Platelet ADP Receptor and $\alpha_2$-Adrenoreceptor Interaction

EVIDENCE FOR AN ADP REQUIREMENT FOR EPINEPHRINE-INDUCED PLATELET ACTIVATION AND AN INFLUENCE OF EPINEPHRINE ON ADP BINDING*

(Received for publication, October 28, 1985)

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The nucleotide affinity analog 5'-p-fluorosulfonylbenzoyl adenosine (FSBA) is a potent irreversible inhibitor of ADP-mediated platelet activation. Utilizing this compound, the role of ADP in epinephrine-mediated platelet activation was evaluated. Pretreatment of platelets with FSBA under conditions producing covalent incorporation was able to completely block epinephrine-stimulated aggregation of human platelets. In addition, the exposure of latent fibrinogen-binding sites by epinephrine was also inhibited in platelets modified by FSBA. The inhibition of epinephrine-mediated activation of the cells was time-dependent, reflecting the need for covalent modification of the ADP receptor by FSBA. The inhibitory effect of FSBA was not due to effects on the affinity of binding methyl $[^3H]$yohimbine or the number of platelet $\alpha_2$-adrenergic receptors. Studies of the effect of epinephrine on the ability of ADP to protect against FSBA incorporation demonstrated that epinephrine can increase the affinity of ADP for its receptor 10-fold without affecting the total amount of FSBA covalently bound. This effect of epinephrine is mediated through the $\alpha_2$-adrenoreceptor since the effect can be reversed by the competitive antagonist, methyl yohimbine.

These results suggest that promotion of platelet aggregation and the exposure of fibrinogen receptors by epinephrine is dependent on ADP. The mechanism by which epinephrine renders low concentrations of ADP effective appears to be mediated by an increased avidity of the ADP receptor for the nucleotide.

Platelet aggregation can be induced by a variety of putative physiological agonists including ADP, epinephrine, collagen, thrombin, and prostanooids. Pairs of these agonists appear to act in a synergistic manner in the induction of aggregation and secretion; however, the biochemical basis for this synergism remains unclear. Huang and Detwiler (1) have classified the synergistic response of the cells in terms of exhibition of characteristics of one or the other aggregating agent. Since epinephrine by itself is required in supraphysiological concentrations (2) for platelet aggregation, its activation of platelets, to be relevant in vivo, must involve cooperative effects. Plow and Marguerie (3) have reported that enzymes which metabolize ADP were able to inhibit epinephrine-mediated aggregation and fibrinogen binding to platelets. Peerchke (4) has demonstrated that epinephrine-mediated fibrinogen binding can be influenced by ADP in the media. Morinelli et al. (5) have demonstrated a role for ADP in platelet activation by prostaglandin analogs azo-PGHa and U46619. These studies support a role for ADP in epinephrine-mediated platelet aggregation; however, they provide little specific mechanistic information.

Studies from our laboratory have identified a membrane protein (M, 100,000) on the surface of intact platelets which is specifically and uniquely covalently labeled by 5'-p-fluorosulfonylbenzoyl adenosine (FSBA) (6-8), an analog of ADP and ATP; only one radioactive protein band is detected upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of membranes isolated after labeling of intact platelets with FSBA. Incorporation of this affinity reagent inhibits platelet shape change induced by ADP (7, 9), as a function of concentration as well as platelet aggregation and fibrinogen binding stimulated by the nucleotide (7). The target protein is closely associated with actin in the membrane (8) and is a candidate for the receptor-mediating ADP-induced platelet activation but distinct from that receptor which modulates ADP effects on adenylate cyclase (9).

The receptor for epinephrine on the platelets has been studied and classified as an $\alpha_2$-adrenergic site (10). Quantification of the receptors and their binding constants has been achieved utilizing the reversible binding of the specific $\alpha_2$ antagonist methyl $[^3H]$yohimbine (11).

