Direct Evidence for the Interaction of the Nucleotide Affinity Analog 5′-p-Fluorosulfonylbenzoyl Adenosine With a Platelet ADP Receptor

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Previous reports have indicated that the nucleotide affinity analog 5′-p-fluorosulfonylbenzoyl adenosine (FSBA) at concentrations between 40 and 100 μmol/L and at times greater than 20 minutes covalently modifies a single protein component on the external platelet membrane surface and that adenosine diphosphate (ADP) protects against this reaction. That this protein is an ADP receptor linked to platelet activation is shown by FSBA inhibition of ADP-mediated platelet shape change, aggregation, and fibrinogen receptor exposure. In this report, further evidence for the interaction of FSBA with the ADP receptor on platelets is provided by the observation that FSBA at high concentrations (100 to 500 μmol/L) behaves as a week agonist to produce platelet shape change within one minute as detected by spectroscopic assay and scanning electron microscopy with concomitant phosphorylation of the light chain of platelet myosin. The specificity of FSBA as an agonist is demonstrated by inhibition of FSBA-induced shape change by ATP and the covalent incorporation of SBA as well as the failure of 5′-fluorosulfonylbenzoyl guanosine (FSBG) to cause shape change. In contrast, incubation of platelets with low concentrations of [3H]-FSBA (40 mol/L) is not associated with stimulation of platelet shape change or myosin light chain phosphorylation.

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N EARLY RESPONSE of human blood platelets to various agonists is a morphological change in which they are transformed from discs to spiny spheres. The latter are manifested by the appearance of cytoplasmic projections or pseudopodia. This transformation is seen morphologically by scanning electron microscopy with concomitant decreased light transmission of stirred platelet suspensions. Adenosine diphosphate (ADP), a potent inducer of platelet shape change and aggregation, possibly effects these responses of binding of the nucleotide to a cell surface receptor. The chemical identity of this receptor is unknown, although recent evidence suggests that it is distinct from an ADP receptor linked to adenyly cyclase.

The nucleotide affinity analog, 5′-fluorosulfonylbenzoyl adenosine (5′-FSBA) has been demonstrated to modify covalently the nucleotide binding domains of more than 30 enzymes representing active and allosteric sites as well as the ATP binding sites of actin and myosin on the internal surface of the platelet membrane. Bennett et al have shown that FSBA is covalently incorporated into a single polypeptide (M, 100,000), aggregin, on the external surface of the platelet membrane. The labeling of this protein demonstrates considerable specificity, since it is protected by ADP and its competitive inhibitor ATP but not by adenosine, AMP, or epinephrine. Concomitant with this modification, a rapid inhibition of ADP-induced shape change of platelets in plasma was observed. This inhibition was progressive with time of incubation with FSBA. Figures et al showed that FSBA treatment of intact washed platelets resulted in the inhibition of ADP-mediated aggregation as well as exposure by ADP of latent fibrinogen binding sites. The labeled protein is thus an attractive candidate for a nucleotide binding site that mediates shape change and aggregation as well as exposure of fibrinogen receptors in human platelets.

The designation of the FSBA-sensitive protein as the ADP shape change receptor has been to this point based on the correlation between increased incorporation of the radiolabeled form of FSBA and enhanced inhibition of ADP-related responses of the cell. This communication presents direct evidence that FSBA is capable of interacting with an ADP receptor on the platelet cell surface.

MATERIALS AND METHODS

Materials. FSBA was prepared by the method of Colman et al. The synthesis involves condensation of adenosine with p-fluorosulfonylbenzoyl chloride (Sigma Chemical Co, St Louis). For synthesis of 5′-[3H]-FSBA, [2-3H]-adenosine (New England Nuclear) was used. The radiolabeled compound had a specific radioactivity of 20 Ci/mol. FSBA concentrations were determined spectrophotometrically in ethanol using a molar extinction coefficient of 1.35 × 104 L/mol/cm at 259 nm. 5′-fluorosulfonylbenzoyl guanosine (FSBG) was synthesized by the method of Pai et al. The concentrations of FSBG were determined spectrophotometrically using an extinction coefficient of 7.3 × 104 L/mol/cm at 275 nm. Both compounds were stored over dessicant at −30°C and solutions in dimethyl formamide (DMF) prepared on the day of use. The final concentration of DMF used in every experiment was less than 2.0%, which had no influence on platelet shape change. Equine collagen...
fibrils were purchased from Hormon-Chemie, Munich, Germany. All other chemicals were of reagent grade or better.

