). A single protein was identified with a molecular weight of 180,000. Although it is likely that this protein binds AGEPC it is possible that it binds HSA or Sepharose and was eluted non-specifically from the column by AGEPC. To examine this possibility, one-half of the platelet membrane preparation shown in Fig. 1 was applied to a control column containing HSA-Sepharose alone and the column was developed with AGEPC in parallel with the AGEPC-HSA Sepharose column depicted in Fig. 2. The small quantity of protein recovered from the HSA-Sepharose column was concentrated and 1 μg was subjected to SDS-polyacrylamide gel electrophoresis. Two proteins of mol. wt. greater than 200,000 and multiple smaller proteins were seen but none corresponded to the 180,000 protein which eluted from the AGEPC-HSA-Sepharose column. Thus this protein apparently binds AGEPC and may represent a specific platelet receptor for AGEPC.

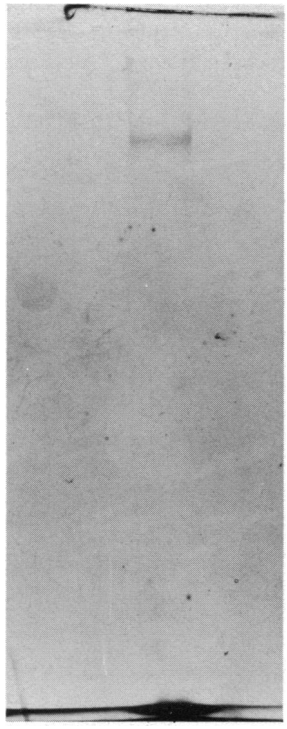

Figure 3. SDS-polyacrylamide gel electrophoresis of a human platelet membrane protein which binds AGEPC. One microgram of the eluate depicted in Fig. 2 was subjected to electrophoresis in a 5% gel.

DISCUSSION

Previous studies have indicated that the phospholipid mediator AGEPC activates human platelet and polymorphonuclear leucocyte function by interacting with specific high affinity receptors (Valone *et al.*, 1982). The current investigations present the isolation by affinity chromatography of a 180,000 mol. wt. platelet membrane protein which may be the high affinity receptor for AGEPC. Human platelet membranes were isolated from disrupted platelets by chromatography on WGA-Sepharose (Fig. 1). The membranes were then dissolved with 5% SDS and the solubilized membrane proteins were subjected to affinity chromatography on a column of AGEPC-HSA-Sepharose (Fig. 2). A single protein with a mol. wt. of 180,000 was recovered when the column was developed with AGEPC (Fig. 3). In contrast, this protein was not recovered in control experiments in which solubilized platelet membrane proteins were applied to a column of HSA-Sepharose which was also developed with AGEPC or in which platelet membranes were applied to a column of AGEPC-HSA-Sepharose which was developed with methanol. Thus the protein isolated from the AGEPC-HSA-Sepharose column binds AGEPC rather than HSA or Sepharose and may represent a specific receptor for AGEPC. The possibility that this protein is a transport protein or phospholipase cannot be excluded by this data however. The mol. wt. of the AGEPC-binding protein is greater than that of the majority of platelet membrane glycoproteins (Berndt & Phillips, 1981a) but falls within the range of the glycoprotein I complex (mol. wt. = 210,000 and 150,000) (Nachman, Kinoshita & Ferris, 1979). The structural and functional relationships between the AGEPC-binding protein and these other platelet proteins are yet to be elucidated. The AGEPC-binding protein is distinct from previously isolated platelet membrane receptors including the substrate for thrombin (mol. wt. = 82,000) (Berndt & Phillips, 1981b), the IgG-Fc receptor (mol. wt. = 255,000 (Cheng & Hawiger, 1979) and the glycoprotein I complex receptor for bovine von Willebrand factor (mol. wt. = 210,000 and 150,000).

Recently, an assay for fluid phase binding proteins for AGEPC was developed based on the capacity of antisera to HSA to precipitate AGEPC which is bound to HSA (Valone, 1984). Preliminary studies indicated that a component of platelet plasma membranes binds AGEPC: a 0·2% SDS extract of WGA-Sepharose-purified human platelet membranes diminished precipitation of [^{3}H]-AGEPC by antisera to HSA, suggesting that a component of platelet membranes competes with HSA for binding of AGEPC. Although this assay demonstrated the presence of a platelet membrane component which binds AGEPC, the low specific activity of the currently available radiolabelled AGEPC precludes assessment of the presence of the binding protein in the small quantity of material recovered from the AGEPC-HSA-Sepharose column. The development of a specific, more sensitive assay for isolated AGEPC-binding proteins has been complicated by the unique phospholipid structure of AGEPC which necessitates a carrier protein for solubilization of AGEPC in aqueous solutions and which makes AGEPC highly soluble in standard detergents. Thus, further characterization of the 180,000 mol. wt. protein recovered from the AGEPC-HSA-Sepharose column requires production of antisera to the protein which can then be utilized both to examine the function of this protein and to isolate greater quantities of it for additional *in vitro* characterization.

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