The number of potential mechanisms which explain agonist synergism is sizable. One potential mechanism emphasizes interaction among receptor sites on the cell surface. In such a mechanism, the binding of a given agonist to its respective receptor could modify the interaction of a second agonist with its receptor. Thus, an induced change in the number of receptors or the lowering of a binding constant could explain the increased sensitivity of the cell. Using FSBA and methyl

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* This work was supported by Specialized Center for Research in Thrombosis Grant HL14217 from the National Heart, Lung, and Blood Institute of the National Institutes of Health, Grant DMB-8302963 from the National Science Foundation, American Heart Association Grant-in-Aid 582424101 from the North Central Pennsylvania Chapter, and American Heart Association Special Investigatorship 582423701 from the Southeastern Pennsylvania Chapter. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PG, prostaglandin; FSBA, 5'-p-fluorosulfonylbenzoyl adenosine; DMF, N,N-dimethylformamide; SDS, sodium dodecyl sulfate.
yohimbine as probes, we have studied the interaction of ADP and epinephrine receptors on the platelet cell surface.

**MATERIALS AND METHODS**

FSBA was prepared by the method of Colman et al. (12). The synthesis involves the condensation of adenosine with methyl fluorosulfonylbenzoyl chloride. For the synthesis of radiolabeled FSBA, [2-3H]adenosine (New England Nuclear) was utilized. The radiolabeled compound had a specific radioactivity of 13.6 Ci/mol. FSBA concentrations were determined spectrophotometrically in ethanol using a molar extinction coefficient of $1.35 \times 10^4$ M$^{-1}$ cm$^{-1}$ at 259 nm (12).

Methyl [3H]yohimbine was obtained from New England Nuclear. The compound had a specific radioactivity of 80 Ci/mmol. Unlabeled methyl yohimbine was obtained from Aldrich.

All other chemicals were of reagent grade or better.

**Platelet Preparation Procedures**—Platelet-rich plasma was prepared from whole blood anticoagulated with sodium citrate (0.085 M), citric acid (0.079 M), and dextrose (0.180 M) by differential centrifugation in 50-ml tubes $(120 \times g, 15 \text{ min}, 37 \degree C)$. Washed platelets were prepared from platelet-rich plasma by the method of Mustard et al. (13). Gel-filtered platelets were prepared by filtration of platelet-rich plasma over a Sepharose CL-2B column $(1.25 \times 40 \text{ cm})$ (14).

Binding of Platelets with FSBA—Labeling of washed and gel-filtered platelets with FSBA was carried out as described by Figures et al. (7) as modified by Mills et al. (9).

Preparation of [3H]-Fibrinogen and Receptor-binding Assay—Human fibrinogen (Kabi, Stockholm, Sweden) was purified by ammonium sulfate precipitation as described by Niewiarowski et al. (15). The purified fibrinogen was labeled with [3H] by the iodine monochloride technique resulting in specific radioactivities of 1–50 mCi/nmol fibrinogen. The binding of [3H]-fibrinogen to platelets was performed using the silicone oil centrifugation technique described by Niewiarowski et al. (16). Methyl Yohimbine Binding to Platelet $\alpha_2$-Adrenergic Receptors—The assay of the number and affinity of $\alpha_2$-adrenergic sites on the platelet surface was carried out by the method described by Macfarlane (11). The method utilizes a silicone oil technique to separate bound and free ligand.

Analysis of FSBA Incorporation into Intact Platelets—The incorporation of [3H]FSBA into the platelet ADP receptors takes advantage of the covalent nature of the receptor-analog complex. The reactions are started by addition of [3H]FSBA to platelet suspensions. The reaction was stopped by addition of dithiothreitol (0.2 M), which had been activated with epinephrine. Platelets pre-treated with (B) FSBA (100 $\mu$M) dissolved in carrier solvent (DMF) or (A) carrier solvent alone (0.1%). All incubations were carried out in the presence of adenosine deaminase (2 units/ml). The cells were incubated for 40 min at 37 °C. Following the incubation period, the cells were placed in stirred aggregometer cuvettes. At $\downarrow$ fibrinogen (1 mg/ml) and epinephrine (1 $\mu$M) were added and aggregation profiles were recorded.

