2-Methylthioadenosine[beta-32P]diphosphate. An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets.

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Research Article

2-Methylthio-ADP and its radioactive analogue [beta-32P]2-methylthio-ADP were synthesized and used to investigate platelet receptors for ADP. 2-Methylthio-ADP induced platelet aggregation and shape change, and inhibited cyclic AMP accumulation in platelets exposed to prostaglandin E1. Compared with ADP, 2-methylthio-ADP was 3-5 times as active as an aggregating agent and 150-200 times as active as an inhibitor of cyclic AMP accumulation. Binding of [beta-32P]2-methylthio-ADP to platelets was measured after centrifuging them through silicone oil to separate platelets from their suspension medium. Binding was reversible, saturable, and specific, with between 400 and 1,200 sites/cell in different platelet preparations. There was no evidence for a second class of binding sites with different affinity. The second order association rate constant was approximately 3.5×10^{6} M-1 S-1, and the first order dissociation rate was 0.024 s-1, both measured at 23 degrees C. The dissociation equilibrium constant (approximately 15 nM) was about three times higher than the concentration giving half-maximal inhibition of prostaglandin E1-stimulated cyclic AMP accumulation in platelet-rich plasma. Binding was inhibited by ADP (Ki = 3.5 microM), ATP (7 microM), 2-azido-ADP (0.12 microM), inosine diphosphate (IDP, 150 microM), guanosine diphosphate (GDP, 350 microM), and AMP (800 microM). Binding of 2-methylthio-ADP was also blocked by the non-cell-penetrating thiol reagent, p-mercuribenzene sulphonate, a reagent that blocks the inhibition of adenylate cyclase by ADP, but which does […]

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AN AGONIST AND RADIOLIGAND FOR THE RECEPTOR THAT INHIBITS THE ACCUMULATION OF CYCLIC AMP IN INTACT BLOOD PLATELETS

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INTRODUCTION

ADP has two distinguishable effects on blood platelets (1): firstly, it induces aggregation of the cell by stimulating a change in their shape and exposure of fibrinogen-binding sites, and, secondly, it inhibits the adenylate cyclase of membrane preparations of platelets and prevents the accumulation of cyclic AMP by intact platelets (2). Inhibition of aggregation by prostaglandin E$_1$ (PGE$_1$),[1] prostacyclin (PGI$_2$), and adenosine is adequately explained by ability of these agents to stimulate the accumulation of cyclic AMP by platelets (1, 3), but it is now accepted that the converse is not correct; that is, the induction of aggregation by ADP for instance is not due to a reduction in intracellular cyclic AMP (3). We have studied the mechanism by which these effects of ADP are mediated.

Extracellular ATP is a specific, competitive antagonist of the pharmacological actions of ADP on platelets, and has little or no effect on aggregation induced

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[1] *Abbreviations used in this paper:* GDP, guanosine diphosphate; IDP, inosine diphosphate; PGE$_1$, prostaglandin E$_1$.

by other agents (4). The inhibition of the accumulation of cyclic AMP conforms accurately to that predicted by a simple receptor-mediated process, and this inhibition is rapid in onset and decays promptly when ADP is replaced with ATP (5). A nonpenetrating sulphydryl reagent, p-mercuribenzene sulfonate, blocks the action of ADP on cyclic AMP accumulation, and apparently competes with ADP for occupation of the same site, suggesting that this receptor for ADP has a thiol in or near the binding pocket. Interestingly, this reagent does not influence the induction of the shape change by ADP, which led us to postulate that the aggregating action of ADP is mediated by a separate pharmacological mechanism (6). We have attempted to label platelet ADP receptors with a pharmacologically active photolabile analogue of ADP, namely, 2-azido-ADP. Although this reagent did not label the receptors covalently, it had high enough affinity for us to demonstrate the existence of receptors by an equilibrium-binding technique using intact platelets. We found ∼500 binding sites/cell, a value much lower than reported by others using less direct techniques (7).

The binding of 2-azido-ADP was competitively blocked by ADP and ATP to a greater extent than by other natural nucleotides. The binding was also blocked by prior incubation of the platelets with p-mercuribenzene sulfonate, which suggests that the site we detected represents the receptor responsible for the inhibition of adenylate cyclase rather than the site responsible for induction of aggregation. Azido purines are known to undergo slow transitions to tetrazole forms, and 2-azido-ADP exists in neutral solution as a mixture of about equal parts of azido and tetrazole forms, making it difficult to purify and use (7).

Many analogues of ADP have been synthesized and tested for their ability to induce aggregation (3). The most potent of these is 2-methylthio-ADP (8). We report here the synthesis of the radioactive analogue of this compound and its use as a radioligand for the ADP receptors on intact platelets.

METHODS

Platelet-rich plasma. Platelet-rich plasma was prepared by centrifugation of normal donors' blood anticoagulated with 1:10 vol 128 mM trisodium citrate (4). For some experiments the platelets were washed by two successive albumin density gradient centrifugations (9) followed by Sepharose 2B gel exclusion chromatography into a buffer containing 140 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM NaH_2PO_4, 100 mM glucose, and 15 mM Tris-HCl pH 7.4 (7). Platelet aggregation was recorded turbidimetrically (4); the shape change was recorded in the same apparatus using platelets preincubated at 37°C for 30 min and to which 1:10 vol 100 mM NaEDTA pH 7.4 was added immediately before the aggregating agent.

