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Agonist and Antagonist Effects of Diadenosine Tetraphosphate, a Platelet Dense Granule Constituent, on Platelet P2Y₁, P2Y₁₂ and P2X₁ Receptors

Hung Chang^{a,b}, Ivan B. Yanachkov^c, Alan D. Michelson^{a,d}, YouFu Li^a, M.R. Barnard^{a,d}, George E. Wright^c, and Andrew L. Frelinger III^{a,d,*}

^aCenter for Platelet Function Studies, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA

^bHematology Division, Chang Gung Memorial Hospital, Chang Gung University, Taipei, Taiwan

^cGLSynthesis Inc., Worcester, MA

^dCenter for Platelet Research Studies, Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, MA

Abstract

Introduction—Diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A) is stored in platelet dense granules, but its effects on platelet function are not well understood.

Methods and Results—We examined the effects of Ap₄A on platelet purinergic receptors P2Y₁, P2Y₁₂ and P2X₁. Flow cytometry was used to measure the effects of Ap₄A in the presence or absence of ADP on: a) P2Y₁₂-mediated decrease in intraplatelet phosphorylated vasodilator stimulated phosphoprotein (VASP), b) P2Y₁-mediated increase in platelet cytosolic Ca²⁺, and c) P2X₁-mediated intraplatelet entry of extracellular Ca²⁺. ADP-stimulated platelet shape change (P2Y₁-mediated) and aggregation (P2Y₁- and P2Y₁₂-mediated) were measured optically. Ap₄A inhibited 3 μM ADP-induced: a) platelet aggregation (IC₅₀ 9.8 ± 2.8 μM), b) P2Y₁-mediated shape change, c) P2Y₁-mediated increase in platelet cytosolic Ca²⁺ (IC₅₀ 40.8 ± 12.3 μM), and d) P2Y₁₂-mediated decrease in VASP phosphorylation (IC₅₀ >250 μM). In the absence of added ADP, Ap₄A had agonist effects on platelet P2X₁ and P2Y₁₂, but not P2Y₁, receptors.

Conclusion—Ap₄A, a constituent of platelet dense granules, is a) an antagonist of platelet P2Y₁ and P2Y₁₂ receptors, where it inhibits the effects of ADP, and b) an agonist of platelet P2X₁ and P2Y₁₂ receptors.

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***Corresponding author:** Andrew L. Frelinger, PhD, Children's Hospital Boston, 300 Longwood Avenue, Karp 07212, Boston, MA 02115-5737. andrew.frelinger@childrens.harvard.edu Phone: (617) 919-2537. Fax: (617) 730 4632.

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Conflict of Interests Statement

Drs. Yanachkov and Wright are employees of GLSynthesis, Inc. Drs. Michelson and Frelinger have been principal investigators on research grants to the University of Massachusetts Medical School from Arena Pharmaceuticals, GLSynthesis, Lilly/Daiichi Sankyo, and Sanofi Aventis/Bristol-Myers Squibb. Dr. Michelson has been a consultant to Lilly/Daiichi Sankyo and Sanofi Aventis/Bristol-Myers Squibb.

Keywords

ADP; diadenosine tetraphosphate; platelets; purinergic receptors; platelet aggregation

Introduction

Platelets express three purinergic receptors, P2X₁, P2Y₁ and P2Y₁₂ (Fig. 1). [1,2] P2X₁ receptors are activated by adenosine 5'-triphosphate (ATP) while P2Y₁ and P2Y₁₂ receptors are both activated by adenosine 5'-diphosphate (ADP) [1,2]. The P2X₁ receptor is a ligand-gated ion channel which upon activation triggers fast influx of extracellular Ca²⁺ into the cytoplasm and transient platelet shape change (Fig. 1) [1–4]. P2Y₁ and P2Y₁₂ are G-protein coupled receptors, P2Y₁ being coupled to G_q and P2Y₁₂ to G_i [1,2]. Upon activation, P2Y₁ triggers Ca²⁺ mobilization from the platelet dense tubular system, shape change, and reversible platelet aggregation (Fig. 1) [1,2]. Activation of P2Y₁₂ leads to inhibition of the adenylyl cyclase-dependent production of cytoplasmic cyclic adenosine 5'-monophosphate (cAMP) and propagation of stable platelet aggregation [1,2]. cAMP activates protein kinase A which then phosphorylates vasodilator stimulated protein (VASP) [2,5], a modulator of platelet cytosolic proteins (Fig. 1). Both P2Y₁ and P2Y₁₂ play major roles in the amplification and stabilization of platelet activation. The exact physiological role of P2X₁ is less clear, but it plays a role in the enhancement of the effect of low levels of primary platelet activators and in high shear stress activation [1,2].

Diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A) and other diadenosine polyphosphates are naturally occurring compounds that are ubiquitous in mammalian tissues [6], including human platelets [7,8]. They may serve as neurotransmitters [9] and modulators of vascular tone [10]. There is growing evidence that Ap₄A plays a role in systemic diseases such as diabetes mellitus and hypertension [11,12]. In platelets, Ap₄A is stored in dense granules, and is therefore released along with ADP and ATP upon platelet activation [8,13]. Ap₄A and its analogs are known to inhibit ADP-induced platelet activation [14,15]. Ap₄A analogs inhibit the ADP-induced platelet release reaction, calcium mobilization, thromboxane production and platelet factor 3 activities [14]. However, these studies [14,15] were performed before all three platelet purinergic receptors were cloned and their functions characterized. Therefore, the mechanism by which Ap₄A inhibits ADP-induced platelet activation and its possible effects on P2Y₁ and P2Y₁₂ are unknown. Diadenosine polyphosphates are potent agonists of P2X receptors expressed on a variety of human and rat cell types [16,17]. Although human platelets express P2X₁ receptors [18–20], whether Ap₄A is an agonist via platelet P2X₁ is unknown. The goal of the present study was, therefore, to elucidate the effects of Ap₄A on signaling through P2Y₁, P2Y₁₂ and P2X₁ receptors on human platelets. We demonstrate that Ap₄A, a known constituent of platelet dense granules, is: a) an antagonist of platelet P2Y₁ and P2Y₁₂ receptors, where it inhibits the effects of the agonist ADP, b) an agonist of P2X₁ receptors, and c) a partial agonist of P2Y₁₂ receptors.

Materials and Methods

Chemicals and reagents

Ap₄A was synthesized by a novel method (to be published) and was >98% pure by reverse phase HPLC. MRS2179, MRS2159, probenecid, adenosine 5'-(β,γ-methylene)triphosphate (β,γ-CH₂-ATP) and apyrase (grade VII) were purchased from Sigma-Aldrich (St. Louis, MO). D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was purchased from Calbiochem (EMD Biosciences, La Jolla, CA). FLUO-4 was from Invitrogen (Carlsbad, CA), ADP was from Bio/Data (Horsham, PA), CD41-phycoerythrin (PE)-Cy5 was from Beckman Coulter (Fullerton, CA), and AR-C69931 was from AstraZeneca (Charnwood, UK).

Blood collection and sample preparation

Human blood samples were taken from healthy volunteer donors who had been free from aspirin or other non-steroidal anti-inflammatory drugs for more than 7 days. IRB-approved written informed consent was obtained before blood collection. Unless otherwise specified, blood was drawn from antecubital veins into 3.2% sodium citrate tubes. Whole blood was used in VASP phosphorylation and P2Y₁ cytosolic Ca²⁺ assays. For platelet aggregation tests, the blood was centrifuged at 110 g for 12 minutes and platelet-rich plasma (PRP) was immediately removed. The remaining samples were further centrifuged at 1650 g for 10 minutes to obtain platelet-poor plasma (PPP), which was used as a reference. For assays of platelet P2X₁ receptor functions, whole blood was drawn into tubes containing PPACK (0.3 mM, final concentration) and apyrase (1.8 μM final concentration). The samples were then centrifuged and processed to retrieve PRP and PPP.

