Two Mechanisms for Inhibition of ADP-induced Platelet Shape Change by 5'-p-Fluorosulfonylbenzoyladenosine

CONVERSION TO ADENOSINE, AND COVALENT MODIFICATION AT AN ADP BINDING SITE DISTINCT FROM THAT WHICH INHIBITS ADENYLYL CYCLASE*

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The interaction of ADP with platelets leads to shape change, exposure of fibrinogen binding sites, and aggregation, all of which have been shown to be inhibited by 5'-p-fluorosulfonylbenzoyladenosine (FSBA), an alkylating analogue of adenine nucleotides which binds covalently to a 100-kDa polypeptide in intact platelet membranes (Figures, W. R., Niewiarowski, S., Morinelli, T., Colman, R. F., and Colman, R. W. (1981) J. Biol. Chem. 256, 7789–7795). In plasma, FSBA can break down to adenosine which stimulates adenylate cyclase. To distinguish between direct effects of FSBA and the actions of adenosine, we have washed platelet suspensions and adenosine deaminase. We studied the effects of FSBA on shape change and cyclic AMP metabolism, and on the binding of 2-methylthio-ADP, which mimics the effects of ADP on cyclic AMP metabolism at concentrations too low to activate platelets.

Inhibition of ADP-induced shape change of platelets incubated with FSBA for 2 min in platelet-rich plasma was greatly reduced by adenosine deaminase. In the presence of a phosphodiesterase inhibitor, 100 μM FSBA increased platelet cyclic AMP to the same extent as did 10 μM adenosine. These effects were inhibited by theophylline, an adenosine receptor antagonist, and by adenosine deaminase. Incubation of washed platelets for 60 min with FSBA and adenosine deaminase caused a concentration-dependent inhibition of ADP-induced shape change. Inhibition closely paralleled the covalent incorporation of 3H from tritiated FSBA into platelet membranes. Under these conditions, FSBA did not block inhibition of cyclic AMP accumulation by ADP, nor did it block the binding of 2-methylthio-ADP. We conclude that part of the inhibition of shape change caused by brief exposure to FSBA is due to adenosine, but at longer times shape change is inhibited in association with covalent incorporation of sulfonylbenzoyladenosine. This effect of FSBA is independent of adenosine and occurs at a site distinct from that at which ADP inhibits adenylate cyclase.

ADP is a major regulator of platelet behavior. At micromolar concentrations, ADP induces shape change, aggregation, and release of the contents of platelet dense bodies and α-granules (1). ADP is also an inhibitor of the adenylate cyclase activity of platelet membranes (2, 3), and in intact cells it antagonizes the effect of agents that increase cyclic AMP accumulation (4, 5). ADP does not penetrate the cell membrane, but induces these responses by interacting with specific receptors on the cell surface (6).

It is not clear whether a single receptor can account for these diverse effects of ADP, or whether more than one receptor is involved. Several analogues of ADP with different substituents in the 2-position of the purine ring have potencies as antagonists of cyclic AMP accumulation that differ widely from their potencies as aggregating agents (7, 8). Moreover, the mercuration thiol reagent, p-mercuribenzenesulfonate, which does not readily penetrate cell membranes, can block the effect of ADP on adenylate cyclase without inhibiting the shape change. This reagent also blocks the binding of radiolabeled ADP analogues including 2-methylthio-ADP (7, 8).

5'-p-Fluorosulfonylbenzoyladenosine (FSBA) is a reactive derivative of adenosine which is structurally related to ADP and ATP. It reacts covalently with nucleophilic residues in the region of ADP or ATP binding sites on a large number of different enzymes (9, 10). FSBA also inhibits platelet shape change (11), aggregation, and fibrinogen binding (12) induced by ADP. When [3H]FSBA is incubated with human platelets, a single band of membrane protein (Mr = 100,000) becomes covalently labeled (13). Since 1 mM ADP reduces the labeling of this protein by 50%, it appears to be a candidate for an ADP receptor mediating platelet activation. Intact platelets, washed (12) or gel filtered (13) to remove plasma protein, incorporate about 40,000 molecules of FSBA/cell.

