Binding and Internalization of Platelet-activating Factor 1-O-Alkyl-2-acetyl-sn-glycero-3-phosphocholine in Washed Rabbit Platelets*

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The binding profile of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC, platelet-activating factor) to washed rabbit platelets was investigated through the use of structural analogs of AGEPC, e.g., U66985, which specifically suppressed AGEPC biological activities on rabbit platelets. This interaction of AGEPC with platelets could be divided into three different components termed A, B, and C. Component A was considered as one of high affinity (K_a = 0.5 x 10^-9 M) and with a low capacity (about 400 sites/platelet). The binding of AGEPC to component A was reversible and was blocked by the inhibitory analogs of AGEPC. This was considered to be the AGEPC receptor site(s). Component B was irreversible in nature and was presumed to be associated with internalization of AGEPC. This was sensitive to the structural inhibitors. Component C was not affected by the inhibitors and probably represented a nonspecific binding to the lipid layer of the membrane. The binding profile of 1-O-alkyl-2-(lyso)-sn-glycero-3-phosphocholine, a biologically inactive and noninhibitory analog of AGEPC, was observed to consist of a single component and was (also) unaffected by the inhibitors.

Internalization of AGEPC into rabbit platelets was further examined by the bovine serum albumin extraction method, which was originally developed by Mohandas et al. (Mohandas, N., Wyatt, J., Mel, S. F., Rossi, M. E., and Shohet, S. B. (1982) J. Biol. Chem. 257, 6537-6543). AGEPC was instantly taken up by the cell and internalized into its membrane, where it remained and was not released into cytosol. The internalization of AGEPC was suppressed by pretreating the cells with AGEPC analogs.

In platelets desensitized to AGEPC, no down-regulation of the receptor site(s) was observed. The internalization of AGEPC in the desensitized cells was clearly enhanced and this was obvious even in the presence of the AGEPC inhibitor(s). Even in the presence of the inhibitors, effective internalization of AGEPC was also evident in thrombin-treated cells. These results suggested that the internalization of AGEPC was irreversibly enhanced in the platelets which were activated by AGEPC itself as well as by thrombin.

AGEPC (platelet-activating factor) is a phospholipid chemical mediator which exhibits potent biological activities in a variety of cells as well as tissues (1-4). Its structure has been characterized as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (5-7). The structural specificity for the biological effects of AGEPC on target cells has suggested the presence of specific receptor(s) for this lipid on the membrane. Actually, specific binding of AGEPC has been reported in various cells and tissues as well as in membrane preparations (8-16). It was presumed to represent binding to specific receptor(s) on the surface membrane. In these studies, considerable binding of AGEPC to a low affinity, infinite capacity site had been observed and this was assumed to represent nonspecific binding. AGEPC was presumably incorporated into the lipid layer of the membrane, since it was structurally similar to a variety of lysophospholipids which were observed to dissolve in various cell membranes (17).

On interaction with target cells, AGEPC has been shown to be metabolically inactivated to 1-O-alkyl-2-long chain fatty acylphosphatidylcholine (alkyacyl-PC), which appears to contain exclusively arachidonic acid (18-21). This metabolic processing had been considered as not related to the activation process of the target cells, since the metabolic reaction was considerably slower than the biological effect of AGEPC (22, 23). Recently we have characterized certain structural analogs of AGEPC as specific inhibitors of AGEPC action on washed rabbit platelets (24). Through the use of these inhibitors, the biochemical behavior of AGEPC on interaction with rabbit platelets had been investigated and it was observed that the metabolism of AGEPC was enhanced upon the activation of the cells with AGEPC itself as well as other agonists (21). In this current paper, a preliminary study (25) has been further extended to an inquiry into the biochemical characteristics of the binding and internalization of AGEPC in rabbit platelets.