ADP-induced platelet aggregation and shape change

Light transmission platelet aggregation was performed as previously described[21]. ADP (3 μM) in the presence or absence of various concentrations of Ap₄A was added to PRP, and the aggregation response was recorded for a total of six minutes in a Chrono-log® aggregometer running the Aggro/Link software (Chrono-log®, Havertown, PA).

For observation of ADP-induced platelet shape change, EDTA (final concentration 10 mM) was mixed with PRP to allow ADP-induced shape change but to avoid platelet-platelet aggregation. After obtaining a stable baseline tracing in the aggregometer ADP (3 μM), in the presence or absence of various concentrations of Ap₄A, was added to induce platelet shape change, and the tracing recorded for a total of 4 minutes.

Platelet aggregation measured by microplate reader

The 96-well microplate method for the detection of platelet aggregation is a simple, rapid, low volume method to simultaneously measure platelet aggregation of multiple samples, thereby avoiding the variable of platelet aging[22,23]. In brief, ADP (5 μL, final concentration 3 μM) and Ap₄A (5 μL, at various concentrations) were added to each well of a flat bottom 96-well Immulon microplate (Thermo Fisher Scientific, Waltham, MA). PRP (90 μL) was then added to each well with a multi-channel pipette, and light transmission was recorded with a Molecular Devices microplate reader, running SOFTmax Pro 4.0 software at 580 nm wavelength under the kinetic mode, with intermittent shaking and readings every 11 seconds at 37°C. Results of platelet aggregation measured by the microplate method have been demonstrated to be comparable to results of conventional platelet aggregation[22,23]. Similarly, we observed comparable Ap₄A inhibition of ADP-stimulated platelet aggregation by these two methods in the present study (Figure 2A and 2B). Since the microplate method avoids possible aging artifacts, results from this method were used to calculate the IC₅₀ for Ap₄A.

P2Y₁₂-mediated VASP phosphorylation assay

VASP phosphorylation (Fig. 1) was measured by flow cytometry[5] using a kit (BioCytex, Marseilles, France) essentially according to the manufacturer's recommendations. In brief, Ap₄A or vehicle (2 μL) was added to each set of assay tubes, followed by prostaglandin E₁ (PGE₁, 9 μL) or the same volume of PGE₁ plus ADP. Citrated whole blood (10 μL) was then added to each tube, and the samples were incubated for 10 minutes at room temperature. The samples were then fixed, permeabilized, and labeled with a fluorescently conjugated monoclonal antibody (clone 16C2) directed against the serine 239 phosphorylated form of VASP and, as a platelet identifier, CD61. Analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The platelet reactivity index (PRI) was calculated after subtracting background fluorescence, according to the manufacturer's

recommendations using the formula $PRI = [(MFI_{(PGE1)} - MFI_{(PGE1+ADP)}) / MFI_{(PGE1)}] \times 100$, where MFI is the mean fluorescence intensity.

HEPES-saline buffer was added as a control (i.e. no antagonist, 0% inhibition). The possible agonist property of Ap₄A on P2Y₁₂ was tested by adding Ap₄A to the tube incubated with PGE₁ in the absence of ADP.

To evaluate degradation and the possibility of generating biologically active metabolites of Ap₄A in the VASP assay, Ap₄A was incubated with whole blood and the PGE₁ reagent from the VASP kit for 0, 3, 6 and 10 minutes. The mixture was centrifuged at 13,600 g for 1 minute and the supernatant retrieved and stored at -80°C until analysis. Prior to analysis by high performance liquid chromatography (HPLC), proteins were removed by perchloric acid precipitation and the samples centrifuged (13,000 g) and filtered (0.45 μ nylon).

P2Y₁-mediated cytosolic calcium increase measured by flow cytometry with the calcium indicator FLUO-4

The ADP-dependent, P2Y₁-mediated increase in platelet cytosolic calcium was measured by detecting changes in FLUO-4 fluorescence (Fig. 1), as previously described[24]. In brief, one part of citrated whole blood was added to 9 parts of a loading solution consisting of FLUO-4 (5 μM final concentration), CD41-PE-Cy5 (1:176 dilution in HEPES-saline buffer [10 mM HEPES, 0.15 M NaCl, pH 7.4]) and probenecid (1 mM final concentration), and the mixture was incubated for 30 minutes at room temperature. Fifteen μL of this mixture were added to 525 μL of HEPES-saline buffer, and the mixture analyzed by a FACSCalibur flow cytometer. After obtaining a 30 second baseline recording, the acquisition was paused, and 60 μL of either ADP (3 μM final concentration), Ap₄A at various concentrations, or ADP (3 μM final concentration) plus Ap₄A at various concentrations were quickly added, the sample mixed, and the acquisition resumed (total pause time less than 10 seconds). FLUO-4 fluorescence was monitored for a total of 2 minutes. FLUO-4 fluorescence was plotted vs. time using FlowJo version 7.2.2 (Tree Star, Ashland, OR) software. The mean FLUO-4 fluorescence of the baseline 30 second interval and of 10 second post-stimulant intervals were calculated. The cytosolic Ca²⁺ increase was calculated as the ratio of the maximal post-stimulant FLUO-4 fluorescence to the baseline FLUO-4 fluorescence. The percent inhibition of ADP-induced calcium increase due to the addition of Ap₄A was calculated relative to ADP (3 μM) plus vehicle (HEPES-saline).

P2X₁-mediated entry of extracellular calcium measured by flow cytometry with the calcium indicator FLUO-4

P2X₁ receptors are ligand-gated ion channels which, when triggered, cause cytosolic Ca²⁺ to increase by allowing entry of extracellular Ca²⁺ (Fig. 1). Measurement of P2X₁-mediated entry of extracellular Ca²⁺ with FLUO-4 differed from measurement of P2Y₁-mediated increase in cytosolic Ca²⁺ from intracellular stores in that 1) blood was collected in PPACK (0.3 mM) rather than sodium citrate, to preserve physiologic extracellular Ca²⁺ levels, 2) apyrase (1.8 μM final concentration) was added immediately upon blood collection to degrade any ATP present, thereby avoiding P2X₁ receptor desensitization.[1,25], 3) prior to loading platelets with FLUO-4, PRP was separated from erythrocytes in order to minimize erythrocyte-derived nucleotides, and 4) FLUO-4-loaded platelets were diluted and analyzed in buffer containing Ca²⁺ (2 mM) rather than in Ca²⁺-free buffer to provide physiologic levels of extracellular Ca²⁺. Platelets in apyrase-treated, PPACK-anticoagulated PRP were loaded with FLUO-4 for 30 minutes in the presence of probenecid (1 mM), diluted in HEPES-saline buffer containing 2 mM CaCl₂, and then baseline FLUO-4 fluorescence was obtained. P2X₁-dependent entry of extracellular Ca²⁺ was demonstrated by increased FLUO-4 fluorescence upon addition of the specific P2X₁ receptor agonist β,γ-CH₂-ATP (20 μM), and the absence of this increase when

platelets were resuspended in 1 mM EGTA, HEPES-saline buffer, or prepared without addition of apyrase to preserve P2X₁ activity. FLUO-4 fluorescence after addition of various concentrations of Ap₄A to this system was used to evaluate agonist activity on P2X₁. In addition, the ability of Ap₄A (50 μM) to block P2X₁ activation by β,γ-CH₂-ATP (20 μM) was tested. To ensure that the increase of cytosolic Ca²⁺ in these experiments was not due to spurious P2Y₁-mediated Ca²⁺ increase, some experiments were done in the presence of 1 mM EGTA in HEPES-saline buffer, MRS2179 (100 μM, a P2Y₁ receptor antagonist), [26,27] or both.