FSBA contains a labile ester linkage that can be hydrolyzed by acid or base catalysis, and which may be susceptible to the action of plasma esterases. One product of hydrolysis is adenosine, which inhibits platelet aggregation and shape change by stimulating adenylate cyclase (14, 15). We have therefore investigated the effects of FSBA on cyclic AMP metabolism and on ADP-induced shape change in platelet-rich plasma

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1 The abbreviations used are: FSBA, 5'-p-fluorosulfonylbenzoyladenosine; FSBG, 5'-p-fluorosulfonylbenzoylguanosine; RA233, 2,6-bis(diethanolamino)-4-piperidinopurin-9(6),9(6)pyrimidinone; DMF, N,N-dimethylformamide; SQ26538, 8(R),9(S),11(R),12(S)-9-oxo-9,11-epoxy-5(Z),13(E)-15(R)-hydroxyprostaglandin D; PG, prostaglandin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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and in washed platelet suspensions, and used adenosine deaminase to distinguish between direct effects and those due to adenosine.

**EXPERIMENTAL PROCEDURES**

Platelet-rich plasma was prepared from the citrated blood of normal volunteers (16). Platelet adenine nucleotides were labeled by incubating platelet-rich plasma for 1 h at 37 °C with 0.1 μCi/ml [U-3H]adenine, or washed platelets with 1 μCi/ml [2-3H]adenine for 1 h before the final resuspension. Radioactive cyclic AMP was separated from other nucleotides by chromatography on Dowex AG 50 (Bio-Rad) followed by two successive precipitations with ZnSO4 and Ba(OH)2, or by a two-column procedure (17) which gives lower basal levels. Platelets were washed by a modification of the procedure of Mustard et al. (18). Heparin was omitted from the first washing solution, which instead was supplemented with one-fifth volume of 3.8% NaCl, citrate and acidified to pH 6.5, thus conforming to the ionic composition of citrated plasma acidified to pH 6.5 with citric acid.

The platelets were finally resuspended in a medium containing Na+ (130 mM), K+ (2.7 mM), Mg2+ (2 mM), Ca2+ (2 mM), Cl- (130 mM), HCO3- (12 mM), PO43- (1 mM), HEPES (10 mM), glucose (560 mM), and bovine serum albumin (3.5 mg/ml), at pH 7.4 and a platelet count of 3–5 × 10^8/ml.

Shape change was measured in platelet-rich plasma diluted with an equal volume of 5 mM EDTA in isotonic saline to prevent aggregation, using a Chrono-Log Lumiaggregometer at 37 °C, and was expressed as the maximum change in optical density in millivolts measured 20 s after the addition of ADP. Washed platelet suspensions were diluted to 0.5–1.0 × 10^9/ml.

Platelets were prepared for scanning electron microscopy by adding 2.5% glutaraldehyde in Tyrodes buffer. The samples were fixed for 2 h in room temperature and then were kept overnight at 4 °C.

The next day the platelets were deposited with gentle suction (10 mm Hg) at a 1-μm pore nucleopore membrane. The membrane and supernatants carefully aspirated. The cells were then sedimented and the supernatants carefully removed and counted in ACS-II scintillation mixture (Amersham Corp.).

Antagonists to adenosine were added at the initiation of the reaction, and the mixture incubated for 2 min at 37 °C, or at 30 min at room temperature, following which the binding was assayed as previously described (9). For measurements of stability in plasma, samples were diluted at intervals into equal volumes of cold ethanol. [2,5-3H]FSBA (1.4 × 10^6 cpm/mmol) was prepared as previously described (9). For measurements of stability in plasma, samples were diluted at intervals into equal volumes of cold ethanol and applied as 10-μl spots to silica gel TLC sheets and run upwards in methyl ethyl ketone-acetone-water (65:20:15, v/v,v). UV-absorbing spots were scraped off and counted in a Triton-based counting mixture. The Rf values for FSBA and adenosine were 0.81 and 0.48, respectively.