**RESULTS**

Inhibition of Epinephrine-induced Platelet Activation of FSBA—Gel-filtered platelets were treated with FSBA in DMF or DMF alone in the presence of adenosine deaminase to obviate any effects of adenosine arising from the degradation of FSBA. FSBA-treated cells failed to aggregate in response to epinephrine (1 $\mu$M) whereas control cells exhibited optimal aggregation at the same concentration (Fig. 1). Even concentrations of epinephrine as high as 20 $\mu$M failed to aggregate FSBA-treated cells (data not shown).

The time course of this inhibition is shown in Fig. 2. The inhibition of epinephrine-mediated aggregation by FSBA (40 $\mu$M) was progressive with time, with an observed half-life of approximately 17 min. This time dependence of inhibition is similar to that of covalent incorporation of FSBA into platelet membranes (9).

In addition to the inhibition of epinephrine-mediated aggregation, FSBA also inhibited fibrinogen binding to cells which had been activated with epinephrine. Platelets pretreated with FSBA (100 $\mu$M, 20 min, 37 °C) in DMF (Fig. 3) failed to bind fibrinogen while those pretreated with DMF (0.02%) showed the expected binding.
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Effect of FSBA on the Availability of the Platelet α2-Adrenergic Receptor—The inhibitory effect of FSBA could be explained by the ability of the nucleotide analog to sterically block the accessibility of epinephrine to the α2 binding sites on the platelet. In order to test this possibility, we studied the effect of FSBA on the binding of a specific α2 antagonist [3H]methyl yohimbine to the α2 receptor. Gel-filtered platelets were pretreated with FSBA (60 μM, 20 min, 37°C) or carrier (DMF) alone. The cells treated with FSBA were unable to aggregate in response to epinephrine as compared to a full response with control (DMF-treated) cells. We then measured the binding of methyl [3H]yohimbine in FSBA-treated and control cells over a range of methyl [3H]yohimbine concentrations (0–10 nM). The data are shown as binding isotherm and analyzed by the method of Scatchard (16) (Fig. 4, A and B). Very little difference in either the total number of sites or the Kd was observed between control (Kd = 1.4 nM, binding sites/platelet = 200) and FSBA (Kd = 1.6 nM, binding sites/platelet = 213)-treated cells.

Unidirectional Dependence of Epinephrine-induced Platelet Aggregation on Occupancy of the ADP Receptor by ADP—The interaction between ADP and epinephrine in a synergistic manner in the mediation of platelet aggregation is well known. The inhibition of FSBA of epinephrine-induced aggregation indicates that the response to the epinephrine is dependent on the binding of ADP to its receptor. We questioned whether an ADP response was similarly dependent on the occupancy of the α2-receptor sites. Methyl yohimbine (20 nM) was able to totally inhibit the aggregation of gel-filtered platelets by epinephrine (10 μM). At this concentration of methyl yohimbine, no effect on ADP (10 μM)-induced aggregation was noted. Even at equimolar concentrations of methyl yohimbine and ADP (10 μM), no effect on platelet aggregation was observed (data not shown).

Effect of Epinephrine and ADP on Washed Platelets—Platelets washed by repeated centrifugation in the presence of apyrase respond well to thrombin (0.1 unit/ml) and ADP (1 μM); however, these cells fail to respond to epinephrine even at high concentrations (10 μM). The reason for the inability of the platelets to respond under these conditions has not been apparent, but the observation could be explained by the fact that the washing procedure serves to remove extracellular ADP which is required to support epinephrine-induced responses. When 0.25 μM ADP (a concentration insufficient to produce platelet aggregation) was added, epinephrine (10 μM) produced complete aggregation.