Statistical analysis

The results were analyzed by GraphPad Prism software, version 4.00 for Windows (GraphPad Software, San Diego, CA). All results are expressed as mean ± standard error of the mean (SEM). Student's t-test was used to determine statistical significance when two groups of data were compared. One way ANOVA and Bonferroni's multiple comparison test were used when three or more groups of data were compared.

Results

Inhibition of ADP-induced platelet aggregation

Ap₄A inhibited maximal ADP-induced platelet aggregation and, at a high concentration (100 μM), prevented secondary aggregation and resulted in disaggregation (Fig. 2A). Dose-dependent inhibition of ADP-induced platelet aggregation determined by the 96-well microplate method is shown in Fig. 2B. The IC₅₀ was 9.8 ± 2.8 μM.

Agonist and antagonist properties of Ap₄A on P2Y₁₂ receptors

The unstimulated baseline VASP phosphorylation MFI was obtained by treating platelets with PGE₁ in all experiments (Fig. 3). As expected, the addition of 3 μM ADP reduced VASP phosphorylation (Fig. 3, open bar). Ap₄A was added to attenuate this ADP effect as a potential antagonist (in comparison with PGE₁+ADP) or to reduce VASP phosphorylation by itself as a potential agonist (in comparison with PGE₁ alone). VASP phosphorylation in the presence of 15.6 μM Ap₄A (Fig. 3, hash-marked grey bar) was significantly reduced compared to that obtained with PGE₁ alone (Fig. 3, grey bar, *p* < 0.01), suggesting that Ap₄A acts as an agonist of P2Y₁₂. However, increasing the concentration of Ap₄A up to 250 μM did not further reduce PGE₁-stimulated VASP phosphorylation (*p* > 0.05 for the comparison among three Ap₄A concentrations tested), suggesting that Ap₄A is only a partial agonist of P2Y₁₂. Addition of the specific P2Y₁₂ antagonist AR-C69931 (10 μM) blocked Ap₄A-induced reduction of VASP phosphorylation (data not shown), indicating that this agonist effect of Ap₄A is mediated through P2Y₁₂. HPLC analysis of Ap₄A incubated with whole blood under the same conditions used in the VASP assay for up to 10 min (*i.e.*, the incubation time of the VASP assay) showed no loss of Ap₄A (data not shown). Likewise, the normal degradation products of Ap₄A, AMP and ATP, were low and did not increase over time and ADP was not detected (data not shown). Thus, the agonist effect of Ap₄A on P2Y₁₂ did not appear to be mediated by a metabolite of Ap₄A.

In the presence of increasing concentrations of Ap₄A, there was a dose-dependent blockade of ADP's effect, resulting in higher levels of phosphorylated VASP (Fig. 3, hash-marked open bars). The calculated PRI for the inhibition of VASP phosphorylation induced by ADP relative to the maximal VASP phosphorylation without ADP was 89.5 ± 2.1%, 74.7 ± 1.0%, 61.5 ± 9.4%, and 48.9 ± 6.2% for 0, 15.6, 62.5, and 250 μM Ap₄A, respectively. Thus, the IC₅₀ for Ap₄A inhibition of the ADP-induced, P2Y₁₂-mediated response was >250 μM.

Agonist and antagonist properties of Ap₄A on P2Y₁ receptors

ADP stimulation of P2Y₁ results in platelet shape change which can be measured by the change in transmittance in an aggregometer. Ap₄A inhibited 3 μM ADP-induced platelet shape change in a dose-dependent manner (Fig. 4A, estimated IC₅₀ 200 μM). ADP stimulation of P2Y₁ also results in increases in cytosolic Ca²⁺ (Fig. 1). Therefore, we evaluated the effect of Ap₄A on the ADP-induced increase in cytoplasmic Ca²⁺. In platelets prepared in the absence of apyrase, *i.e.* without preserving the activity of P2X₁, and without addition of extracellular Ca²⁺, 3 μM ADP produced an approximately 3.5-fold increase in FLUO-4 fluorescence and this increase was inhibited by Ap₄A in a dose-dependent manner (Fig. 4B), with an IC₅₀ of 40.8 ± 12.3 μM. That a higher concentration of Ap₄A was required for 50% inhibition ADP-stimulated platelet shape change (~200 μM) than was required for inhibition of ADP-stimulated increase in cytoplasmic Ca²⁺ from intracellular stores (~40 μM) is not surprising since platelet shape change is likely triggered by a low threshold increase in cytoplasmic Ca²⁺, and thus would require an amount of Ap₄A that reduces the cytoplasmic Ca²⁺ increase by greater than 50%. Thus, as evidenced by two independent assays, platelet shape change and increase in cytosolic Ca²⁺, Ap₄A is an antagonist of P2Y₁ receptors.

Under the same conditions, but in the absence of ADP, Ap₄A at concentrations up to 250 μM did not induce an increase in cytoplasmic Ca²⁺ as measured by the FLUO-4 fluorescence ratio in comparison with HEPES-saline vehicle control (1.18 ± 0.09 vs. 1.09 ± 0.06, mean ± SEM, n = 5, p = 0.161). Thus, Ap₄A is not an agonist of P2Y₁.

Agonist and antagonist properties of Ap₄A on P2X₁ receptors

Platelet P2X₁ function was not a factor in the platelet aggregation, shape change, P2Y₁ release of internal Ca²⁺, and P2Y₁₂-mediated VASP assays described above since activity of P2X₁ was not preserved under the conditions used to isolate platelets for those assays. However, when platelets were prepared using protocols to protect P2X₁ from desensitization and suspended in buffer containing 2 mM Ca²⁺, Ap₄A at 0.1 μM and higher concentrations induced an increase in platelet intracellular Ca²⁺ (Fig. 5A). For Ap₄A concentrations between 0.1 and 10 μM, this increase was entirely due to the P2X₁-mediated entry of extracellular Ca²⁺, because it was eliminated when platelets were resuspended in buffer containing EGTA instead of Ca²⁺ (Fig. 5). Moreover, failure of the P2Y₁ antagonist MRS2179 at 100 μM to inhibit the Ap₄A-stimulated rise in Ca²⁺ (Fig. 5A) indicated that this rise was not due to direct or indirect stimulation of P2Y₁. Similarly, the majority of the cytoplasmic Ca²⁺ rise produced by 50 μM Ap₄A was eliminated in the presence of EGTA and could not be blocked by MRS2179. However, there was a small, but statistically significant component of the Ca²⁺ increase at 50 μM Ap₄A that was not blocked by EGTA but was blocked by MRS2179, indicating some P2Y₁-mediated contribution at this high Ap₄A concentration. The biphasic dose-response observed for Ap₄A (Fig. 5A) was also observed with the specific P2X₁ receptor agonist β,γ-CH₂-ATP (data not shown). Finally, MRS2159, a selective P2X₁ antagonist [28], added in parallel with Ap₄A, dose-dependently blocked Ap₄A-stimulated Ca²⁺ entry into platelets (Fig. 6), demonstrating that the Ap₄A-induced rise in platelet cytoplasmic Ca²⁺ requires P2X₁. Taken together, these data show that Ap₄A is an agonist of platelet P2X₁ receptors.

When 50 μM Ap₄A was added to a submaximal concentration of a selective P2X₁ agonist, 20 μM β,γ-CH₂-ATP, the post-stimulation FLUO-4 MFI was higher than with 20 μM β,γ-CH₂-ATP alone (4.43 ± 0.20 vs. 3.57 ± 0.47, p = 0.034, n = 3). Thus, there was no evidence that Ap₄A is a P2X₁ antagonist, and the enhanced response suggested that Ap₄A augmented the P2X₁ agonist effect of 20 μM β,γ-CH₂-ATP.