5'-Fluorosulfonylguanosine (FSBG) was prepared and analyzed as described by Pal et al. (19) and dissolved in dimethyl sulfoxide. Its concentration was determined from its absorbance using an extinction coefficient of 7.2 × 10^3 M^-1 cm^-1 at 270 nm.

Incorporation of 'H from [5-3H]FSBA into platelet membranes was measured by incubating washed platelets with [2-3H]FSBA (1.35 × 10^6 cpm/mmol) for 60 min. The cell suspension (1 ml) was added to 100 μl of 2 mM dithiothreitol to stop the reaction by eliminating excess FSBA. The cells were then sedimented and the supernatants carefully aspirated. When intact platelets were labeled under similar conditions, all of the bound 'H was associated with a single 100-kDa protein (11, 13). Covalent incorporation of 'H into platelet membranes was blocked by 10 mM ADP or ATP. We used 10 mM ADP to determine nonspecific binding. The pellets were solubilized in 0.04 M phosphate buffer at pH 7.2 containing 0.5% w/v sodium dodecyl sulfate, 8 M urea, and 0.01 M EDTA. To remove unbound ligand, the solutions were dialyzed in a multiple cell dialysis chamber (Bethesda Research Laboratories) against 0.01 M phosphate buffer at pH 7.2 containing 0.1% sodium dodecyl sulfate and 0.01 M EDTA using a 50,000-dalton cutoff Spectrapore membrane (Fisher). After 72 h the samples were removed and counted in ACS-II scintillation mixture (Amersham Corp.).

Adenosine deaminase from calf intestinal mucosa was obtained as the lyophilized powder from Sigma and stored at 4 °C as a solution containing 1000 units/ml in Tris-buffered saline at pH 7.4. Theophylline was used as the ethyleneimine complex (aminophylline), obtained from Sigma. The thromboxane A2 analogue, SQ 29,569, was a gift from Dr. D. N. Harris, The Squibb Institute for Medical Research, Princeton, NJ, and prostaglandins E2 and I2 were gifts from Dr. J. F. Pike, Upjohn Ltd, Kalamazoo, MI. Human thrombin was a gift from Dr. J. Fenton, New York State Department of Health, Albany, N.Y.

2-Methylthio-ADP labeled with 32P in the β-phosphate was prepared at a specific activity of 5000 Ci/mmol and purified by high performance liquid chromatography on a Whatman Partisil SAX anion exchange column (8). Binding of 2-methylthio-ADP to platelets was studied by centrifugation through a layer of silicone oil of suitable density and a swing-out head attachment for the Eppendorf Microfuge (7, 20).

**RESULTS**

Fig. 1 shows the effect of incubating platelet-rich plasma for 2 min with increasing concentrations of FSBA on the shape change induced by 2 μM ADP. Inclusion of 2 μl/ml adenosine deaminase during the incubation blocked inhibitory effects of up to 40 μM FSBA and up to 10 μM adenosine. Adenosine deaminase itself caused a small but consistent enhancement of shape change, possibly as a result of elimination of adenosine present in the platelet-rich plasma.