Adenosine deaminase. Since FSBA can hydrolyze to adenosine, which inhibits platelet shape change, adenosine deaminase (Sigma) was added to washed platelets just before all incubations with FSBA. The concentration used (2 U/mL) is sufficient to eliminate the inhibitory action of adenosine (400 μmol/L) on platelet shape change.

Preparation of appyrase. Potato appyrase was prepared using the method of Molnar and Lorand.8 These preparations contain both adenosine diphosphatase (ADPase) and adenosine triphosphatase (ATPase) activities as well as a platelet aggregating lectin. The lectin was separated from the appyrase using fetuin-agarose affinity chromatography.9

ATP purification. The disodium salt of equine muscle adenosine triphosphate (ATP) (Sigma) was dissolved in cold (4°C) deionized water. The ATP was separated from any ADP on a Dowex 50X8-2 column in the cold. Fractions were collected in an equal volume of phosphate (0.24 mol/L) and EDTA (1 × 10−4 mol/L) buffer. The concentration of the ATP was determined by optical density ε260 = 15.3 × 10^3 L/mol/cm−1.

Preparation of washed platelets. Platelet-rich plasma (PRP) was prepared from 9 vol of whole human blood and 1 vol of 3.8% sodium citrate solution by differential centrifugation (120 × g, 30 minutes, 23°C). Platelets were separated from plasma by centrifugation (1,800 × g, 20 minutes, 23°C). The platelet pellet was resuspended in a volume equal to the original PRP volume with a diluted Tyrode’s buffer (buffer A, 4 vol of Tyrode’s buffer with 2.0 mmol/L magnesium chloride diluted with 1 vol of 3.8% sodium citrate adjusted to pH 6.5 with 1 mol/L citric acid). This platelet suspension was incubated in the presence of 3 μL appyrase/mL platelet suspension at 37°C for 20 minutes and then sedimented as with the PRP. The pellet was again resuspended in buffer A, incubated with appyrase, and sedimented. After centrifugation, the platelet pellet was resuspended to the original PRP volume with a modified Tyrode’s buffer (buffer B: Tyrode’s buffer with 2.0 mmol/L magnesium chloride and 2.0 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid, pH 7.4). The platelet suspension was adjusted to a platelet count of 1 to 2 × 10^9/mL and kept at 23°C until used.

Measurement of platelet shape change. The washed platelet suspension (0.25 mL) was diluted with an equal volume, 5mmol/L EDTA in 0.15 mol/L NaCl, pH 7.4, to a final platelet count of 0.5 to 1.0 × 10^9/mL just before adding the compounds to be tested in a cuvette of a Chronolog Lumi-Aggregometer (Chronolog Corp, Havertown, PA). The reference channel contained suspended platelets that had been incubated with 10 μmol/L ADP. The compounds were added in volumes of 25 to 50 μL and the change in light transmittance recorded in a chart recorder. The decrease of light transmittance was measured, and the greatest decrease in light transmittance was defined at 100% of maximum shape change. All other measurements for a specific test compound on platelet suspension are given in relation to this value. The rate of decrease in light transmittance was measured with the slope of tracings. The highest rate of change was defined as 100% of maximum rate of shape change, and all other rates for a specific compound and platelet suspension are expressed in terms of the maximum. ATP release was also monitored simultaneously using firefly luciferase in selected experiments.

Observation of shape change by scanning electromicroscopy. Platelets were prepared for scanning electron microscopy by adding 2.5% glutaraldehyde in Tyrode’s buffer. The samples were fixed for 30 minutes at room temperature and kept overnight at 4°C. The next day, platelets were deposited with gentle suction (10 mm Hg) on a 1-μm pore nucloepore membrane. The membrane and cells were then post-fixed in 1% osmium tetroxide, dehydrated with graded alcohol, dried at the critical point, and coated with gold. Photomicrographs were made at ×5,000 magnification and 100 to 120 platelets from each sample were classified as discoid, spiny sphere, or intermediate form. All samples were coded to reduce subjective bias.