Accumulation of cyclic AMP. This was determined ra-

diochemically using intact platelets prelabeled for 60–90 min with 0.5 μM [^{14}C]adenine (4). After appropriate additions and incubation, perchloric acid containing cyclic [^{3}H]AMP as a recovery standard was added, and the cyclic AMP was purified by chromatography on Dowex 50 H$^+$; adsorption of other nucleotides and nucleosides was done by two precipitations with Ba(OH)$_2$ and ZnSO$_4$ (4). Results are expressed as the percentage of intracellular ^{14}C present as cyclic AMP.

Binding of 2-methylthio-[β-^{32}P]ADP to platelets. A centrifugal method (7) was used for this determination. After appropriate incubations of the platelets with 2-methylthio-[β-^{32}P]ADP plus other additions, the suspension (50–200 μl) was layered over 20 μl silicone oil (10) in a microsedimentation tube (No. 702, Walter Sarstedt, Inc., Princeton, NJ) and centrifuged for 1 min at 8,000 g in a microfuge (Fisher Scientific Co., Pittsburgh, PA) equipped with appropriate inserts. Control studies using [^{14}C]adenine-prelabeled platelets showed that the majority of platelets were pelleted in this fashion in ∼5 s. The tips of the centrifuge tubes were cut off through the silicone layer, and the platelets were recovered by inverting the tip and centrifuging the pellet into 100 μl 1% sodium dodecyl sulfate. The radioactivity in the solubilized pellet and a sample of the supernatant was determined. Nonspecific binding was defined as the radioactivity found in the pellet when platelets were incubated with an excess (1 mM) of ADP with the 2-methylthio[β-^{32}P]ADP. In some experiments 50 μl of 1 M perchloric acid containing 20 mg/ml sucrose was layered beneath the silicone oil. These perchloric acid extracts were neutralized with K_2CO_3 and electrophoresed on a Shandon L24 apparatus (Shandon Southern Instruments, Inc., Sewickley, PA) using 3MM paper (Whatman Inc., Clifton, NJ) moistened with 50 mM Na citrate, pH 4.05. The relevant spots, identified by coelectrophoresis of standards and visualized under 254 nm light, were cut out and their radioactivity determined by scintillation counting.

High pressure liquid chromatography. This was performed using a Waters 6000 gradient-forming pump (2 ml/ mm, Waters Instruments, Inc., Rochester, MN), a Whatman Partisil 10 × 25-SAX anion exchange column (Whatman Inc.) and an Altex monitor (Altex Scientific Inc., Berkeley, CA) operated at 254 nm. Using a 10-min linear gradient of from 10 to 700 mM NaH_2PO_4, 2-methylthio-AMP eluted at 250 mM and 2-methylthio-ADP at 652 mM NaH_2PO_4.

Synthesis of 2-methylthio-ADP. Adenosine N^1-oxide (Sigma Chemical Co., St Louis, MO) was hydrolyzed with NaOH to 5-amino-1-D-ribofuranosylimidazole-4-carboxamidoxime and cyclized with carbon disulfide to 2-thioadenosine (11). Recrystallization from water gave golden yellow needles, mp 196–199°C (decomposed). Analysis calculated for $C_{10}H_{13}N_5O_4S\cdot H_2O$: C, 37.85; H, 4.72; N, 22.07; S, 10.11. Found: C, 37.94; H, 4.91; N, 21.95; S, 10.03.

The 2-thioadenosine (317 mg, 1 mmol) was dissolved in water (2 ml), methanol (2 ml), and 1 N NaOH (1 ml) and cooled on ice. Methyl iodide (0.4 ml, 6 mmol) was added and the mixture stirred at 0°–5°C for 3 h (11). The separated product was collected by filtration, washed with water, and dried (290 mg, 92.5%). Recrystallization from methanol gave 2-methylthioadenosine that migrated as a single spot on thin-layer chromatography on silica gel using chloroform/ methanol 4:1, or ethylacetate/methanol 4:1. Yield 235 mg, 75%. mp 227°–228°C. Analysis calculated for $C_{11}H_{15}N_5O_4S$: C, 42.16; H, 4.83; N, 22.35; S, 10.23. Found: C, 42.07; H, 4.90; N, 22.44; S, 10.33.