Discussion

The major findings of this study are that Ap₄A, a known constituent of platelet dense granules, is: a) an antagonist of platelet P2Y₁ and P2Y₁₂ receptors, where it inhibits the effects of the agonist ADP, b) an agonist of P2X₁ receptors, and c) a partial agonist of P2Y₁₂ receptors. These stimulating and inhibiting activities are summarized in Table 1.

Antagonist effects of Ap₄A on platelet P2Y₁ and P2Y₁₂ receptors

Previously published studies showed that Ap₄A and its analogs inhibit ADP-induced platelet aggregation and several aspects of platelet activation including release action, cytoplasmic calcium mobilization, thromboxane production, fibrinogen binding, and platelet factor 3 activities[14]. This inhibition of platelet functions was considered to be mediated through the P_{2T} pathway[14], a poorly defined term used before the currently known purinergic receptors, P2X₁, P2Y₁ and P2Y₁₂, were cloned and their functions well characterized. In the current study, consistent with these previous reports [14], we found that Ap₄A inhibits 3 μM ADP-induced platelet aggregation. The platelet disaggregation that was observed with 100 μM Ap₄A (Fig. 2) suggests that Ap₄A inhibited the P2Y₁₂ pathway. On the other hand, we also found that Ap₄A inhibits 3 μM ADP-induced platelet shape change (Fig. 4A), which is mainly mediated by P2Y₁[1,25].

ADP-induced platelet signaling through P2Y₁ and P2Y₁₂ receptors can be examined by specific assays, i.e. ADP-induced calcium increase for P2Y₁ receptors and ADP-induced VASP phosphorylation decrease for P2Y₁₂ receptors (Fig. 1). In the present study, 3 μM ADP-induced platelet cytosolic Ca²⁺ rise was inhibited by Ap₄A with an IC₅₀ of 40.8 μM (Fig. 4B). This result confirmed that Ap₄A is a P2Y₁ antagonist. In addition, the decrease in ADP-induced VASP phosphorylation was inhibited by Ap₄A in a dose-dependent manner (Fig. 3). While Ap₄A inhibition of ADP-stimulated, P2Y₁-mediated increase in cytosolic calcium was measured within seconds of adding Ap₄A and ADP to cells, the P2Y₁₂ mediated VASP response was measured 10 minutes after adding Ap₄A to whole blood, raising the possibility that metabolites of Ap₄A, and not Ap₄A *per se* may be responsible for effects on P2Y₁₂. However, this appears unlikely since HPLC analysis showed no breakdown of Ap₄A when incubated under VASP assay conditions. Taken together, these results indicate that Ap₄A is an antagonist for both P2Y₁ and P2Y₁₂ receptors. Such dual specificity on platelet P2Y₁ and P2Y₁₂ receptors has been recently reported for other compounds[29,30].

Although 3 μM ADP was used as the stimulant in all experiments, the IC₅₀ for inhibition of platelet aggregation (9.8 ± 2.8 μM) was 4-fold lower than the IC₅₀ for the inhibition of the P2Y₁-mediated increase of cytosolic Ca²⁺ (40.8 ± 12.3 μM) and more than 25-fold lower than the IC₅₀ for the P2Y₁₂-mediated decrease in VASP phosphorylation (>250 μM). Our results are similar to the findings of Cattaneo *et al.* [29] who reported that both MRS2298 and MRS2496 inhibited ADP-induced platelet aggregation, shape change and P2Y₁-mediated Ca²⁺ increase but, at higher concentrations, these compounds also partially inhibited ADP-induced decrease of cAMP, possibly via the P2Y₁₂ pathway. Furthermore, Cattaneo *et al.* [29] reported that the IC₅₀s of MRS2298 and MRS2496 for inhibition of platelet aggregation were lower than those for cytosolic Ca²⁺ increase and shape change. These data suggest synergism between P2Y₁ and P2Y₁₂ antagonism. Indeed, the synergism of antagonism between P2Y₁ and P2Y₁₂ receptors using selective antagonists for each receptor has been well documented *in vitro*[31]. The present findings and those of Cattaneo *et al.*[29] demonstrate that synergism of inhibition may occur for a single compound with dual receptor antagonist properties. However, the present findings are the first report of an endogenous molecule released by platelets that has a dual and synergistic inhibitory effect on both P2Y₁ and P2Y₁₂ receptors. This synergism may be explained, at least in part, by the recently reported reciprocal cross-talk between P2Y₁ and P2Y₁₂ receptors[32,33].

Agonist effects of Ap₄A on platelet P2Y₁₂ but not P2Y₁ receptors

In addition to the inhibitory effects of Ap₄A on P2Y₁ and P2Y₁₂ pathways, we demonstrated that Ap₄A attenuated PGE₁-stimulated VASP phosphorylation (Fig. 3). This attenuation was prevented by AR-C69931, demonstrating specificity for P2Y₁₂. These data indicate that Ap₄A either directly or indirectly (*e.g.*, via metabolites) results in platelet P2Y₁₂ activation. However, our study of metabolites after incubation with whole blood suggests such effects result directly from Ap₄A rather than its metabolites.

The apparently opposing effects of Ap₄A, as a weak agonist of P2Y₁₂, causing a decrease in VASP phosphorylation, and at the same time as a partial antagonist of P2Y₁₂, blocking ADP-stimulated decrease in VASP phosphorylation are both explained by a weak and inefficient interaction of Ap₄A with P2Y₁₂. When added alone, the effect of Ap₄A on VASP phosphorylation is maximal at 15.6 μM, suggesting saturation of Ap₄A binding to P2Y₁₂ at this Ap₄A concentration and higher. However, even with saturating concentrations of Ap₄A, only small decreases in VASP phosphorylation were observed, indicating the Ap₄A-P2Y₁₂ interaction triggers only a weak downstream signal. In contrast, ADP by itself causes a large decrease in VASP phosphorylation, suggesting efficient coupling of ADP-P2Y₁₂ with downstream events. Thus, the interaction of Ap₄A with P2Y₁₂, while causing weak downstream signals, prevents ADP from triggering stronger signals with larger decreases in VASP phosphorylation. The fact that very high concentrations of Ap₄A (250 μM) are required to block the ADP effect on VASP suggests that Ap₄A binding is reversible and that even binding of small amounts of ADP, as might occur over the 10 minute VASP assay incubation is sufficient to trigger a strong P2Y₁₂ coupled response.

Although Ap₄A and other diadenosine polyphosphates have been reported to activate P2Y₁ receptors in other cell types[34,35], their agonist activity on platelet P2Y₁ receptors is unknown. We found that Ap₄A stimulation did not result in P2Y₁-mediated increase of platelet cytosolic Ca²⁺ and, therefore, does not act as a platelet P2Y₁ receptor agonist.

Agonist effects of Ap₄A on platelet P2X₁ receptors

It is well known that Ap₄A is an agonist for P2X receptors in rat vas deferens and human urinary bladders[17,36]. In platelets, Sage *et al.*[4] showed that Ap₄A may induce a rise in cytosolic Ca²⁺ and suggested that this was mediated by P2X₁. Such an Ap₄A-induced platelet cytosolic Ca²⁺ rise was also observed in our study with extracellular Ca²⁺ available, but was obliterated when extracellular Ca²⁺ was removed by the chelating agent EGTA (Fig. 5A). These data confirm that the cytosolic Ca²⁺ increase resulted solely from an influx of extracellular Ca²⁺, *i.e.* a P2X-mediated effect (Fig. 1). Moreover, MRS2159, a selective P2X₁ antagonist[28], blocked the Ap₄A-induced influx of extracellular Ca²⁺, demonstrating Ap₄A is a platelet P2X₁ agonist. Although Ap₄A and other adenosine polyphosphates can be both agonists and antagonists of P2Y₁ and P2Y₁₂ receptors, the present study has demonstrated that Ap₄A functions only as an agonist, not an antagonist, for platelet P2X₁ receptors.