EXPERIMENTAL PROCEDURES

Materials

1-O-Hexadecyl- and 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine were purchased from Novabiochem AG (Laufelfingen, Switzerland). 1-O-[10,11-3H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine was obtained from Amersham International, Little Chalfont, Buckinghamshire, England. U66985, which specifically suppressed AGEPC biologic activity on rabbit platelets (24), was obtained from Sigma Chemical Co., St. Louis, Missouri. U68043, which specifically suppressed AGEPC biologic activity on rabbit platelets (25), was obtained from NEN Research Products, now part of DuPont, Wilmington, Delaware. U66982, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; CV3988, 1-(N-n-hexadecylcarbamoyloxy)-2-methoxypropyl-thiazolioethyl phosphate; U66988, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoric acid-10'-trimethylammonium decyl ester; U66985, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoric acid-6'-trimethylammonium hexyl ester; U68043, 1-(N,N-pentadecylcarbamoyloxy)-2-methoxy-rac-glycero-3-phosphocholine; [3H]-180-AGEPC, 1-O-[10,11-3H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine; [3H]-180-lyso-AGEPC, 1-O-[10,11-3H]octadecyl-2-(lyso)-sn-glycero-3-phosphocholine.

1 The abbreviations and analogs used are: AGEPC, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; lyso-AGEPC, 1-O-alkyl-2-(lyso)-sn-glycero-3-phosphocholine; BSA, bovine serum albumin; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PC, phosphatidylcholine; CV3988, rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl-thiazolioethyl phosphate; U66988, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoric acid-10'-trimethylammonium decyl ester; U66985, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoric acid-6'-trimethylammonium hexyl ester; U68043, 1-(N,N-pentadecylcarbamoyloxy)-2-methoxy-rac-glycero-3-phosphocholine; [3H]-180-AGEPC, 1-O-[10,11-3H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine; [3H]-180-lyso-AGEPC, 1-O-[10,11-3H]octadecyl-2-(lyso)-sn-glycero-3-phosphocholine.
line ([H]-18:0-AGEPC, 67 Ci/mmol) was obtained as described previously (24). 1-O-(10,11-[H]octadec-2-lyso)-sn-glycero-3-phosphocholine ([H]-18:0-lyso-AGEPC) and non-labeled 18:0-lyso-AGEPC were prepared by mild alkaline treatment of corresponding parent compounds followed by purification on thin layer chromatography and on high performance liquid chromatography. CV3988 (rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate) was kindly provided by Dr. Z. Suzuoki, Takeda Chemical Ind., Osaka, Japan. The following analogs of AGEPC were generous gifts of the Lipid Research group (Dr. D. E. Ayer), The Upjohn Company; 1-O-octadecylglycero-3-phosphoric acid-trimethylammonium decyl ester (U66982), 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphoric acid’-trimethylammonium hexyl ester (U66985) and 1-(N-n-pentadecylcarbamoyloxy)-2-methoxy-rac-glycero-3-phosphoric acid (U66943). AGEPC and AGEPC analogs were all dissolved in BSA (0.125%-saline solution and added to a platelet suspension (100-fold incubation). Bovine serum albumin was purchased from Aldrich. All other reagents were of highest grade available.

Methods

Rabbit Platelet Preparation—Preparation of washed rabbit platelets was performed as described previously (26). Platelets desensitized to AGEPC were prepared as outlined elsewhere (21, 24).

Binding of AGEPC to Platelets—The analysis of the binding pattern of AGEPC to platelets was performed essentially by the method of Mohandas et al. (27). Washed rabbit platelets were suspended in Tyrode’s solution-gelatin buffer (pH 7.2) containing 0.1 mM EGTA and 0.1% BSA at a concentration of 1 × 10^11 cells/ml. 1.2 ml of the suspension was then added to a polycarbonate tube which contained [H]-18:0-AGEPC and appropriate amounts of unlabeled AGEPC. AGEPC and its analogs were dissolved in BSA (1.25 mg/ml)-saline solution and were introduced into each tube. In some experiments, AGEPC analogs were included in addition to [H]-18:0-AGEPC and unlabeled AGEPC. [H]-lyso-AGEPC and unlabeled lyso-AGEPC were included instead of AGEPC in the analysis of the binding of lyso-AGEPC. Details are provided in the corresponding figure legends. After a 10-min incubation at 25°C, two 0.5-ml aliquots of the suspension were removed and layered on 50 μl of a mixture of dibutyphthalate and dioctyl phthalate (3:1, v/v) in a microcentrifuge tube and quickly centrifuged in an Eppendorf Centrifuge 3200 at 10,000 × g for 1 min. The supernatant together with the mixture of phthalate was carefully removed and 50 μl of a mixture of 1% Triton X-100 and 10% saline solution and 2% bovine serum albumin was added. The cell pellet was resuspended in 200 μl of 1% Triton X-100 and transferred into a vial. The centrifuge tube was rinsed once with 200 μl of 1% Triton X-100, the wash was combined with the cell suspension, and the radioactivity was counted.