Mechanism of ADP-Epinephrine Interaction in Platelet Activation—The dependence of epinephrine-induced platelet response on ADP as well as available unoccupied ADP receptors suggested that the interaction was occurring at the receptor level. Two potential mechanisms by which epinephrine can increase the effectiveness of low levels of ADP were studied. The first possibility is that epinephrine mediates an increase in the number of available ADP receptors on the cell surface which would be reflected by an increase in the incorporation of [3H]FSBA in intact platelets. However, the data in Table I indicate that at saturating levels of FSBA (100 μM, 37°C, 40 min), there was no net increase in the number of ADP ([3H]FSBA)-binding sites on the cell surface in the presence of epinephrine (10 μM). A second potential mechanism by which epinephrine might increase ADP effectiveness could be a decrease in the Kd of the receptor for ADP due to the binding of epinephrine to its receptor. ADP is able to compete with FSBA for receptors on the cell surface prior to the covalent binding of the affinity reagent (17). A shift in the Kd of the ADP receptor for ADP would be reflected in an in-

![FIG. 3. Effects of FSBA on epinephrine-induced 125I-fibrinogen binding.](https://example.com/figure3.png)

![FIG. 4. Effect of FSBA on the availability of platelet α2-adrenergic receptors.](https://example.com/figure4.png)

**Table I**

| [3H]FSBA | Epinephrine | [3H]FSBA incorporation pmol/10⁶ cells |
|----------|------------|-----------------------------------|
| 50 μM    | 0 μM       | 14.4                              |
| 50 μM    | 10 μM      | 14.0                              |

The data were plotted by the method of Scatchard (7) (Fig. 3). The average non-specific binding was 25% of the total bound. The data represent the average of 3 individual experiments.
creased ability of ADP to protect against \(^{3}H\)FSBA incorporation provided that a similar effect of epinephrine on the binding of FSBA was absent. The rate of FSBA covalent incorporation into intact platelets is linear over 5 min using the dialysis procedure described under “Materials and Methods” (Fig. 5), but the rate decreases with longer incubation (data not shown). A rate of covalent incorporation of \(^{3}H\)FSBA of 3000 molecules/platelet/min was observed in this particular experiment. The rate of incorporation was not altered in the presence of epinephrine (10 \(\mu\)M).

This lack of effect of epinephrine on covalent incorporation of FSBA allows for an evaluation of the effect of epinephrine on ADP binding directly. The covalent incorporation of \(^{3}H\)FSBA into intact platelets can be modified by addition of various concentrations of ADP to the incubation mixture (Fig. 6). The initial rate (0–5 min) of \(^{3}H\)FSBA incorporation into intact platelets was not affected at 20 \(\mu\)M ADP, whereas the rate progressively declined above 100 \(\mu\)M and reached 0 at 100 \(n\)M ADP. In the same experiment, epinephrine (10 \(\mu\)M) increased the ability of 10 \(\mu\)M ADP to inhibit the initial rate of \(^{3}H\)FSBA incorporation equivalent to the effect of 300 \(\mu\)M ADP (Fig. 6). The degree of epinephrine enhancement of ADP protection decreased with increasing ADP concentrations as the protection curves converged at 10 mM ADP. Although this behavior suggests competition, the complexity of this system precludes rigorous testing of this hypothesis. These results indicate that epinephrine increases the ability of the receptor to bind ADP.

In order to demonstrate that the enhancement of ADP protection was specific to the interaction of epinephrine with the \(\alpha_2\) receptor, a rate experiment was carried out in the presence and absence of methyl yohimbine (10 nM). When methyl yohimbine was added to the reaction mixture, the enhancement of the protection conferred by 20 \(\mu\)M ADP by epinephrine (10 \(\mu\)M) disappeared (Fig. 7). The results of this experiment demonstrate that methyl yohimbine can block the “potentiating” effect of epinephrine.