2-Methylthioadenosine (66 mg, 200 μmol) was added to a precooled solution of freshly distilled phosphoryl chloride

(120 mg, 800 μmol) and dry triethylphosphate (1 ml) (12). The reaction mixture was stirred with exclusion of moisture at 0°C. The nucleoside dissolved within 15 min and the clear solution was stirred for an additional 3.5 h at 0°C. The reaction mixture was poured into stirred diethyl ether (100 ml) and the precipitate was collected by centrifugation. It was washed twice with 100 ml diethylether and then dissolved in cold water, adjusted to pH 7 with 2 N NaOH, and applied to a 2.5-ml column of Dowex AG 50 H$^+$. After washing with water (50 ml), the 2-methylthio-AMP was eluted with 45% acetic acid (25 ml) and evaporated *in vacuo* and coevaporated twice with water (5 ml). Addition of water (0.5 ml) gave crystalline 2-methylthio-AMP, which was collected by filtration and dried under vacuum over P$_2$O$_5$. Yield 45 mg (55%). Analysis calculated for C$_{11}$H$_{16}$N$_5$O$_7$PS.H$_2$O: C, 32.13; H, 4.40; N, 17.02; P, 7.53; S, 7.80. Found: C, 31.97; H, 4.44; N, 16.82; P, 7.59; S, 8.12. This material migrated as a single spot on high voltage electrophoresis and as a single peak on high pressure liquid chromatography.

2-Methylthio-ADP and 2-methylthio-[β-^{32}P]ADP were prepared from the monophosphate using N,N'-carbonyldiimidazole and tri-n-butylammonium phosphate as previously described for 2-azido-ADP (7) and were purified either by paper electrophoresis or by high pressure liquid chromatography. The specific activity of the final product was between 2,000 and 10,000 Ci/mol. High pressure liquid chromatography of a typical preparation showed that ∼90% of the radioactivity coeluted with 2-methylthio-ADP and the remainder eluted with orthophosphate.

Chemical analyses. All chemical analyses were performed by Galbraith Laboratories, Knoxville, TN.

Materials. PGE$_1$ was a gift from Dr. J. E. Pike, Upjohn Co., Kalamazoo, MI, and the phosphodiesterase inhibitor RA233 (2,6bis-(diethanolamino)-4-piperidinopyrimido-[5,4,d]-pyrimidine) was a gift from Dr. Bell, Boehringer Corp., Isleworth, UK. 2-Azido-ADP was prepared as previously described (7). p-Mercuribenzene sulphonate, ADP, ATP, IDP, GDP, and AMP were purchased from Sigma Chemical Co. Other materials were of the highest grade available commercially. [U-^{14}C]Adenine, sp act 286 Ci/mol was purchased from Amersham Corp., Arlington Heights, IL, and cyclic [2-^{3}H]AMP was purchased from ICN K&K Laboratories Inc., Plainview, NY.

RESULTS

2-Methylthio-ADP was a powerful aggregating agent for human platelets. Comparison of the rates of primary aggregation induced by 2-methylthio-ADP showed that this agent was equivalent in potency to about a fourfold higher concentration of ADP (Fig. 1A). The aggregation was preceeded by a shape change. As we had found with 2-azido-ADP (7), disaggregation was less prompt and biphasic aggregation was more prominent with 2-methylthio-ADP than with natural ADP. 2-Methylthio-ADP was also a powerful inhibitor of the accumulation of cyclic AMP in intact platelets exposed to PGE$_1$ and a phosphodiesterase inhibitor. Saturating concentrations inhibited this accumulation by ∼90% and the effect was half maximal at ∼6 nM in platelet-rich plasma (2 nM in washed platelets), a value more than two orders of magnitude lower than that for ADP (e.g., Fig. 1B).
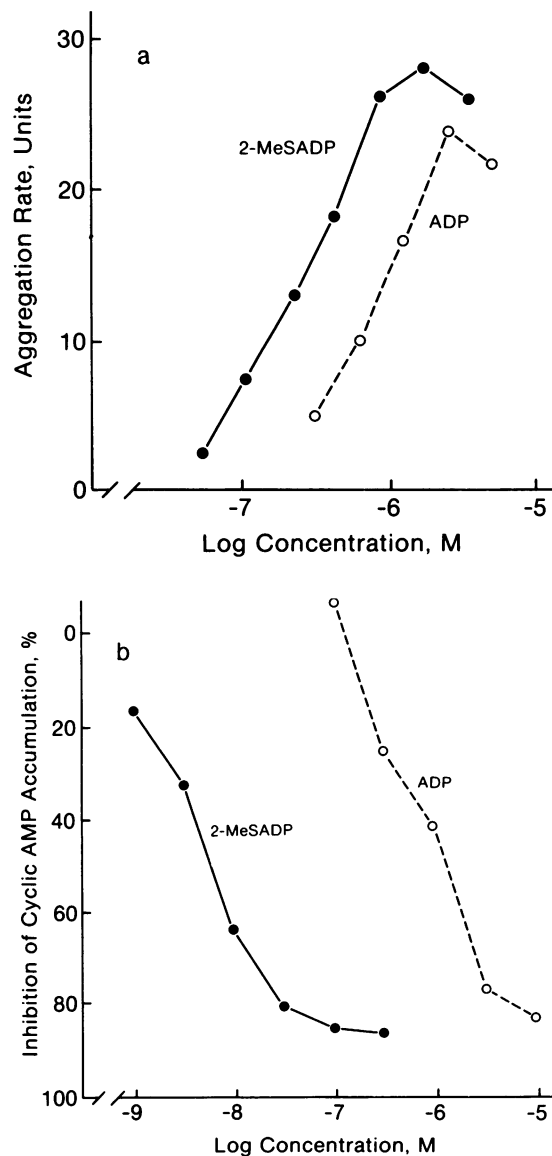