Possible pathophysiologic roles of Ap₄A

The plasma concentration of Ap₄A has been reported to range from 0.33 – 1.0 μM[8,10,13]. However, in the platelet-rich microenvironment where a thrombus is formed or high shear force is generated by vessel damage, the local concentration of Ap₄A may reach up to 100 μM as a result of dense granule release by involved platelets[8,10,13]. The present study has demonstrated that 0.1–1 μM Ap₄A has significant agonist effects on platelet P2X₁ receptors. In addition, Ap₄A inhibits ADP-induced aggregation and cytosolic Ca²⁺ increase with IC₅₀s of 9.8 ± 2.8 and 40.8 ± 12.3 μM, respectively. Although the concentrations of Ap₄A required to antagonize ADP effects on platelet P2Y₁ and P2Y₁₂ receptors are high compared to ADP, it is well known that Ap₄A and other diadenosine polyphosphates are more stable than ADP

and ATP[10]. Whereas ADP is rapidly inactivated by ecto-nucleotidase[37], Ap₄A is more resistant to hydrolysis[10] and therefore attains higher concentrations than ADP in plasma and in the local thrombotic microenvironment. Whether this high concentration can be reached in any pathophysiological conditions is unclear therefore the pathophysiologic role of high concentration Ap₄A is speculative.

Pharmacologic Perspectives

Ap₄A is the backbone of some chemical derivatives being developed as potential antiplatelet agents. *In vitro* antiplatelet[14,38] and *in vivo* antithrombotic effects[39,40] of Ap₄A and its derivatives have been reported, and antithrombotic potency is improved after certain chemical structure modifications[14,38]. The current study shows that Ap₄A has antagonist effects for platelet P2Y₁₂ receptors, the target of several effective antithrombotic agents including clopidogrel and prasugrel[41]. Ap₄A derivatives, while different in potencies and selectivity towards platelet receptors (results to be published), may have antiplatelet effects comparable to clinically available drugs such as clopidogrel. In clinical studies[5], standard doses of the widely-used, FDA-approved P2Y₁₂ antagonist clopidogrel reduced VASP PRI to 61.1 ± 17% mean ± SD) of maximal. In the present study, 62.5 μM Ap₄A resulted in a similar reduction of VASP PRI, to 61.5 ± 9.4%. Thus, Ap₄A released as a result of initial platelet activation may block subsequent activation of platelets to the same degree as pharmacological doses of clopidogrel. Furthermore, in the present study 250 μM Ap₄A reduced VASP PRI to 48.9 ± 6.2%.

The potential advantage of Ap₄A and its derivatives as therapeutic antiplatelet agents is that, unlike clopidogrel, prasugrel, or other P2Y₁₂ antagonist in development, they also have, as demonstrated in the present study, a synergistic inhibitory effect on platelet P2Y₁ receptors. One potential concern of using Ap₄A or its derivatives as antithrombotic agents might be their action on platelet P2X₁ receptors. Recent studies indicate that P2X₁ has an important role in platelet activation, particularly under conditions of shear stress[19]. Nevertheless, the present study on the mechanism of platelet inhibition by Ap₄A provides a framework for future development of Ap₄A-derived antiplatelet agents. Chemically modified Ap₄A derivatives may be compared to Ap₄A for their selective potency on platelet purinergic receptors.

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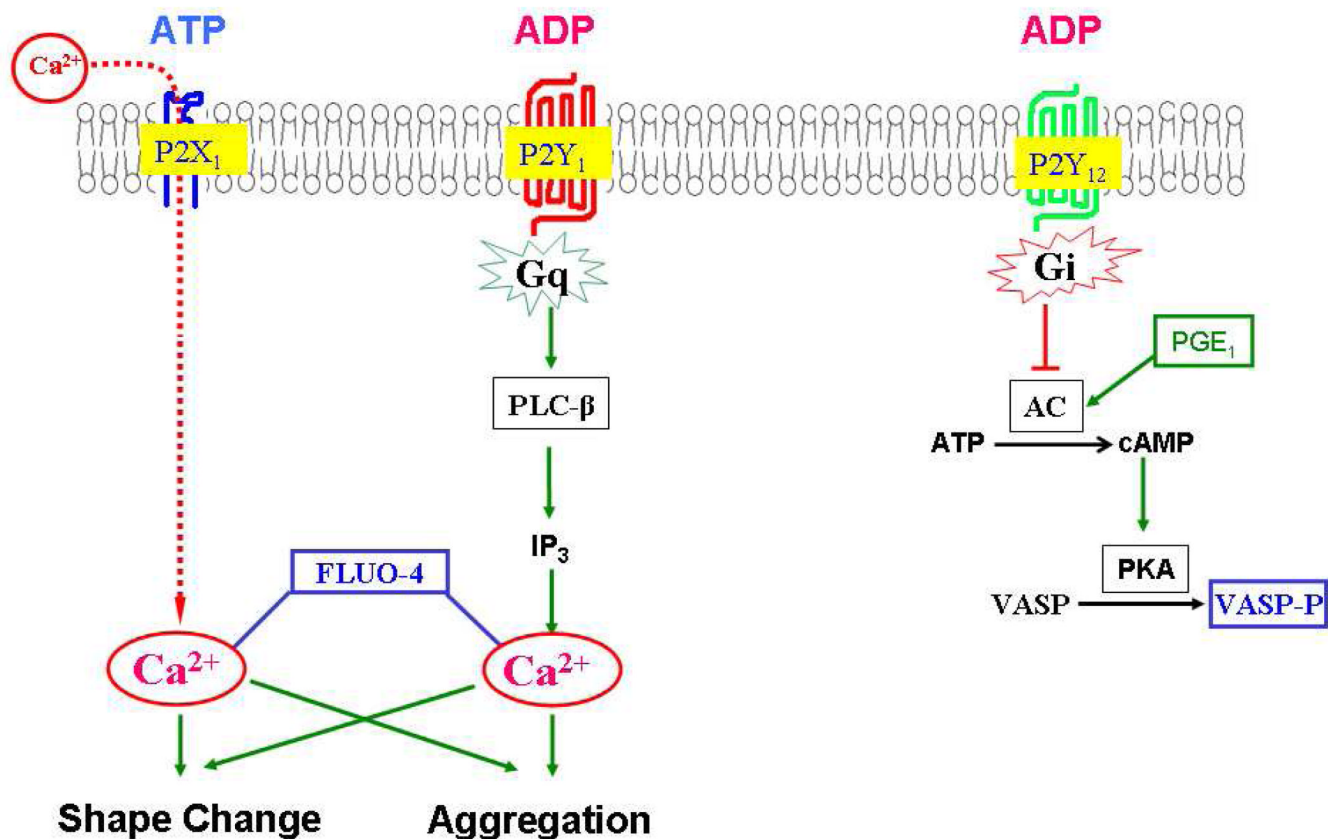


Figure 1. Platelets express three purinergic receptors, P2Y₁, P2Y₁₂ and P2X₁
 The ligand is ADP for P2Y₁ and P2Y₁₂, and ATP for P2X₁. P2X₁ is a ligand-gated ion channel which allows extracellular Ca²⁺ to shift into the cytoplasm upon activation. ADP binding to the P2Y₁ receptor induces activation of phospholipase C β (PLC-β), which, via inositol triphosphate (IP₃), subsequently leads to release of cytoplasmic Ca²⁺ pools. Both P2X₁ and P2Y₁ activation cause cytosolic Ca²⁺ increase, which in the present study was measured by flow cytometry with the Ca²⁺ indicator, FLUO-4. ADP binding to platelet P2Y₁₂ receptors results in a decrease of cytoplasmic cAMP by inhibiting adenylyl cyclase. cAMP subsequently leads, via protein kinase A (PKA), to phosphorylation of vasodilator stimulated phosphoprotein (VASP), which was measured by flow cytometry.

