ADP-Induced Platelet Shape Change and Mobilization of Cytoplasmic Ionized Calcium Are Mediated by Distinct Binding Sites on Platelets: 5'-p-Fluorosulfonylbenzoyladenosine Is a Weak Platelet Agonist

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ADENOSINE DIPHOSPHATE (ADP) induces a number of responses in platelets, including shape change from disc to sphere, aggregation, and secretion of granule contents. It inhibits adenylate cyclase activity of platelet membranes and the accumulation of cyclic AMP in intact platelets induced by prostaglandins such as prostaglandin E1 (PGE1). ADP does not penetrate the intact cell membrane, and the various responses are considered to be mediated by its interaction with specific binding sites on the platelet surface. The interaction of ADP with the putative membrane binding sites has been probed using several compounds, including ATP, ADP analogs, and the thiol reagent, 5'-p-fluorosulfonylbenzoyladenosine (FSBA). Platelet shape change and the antagonism of adenylate cyclase activity by ADP are inhibited by ATP. The ADP analogs 2-azido-ADP and 2-methylthio-ADP are three- to fivefold more potent than ADP as aggregating agents, but 10 to 200-fold more potent as inhibitors of PGE1-stimulated cyclic AMP accumulation in intact platelets. In contrast, the nonpenetrating, mercurial thiol reagent pCMBS does not inhibit shape change but blocks the effect of ADP on adenylate cyclase and antagonizes the platelet binding of both the above-mentioned ADP analogs.

5'-p-Fluorosulfonylbenzoyladenosine (FSBA) interacts covalently with nucleophilic residues in the region of ADP or ATP binding sites on enzymes and platelets. It is covalently incorporated into a 100-kilo Dalton (kD) membrane protein in intact platelets and inhibits ADP-induced shape change, aggregation, and fibrinogen binding, but does not block the inhibitory effect of ADP on cyclic AMP accumulation. Furthermore, FSBA does not block the platelet binding of 2-methylthio-ADP. Based on evidence from studies with ADP antagonists and analogs, platelets have been proposed to possess at least two different types of binding sites for ADP, one mediating the shape change and the second the inhibition of adenylate cyclase.

Platelet stimulation with ADP results in several responses, including shape change, increase in cytoplasmic ionized calcium concentration ([Ca2+]i), and inhibition of adenylate cyclase. FSBA (50 to 200 μmol/L) induced a dose-dependent rise in [Ca2+]i, indicating that it is a weak platelet agonist. Under conditions of covalent labeling of the ADP binding sites, FSBA (50 to 100 μmol/L) did not inhibit the ADP-induced increase in [Ca2+]i, or its inhibition of adenylate cyclase, whereas pCMBS (up to 1 mmol/l) abolished both these responses but not shape change. These findings suggest that ADP-induced Ca2+ mobilization and inhibition of adenylate cyclase are mediated by platelet binding sites distinct from those mediating shape change.

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quin2 ester (20 μmol/L final concentration) at 37°C for 30 minutes. Final concentration of DMSO was 0.1%. The suspension was then centrifuged at 1,000 g for 15 minutes at room temperature. Hirudin (0.05 U/mL, final concentration) and apyrase (10 μg/mL, final concentration) were added prior to centrifugation to prevent platelet activation. The sedimented platelets were resuspended to a concentration of 2 x 10^7 cells/mL in Hepes buffer, pH 7.4 (Hepes 10 mmol/L, NaCl 145 mmol/L, KCl 5 mmol/L, MgSO4 1 mmol/L, glucose 0.1%) in the presence of apyrase (10 μg/mL, final concentration). Before each experiment, the cells (2-mL aliquots) were equilibrated at 37°C for 3 minutes. The external calcium concentration was adjusted by addition of 10 μL of 200 mmol/L CaCl2 (1 mmol/mL final concentration) followed by incubation for 2 minutes at 37°C. During the experiments, 2-mL aliquots of platelet suspensions were continuously stirred in a quartz cuvette (Fisher, Springfield, NJ) thermostatted at 37°C. Small aliquots (5 to 10 μL) of agents were added, and fluorescence was recorded over time. The fluorescence signal was recorded at 492 nm (excitation at 339 nm) using a Perkin Elmer LS-5 Spectrophotofluorimeter (Oatbrook, IL). The calibration of the fluorescence signal as a function of Ca^{2+} was performed as described by Tsien and co-workers based on the equation:

\[ F = F_{\text{max}} + \frac{(F_{\text{max}} - F_{\text{sat}})(\text{Ca}^{2+}/kd)}{1 + \text{Ca}^{2+}/kd} \]

where \( F_{\text{max}} \) and \( F_{\text{sat}} \) represent fluorescence at a very low and a very high calcium concentration, respectively. For estimation of \( F_{\text{max}} \), we added a high concentration of ionomycin (400 to 800 mmol/L) in the presence of 1 mmol/L external Ca^{2+}. \( F_{\text{sat}} \) was calculated as follows: \( F_{\text{sat}} = AF + (F_{\text{max}} - AF)/6 \), where \( AF \) is autofluorescence of the cells after the addition of 1 mmol/mL MnCl2 to quench the dye fluorescence.

For experiments with fura-2, the PRP was incubated with fura-2 ester (5 μmol/L final concentration) for 30 minutes at 37°C and resuspended in Hepes buffer as described above for quin2-loaded platelets. Binding of Ca^{2+} to fura-2 results in a shift in peak fluorescence emission (510 nm) following excitation at 340 and 380 nm.

**Treatment of platelets with FSBA or pCMBS.** All experiments with FSBA were carried out in the presence of 2 U/mL adenosine deaminase to degrade the contaminating adenosine, which inhibits platelets in a time-dependent and saturable manner with 80% of maximal incorporation by 30 minutes. Therefore, platelets were incubated for 30 minutes at 37°C with the analog to permit covalent labeling of the ADP binding site. For experiments with pCMBS, platelet suspensions were incubated for 2 minutes because of its rapid onset of effect. In experiments involving measurement of [Ca^{2+}], levels, platelets were loaded with quin2 or fura-2 prior to treatment with FSBA or pCMBS.

**Studies on shape change and accumulation of cyclic AMP.** Platelet suspensions were prepared from PRP as described above. Platelet shape change was studied in the presence of 2 mmol/L EDTA and was recorded using a Chrono-Log Aggregometer (Chrono-Log, Haverton, PA) as a change in optical density.

The studies on the accumulation of cyclic AMP were performed by incubating platelets, pretreated with either FSBA for 30 minutes or pCMBS for 2 minutes, with the stable prostaglandin I2 derivative, ZK 36,374 (50 nmol/L) for 2.5 minutes at 37°C in the presence of theophylline (7 mmol/L). Various concentrations of ADP were then added, and platelets were incubated for an additional 2.5 minutes before extraction with 20% trichloroacetic acid (TCA). The cyclic AMP levels were measured using a radioimmunoassay (RIANEN platelets with increasing concentrations of FSBA in the presence of adenosine deaminase. In this experiment, platelet-CAMP KIT, Dupont, Wilmington, DE) in the supernatants after lyophilization.

**RESULTS**

**Effect of ADP and FSBA on cytoplasmic ionized calcium concentration.** Addition of ADP to quin2-loaded platelets resulted in a prompt increase in [Ca^{2+}], (Fig 1) from a basal level of ~80 nmol/L to peak levels of 270 ± 91 nmol/L (mean ± SD) and 292 ± 102 nmol/L on stimulation with 5 and 25 μmol/L ADP, respectively (n = 22). With FSBA at concentrations >50 μmol/L and in the presence of adenosine deaminase (2 U/mL), there was a dose-dependent rise in [Ca^{2+}], which was slower than that noted with ADP. Adenosine deaminase by itself did not induce any changes in [Ca^{2+}]. In the absence of adenosine deaminase, the rise in [Ca^{2+}], on addition of FSBA was lower (not shown); this is in line with previous studies demonstrating a rapid platelet inhibitory effect of FSBA due to the presence of adenosine in the FSBA preparations.