FIGURE 1 (a) Platelet aggregation induced by ADP and 2-methylthio-ADP added at the concentrations shown. The slope of the steepest part of the aggregation trace was measured in arbitrary units. (b) Inhibition of cyclic AMP accumulation by ADP and 2-methylthio-ADP. Platelet-rich plasma (0.5 ml), which had been prelabeled with [^{14}C]adenine, was incubated with PGE$_1$ (2 μM), RA233 (200 μM), EDTA (10 mM), plus the concentration of ADP or 2-methylthio-ADP shown for 2 min and its content of [^{14}C]cyclic AMP was determined. The degree of inhibition of cyclic AMP accumulation was calculated after subtraction of the basal level.

Thus, there is a marked discrepancy between the ratio of the potencies of these two agents as inhibitors of cyclic AMP accumulation (∼200-fold) and inducers of aggregation (about fourfold).
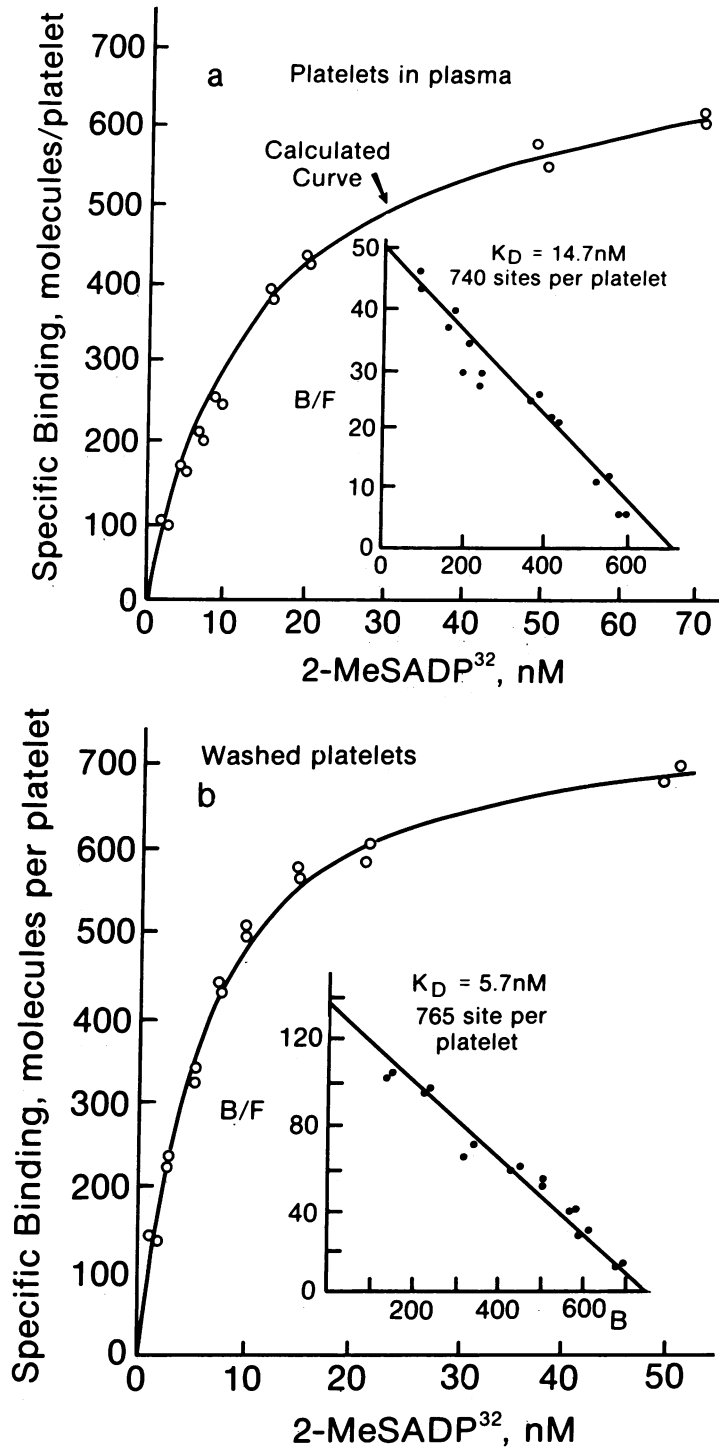
FIGURE 2 Binding of [^{32}P]2-methylthio-ADP to platelets in their own plasma (a) and to washed platelets (b) after incubation at 37°C for 90 s. The number of molecules bound per platelet (B) was calculated after subtraction of nonspecific binding. The inserts are Scatchard plots of the data. F represents the free concentration (nanomolar) of 2-methylthio-ADP. In this figure and subsequent figures, each point represents a single determination of a representative experiment.