We compared the ability of adenosine and of FSBA to stimulate cyclic AMP formation in intact platelets in platelet-rich plasma under a variety of conditions. In the presence of RA235, a phosphodiesterase inhibitor of the pyrimidopurine type, both adenosine and FSBA caused increases in cyclic AMP levels, although FSBA was about 10-fold less active than adenosine (Fig. 2). Increasing the incubation time from 1 to 10 min enhanced the effects of FSBA and adenosine. These results could be due to a weak interaction of FSBA itself with the adenosine receptor, to the presence of about 10% of adenosine as a contaminant or to hydrolysis of FSBA. We compared the effect of adenosine deaminase on the increase in cyclic AMP accumulation occurring during 1- and 10-min inductions with adenosine and FSBA. The results showed that both adenosine and FSBA increased the accu-

![Fig. 1. Maximum extent of shape change induced in human platelets in diluted platelet-rich plasma by incubation for 2 min with increasing concentrations of FSBA in the presence of 2 units/ml adenosine deaminase (0) or in the absence of the enzyme (O). Shape change was expressed as the deflection of the aggregometer tracing measured 20 s after the addition of 2 μM ADP. EDTA was present in all samples at 2 mM.](image-url)
FIG. 2. Formation of cyclic AMP in platelets incubated with adenosine (A) or with FSBA (B) in the presence of 0.4 mM RA233. Samples (0.5 ml) of platelet-rich plasma, prelabeled by incubation with [3H]adenine, were incubated for 1 min ( ), or 10 min (O) with ( ) or without ( ) 1.75 units/ml adenosine deaminase in Tris buffer. The final concentration of DMF added with FSBA was not more than 10 μl/ml plasma.

FIG. 3. Accumulation of cyclic AMP in platelets stimulated by adenosine or by FSBA is inhibited by theophylline. Platelet-rich plasma prelabeled with [14C]adenine was incubated for 10 min with ( ) adenosine or ( ) FSBA at the indicated concentrations. FSBA was added as a solution in DMF (up to 0.6% v/v). All tubes contained 0.4 mM RA233. The data points represent the means of duplicate measurements, with ( ) or without ( ) theophylline (0.2 mM) added in addition to the other reagents.

mulation of cyclic AMP, and that both effects were greater at 10 min than at 1 min. Adenosine deaminase blocked both responses. We measured the effect of adenosine deaminase on FSBA spectrophotometrically at 265 nm. FSBA was not a substrate for adenosine deaminase, nor did it inhibit adenosine deamination. Thus the effect of adenosine deaminase is due to removal of adenosine.

The inclusion of an adenosine receptor antagonist, theophylline, during a 10-min incubation, also inhibited the effects of both adenosine and FSBA (Fig. 3), confirming that the effect is mediated by adenosine. FSBA was again 10-fold less active than adenosine.

Tritiated FSBA was incubated at 37 °C with citrated human plasma at an initial concentration of 100 μM, and samples were taken at intervals to measure the amount of FSBA remaining intact by thin layer chromatography of ethanol extracts. Radioactivity as FSBA disappeared with a half-life of 10 min.

To study the effects of FSBA in the absence of adenosine, experiments were performed with washed platelet suspensions to eliminate the effect of plasma esterases, and with adenosine deaminase to remove any adenosine initially present. Inhibition of ADP-induced shape change was seen under these conditions over a range of 25-45 μM FSBA (Fig. 4). The extent of inhibition was proportional to the concentration of FSBA, and it was well correlated with the incorporation of radioactivity into a nondialyzable fraction of platelets when exposed under similar conditions to 3H-labeled FSBA. The inhibition was not competitive in nature, as it could not be overcome by increasing the concentration of ADP. Also it was necessary to preincubate the platelets with FSBA for 10–20 min before the full effect was observed.

The specificity of the effects of FSBA were examined by comparing it with the analogous derivative of guanosine, FSBG. Washed platelets incubated for 60 min with 40 μM FSBG showed no inhibition of shape change, while under the same conditions (Fig. 4), FSBA caused 90% inhibition. The effect of FSBA was relatively specific for ADP, as washed platelets incubated for 30–90 min with 100 μM FSBA were completely insensitive to ADP at concentrations up to 200 μM, but were still able to change shape when treated with either human thrombin (0.15 units/ml) or the thromboxane A2 analogue, SQ26538 (0.6 μM). Lower concentrations of thrombin (0.025 units/ml) and SQ26538 (0.1 μM) were partly inhibited.