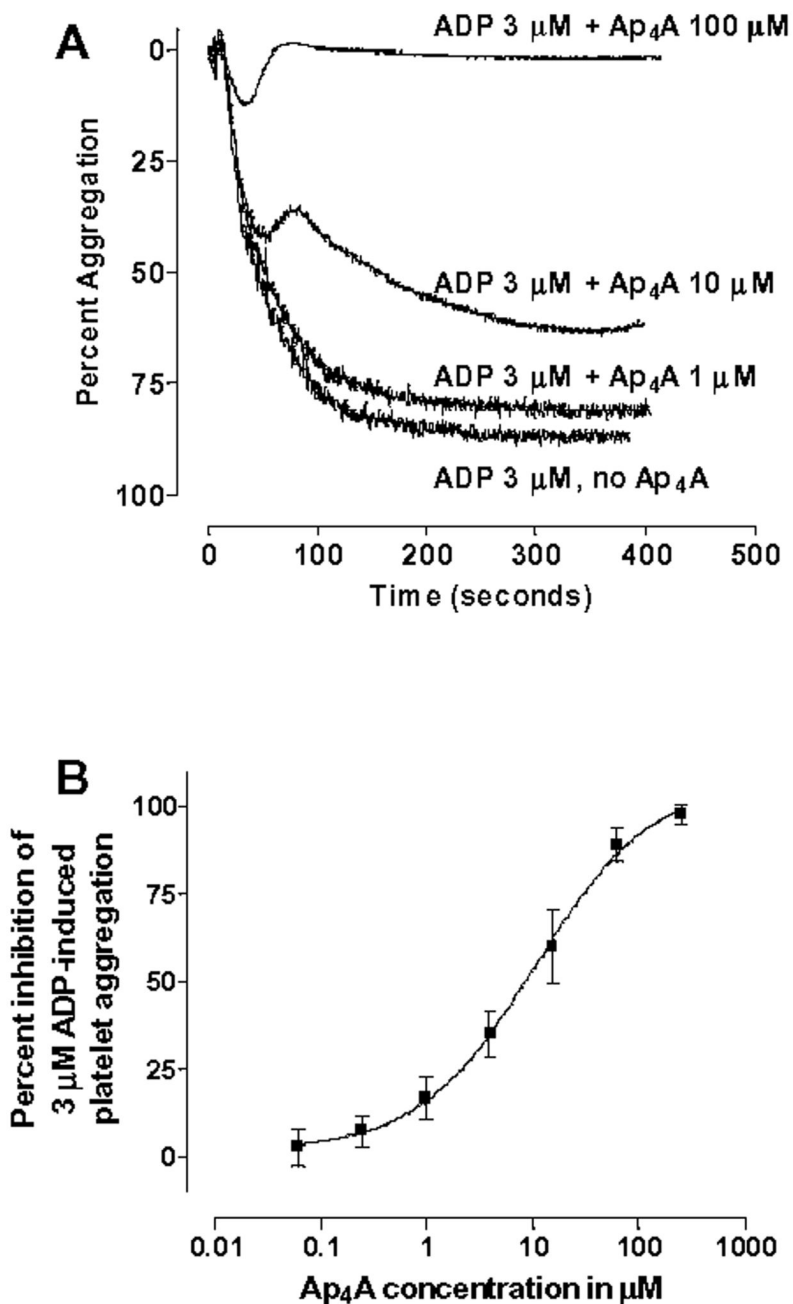


Figure 2. Ap₄A inhibits ADP-induced platelet aggregation

A, Platelet aggregation induced by 3 μ M ADP was inhibited by Ap₄A in a dose-dependent manner. At 100 μ M Ap₄A complete platelet disaggregation was observed. The experiments were performed in a Chrono-log® aggregometer. B, Dose response curve for Ap₄A inhibition of 3 μ M ADP-induced platelet aggregation (mean \pm SEM, n = 3). The results were obtained with the 96-well microplate reader method.

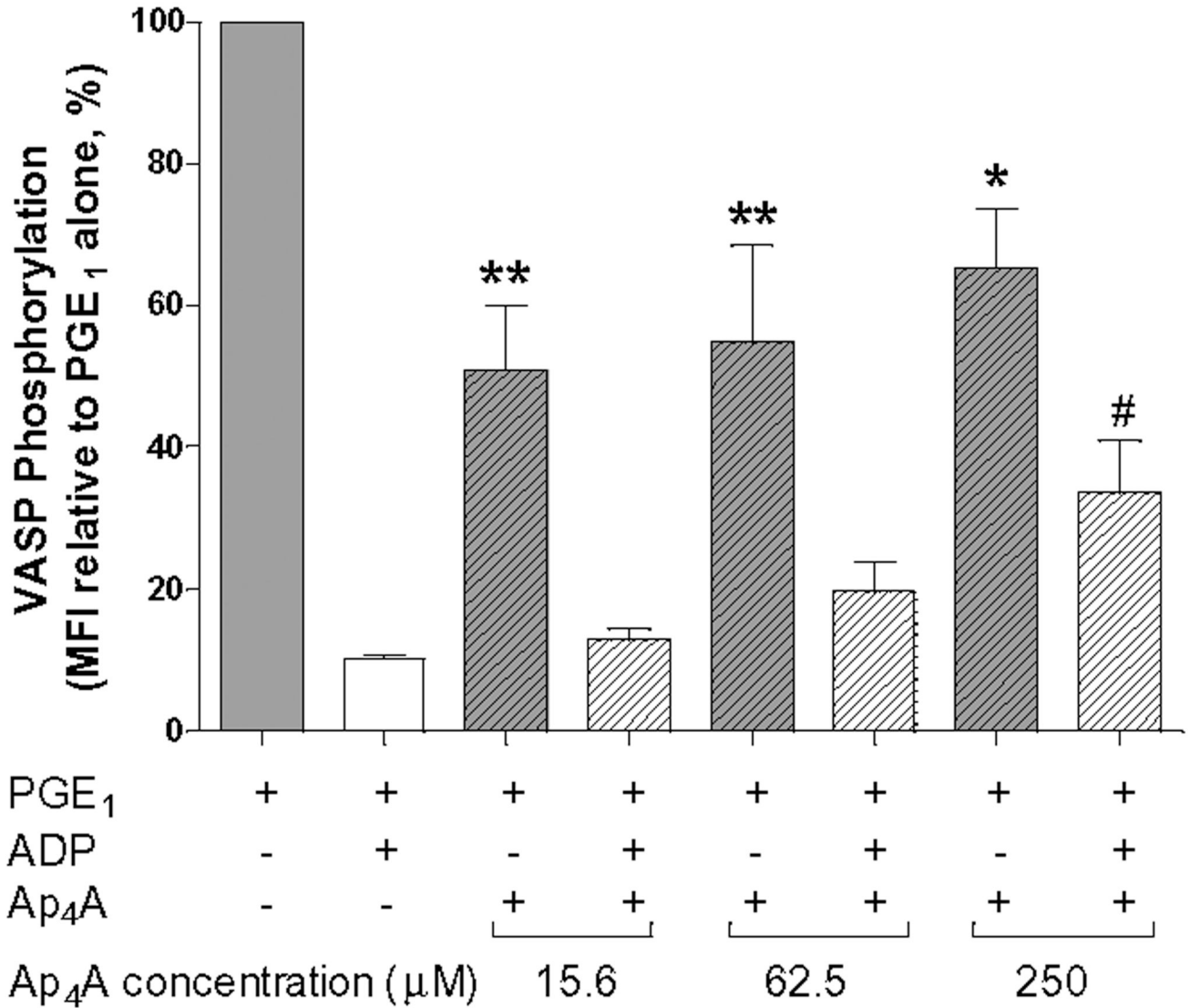


Figure 3. Ap₄A reduces VASP phosphorylation and attenuates the ADP-induced decrease in VASP phosphorylation

Platelets were treated with PGE₁ in every experiment, and the resultant MFI was used as unstimulated baseline (see Materials and Methods for details). As expected, the addition of 3 μM ADP reduced VASP phosphorylation (open bar). This ADP-induced decrease in VASP phosphorylation was attenuated by Ap₄A in a dose dependent manner (hash-marked open bars), # p < 0.05 compared with PGE₁ plus ADP (open bar). Ap₄A in the absence of ADP addition resulted in a decrease in VASP phosphorylation but not in a dose-dependent manner (hash-marked grey bars, * p < 0.05, ** p < 0.01 compared with PGE₁ alone). There was no statistically significant difference among the three tested concentrations of Ap₄A. The data (mean ± SEM, n = 3) were analyzed by one way ANOVA and Bonferroni's multiple comparison test.