When radioactive 2-methylthio-ADP was incubated with platelets, radioactivity rapidly became associated with them. The concentration dependence of this association is illustrated in the typical experiment shown in Fig. 2. The binding was clearly saturable. After subtracting nonspecific binding, we plotted the data according to Scatchard (insert). The straight line indicates binding to 740 sites with a dissociation constant of 14.7 nM. The conformity of the data to the computer drawn curve shows that the data are adequately described as a simple bimolecular binding model, which assumes that all sites are equivalent and non-interactive. On one occasion we estimated the number of binding sites and the affinity for 2-methylthio-ADP of the platelets from three separate donors in their own plasma (Fig. 3). Although there was a range of values for the number of sites, the affinity did not significantly differ in the three batches of platelets.

The rate of binding was rapid—indeed too rapid at 37°C for us to measure accurately with our present techniques. At 23°C we were able to estimate the forward rate (Fig. 4) as well as the reverse rate. The time scale of this figure is the interval between adding the reagent and starting the centrifuge. It takes a further 5 s or so to centrifuge the platelets through the silicone oil, so that the true time of exposure of the platelets to the reagents is longer than indicated on the time scale. The kinetically derived dissociation constant from these data (~7 nM) is close to that observed by equilibrium methods.

Measurements of the off rate is easier to perform than measurement of the on rate, because it can be done by adding an excess of nonradioactive ADP, which does not have to be completely mixed or equil-
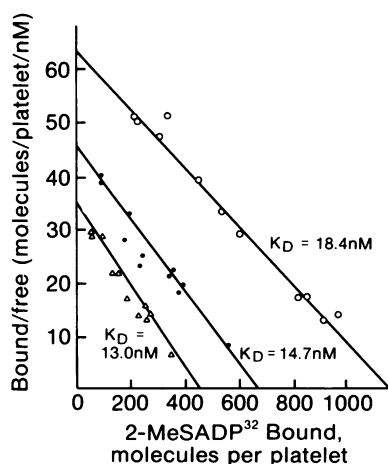
ibrated with other components of the system. We were therefore able to measure this rate at several temperatures (Fig. 5a). The dissociation appeared to be mono-exponential and was temperature dependent. The empirically derived activation energy for dissociation as revealed by the Arrhenius plot (insert) was ~15 kcal/mol. We also determined the effect of temperature on the equilibrium constant of binding (Fig. 5b). As can be seen, increasing the temperature resulted in a small decrease in the apparent number of receptors, but there was no discernible change in the affinity, indicating that the binding involved no net change in enthalpy.

The number of observed binding sites was not influenced by the pH of the suspending medium in the range of 6.0–8.0, but at pH 6.0 there was some decrease in the affinity (Fig. 6). As we previously showed for 2-azido-ADP, the binding of 2-methylthio-ADP was blocked by prior incubation of the platelets with the nonpenetrating thiol-complexing agent, p-mercuribenzene sulfonate (Fig. 7). This experiment was performed in plasma, which contains ~0.5 mM protein thiol. Concentrations of p-mercuribenzene sulfonate higher than this value abolished binding of 2-methylthio-ADP. We also determined the influence of other nucleotides on the binding of 2-methylthio-ADP. The results were compatible with competitive interactions with the following mean inhibitory constants (K_i) 2-azido-ADP 0.12 μM, ADP 3.5 μM, ATP 7.0 μM, IDP 150 μM, GDP 350 μM, AMP 800 μM (data not shown).

To determine the chemical nature of the bound radioactivity, we incubated platelets with radioactive 2-methylthio-ADP, then centrifuged them through silicone oil into perchloric acid, and analyzed the neutralized extracts by paper electrophoresis. In one such experiment, radioactivity in the supernatant migrated as 2-methylthio-ADP (71%), orthophosphate (21%), and material that ran with 2-methylthio-ATP (8%). The extract of the platelet pellet migrated as the diphosphate (87%) and triphosphate (13%), and no orthophosphatelike activity was specifically bound. This experiment required that we load the electrophoresis system so heavily that distorted spots were obtained, but it nevertheless shows that platelets do not phosphorylate the diphosphate bound to the receptor to any substantial degree.