Internalization of AGEPC Molecule into Platelets—Internalization of the AGEPC molecule was assayed by the technique originally developed by Mohandas et al. (27). This protocol was first applied by these investigators to the assay of the internalization of exogenous lysophosphatidylcholine across the erythrocyte membrane and has been developed in other studies (28-31). On the basis of the observation that BSA has an affinity for AGEPC as well as lysophosphatidylcholine (32), this technique was applied in this study. The assay protocol is based on the assumption that washing the cells with a concentrated albumin solution extracted and preferentially removed AGEPC accessible from the outer layer of the platelet surface membrane, whereas AGEPC which was retained in the cells after this albumin washing would mostprobably have been internalized into the cells and thus was inaccessible to the albumin washing. AGEPC which may reside on the receptor site on the cell surface after BSA washing was not significant, as will be outlined later. The total uptake of AGEPC was measured after washing the cells with the corresponding buffer alone.

Experimentally, washed rabbit platelets were suspended in a Tyrode’s solution-gelatin buffer (pH 7.2) containing 2.0 mM EDTA at a concentration of 2.5 × 10^11 cells/ml and prewarmed at 37°C for 5-10 min. To 1.2 ml of the suspension, [H]-AGEPC was added and the suspension was quickly mixed and incubated at 37°C for 1 min or for indicated time intervals as described in the figure legends. Two 0.5-ml aliquots of the suspension were then quickly removed and layered on 50 μl of a mixture of dibutyphthalate and dioctyl phthalate (3:1, v/v) in a microcentrifuge tube and centrifuged in an Eppendorf Centrifuge 3200 at 10,000 × g for 1 min. The radioactivity levels in the cell pellet and the buffer medium were determined as described above.

In another set of platelet suspensions, 0.5% BSA was introduced 1 min, or at indicated time intervals, after the addition of [H]-AGEPC, and the suspension incubated at 37°C for 1 min for an optimal extraction of AGEPC molecules by BSA. Subsequently, 0.5% BSA was used for washing the cells, since AGEPC was optimally extracted from the cells at this concentration of BSA. The radioactivity level in the cell pellet and the buffer medium were then measured as described above. The percentage of [H]-AGEPC which was retained-lysophosphatidic acid-3 cells after BSA washing was thus determined. This value was assumed to represent the degree of internalization of AGEPC molecule into the platelets.

Data Presentation—Unless otherwise stated, all data are presented as the mean ± half-range of duplicate determinations and are representative of at least two separate experiments.