Figure 2 shows the [Ca^{2+}], in response to incubation of
lets were incubated at 37°C with FSBA without stirring; at various intervals \([Ca^{2+}])_{1}\), were measured in aliquots after equilibration with \(CaCl_2\) (1 mmol/L). With 50 to 200 \(\mu\)mol/L FSBA, there was a slow, dose-dependent increase in the \([Ca^{2+}])_{1}\), followed by a gradual return to lower levels over 20 to 30 minutes. No such increase was noted at FSBA concentrations <50 \(\mu\)mol/L. To demonstrate that the increase in fluorescence noted with FSBA reflected platelet activation and increase in \([Ca^{2+}])_{1}\), we studied the effect of a stable \(PGI_2\) analog, ZK 36,374 (0.5 \(\mu\)mol/L) on the responses induced by FSBA. Previous studies have demonstrated that the rise in \([Ca^{2+}])_{1}\), on platelet stimulation is inhibited by agents that elevate platelet cAMP. The rise in \([Ca^{2+}])_{1}\), noted with FSBA and ADP were completely inhibited (not shown) by prior exposure of platelets to 0.5 \(\mu\)mol/L ZK 36,374 in the presence of theophylline (7 \(\mu\)mol/L). These studies provide direct evidence that FSBA activates platelets and leads to a rise \([Ca^{2+}])_{1}\). However, the rise was slower than with ADP and, in general, the peak \([Ca^{2+}])_{1}\), were comparable to those achieved with about a tenfold lower concentration of ADP, indicating that FSBA is a weak agonist.

**Effect of FSBA on ADP-induced increase in cytoplasmic ionized calcium concentration and shape change.** Studies with \(^3\)H-sulfonylbenzoyladenosine indicate that maximal incorporation into intact platelets occurs by 60 minutes, with 80% incorporation by 30 minutes. Previous studies using FSBA as an inhibitor of ADP-induced responses have been performed with incubations of 30 to 60 minutes. Incubation of platelets with FSBA (25, 50 \(\mu\)mol/L) for 30 minutes at 37°C abolished ADP (5 \(\mu\)mol/L) induced shape change but did not inhibit the rise in \([Ca^{2+}])_{1}\) (Fig 3). However, with PAF (100 \(\mu\)mol/L), FSBA did not inhibit either shape change or the calcium responses (Fig 3).

To determine the effect of FSBA at various intervals of incubation, platelet suspensions were incubated at 37°C with 100 \(\mu\)mol/L FSBA without stirring, and the response to ADP (5 \(\mu\)mol/L) was recorded at different intervals in separate aliquots (Fig 4). When \([Ca^{2+}])_{1}\), prior to addition of ADP was lower than the peak value induced by FSBA alone, the increment in \([Ca^{2+}])_{1}\), signal due to the ADP was clearly demonstrable. As the \([Ca^{2+}])_{1}\), returned close to basal levels, addition of ADP evoked a rise in \([Ca^{2+}])_{1}\), comparable to that in control platelets incubated at 37°C for 30 minutes with DMF. Following a 30-minute incubation with FSBA, the peak calcium levels on ADP stimulation were only minimally decreased as compared with those in platelets incubated with DMF alone. These studies indicate that under conditions at which FSBA completely inhibits ADP-induced shape change, it does not abolish ADP-induced increase in \([Ca^{2+}])_{1}\).

Studies were also performed using other agonists, PAF (40 \(\mu\)mol/L) and thrombin (0.1 U/mL) (Fig 4). The results were similar to those with ADP in that the peak \([Ca^{2+}])_{1}\), at various time intervals were comparable in the presence or absence of FSBA. These results also indicate that the increment in \([Ca^{2+}])_{1}\), noted on stimulation of platelets at elevated initial \([Ca^{2+}])_{1}\), is not the same as that following stimulation at basal levels. At 30 minutes of incubation with FSBA, the peak levels on stimulation were minimally decreased as compared with that in control platelet incubated with DMF.

In some experiments, the effect of FSBA was studied using platelets loaded with fura-2, a newer \(Ca^{2+}\) indicator that offers a much greater fluorescence than quin2 and obviates some of its drawbacks. Incubation of fura-2-loaded platelets with FSBA (50 and 100 \(\mu\)mol/L) resulted in a rise in \([Ca^{2+}])_{1}\), similar to that noted in quin2 loaded platelets (Fig 5). Moreover, following 30-minute incubation with FSBA, the responses to ADP (25 \(\mu\)mol/L) were not abolished and were similar to those in platelets incubated with DMF alone. On stimulation with ADP, the rise in...
[Ca\(^{2+}\)], was higher in platelets incubated with 50 μmol/L FSBA than with 100 μmol/L FSBA, whereas the [Ca\(^{2+}\)]i prior to ADP stimulation were higher with the latter. These findings support the conclusions from experiments with quin2-loaded cells that FSBA does not inhibit the rise in [Ca\(^{2+}\)]i induced by ADP.