DISCUSSION

A large number of synthetic or naturally occurring analogues of ADP have been tested for their ability to induce platelet aggregation or to inhibit the accumulation of cyclic AMP in intact platelets. As a result we know that modifications in the pyrophosphate or ribose moiety reduce the activity of the resultant com-
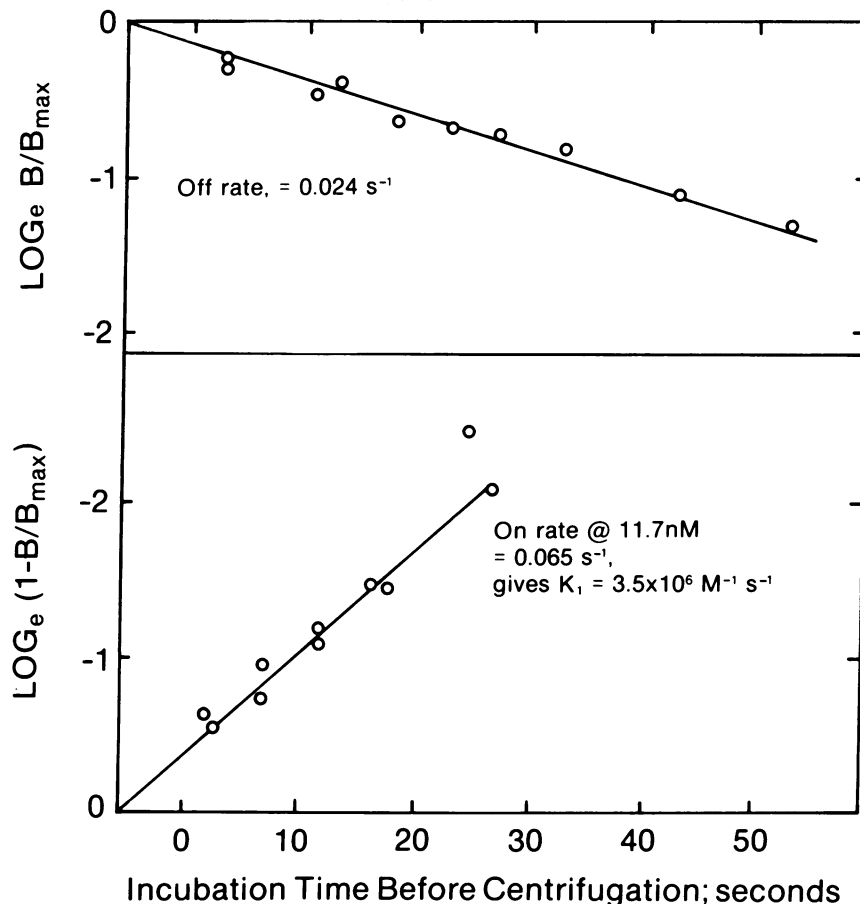

FIGURE 3 Binding of [32P]2-methylthio-ADP to platelets in their own plasma prepared from three different donors. Scatchard plots are shown of the data, which were obtained at 37°C.

FIGURE 4 Logarithmic progress plots of the association and dissociation of [^{32}P]2-methylthio-ADP. The on-rate (lower panel) was measured by centrifuging the platelets at the time indicated after adding 11.7 nM [β-^{32}P]2-methylthio-ADP. B, amount specifically bound; Bmax, amount bound after 10-min incubation. The off-rate (upper panel) was determined by incubating the platelets with 20 nM [^{32}P]2-methylthio-ADP for 5 min; 10 mM ADP was then added, and the sample was centrifuged after the indicated interval. B, amount specifically bound; Bmax, amount bound before addition of nonradioactive ADP. The calculated K_D from this experiment is 6.9 nM.

pound, and the only permissible change in the purine nucleus is substitution in the 2-position. A thorough review of this subject has recently been published by Haslam and Cusack (3). In general, compounds with high activity as aggregating agents are also potent inhibitors of cyclic AMP accumulation, and ATP, which competitively inhibit aggregation induced by ADP, also inhibits the action of ADP on cyclic AMP accumulation.

2-Methylthio-ADP was first synthesized by Gough et al. (8), and shown to be a potent inducer of the aggregation of sheep platelets. We used a more direct route for this synthesis and prepared the radioactive analogue of this compound 2-methylthio-[β-^{32}P]ADP for use as a radioligand. The technique we used for the 5'-phosphorylation of 2-azido-adenosine (phos-