RESULTS

Binding of AGEPC and Lyso-AGEPC to Washed Rabbit Platelets—Scatchard analysis (27) of previously (21), AGEPC was metabolized to lyso-AGEPC and phosphatidylcholine in washed rabbit platelets, particularly at 37°C. This metabolic pathway was much more evident in rabbit platelets than in washed human platelets (33). In any study of the binding of AGEPC to washed rabbit platelets, such metabolic conversions made the results difficult to interpret. Therefore, the incubation temperature for the binding experiment was decreased to 25°C to suppress these metabolic changes. In preliminary studies of the binding of 1 × 10^10 M AGEPC at 25°C, the percent of total binding of AGEPC was 14.05, 18.20, 20.33, 25.09, and 27.25% at 1, 2, 3, and 7 min, respectively, under the procedures described under “Experimental Procedures.” Since further incubation brought about metabolism of AGEPC, the incubation was stopped at 5 min, at which point total binding apparently reached an almost steady state level. In preliminary experiments, addition of CaCl₂ (1.8 mM) increased the nonspecific binding of AGEPC (see below) without affecting specific binding (also see below). Hence, CaCl₂ was not included in the incubation mixtures in subsequent studies. Fig. 1 shows the Scatchard plot of the binding of [H]-18:0-AGEPC to washed rabbit platelets. The binding of AGEPC to the platelets apparently consisted of three components of different properties (Fig. 1). These were considered to have different affinity and capacity characteristics. The apparent Kd value of the higher affinity and lower capacity component (A in Fig. 1) was approximately near 0.5 × 10^-8 M and the number of these sites was calculated to be approximately 400/platelet as shown in Fig. 1. This site was considered to be the receptor(s) associated with the agonist biological response. The second component had lower affinity and higher capacity features (B in Fig. 1). In the presence of 5 × 10^-7 M U66985, which almost completely inhibited the biological responses evoked by AGEPC at concentrations up to 10^-8 M, binding profile was composed of a single component (see legend to Fig. 1). This result was consistent with a nonsaturable binding site which was assumed to be a nonspecific one (C in Fig. 1). Inasmuch as component B was considered not to be the same as the nonspecific binding site C, the nature of the differences between components B and C was investigated (see below).

The binding of [H]lyso-AGEPC to platelets was also measured and the data are given in Fig. 2. The Scatchard analysis of the lyso-AGEPC binding was consistent with a nonsaturable (nonspecific) binding site, and the presence of U66985 had no effect on this binding, whereas this inhibitor did affect the binding of AGEPC, as shown in Fig. 1. Interestingly, the nonspecific binding of AGEPC was greater than that of lyso-AGEPC and was probably due to an increase in hydrophobicity.
of AGEPC attributable to the acetyl moiety at the sn-2 position.

Inhibition of Binding of AGEPC by Various Structural Analogos of AGEPC—The effects of various analogs of AGEPC on the binding of AGEPC was investigated and the results are given in Fig. 3. U66985, CV3988, and U66982 effectively inhibited the binding of AGEPC in a dose-dependent manner, whereas U68043 had a marginal effect. The nonspecific binding sites (component C) was subtracted from total sites was in equilibrium with the specific binding sites (component A). The amount of AGEPC bound specifically to component A (specific binding site(s)), the amount of AGEPC bound irreversibly (component B), the amount of unbound AGEPC, and the amount of AGEPC attributable to the acetyl moiety at the sn-2 position. It was assumed that AGEPC in the nonspecific binding sites was in equilibrium with the specific binding sites (component A). Extrapulated values for $K_d$ and number of the sites were approximately $0.5 \times 10^{-9}$ M and 400/platelet, respectively.

Reversibility of AGEPC Binding—The capability of U66985 in dissociating AGEPC previously bound to platelet cells was examined and the data are presented in Fig. 4. AGEPC at two different concentrations was allowed to bind to platelets for 5 min at 25 °C. $10^{-6}$ M U66985 was then added to examine replacement of AGEPC molecules bound to the cells. At initial concentrations of $1 \times 10^{-9}$ M U66985 within 10 min to a steady state of near 15%. This level of the binding (15%, bound/free == 0.17) corresponded to the binding component B in Fig. 1 and was higher than the nonspecific binding component C (bound/free = 0.13).

Thus, binding component A was considered a reversible site and assumed to be the primary receptor site. On the other hand, component B was a nonreversible site and assumed to be associated with internalization of AGEPC (see below).