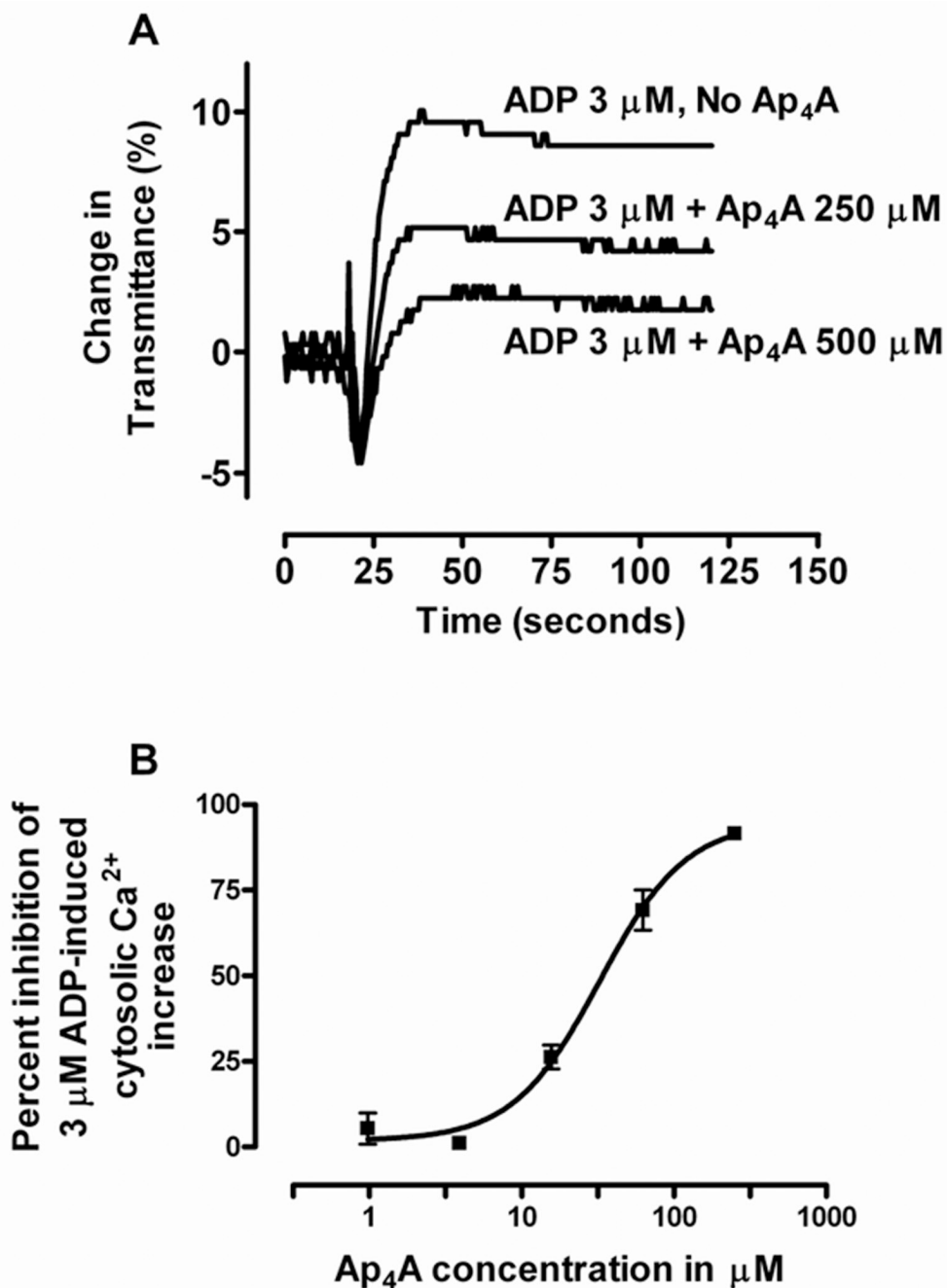


Figure 4. Ap₄A inhibits ADP-induced platelet shape change and cytosolic Ca²⁺ increase
 A, Platelet shape change (upward deflection) induced by 3 μ M ADP was inhibited by Ap₄A in a dose-dependent manner. Note that there was no platelet aggregation (downward deflection) after shape change because EDTA (10 mM) was mixed with PRP. Results shown are representative of 3 such experiments. B, Dose response curve of Ap₄A inhibition of 3 μ M ADP-induced intracellular Ca²⁺ increase, measured by flow cytometry with the calcium indicator FLUO-4 (mean \pm SEM, n = 4).