phoryl chloride/pyridine/H_2O in dimethylformamide, reference 7) gave poor yields of 2-methylthio-AMP. However, we were successful using phosphoryl chloride in triethyl phosphate (12), a procedure specific for 5'-hydroxyl phosphorylation. Most of the experiments reported here were performed with material purified by high voltage electrophoresis, but some of the later ones used high pressure liquid chromatography as a final step. The results were equivalent, and liquid chromatography of the electrophoretically purified 2-methylthio-[β-^{32}P]ADP usually showed a radiochemical purity >90%; the principle radioactive impurity appeared to be orthophosphate. In experiments with washed platelets, we included 1 mM orthophosphate in the suspending medium to prevent the uptake of labeled orthophosphate.
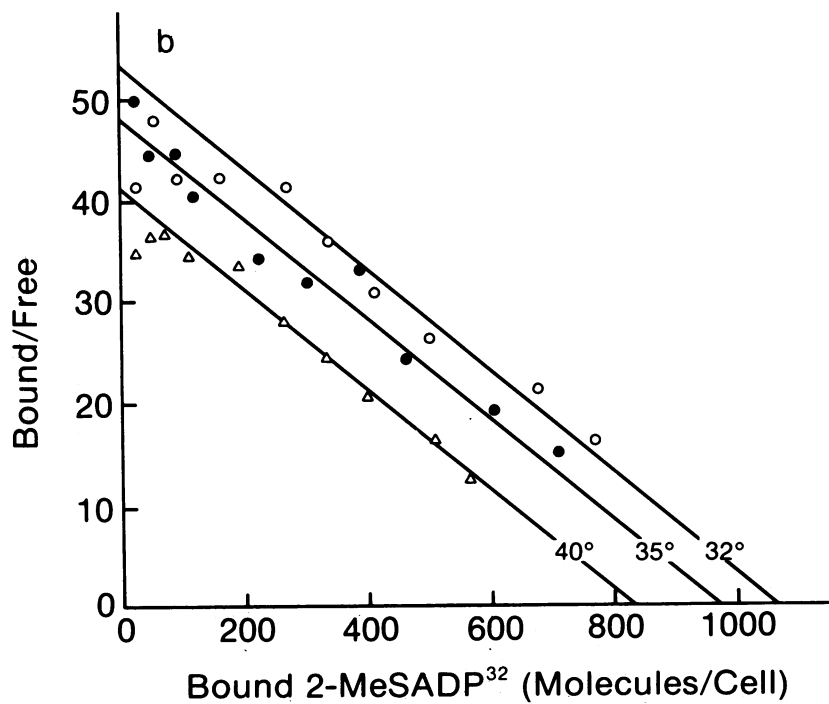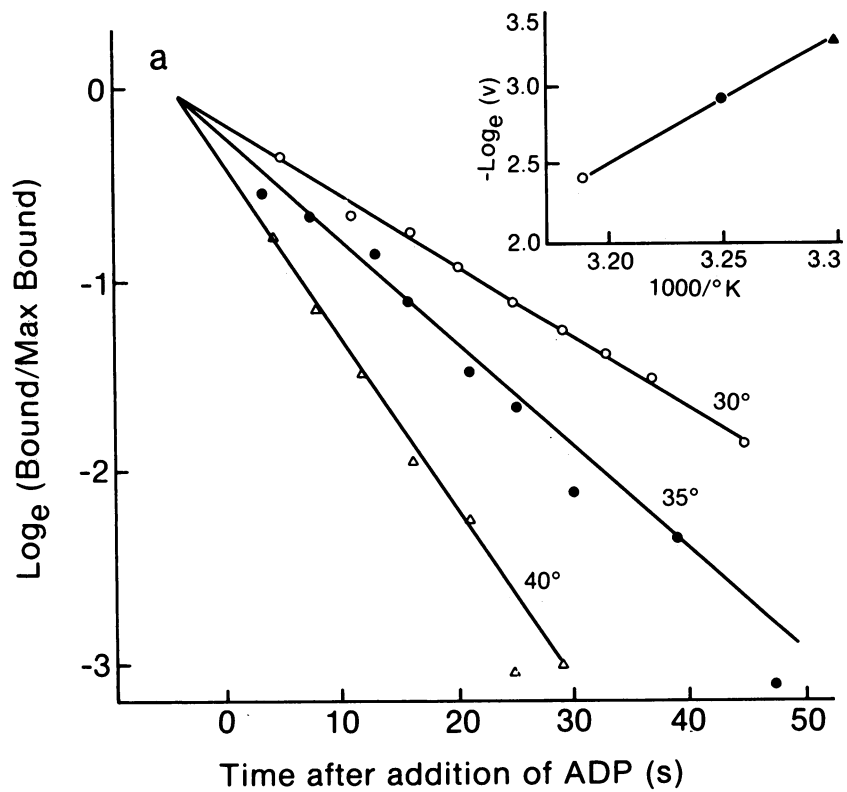
FIGURE 5   Influence of temperature on the rate of dissociation of [^{32}P]2-methylthio-ADP from washed platelets (a; logarithmic progress plots) and on equilibrium binding (b; Scatchard plots). The insert is a Arrhenius plot, indicating an activation energy (Ea) for dissociation of ∼15 kcal/mol.
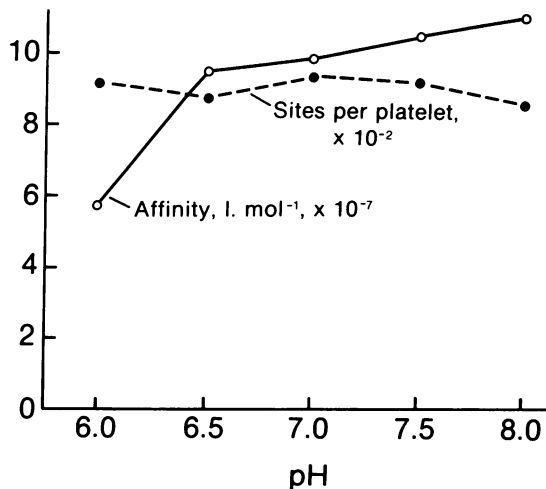
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FIGURE 6 Effect of pH on the binding of [^{32}P]2-methylthio-ADP to washed platelets. The platelets were suspended in a buffer containing 80 mM NaCl, 2 mM HCO$_3$, 20 mM NaH$_2$PO$_4$, 20 mM Tris, 20 mM trisodium citrate, 2 mM MgCl$_2$ and 5 mM KCl, 100 mM glucose. The pH was adjusted with 2 N HCl or NaOH. The platelets were incubated with eight different concentrations of [^{32}P]2-methylthio-ADP between 0.5 and 45 nM, for the construction of Scatchard plots, from which the association constant and number of binding sites were derived graphically.