Internalization of AGEPC into Washed Rabbit Platelets—In subsequent experiments, the internalization of AGEPC into platelets was investigated in detail. The assay protocol for internalization of AGEPC was based on the degree of extraction of AGEPC from cells using high concentrations of BSA as described under “Experimental Procedures.” AGEPC molecules which were retained in cells after BSA washing were considered to represent primarily those molecules which has been transported into the cell sufficiently far so as to be inaccessible to extraction with BSA. AGEPC retained after washing with corresponding buffer alone (no BSA) was considered to represent total AGEPC which was taken up into cells.
In the following internalization assay, high concentrations of AGEPC were used so that the proportion (percent) of AGEPC bound to specific receptor site(s) against total AGEPC remained small and no longer obvious (3). AGEPC which bound to specific receptor site against total AGEPC molecules were not released into cytosol but retained in the cells with a specific inhibitor of AGEPC, U66985. This is consistent with the assumption that the bulk of AGEPC was nonspecifically taken up into the cells under these conditions (data not shown). The biological responses of platelets were actually suppressed significantly in the presence of 0.5% BSA as previously described (32, 34, 35). Thus the proportion of AGEPC remaining on the receptor site after 0.5% BSA washing was considered to be very small, and AGEPC retained in the cells after the washing could be regarded as those molecules internalized into the platelets.

When washed rabbit platelets were incubated with \(3 \times 10^{-6}\) M \(^{3}H\)AGEPC at 37°C for 1, 2, and 3 min and washed with Tyrode’s solution-gelatin buffer alone, as described under “Experimental Procedures,” more than 90% of \(^{3}H\)AGEPC added was retained in the cells (Fig. 5). This total uptake of \(^{3}H\)AGEPC was not appreciably affected by pretreatment of the cells with a specific inhibitor of AGEPC, U66985. This is consistent with the assumption that the bulk of AGEPC was nonspecifically taken up into the cells under these conditions.

When these cells were lysed by sonication and centrifuged at 100,000 \(\times g\) for 1 h to separate particulate and cytosol fractions, 87.3 ± 0.82% (n = 4) of \(^{3}H\)AGEPC was found in the particulate fraction. In the cells pretreated with U66985, 86.4 ± 1.4% (n = 4) was also recovered in the particulate fraction. Thus after being taken up into cells, the bulk of the AGEPC molecules were not released into cytosol but retained in the membrane regardless of pretreatment with U66985.

When the cells were washed with 0.5% BSA, 28, 30, and 39% of the added \(^{3}H\)AGEPC was retained in control cells after 1, 2, and 3 min incubation with \(^{3}H\)AGEPC, respectively, whereas only 12, 14, and 17% was retained in the cells pretreated with U66985 after corresponding times of incubation (Fig. 5). Analysis on thin layer chromatography of \(^{3}H\) radioactivity retained after BSA washings revealed that AGEPC, lysy-GEPC, and alkylacyl-PC were at a level of 96.3, 2.2, and 1.4%, respectively, in nontreated cells and of 99.0, 0.59, and 0.31% in U66985-treated cells after a 1-min incubation. Thus, whereas total uptake of AGEPC into cells was apparently minimally affected by U66985, the internalization of AGEPC was inhibited by pretreatment with U66985. Similar effects on the internalization were also observed with another inhibitor, CV3988 (data not shown). For the investigation of the mechanism of AGEPC internalization and its relation to the receptor site, this phenomenon was further examined in desensitized platelets.

Internalization of AGEPC in Desensitized Platelets—Once rabbit platelets are treated with AGEPC in the absence of Ca\(^{2+}\), they are known to become desensitized to a second stimulation with AGEPC in the presence of Ca\(^{2+}\) (24). Consequently, the characteristics of the binding of AGEPC were explored in the desensitized platelets. The binding percent for 5 \(\times 10^{-11}, 1.5 \times 10^{-10}\), and 2.5 \(\times 10^{-10}\) M AGEPC was 16.87 ± 1.67, 16.06 ± 2.01, and 15.61 ± 1.85% (mean ± S.D., n = 10), respectively, in control, nondesensitized cells. In desensitized cells, the binding percent was 17.56 ± 1.58, 16.11 ± 2.69 and 15.24 ± 1.92% (mean ± S.D., n = 10), respectively. The binding in the desensitized cells was apparently nearly the same or even slightly higher than in control cells. In Table I, the different components of AGEPC binding (A, B, and C, as described above) were examined in control and desensitized platelets. Component A in desensitized cells appeared to be slightly higher than in control cells; component B was almost the same in both types of cells. Thus no down-regulation of the receptor site(s) was observed in desensitized cells. Component C was appreciably higher in desensitized cells than in control cells and this was presumed due to the irreversibly increased internalization of AGEPC in the desensitized cells, as well as described below.