Covalent incorporation of [3H]-SBA into platelet membrane protein. Previous studies have indicated that FSBA (100 μmol/L) modifies a single polypeptide of M, 100,000, aggregin, on the platelet membrane when intact cells were labeled as assessed by SDS polyacrylamide electrophoresis.6 Using identical methods, similar labeling of a single component was observed when FSBA (400 μmol/L) was incubated with washed platelets for 60 minutes, but the incorporation was greater compared with an aliquot of the same suspension incubated with 100 μmol/L [3H]-FSBA (Fig 1). Covalent modification of platelet membrane protein by [3H]-SBA was measured using a dialysis method. Washed platelets were incubated with [3H]-SBA for specific time periods. The cell suspension (1 mL) was added to a solution of diithiothreitol (DTT) (100 μL, 2 mol/L) to stop the reaction by hydrolysis of excess FSBA. The cells were then sedimented and supernatants carefully aspirated. The pellets were then solubilized in phosphate buffer (0.04 mol/L, pH 7.2) containing SDS (2.0%), urea (8 mol/L), and EDTA (0.10 mol/L). In order to remove unbound ligand, the solutions were dialyzed in a multiple

![Image](image_url)
cell dialysis chamber (BRL Laboratories, Model #1200MD) against phosphate buffer (0.1 mol/L, pH 7.2) containing SDS (0.1%) and EDTA (0.001 mol/L) using a 50,000 Dalton (D) cutoff membrane (Spectraper-Fisher Scientific). Dialysis was complete when the levels of radioactivity in a reference sample (identically prepared but not containing platelets) reached background levels. The samples were then removed and counted in a liquid scintillation cocktail (ACS-II, Amersham), in a Beckman Model 330 counter.

Measurement of platelet myosin phosphorylation. In order to measure light chain myosin phosphorylation, an equal volume of cold 0.6 N HClO₄ was added to the platelet sample in the aggregometer. The protein pellet was separated by centrifugation (12,000 × g, three minutes) and washed once with 1 mL of H₂O. Samples were electrophoresed on alkaline-urea polyacrylamide gels as previously described. The percentage phosphorylation was determined by scanning the stained gel and measuring of the relative area of phosphorylated and dephosphorylated myosin light chain. The data were collected using a TRS-80 model III microcomputer and areas determined by a computer program.

RESULTS

Induction of platelet shape change by FSBA. Platelets were induced to undergo shape change by addition of ADP or FSBA. The rate and extent of shape change, measured spectrophotometrically, were very similar except that ADP induced maximum shape change at 30 seconds and FSBA (190 μmol/L) at 60 seconds (Fig 2A). Shape change was confirmed by direct examination of platelets by scanning
INTERACTION OF FSBA WITH PLATELET ADP RECEPTOR

Electromicrography (Fig 2B). Control platelets (frame A) were discoidal in shape, while ADP and FSBA-treated cells were spheroidal with numerous pseudopodia (frames B and C). The stimulation of shape change by FSBA (400 μmol/L) was not due to induction of the release reaction, since release of ATP during shape change by FSBA (190 μmol/L) was less than 0.06 μmol/L. At 400 μmol/L the release of ATP was still less than 0.1 μmol/L. In contrast, thrombin (1 U/mL) released greater than 5 μmol/L ATP under these conditions. Furthermore, apyrase at a concentration that completely inhibited shape change induced by ADP (0.5 μmol/L) failed to inhibit shape change induced by FSBA (400 μmol/L). The effect of FSBA was not due to stimulation of PGH₂ or thromboxane A₂ synthesis. Indomethacin (10 μmol/L), which completely inhibited shape change induced by collagen (4 μg/mL), failed to inhibit shape change induced by ADP (1 μmol/L), FSBA (400 μmol/L), or thrombin (1 U/mL).

Comparison of the relative potencies of ADP and FSBA in the induction of platelet shape change. ADP induces shape change (Fig 3) at concentrations above 0.05 μmol/L with an EC₅₀ = 0.43 μmol/L. FSBA induces platelet shape change at concentrations above 100 μmol/L with an EC₅₀ = 220 μmol/L. This difference in potencies may reflect the relative ability of the agonist to bind to the receptor due to their differing charge and structure.

Effect of FSBG on platelet shape change. To ascertain whether the effect of FSBA was specific and in particular the role of the sulfonyl fluoride group, a structural analog of FSBA, FSBG (differing only in the nature of purine base) was tested for the ability to induce platelet shape change. FSBG has no measurable effect on platelets even at concentrations of 500 μmol/L when dissolved in similar final concentrations (<2.0%) of DMF.