As anticipated, 2-methylthio-ADP was a powerful aggregating agent, being some four times as powerful as ADP on a concentration basis when we measured the rate of primary aggregation. The aggregation traces did differ in form, and aggregation induced by this reagent had less tendency to reverse and more tendency to progress to biphasic aggregation than does aggregation induced by ADP. 2-Methylthio-ADP was also a potent inhibitor of the accumulation of cyclic AMP within intact platelets exposed to PGE$_1$. The maximum degree of inhibition was ~90%, and half-maximal inhibition (K_{effect}) occurred at ~5 nM in platelet-rich plasma and 2 nM in platelets suspended in buffer. This compound is therefore substantially more potent than other inhibitors of the adenylate cyclase of intact cells (14). There is a major disparity between the K_{effect} for aggregation and for inhibition of the adenylate cyclase (250 nM vs. 5 nM). We cannot reconcile this difference on methodological grounds, since both assays were performed in platelet-rich plasma with similar concentrations of Na$^+$ and Mg^{2+}, and the minor differences in the duration of exposure to the reagent are unlikely to be of major importance since 2-methylthio-ADP rapidly equilibrates with its receptor.

The analysis of the binding of radioligands to membrane preparations frequently reveals the existence of more than one class of binding site, as well as a nonsaturable component. Using 2-methylthio-ADP and intact platelets, the degree of nonsaturable binding (which can be expressed mathematically in terms of an equivalent volume of supernatant) was no more than expected from the volume of the bulk phase known to be trapped in the interstices of the platelets. After subtracting this component (revealed by the inclusion of an excess of ADP to block specific binding, and amounting to no <20% of the total pellet radioactivity), the data were well described by the Langmuir isotherm for the binding of a ligand to noninteractive sites of uniform affinity. We found no evi-
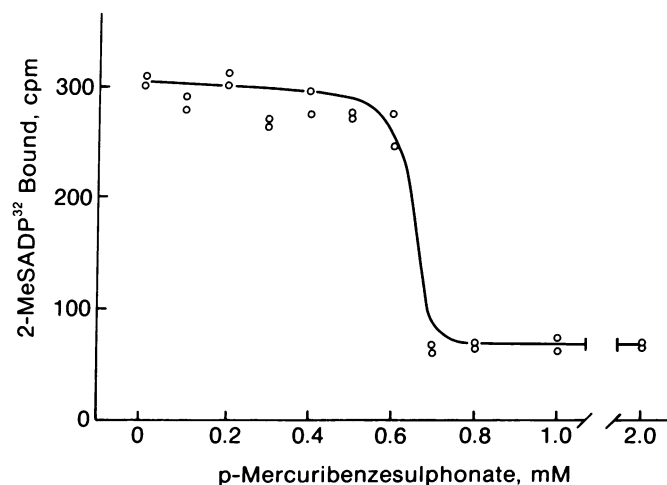

FIGURE 7 Influence of p-mercuribenzene sulphonate on binding of [β-^{32}P]2-methylthio-ADP to platelets in their own plasma. The platelets were incubated with the p-mercuribenzene sulphonate for 5 min, then with [^{32}P]2-methylthio-ADP (28.5 nM) for a further 4 min. The platelets were then sedimented through silicone oil and the pellet count was determined.

dence for the two classes of agonist binding sites predicted by the floating or mobile receptor hypothesis (15), but the dissociation constant (K_D) for 2-methyl-thio-ADP (15 nM in plasma, 5 nM in saline) was about three times higher than the K_{effect} on the adenylate cyclase. These results are similar to those we have reported with the α_2-adrenergic receptor (16); they can be accounted for by assuming that these receptors act catalytically to switch the adenylate cyclase to a stable "off" state (17).

We found 400–1,200 binding sites for 2-methylthio-ADP per platelet, which is compatible with the number of sites we found for 2-azido-ADP, but is higher than the number of α_2-adrenergic sites identified with [³H]yohimbine (~200, references 16 and 18). The number of ADP receptor sites we report is far lower than those reported by others using radioactive ADP as the ligand (3).

There is substantial evidence that the site to which 2-methylthio-ADP binds is the ADP receptor responsible for the inhibition of cyclic AMP accumulation by the platelet. Binding occurred in the absence of free divalent cation: the radioligand was displaced by ADP, ATP, and by 2-azido-ADP with the potency ratios expected from their effects on cyclic AMP accumulation and other closely related nucleotides IDP, GDP, and AMP were much less effective; the nonpenetrating thiol reagent, p-mercuribenzene sulfonate, which blocks the effect of ADP on cyclic AMP accumulation without blocking the ability of ADP to induce the platelet shape change, inhibits the binding of both 2-methylthio-[³²P]ADP and 2-azido-[³²P]ADP (7). We take this to indicate that both these ligands bind to the receptor that regulates the adenylate cyclase. Equilibrium binding measurements failed to detect any interaction of these ligand with the platelets that could be specifically associated with their ability to induce shape change and aggregation.

It has been suggested that the ADP receptor for aggregation is an ectoenzyme that generates a pharmacological signal when it phosphorylates the diphosphate agonist to the corresponding triphosphate (13). We extracted the bound radioactivity from the receptor to see if it was converted to the triphosphate, and found no evidence that it was. Further experiments are under way to determine the extent to which platelets can phosphorylate extracellular 2-methylthio-ADP, but preliminary evidence shows that if this does occur, it does so only slowly (19).

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