**DISCUSSION**

The ability of combinations of platelet agonists to promote aggregation to platelets in what appears to be a synergistic manner is generally recognized. These studies are based on the ability of low levels of various agonists to act in a cooperative manner, thus producing responses greater than the expected response for each agonist acting independently. The biochemical basis for this synergy is not well understood. Studies reported here show that FSBA when incubated with platelets can block the known exposure of fibrinogen receptors by epinephrine (18). These results suggest a role for ADP in the epinephrine-activated system. In assessing the apparent synergism between ADP and epinephrine, the potential contamination of platelet preparations by ADP must be considered. Huang and Detwiler (19) have shown that platelet-rich plasma contains at least 12 nM ADP. Other reports have indicated ADP concentrations in plasma to range from 80 to 120 nM (20, 21). In addition, platelets prepared by gel filtration have been reported with contaminating ADP concentrations as high as 1 \(\mu\)M (22). This situation renders the measurement of pure epinephrine effects difficult unless ADP is removed from the system. Peerche (4) has studied epinephrine-mediated fibrinogen binding in platelets pretreated with aspirin in order to block thromboxane production and the release of ADP from dense granules. Cells prepared in buffers containing apyrase or creatine phosphate and creatine kinase to remove contaminating ADP exhibited a large decrease in the amount of fibrinogen bound when stimulated with epinephrine compared to cells prepared in the absence of ADP, scavengers. The conclusion of that study, that epinephrine was able to expose a limited number of fibrinogen receptors in the absence of ADP, was based on the assumption that the ADP-metabolizing enzymes were effectively removing all of
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the ADP from the system. However, “potentiating” concentrations may still exist under the stated conditions. Creatine kinase and potato apyrase have been reported with $K_m$ values of 50 nM (23) and 240 nM (24), respectively. Since ADP concentrations as low as 50 nM can potentiate aggregation by epinephrine (25), the results of experiments using apyrase or creatine kinase must be evaluated with caution.

Another approach to the study of the ADP independence of platelet responses is to block the effects of ADP at the receptor level. FSBA, a nucleotide affinity analog, has been shown to inhibit ADP-induced platelet shape change (6), aggregation (7), and fibrinogen binding (7). Concomitant with the inhibition of these responses, FSBA modifies a single 100,000-dalton protein component of the platelet plasma membrane (6-8). This protein appears to represent the ADP receptor-mediated platelet shape change and fibrinogen receptor exposure. The use of the FSBA obviates the need for removing trace amounts of ADP from platelet preparations as cells treated with this compound are totally refractory to ADP. The specificity of this protein for ADP has been demonstrated. While the covalent binding of FSBA is blocked by ADP (6), neither epinephrine, adenosine, nor thrombin are effective. The high concentration of ADP necessary to effect protection against FSBA incorporation is most likely due to the fact that FSBA forms a covalent complex with the target protein. Thus, high occupancy levels of the receptor by ADP are probably necessary in order to keep molecules of FSBA from interacting with the receptor. A recent study from our laboratory (9) indicates that inhibition of platelet shape change by FSBA is concentration dependent. The covalent modification of the platelet membrane protein shows a similar concentration dependence. While the time course of the incorporation is linear over 0-5 min of incubation (Figs. 5 and 7) by 10 min, the rate decreases and saturation occurs by 60 min (6). Thus, we have confined ourselves to measurement of the initial rate of incorporation up to 5 min.

FSBA exhibits considerable specificity for an adenosine nucleotide receptor on the platelet cell surface, mediating shape change, aggregation, and fibrinogen binding. In contrast, cells treated with the guanosine analog (5'-fluorosulfonyl guanosine) (9) are not inhibited. In addition, FSBA has no effect on the ADP receptor responsible for the lowering of the PGF$_2$-stimulated cyclic AMP levels (9). All of these studies were carried out in the presence of adenosine deaminase to eliminate any effects of adenosine arising from the breakdown of FSBA during incubations.