The results obtained by measuring shape change responses by the optical density changes recorded in the aggregometer were confirmed by direct observation of scanning electron micrographs made on samples of platelets fixed in glutaraldehyde at the height of the shape change and deposited onto
washed platelets were incubated with 3H-labeled FSBA under similar conditions, and the amount of label incorporated into nondialyzable material (\(\text{FSBA}^{3H}\)) was determined (see "Experimental Procedures").

In a separate experiment, platelets were incubated with FSBA or with the solvent, DMF at 0.5% v/v, in the presence of adenine deaminase. Adenine deaminase, added to stop the reaction. Results are given as the percentage of 3H radioactivity recovered as cyclic AMP after correction for the recovery of an internal standard of [\(^{3}H\)]cyclic AMP. 0, control; PGI\(_1\), only; \(\bullet\), + ADP 3 \(\mu\)M; \(\triangle\), + ADP 30 \(\mu\)M.

FIG. 5. Scanning electron micrographs of washed platelets demonstrating shape change. The cells were fixed in 2.5% glutaraldehyde and deposited with gentle suction onto nucleopore membranes with 1 \(\mu\)m diameter holes. All samples contained 2 \(\mu\)l/ml of adenine deaminase. A, control. Platelets were stirred in the aggregometer for 5 min and then fixed. (96% disc shaped, optical shape change signal, 0 mV.) B, control platelets fixed 30 s after the addition of 2 \(\mu\)M ADP. (4% disc shaped, optical shape change signal, 55 mV.) C, platelets incubated for 45 min with 0.5% v/v DMF and then for 30 s with 2 \(\mu\)M ADP. (5% disc shaped, optical shape change signal, 84 mV.) D, platelets incubated for 45 min with 40 \(\mu\)M FSBA and then for 30 s with 2 \(\mu\)M ADP. (87% disc shaped, optical shape change signal, 10 mV.)

nucleopore membranes (Fig. 5). Treatment with FSBA reduced the proportion of platelets that lost their disc shape on treatment with ADP to 12% from the control value of 95%.

We have also investigated the effect of FSBA on the mechanism by which ADP inhibits cyclic AMP accumulation in platelets exposed to adenylate cyclase stimulators. Fig. 6 shows the effect of increasing concentrations of ADP on the accumulation of cyclic AMP in intact washed platelets incubated for 30 s with 0.4 \(\mu\)M PGI\(_1\) after 30 min preincubation with FSBA. FSBA did not inhibit the cyclic AMP increase, but at concentrations above 20 \(\mu\)M, it somewhat enhanced the response to PGI\(_1\), an effect that has been observed before with the cell-penetrating sulfhydryl reagent, N-ethylmaleimide (5). Unlike N-ethylmaleimide, FSBA had no detectable effect on the ability of ADP to suppress the stimulation of adenyl cyclase by PGI\(_1\).

The ADP analogue, 2-methylthio-ADP is an aggregating agent and an inhibitor of the accumulation of cyclic AMP in platelets stimulated with PGE\(_2\) or PGI\(_1\) (8). Labeled 2-methylthio-ADP, with \(^{32}\)P in the \(\beta\)-phosphate position, binds selectively and reversibly to a single class of binding sites with an apparent dissociation constant of 5–20 nM and 500–1500 sites/platelet; this binding is specifically and competitively blocked by ADP and by ATP (8). Fig. 7 shows that 10 min incubation of washed platelets with 1 mM FSBA had no effect on the number of binding sites available to 2-methylthio-ADP; the apparent dissociation constant for 2-methylthio-ADP was slightly increased, from 22 to 33 nM.