Subsequently, the internalization of AGEPC was examined in desensitized cells. Fig. 6 shows the retention of \(^{3}H\)AGEPC after washing with buffer alone or 0.5% BSA in control as well as in desensitized cells. There was apparently no difference in total uptake of AGEPC between control and desensitized cells. The internalization of AGEPC, however, was
TABLE I

Binding of AGEPC to desensitized platelets

Control and desensitized platelets were prepared as described under “Experimental Procedures.” Binding of [3H]AGEPC was measured as described in the legend to Fig. 1. Binding percent of components A and B was determined at a concentration of [3H]AGEPC 1 × 10^{-10} and 2 × 10^{-9} M, respectively. Binding component C was measured with 1 × 10^{-10} M [3H]AGEPC plus 10^{-6} M U66985. The total binding percent observed was smaller than that shown in Fig. 1, presumably since platelet cells were incubated at 37 °C for 10 min for desensitization. Data are the mean ± S.D. (n = 4).

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![Fig. 6. Internalization of [3H]AGEPC in desensitized platelets.](image)

The suspension of washed rabbit platelets was incubated at 37 °C for 1 min with 10^{-8} M U66985. 1 × 10^{-9} M [3H]AGEPC was then introduced and the suspension was incubated at 37 °C for 2 min. Total uptake and internalization of AGEPC was determined as in Fig. 5. Total uptake of AGEPC, E, internalization of AGEPC determined by its retention after washing with 0.5% BSA.

considerably greater in desensitized cells, even though the level of internalization was determined after pretreatment of the cells with 10^{-6} M U66985, which blocked the specific binding site (component A), as recorded in Table I. Thus apparently the enhanced internalization of AGEPC was independent of its receptor site(s) in the cells. In platelets which were treated with thrombin, AGEPC was also effectively internalized even in the presence of U66985 (Fig. 7). AGEPC was therefore considered to be efficiently internalized upon treatment of the platelets with thrombin, and this very active internalization had persisted and had been resistant to antagonist in desensitized cells.

**DISCUSSION**

In this study the binding characteristics of AGEPC to washed rabbit platelets were investigated through use of a specific inhibitor of AGEPC, U66985. The binding of AGEPC was separated into three components termed A, B, and C. Component A exhibited a high affinity and a low capacity. It was reversible and was inhibited by the structural analogs of AGEPC which specifically inhibited the biological actions of AGEPC (24). Component A was thus considered to be the putative receptor site. Component B was an irreversible bind-

![Fig. 7. Effect of thrombin on internalization of [3H]AGEPC into platelets.](image)

The suspension of washed rabbit platelets was incubated with saline or thrombin (0.2 units/ml) at 37 °C for 1 min and then with 10^{-6} M U66985 for 1 min. After 1 or 2 min incubation with 10^{-6} M [3H]AGEPC at 37 °C, total uptake (□) and internalization of AGEPC (□) were determined as in Fig. 5.

The internalization of AGEPC, which appears closely associated with binding component B, was investigated by the use of the technique developed by Mohandas et al. (27). This technique was originally applied to assay of internalization of exogenous lyosphatidylcholine (lyso-PC) into the erythrocyte membrane. This assay protocol was applied in this study, since AGEPC has physicochemical characteristics as well as an affinity to albumin similar to that of lyso-PC (32). This case, however, was not identical to that of lyso-PC in the erythrocyte membrane (27), since AGEPC binds to its platelet membrane receptor(s) in contrast to lyso-PC. In the internalization assay, the proportion (percent) of AGEPC bound to receptor site was essentially insignificant compared to that undergoing internalization as described above, and this technique would reflect the latter pool.