Evidence for specific interaction of FSBA with platelet ADP receptor-mediating shape change. In order to further determine whether the induction of platelet shape change by FSBA is due to specific interaction of nucleotide affinity analog with the platelet surface, ADP receptor inhibition studies using ATP were carried out. ATP has been shown to be a specific antagonist of ADP-mediated platelet shape change and aggregation. Platelets were washed as described above, and FSBA-mediated shape change was carried out at various concentrations of FSBA in the presence and absence of ATP (600 μmol/L), a concentration which inhibited 50% shape change induced by ADP (3 μmol/L) and 100% of that induced by 1 μmol/L ADP. As shown in Table 1, ATP proved capable of inhibiting FSBA-mediated shape change to as little as 22% of maximum. Note that at the highest concentration of FSBA, the inhibition by ATP was decreased similar to the competitive mechanism documented for ATP inhibition of ADP aggregation.

Effects of low concentrations of FSBA on platelets. The ability of FSBA to induce platelet shape change at concentrations above 100 μmol/L occurs within one minute after the addition of the compound to stirred platelet suspensions. In contrast, FSBA (40 μmol/L) does not produce shape change even after 60 minutes of incubation. However, FSBA (40 μmol/L) can inhibit ADP-mediated shape change progressively over time with 50% inhibition at 10 minutes and total inhibition of shape change occurring at 20 minutes (Fig 4A). Using different platelet donors in ten additional experiments, the time necessary for total inhibition varied between 15 and 40 minutes. Platelets incubated with FSBA under these conditions failed to show shape change, as assessed by scanning electron microscopy, after stimulation with ADP (10 μmol/L). Platelets stimulated with ADP (10 μmol/L) showed shape change after 60 minutes of incubation with FSBG or DMF equal to that without incubation. Figure 4B displays a time course of covalent incorporation of the compound into platelet membranes. The maximum incorporation was 60.4 pmol SBA/10⁹ platelets. Comparing Figs 4A and 4B, it is evident that 36 pmol SBA incorporated per 10⁹ platelets is sufficient for maximum inhibition.

Correlation of induction of shape change with incorporation of [³H]-SBA at a high concentration of FSBA. When cells were treated with high concentrations of FSBA (400 μmol/L), they changed shape very rapidly (Fig 5), as demonstrated by superposition of the time course of the incorporation of [³H]-SBA into platelet membrane protein as compared with the actual spectrophotometric recording of

![Fig 3. Effect of various concentrations of ADP, FSBA, and FSBG on induced platelet shape change. Each compound was tested at a wide range of concentrations for ability to produce shape change as in Fig 2A. For comparison, each is normalized to the percent of maximum shape change and plotted as a log of concentration. The data are from a single experiment and are representative of three such experiments.](image-url)
Figures 4 and 5. Figure 4 shows the inhibition by FSBA on platelet shape change and correlation with covalent incorporation. Platelets were incubated with FSBA (40 μmol/L) (filled circles), FSBG (40 μmol/L) (open circles), or DMF (x). The 100% point was determined in the presence and absence of DMF and was identical. (A) At various time intervals, aliquots of the reaction mixture were withdrawn and the cells were tested for their ability to undergo shape change in response to ADP (10 μmol/L). (B) An additional aliquot of samples was simultaneously incubated with [3H]-FSBA (40 μmol/L) to determine the extent of covalent incorporation of [3H]-SBA in the membrane receptor protein (see Materials and Methods).

Fig 5. Correlation of incorporation of [3H]-SBA into platelets with induction of shape change by high concentration of FSBA. [3H]-FSBA (400 μmol/L) was incubated with washed platelets at various times. The actual tracing (continuous line, left axis) is presented. Aliquots were withdrawn and assayed for covalent incorporation of [3H]-SBA (filled circles) and expressed on the right axes as both absolute incorporation as well as percent maximum incorporation (as measured in Fig 4B).