**DISCUSSION**

The responses of platelets to ADP are the result of an interaction of the nucleotide, which does not cross the cell...
Our studies suggest that FSBA can undergo hydrolysis to adenosine during incubation with platelet-rich plasma, causing an increase in the accumulation of cyclic AMP that can be detected in the presence of an inhibitor of phosphodiesterase. The increase in cyclic AMP accumulation seen with FSBA was blocked by adenosine deaminase which converts adenosine to the inactive compound, inosine. The absorbance of FSBA at 260 nm was not affected by incubation with adenosine deaminase, indicating that it is not a substrate. The increase in cyclic AMP accumulation seen with FSBA in the presence of the phosphodiesterase inhibitor RA238 was also partly blocked by the Ra receptor antagonist, theophylline. To a large extent the inhibition of shape change in platelet-rich plasma was also prevented by adenosine deaminase, indicating that under these conditions the inhibition was due to adenosine. The short half-life of FSBA in plasma (about 10 min) contrasts with a half-life of several hours in buffer at pH 7.4 (10) and clearly limits the usefulness of this reagent in plasma.

To study the effects of FSBA in the absence of adenosine, we used washed platelet suspensions prepared by a method designed to preserve the morphology and responsiveness of the cells towards inducers of shape change, and included adenosine deaminase to remove any adenosine that may have been formed. FSBA irreversibly inhibited ADP-induced shape change in a time-dependent fashion. The inhibition was proportional to the covalent incorporation of radioactivity from labeled FSBA. Under similar conditions FSBA has been shown to modify covalently a single externally oriented membrane protein (11, 12), suggesting that this protein has a role in platelet activation. A nonselective effect of the reagent is made less likely by the observation that 5'-p-fluorosulfonylbenzoylguanosine caused no inhibition of shape change.

Since ADP inhibits the stimulation of adenylyl cyclase by prostaglandins, we measured the effects of FSBA on cyclic AMP metabolism. FSBA slightly enhanced the stimulation of cyclic AMP accumulation by PGI2 in washed platelets, but it did not inhibit the antagonistic effect of ADP. Incubation of washed platelets with FSBA did not reduce the number of binding sites for the ADP analogue, 2-methylthio-ADP, which binding to the platelet membrane, with a specific receptor or receptors on the external surface of the cell. Evidence that aggregation and inhibition of adenylyl cyclase are mediated by the same receptor has been presented by Cusack and Hourani (21) who synthesized a series of competitive inhibitors of ADP-induced platelet aggregation, that were also competitive inhibitors of the effect of ADP on cyclic AMP accumulation. A good correlation between the apparent affinity constants for the two effects was observed, but the compounds examined were all quite similar in structure, being close structural analogues of ATP with ribose and purine moieties intact.

A number of possible explanations have been considered to explain the inhibitory effect of FSBA on the platelet shape change induced by ADP. Among these are (a) a direct effect on the ADP receptor for shape change and aggregation; (b) breakdown to adenosine which stimulates the accumulation of intracellular cyclic AMP by an effect on an adenosine receptor (22); (c) a direct stimulation of the adenosine receptor by FSBA; and (d) binding to an ADP receptor linked to inhibition of adenylyl cyclase. FSBA itself does not appear to enter the platelet (11). However, FSBA is an ester that can undergo spontaneous hydrolysis and may be degraded to the corresponding benzoic acid derivative and adenosine by enzymes in plasma. Adenosine is an inhibitor of platelet aggregation induced by ADP and other agents. Its effect is noncompetitive with respect to ADP, and is mediated by the action of an extracellular receptor through which it stimulates adenylyl cyclase and increases intracellular cyclic AMP levels (6). Effects of adenosine on this receptor are competitively inhibited by the methylxanthine phosphodiesterase inhibitors, theophylline, caffeine, and isobutylmethylxanthine (14, 15, 23). The increase in cyclic AMP in platelets is enhanced by other types of phosphodiesterase inhibitors, including papaverine and pyrimidopyrimidines (14, 23).

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