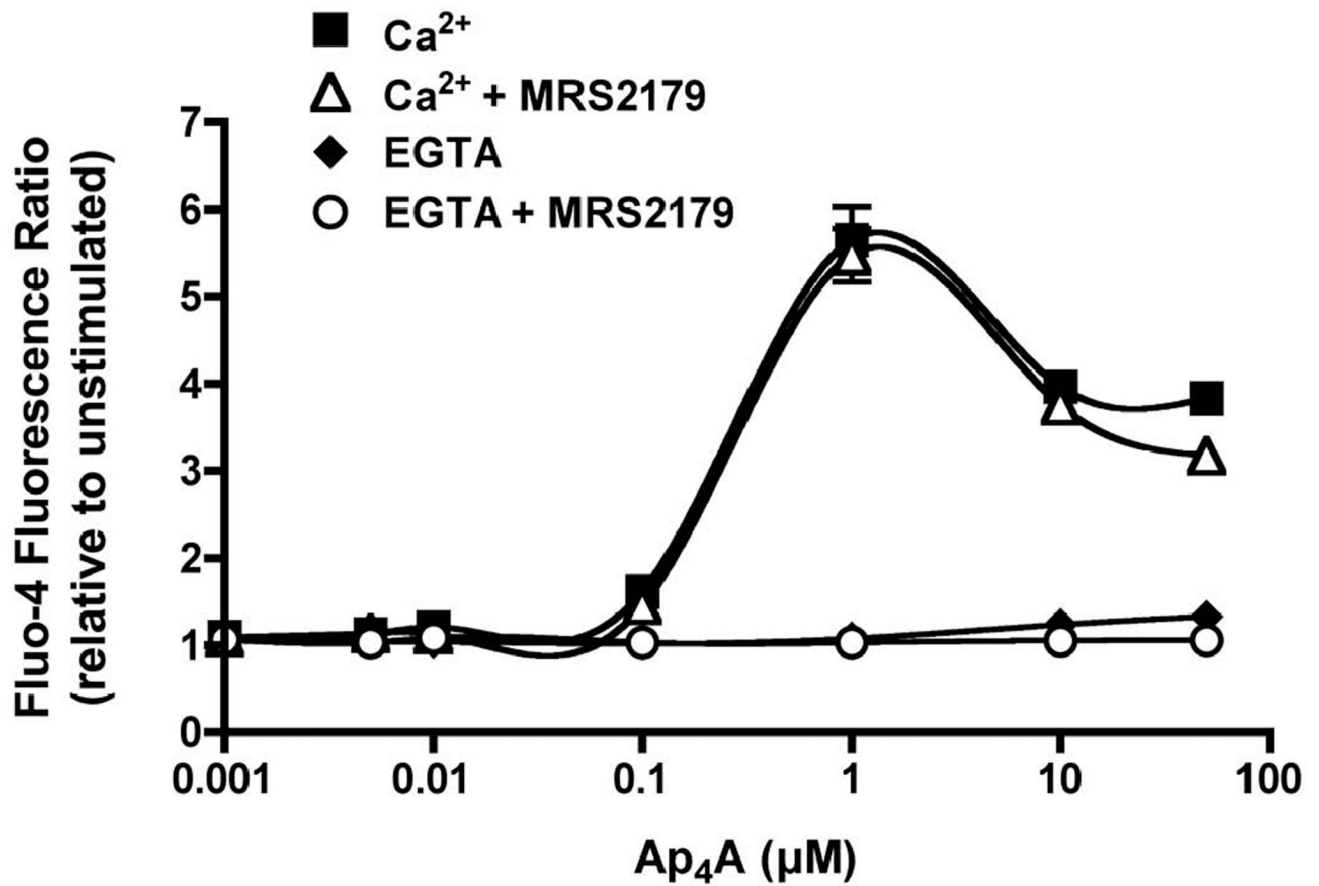


Figure 5. Dose response of Ap₄A-induced, P2X₁-mediated cytosolic Ca²⁺ increase. A
 The experiments were performed under conditions specific for P2X₁ (see Materials and Methods for details), with the indicated buffers: (■) 2 mM Ca²⁺; (◆) 1 mM EGTA, no Ca²⁺; (△) 2 mM Ca²⁺ plus 100 μM MRS2179; (○) 1 mM EGTA plus 100 μM MRS2179, no Ca²⁺. Results are mean ± SEM, n = 4.

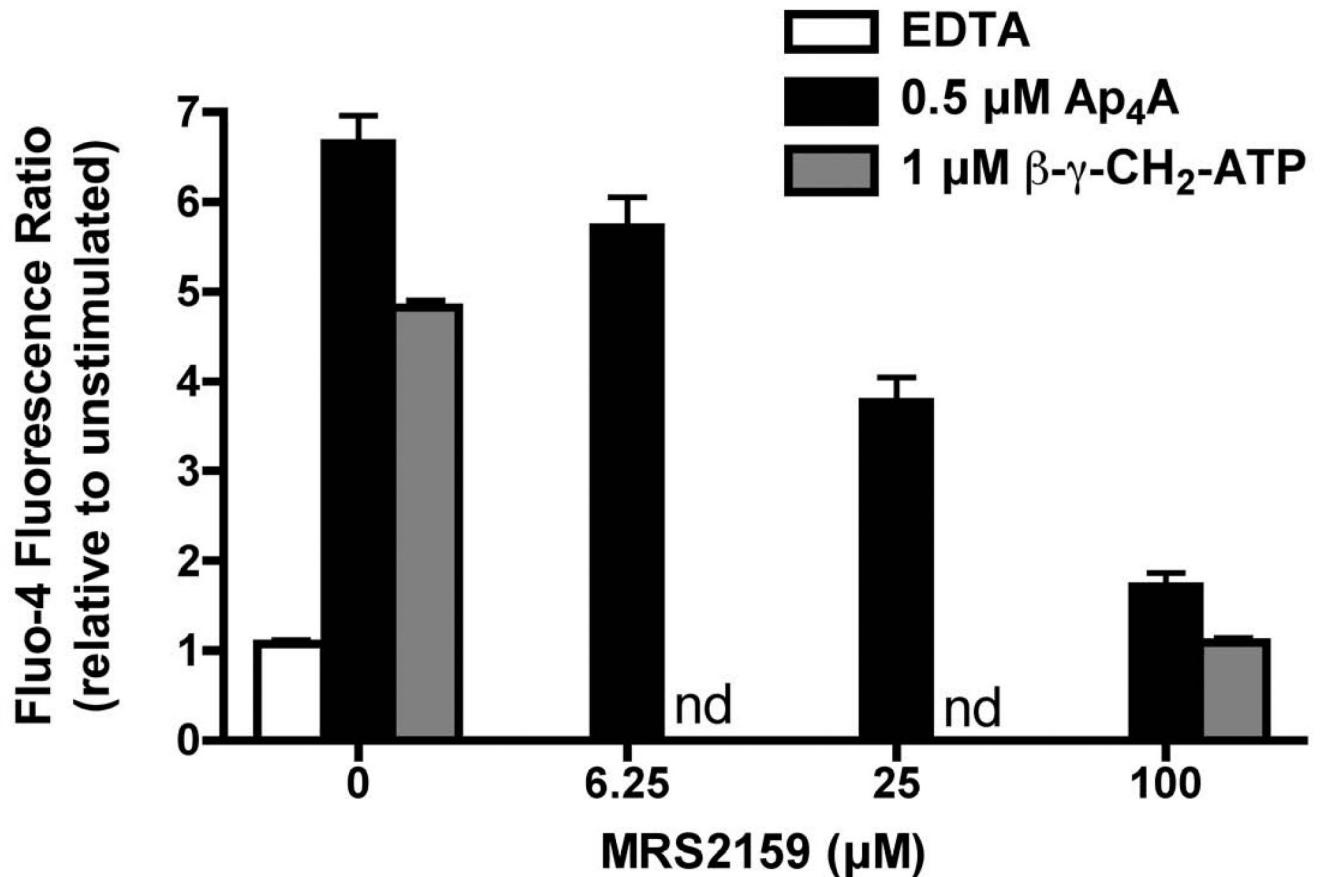


Figure 6. Dose-dependent MRS2159 inhibition of 0.5 µM Ap₄A- and 1 µM β,γ-CH₂-ADP-induced entry of extracellular Ca²⁺ into platelets

FLUO-4-loaded platelets suspended in 2 mM Ca²⁺ buffer were stimulated with either Ap₄A 0.5 µM (black bars) or 1 µM β,γ-CH₂-ATP (grey bars) in the presence of 0, 6.25, 25, or 100 µM MRS2159. Dependence on extracellular calcium is shown by results of control samples suspended in 2 mM EDTA without Ca²⁺ and stimulated with Ap₄A 0.5 µM (open bar). Results are mean ± SEM, n=3. Abbreviations: EDTA, ethylene diamine tetraacetic acid; nd, not done.

TableSummary of Ap₄A platelet stimulating and inhibiting activities.

Assay	Ap ₄ A Agonist Activity	Ap ₄ A Antagonist Activity (inhibition of ADP or β,γ -CH ₂ -ATP)*
Aggregation (P2Y ₁ and P2Y ₁₂ mediated)	no activation at up to 250 μ M	IC ₅₀ 9.8 \pm 2.8 μ M
Shape Change (P2Y ₁ mediated)	no activation at up to 250 μ M	IC ₅₀ ~200 μ M
P2Y ₁ release of internal Ca ²⁺	no activation at up to 100 μ M	IC ₅₀ 40.8 \pm 12.3
P2Y ₁₂ -mediated VASP	maximal at 15.6 μ M	IC ₅₀ >250 μ M
P2X ₁ entry of external Ca ²⁺	EC ₅₀ ~0.3 μ M (biphasic curve)	No inhibition at 50 μ M

* Ap₄A antagonist activity was determined relative to 3 μ M ADP stimulation of platelet aggregation, shape change, P2Y₁ release of internal Ca²⁺, and P2Y₁₂-mediated VASP and relative to 20 μ M β,γ -CH₂-ATP stimulation of P2X₁ entry of external Ca²⁺.