In the presence of a very low concentration of BSA (or in its absence) the bulk of AGEPC in the medium was taken up by cells and instantly incorporated into the outer layer of the surface membrane. Internalization of AGEPC was observed in the presence of U66985, as shown in Fig. 5, and this was assumed to be basal internalization in resting platelets. This basal internalization of AGEPC was thus insensitive to agonist, AGEPC. In activated platelets which were not treated with the antagonist, more AGEPC was observed internalized (Fig. 5). These results suggested that internalization of AGEPC was enhanced when AGEPC bound to its receptor. This internalization of AGEPC that is able to be enhanced by AGEPC was thus agonist-sensitive internalization. AGEPC molecules which were internalized into the inside of the cells appeared to remain in the membrane, not being released into cytosol. The component C in Fig. 1 was assumed to consist of nonspecific incorporation of AGEPC into the outer layer of the surface membrane as well as the basal
internalization. The component B presumably contained in addition the agonist-sensitive internalization of AGEPC that was enhanced by the agonist, AGEPC.

In platelets desensitized to AGEPC, specific binding to the receptor site was apparently the same or slightly higher than in control cells. This result was quite in contrast to studies using human platelets, where the affinity of the specific binding site decreased (36) or the number of the sites diminished (8) upon desensitization. This contradiction may be due to the species difference. The (low \( K_a \)) GTPase in the rabbit platelet membrane was desensitized after pretreatment with AGEPC. The desensitization process, therefore, might originate in the sequence subsequent to the binding of AGEPC to the receptor in rabbit platelets.<ref>1</ref>

Internalization of AGEPC was found to be greater in desensitized platelets even in the presence of U69885. In the presence of U69885, more AGEPC was also found internalized in thrombin-treated platelets. On the basis of these results the mechanism of enhancement of AGEPC internalization might be explained as follows: when platelets are activated by AGEPC, the agonist-sensitive pathway for internalization of AGEPC is also reversibly activated. The inhibitors of AGEPC block the activation of cells by AGEPC and then suppress the promotion of the agonist-sensitive internalization process (Fig. 5). Actually, the inhibitors no longer had an effect on AGEPC internalization when they were introduced after AGEPC (data not shown). In desensitized cells, the agonist-sensitive internalization of AGEPC remains irreversibly enhanced and is no longer suppressed by the inhibitors. AGEPC was thus observed effectively internalized in desensitized cells even in the presence of U69885. Activation of the cells with thrombin also results in a similar consequence.

The characteristics of the agonist-sensitive pathway for internalization and the difference from the basal (agonist-insensitive) internalization pathway are still unknown. The results described above, however, apparently argue against the suggestion that AGEPC was mainly internalized as a ligand-receptor complex. Enhanced internalization of AGEPC may be caused by translocation of AGEPC across the platelet surface membrane. It might be that AGEPC passes through its receptor across the membrane or that a specific component(s) of the membrane mediates the translocation of AGEPC upon cell activation. Whereas the internalization of AGEPC was obviously enhanced as described, that of lyso-AGEPC or U69885 was apparently slow and was not significant in a 1-min incubation time (data not shown).

Lachachi et al. (37) suggested in a study using another inhibitor of AGEPC, BN52021, that the transmembrane process is the limiting step in the metabolism of AGEPC in rabbit platelets and that binding of AGEPC to its external receptor favors its internalization. Our observations concerned with the metabolic conversion of AGEPC to alkylacyl-PC in rabbit platelets (21) and a portion of the results described in this present paper were compatible with this report of Lachachi et al. Our results indicated that internalization of AGEPC is enhanced when AGEPC binds to its receptor on rabbit platelets and that AGEPC is apparently 'internalized independent of its receptor(s) (not as a ligand-receptor complex).

The metabolic inactivation of AGEPC was observed enhanced by AGEPC itself as well as other agonists in rabbit platelets (21). This metabolic enhancement may be associated with stimulated internalization of AGEPC into the platelet cells.

Upon activation of platelets, it has been reported that phosphatidylserine was translocated into the outer layer of the membrane, although it was originally localized in the inner layer (38,39). Some glycoproteins were also reported to appear on the surface of platelet cells when stimulated (40, 41). These results might be related to the enhanced internalization of AGEPC described in this study.

REFERENCES