Effect of pCMBS on ADP and FSBA-induced increase in cytoplasmic ionized calcium concentration and on ADP-induced shape change. Incubation of quin2-loaded platelets with pCMBS (up to 1 mmol/L) alone did not result in an increase in the basal [Ca\(^{2+}\)]. Following a 2-minute incubation with pCMBS, the peak responses to ADP (25 μmol/L) were strikingly inhibited in a dose-dependent manner (Fig 6) with >50% inhibition at pCMBS concentrations >5 μmol/L. Because of the slower rise in peak [Ca\(^{2+}\)], with FSBA, the effect of pCMBS was studied on the [Ca\(^{2+}\)], at 4 minutes after addition of FSBA (100 μmol/L). Incubation for 2 minutes with pCMBS inhibited the rise in [Ca\(^{2+}\)], although the inhibition required higher pCMBS concentrations than with ADP. In contrast, pCMBS (1 mmol/L) did not inhibit ADP-induced shape change (Fig 7).

Effect of FSBA and pCMBS on ADP-induced inhibition of cyclic AMP levels. Platelet suspensions preincubated with FSBA (100 μmol/L) for 30 minutes or with pCMBS (1 mmol/L) for 2 minutes were incubated with ZK 36,374 (50 nmol/L) for 2.5 minutes in the presence of theophylline (7 mmol/L) and then with increasing ADP concentrations for 2.5 minutes. The inhibition of cyclic AMP levels by ADP in platelets incubated with FSBA was comparable to that in platelets incubated with buffer or DMF (Fig 8). Thus, FSBA did not impair the ability of ADP to inhibit adenylate cyclase. The peak levels of cyclic AMP following exposure to ZK 36,374 (but before exposure to ADP) were lower (217 pmol/10⁸ platelet, mean of two experiments in duplicate) in platelets preincubated with FSBA for 30 minutes than in platelets exposed to DMF alone (270 pmol/10⁸ platelet); this reflects the effect of FSBA as a weak agonist in partially inhibiting cAMP levels as well. Preincubation with pCMBS (1 or 100 μmol/L) for 2.5 minutes abolished the effect of ADP in inhibiting the accumulation of cyclic AMP (Fig 8).

**DISCUSSION**

FSBA is a nonpenetrating, adenosine nucleotide affinity analog which covalently modifies only a single polypeptide chain (mol wt 100,000) in intact platelets. It has been used effectively as an antagonist to examine the various ADP-induced platelet responses, including shape change, aggregation, and fibrinogen binding. We demonstrate that FSBA (50 to 200 μmol/L) by itself induces a concentration-dependent increase in [Ca\(^{2+}\)], (Figs 1 and 2) and provide direct evidence that FSBA is a platelet agonist. The agonist role of FSBA is supported by several lines of evidence. At higher concentrations, FSBA induces shape change, myosin phosphorylation and thromboxane A₂ synthesis in platelets (Rao AK, Kowalska A, Disa J, unpublished observations). These effects occurred at concentrations higher than those at which we demonstrate mobilization of intracellular Ca\(^{2+}\). Nevertheless, FSBA is a weak activator of platelets; the concentrations that yield comparable increases in [Ca\(^{2+}\)], are about tenfold higher than those for ADP (Fig 1).
In agreement with previous studies, FSBA abolished ADP-induced shape change but did not inhibit the effect of ADP on PGI2-stimulated cyclic AMP accumulation (Figs 3 and 8). Under conditions that permit covalent labeling of the putative ADP binding sites, FSBA (100 μmol/L) failed to inhibit ADP-stimulated increase in [Ca2+], in platelets loaded with quin2 (Figs 3 and 4) or fura-2 (Fig 5). As expected, incubation with FSBA (100 μmol/L) did not abolish the rise in [Ca2+], induced by PAF or thrombin. With all three agonists studied, the peak responses following the 30-minute incubation with FSBA were minimally reduced as compared with platelets incubated with DMF (Fig 4). We have observed similar findings in other experiments (not shown) in which platelets were stimulated with a second agonist (e.g., thrombin) after an interval (10 to 30 minutes) following initial exposure to a different agonist (ADP or PAF); the peak responses to the second agonist were somewhat blunted as compared with those elicited by the second agonist alone. Thus, the small reduction in the peak [Ca2+], levels noted following platelet incubation with FSBA may reflect incomplete return to the basal state after activation by FSBA. Overall, we interpret these findings to indicate that FSBA does not inhibit ADP-induced elevation in [Ca2+], and that the rise in [Ca2+], induced by ADP is mediated by binding sites on platelets distinct from those mediating shape change.