The inhibition of epinephrine-mediated responses of the cells by FSBA was not due to effects on the $\alpha_2$-adrenergic receptor as FSBA had no effect on the binding of [H$]methyl yohimbine to this receptor (Fig. 4). Moreover, methyl yohimbine, which competes with epinephrine $\alpha_2$-binding sites, had no effect on ADP-mediated aggregation of the cells even when added in 1000-fold excess of the concentration which optimally inhibited epinephrine-induced aggregation. These results indicate that the mechanism of cooperativity between ADP and epinephrine may not be a true synergism but a unidirectional dependence of epinephrine activation on ADP receptor occupancy. Direct evidence for this dependence is that repetitive washing of platelets in the presence of apyrase to degrade ADP released from the cells during the centrifugation process renders these cells insensitive to epinephrine. This effect should not be viewed as potentiation as these cells fail to respond to any concentration of epinephrine in the absence of ADP.

In vitro, the ADP required for epinephrine-induced aggre-

gation could arise in the media due to platelet lysis or release of ADP from platelet storage granules during platelet preparation procedures. Another possibility is that epinephrine causes the release of small amounts of ADP prior to the major release that occurs during the second phase of aggregation. Although evidence exists that platelet preparation procedures result in contamination by ADP (19-22), epinephrine-mediated release of ADP from storage granules during the initial stages of aggregation has not been demonstrated (26).

The basis of ADP-epinephrine effects on platelet activation may be due to a number of mechanisms. At the receptor level, it is possible that the binding of a given agonist (i.e. epinephrine) to its receptor site can effect a change in the binding constant ($K_r$) of another receptor for its ligand (i.e. ADP). This situation would then increase the level of receptor occupancy for the second ligand rendering lower concentrations of the ligand more effective in promoting the response. A second possible mechanism of cooperativity is based on the ability of a ligand to increase the number of available receptors for a second ligand on the cell surface. This situation is known to exist in platelets for the exposure of latent fibrinogen receptors on platelets by ADP, epinephrine, collagen, chymotrypsin, prostaglandin derivatives, and thrombin. Neither of the proposed mechanisms require that the cooperativity be bidirectional, that is one ligand may cause a change in $K_r$ or number of sites for a receptor for a second ligand but the reciprocal situation need not occur.

The current study demonstrates that the dependence of epinephrine on low levels of ADP is not due to a shift of $K_r$ but the combination of the number of ADP receptors as epinephrine did not increase the number of receptors for FSBA on the surface of the cell (Table I). However, an epinephrine-mediated change in the avidity of ADP binding was observed in a study in which ADP was utilized to protect against the incorporation of FSBA (Fig. 6). This study was facilitated by the fact that epinephrine had no effect on the rate of [H$]FSBA incorporation. This effect appears to be specific for interaction of epinephrine with an $\alpha_2$ site as methyl yohimbine, a competitive antagonist, was able to inhibit the epinephrine-mediated effect (Fig. 7). A study of the concentration dependence of ADP on FSBA incorporation demonstrates an epinephrine-mediated shift of 15-fold in the ability of ADP to protect against FSBA binding. Such an increase in the ability of the receptor to bind ADP can be roughly correlated with the ranges of platelet sensitivity to ADP in vitro. Normally, 1-2 $\mu$M ADP can induce platelet aggregation in platelet-rich plasma. However, potentiating concentrations of ADP have been reported in the 50-100 nM range in the presence of a suboptimal dose of epinephrine.

The data presented here are strongly indicative of a central role for ADP in epinephrine-induced platelet aggregation. The term "synergism" as classically applied to the ability of platelet agonists to interact at the suboptimal doses to produce a larger than additive effect is herein viewed as an alteration by epinephrine on the ability of ADP to interact with its receptor. From the present data, the biochemical mechanisms for this interaction appear to be due to an epinephrine-mediated change in the ability of the ADP receptor to bind its ligand, thus enabling a higher level of receptor occupancy at low ADP concentrations. Whether or not other "synergistic" pairs of agonists operate through similar mechanisms remains a subject for further study.

Acknowledgments-We wish to acknowledge Julie Mills and Steven Sargent for their assistance in the early phases of this work and Dr. David Mills for his helpful discussions.
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