The effect of the covalent incorporation of SBA on shape change induced by FSBA. Two possibilities exist to explain the previous observation that at the time when shape change is induced by 400 μmol/L FSBA, insufficient SBA was incorporated to block ADP-induced shape change. Either additional FSBA is noncovalently bound to the same site to induce shape change or FSBA is noncovalently bound to a second site distant from aggregin. To distinguish these alternatives, we incubated washed platelets with FSBA (400 μmol/L) for 40 minutes to insure extensive covalent incorporation, and in parallel the same platelets were incubated with the solvent DMF. FSBA (50 to 400 μmol/L) was then added to the platelets, and the maximum extent of shape change (Fig 6) as well as the maximum rate of shape change (data not shown) were measured. The platelets incubated with DMF underwent shape change, the maximum extent of which was proportional to the log of the FSBA concentration (r = 0.88, P < .01). Similar findings were observed for the maximum rate of shape change (r = 0.88, P < .01). In contrast, over the same range of FSBA concentration shape change was completely inhibited, suggesting that the covalent and noncovalent sites for FSBA were similar.

The effect of FSBA on phosphorylation of platelet myosin. Phosphorylation of myosin light chain is an early response of platelets to stimulation by an agonist and has been shown to correlate with ADP-induced platelet shape change.13 We have investigated whether the high concentrations of FSBA that induce platelet shape change also induce myosin phosphorylation and whether the low doses of FSBA that do not induce shape change also fail to induce myosin phosphorylation. When platelets were stimulated with 400 μmol/L FSBA, shape change occurred, and as in previous experiments, the response was maximal at about 90 seconds. The measurement of myosin phosphorylation (Fig 7A) closely paralleled the shape change response. The phosphorylation was qualitatively similar to that seen with ADP in that after shape change was complete, the level of phosphorylation gradually returned to basal levels (Fig 7B). At 90 seconds about 3 to 4 pmol of FSBA per 10^8 cells would be incorporated into the platelet membrane (Fig 5). At 40 μmol/L, the same incorporation requires five to ten minutes. When platelets were incubated with 40 μmol/L, no shape change or myosin phosphorylation was observed (Fig 7A, open circles). In fact, no phosphorylation was found at times up to 60 minutes when FSBA incorporation was maximal.

The ability of FSBA to block ADP-induced myosin phos-
interaction of FSBA with platelet ADP receptor

Fig 6. Effect of FSBA and FSBG under conditions of covalent incorporation on shape change induced by FSBA. Washed platelets (5 x 10^7/mL) incubated with FSBA (400 μmol/L) (○) or FSBG (□) (100 μmol/L) or DMF (△) for 80 minutes. The platelets were then diluted to 1 x 10^7 with the same suspending buffer and FSBA (50 to 400 μmol/L) was added with stirring and shape change measured as in the Materials and Methods section. Each point is the mean of three separate experiments.

DISCUSSION

The identification of the ADP receptor responsible for the induction of ADP-mediated shape change and aggregation has been studied using a number of approaches. Any measurement of direct binding of ADP to platelet membranes is complicated, since intact platelets metabolize ADP to adenosine, which is transported into the platelets or ATP, which interferes with binding. Born and Feinberg measured 88,000 "high affinity sites" for 14C-ADP on platelets in plasma; however, they did not differentiate between bound 14C-ADP and 14C-adenosine formed from the labeled ADP and taken up by the platelet. Lipps measured the direct binding of radiolabeled ADP to intact platelets and demonstrated that the binding constant of the higher affinity site was in the range of ADP concentration necessary to promote platelet shape change and aggregation. However, since a significant amount of ADP added to the cells was converted to ATP, a known competitive antagonist of ADP, these experiments must be interpreted with caution.

Binding of 14C-ADP to 100,000 sites on isolated plasma membranes was recorded by Nachman and Ferris. However, the sites identified would have included the binding of ADP to nucleotide binding proteins on the inside of the isolated membranes, since Bennett et al., using the adenosine nucleotide affinity analog FSBA, demonstrated the existence

Fig 7. Effect of FSBA on platelet myosin light chain phosphorylation. (A) FSBA was incubated with washed platelets with stirring at 400 μmol/L (△) and 40 μmol/L (○). At various times, aliquots were withdrawn for determination of myosin light chain phosphorylation. (B) Platelets were preincubated with DMF (□) or FSBA, 40 μmol/L (○) for 40 minutes at 37°C. At time 0, ADP (5 μmol/L) was added. At various times, aliquots were withdrawn for determination of myosin light chain phosphorylation. Platelets preincubated without addition of DMF or FSBA behaved similarly to those incubated with DMF (data not shown). These data are representative of three similar experiments.
of at least four nucleotide binding proteins (including actin and myosin) in isolated platelet membranes. An ADP binding protein (M, ~ 60 kDa) has been isolated by Adler and Handin from platelet membranes by freezing and thawing, but no further studies relating this protein to an ADP receptor in intact platelets were performed.