The above conclusions are supported by the studies using pCMBS. This reagent blocked ADP-induced inhibition of cyclic AMP accumulation (Fig 8), but not shape change in platelets (Fig 7). However, at strikingly lower concentrations, pCMBS blocked the rise in [Ca2+], (Fig 6). These findings suggest also that the effect of ADP on cyclic AMP levels and the rise in [Ca2+], are mediated by the same binding site on platelets. The distinct characteristics of the two types of putative ADP binding sites on platelets is supported by observations that FSBA does not inhibit the platelet binding of 2-methylthio-ADP, whereas pCMBS does.

Although the divergence in the effects of various ADP antagonists and analogs on platelet responses has been interpreted as evidence for the existence of different types of ADP binding sites, the data do not exclude other possibilities. For example, a single type of binding site could be coupled to one or other pathways under different conditions, or to both simultaneously if a common protein (e.g., a guanine nucleotide-binding protein) mediates both actions. However, the model invoking the existence of two discrete binding sites provides a simpler explanation for several of the observations. Irrespective of the number of specific ADP binding sites, our studies clearly indicate the existence of distinct membrane-related mechanisms by which ADP induces the different platelet responses. The definitive subdivision of ADP binding sites awaits development of potent and selective inhibitors as has been possible with adrenergic receptors on tissues.

Our studies suggest that FSBA has a weak effect at the ADP binding site mediating the Ca2+ signals as well. First, FSBA by itself induced a rise in [Ca2+], that was inhibited by pCMBS (Fig 6) and second, it had a weak inhibitory effect on cyclic AMP accumulation induced by ZK 36,374. The finding that FSBA acts on both types of binding sites is not unexpected given the structural similarities between FSBA and ADP, and is in accord with observations with other ADP analogs, 2-methylthio-ADP and z-azido-ADP, which also produce effects indicative of interaction with both types of ADP binding sites, albeit with differing degrees in their potencies.

Our studies were performed using quin2 and fura-2 as the [Ca2+] indicators. Another indicator that has been used to study Ca2+ mobilization in platelets is the Ca2+-sensitive photoporphyrin, aequorin. It has been suggested that these indicators detect different pools of [Ca2+], within the platelets and that aequorin may have the capability of more sensitively detecting localized elevations in [Ca2+], not revealed by quin2, which reflects the average [Ca2+]. Therefore, we cannot make conclusions on the relationship between changes in [Ca2+], and the platelet responses until similar studies are performed with aequorin.

In summary, we have provided evidence that FSBA by itself behaves as a weak platelet agonist. We have demonstrated that (a) FSBA inhibits ADP-induced shape change but does not block either elevation in [Ca2+], or inhibition of cyclic AMP levels by ADP; (b) the thiol reagent, pCMBS inhibits ADP-stimulated increase in [Ca2+], and its effect on adenylate cyclase but not shape change. The evidence presented is in accord with the conclusion that ADP-induced elevation in [Ca2+], is mediated by binding sites distinct from those leading to shape change but are probably the same as those mediating inhibition of cyclic AMP accumulation.

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REFERENCES


10. Mills DCB, Macfarlane DE: Attempts to define a platelet ADP receptor with 3H-g-p-mercuribenzene sulphonate (MBS). Thromb Haemostas 38:82a, 1977 (abstr)


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