In an attempt to more clearly define the ADP receptor on the platelet cell surface responsible for activation, Bennett et al used the adenosine nucleotide affinity analog FSBA, which does not penetrate the cell membrane. FSBA (100 µmol/L) inhibited ADP-mediated platelet shape change, and in intact platelets, only aggregin was covalently modified. This study confirms these findings and shows that even at 400 µmol/L FSBA the same polypeptide is modified (Fig 1). Subsequently, Figures et al demonstrated that FSBA (100 µM) was an inhibitor of ADP-mediated aggregation as well as ADP-induced exposure of latent platelet fibrinogen receptors.

The present study further delineates the specificity and consequences of the FSBA-platelet interaction. We have confirmed, both by spectrophotometric assay and scanning electron microscopy (Fig 2), the ability of FSBA (400 µmol/L) to induce platelet shape change and further demonstrated that it induces myosin phosphorylation (Fig 7). FSBA (200 to 400 µmol/L) does function as an agonist similar to ADP, but at a higher concentration (Figs 3, 7). The specificity of the interaction of FSBA and platelets is demonstrated by the failure of FSBG to produce shape change (Fig 3) as well as by the inhibition of FSBA-mediated shape change by ATP, a known competitive inhibitor of ADP responses of platelets (Table 1). FSBA appears to function as a partial agonist, and the protein interacting with FSBA may fulfill the criteria of a receptor with regard to the specificity of binding as well as the correlation of cellular responses with the concentration of FSBA. However, FSBA (40 µmol/L) is also capable of acting as an antagonist of ADP-mediated responses when incubated for prolonged periods of time. The antagonistic effect of FSBA correlates well with the covalent incorporation of the molecule into the platelet membrane protein (Fig 4), with total inhibition occurring at 20 to 40 minutes of incubation with FSBA (40 µmol/L). Bennett et al, using platelets suspended in plasma, observed inhibition of ADP-mediated shape change with FSBA at periods as short as ten seconds. However, such inhibition could be prevented by using washed platelets in the presence of adenosine deaminase, indicating that hydrolytic breakdown of FSBA to adenosine and fluorosulfonylbenzoic acid may be enhanced by plasma esterases. We have therefore carried out all of our incubations in the presence of adenosine deaminase to obviate this effect. As with the agonist effect of FSBA, we have demonstrated that the antagonism of the ADP response by FSBA is specific by comparison with the lack of an effect with FSBG.

The ability of FSBA to act as both an agonist and antagonist in platelets raises the question of its mechanism. As shown in Fig 5, in which 400 µmol/L FSBA is used to induce shape change, the amount of covalent incorporation at two minutes is less (6% of maximum) than that achieved at longer times with 40 µmol/L FSBA (60% of maximum, Fig 4). This result implies that the covalent reaction probably does not induce the platelet activation. The initial noncovalent interaction between FSBA and platelets before covalent incorporation is probably the trigger for shape change response, which only occurs at the high FSBA concentration. That this is probably at the same site is shown by the ability of FSBA (400 µmol/L, 60 minutes) to block FSBA (100 to 400 µmol/L)-induced shape change (Fig 6). Specificity is confirmed by the failure of FSBG under the same conditions (400 µmol/L, 60 minutes) to inhibit FSBA-induced shape change. FSBA (100 µmol/L, 60 minutes) can also completely inhibit shape change produced by a second addition of FSBA (100 µmol/L) and partially (60%) inhibit 200 to 400 µmol/L FSBA (mean of three experiments; data not shown).

The inability of lower concentrations of FSBA to induce myosin phosphorylation appears to be related to the rates of phosphorylation and dephosphorylation. Shape change could therefore be observed only at concentrations of FSBA high enough (> 100 µmol/L) to activate myosin light chain kinase rapidly enough so that the rate of phosphorylation could exceed the rate of dephosphorylation. However, low concentrations of FSBA (40 to 100 µmol/L) that covalently modify the receptor prevent the binding of ADP, which then fails to induce either shape change or myosin phosphorylation.

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