

Adenylic Dinucleotides Produced by CD38 Are Negative Endogenous Modulators of Platelet Aggregation*

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Diadenosine 5',5'''-P₁,P₂-diphosphate (Ap₂A) is one of the adenylic dinucleotides stored in platelet granules. Along with proaggregant ADP, it is released upon platelet activation and is known to stimulate myocyte proliferation. We have previously demonstrated synthesis of Ap₂A and of two isomers thereof, called P18 and P24, from their high pressure liquid chromatography retention time, by the ADP-ribosyl cyclase CD38 in mammalian cells. Here we show that Ap₂A and its isomers are present in resting human platelets and are released during thrombin-induced platelet activation. The three adenylic dinucleotides were identified by high pressure liquid chromatography through a comparison with the retention times and the absorption spectra of purified standards. Ap₂A, P18, and P24 had no direct effect on platelet aggregation, but they inhibited platelet aggregation induced by physiological agonists (thrombin, ADP, and collagen), with mean IC₅₀ values ranging between 5 and 15 μM. Moreover, the three dinucleotides did not modify the intracellular calcium concentration in resting platelets, whereas they significantly reduced the thrombin-induced intracellular calcium increase. Through binding to the purinergic receptor P2Y₁₁, exogenously applied Ap₂A, P18, and P24 increased the intracellular cAMP concentration and stimulated platelet production of nitric oxide, the most important endogenous antiaggregant. The presence of Ap₂A, P18, and P24 in resting platelets and their release during thrombin-induced platelet activation at concentrations equal to or higher than the respective IC₅₀ value on platelet aggregation suggest a role of these dinucleotides as endogenous negative modulators of aggregation.

The dinucleoside diphosphates diadenosine 5',5'''-P₁,P₂-diphosphate (Ap₂A),² adenosine guanosine diphosphate (Ap₂G), and diguanosine diphosphate (Gp₂G) represent a new class of growth-promoting extracellular mediators present in platelet secretory granules (1) and in cardiac myocytes (2), capable of stimulating cardiac myocyte proliferation (1) and believed to play a role in the control of cardiovascular tone (3). The intraplatelet concentration of each one of these dinucleotides has been estimated to be in the range between 30 and 100 μM (1). It has also been shown that the concentration of Ap₂A, Ap₂G, and Gp₂G in the supernatant of platelets stimulated with 0.05 units/ml thrombin is ~60% of the total intraplatelet amount of each dinucleoside diphosphate, suggesting that their primary function is extracellular (1). The enzyme responsible for their synthesis has not been identified, and the effect of these dinucleotides on platelet function has not yet been investigated.

We have recently demonstrated that ADP-ribosyl cyclases from *Axinella polypoides* (Porifera, Demospongiae), *Aplysia californica* (Molluscs), and human CD38 can synthesize three adenylic dinucleotides from cyclic ADP-ribose and adenine (Ade): Ap₂A and two isomers thereof, called P18 and P24, which are characterized by an unusual *N*-glycosidic bond between one adenine and the ribose (C1'-N1 in P18 and C1'-N3 in P24) (4). CD38 is present in human platelets (5, 6), and activation with thrombin induces an increase of CD38 activity associated with the platelet cytoskeleton (7). These results prompted us to investigate the presence of P18 and P24 in human platelets; the effect of Ap₂A, P18, and P24 on platelet aggregation; and possible synergistic or antagonistic effects of these adenylic dinucleotides with thrombin, ADP, or collagen, the most potent physiological platelet agonists.

EXPERIMENTAL PROCEDURES

Materials—If not otherwise indicated, all chemicals were from Sigma and were of the highest purity grade available.

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² The abbreviations used are: Ap₂A, diadenosine 5',5'''-P₁,P₂-diphosphate; Ap₂G, adenosine guanosine diphosphate; Gp₂G, diguanosine diphosphate; NO, nitric oxide; WP, washed platelet; 2MeSADP, 2-methylthioadenosine-5'-diphosphate; Ade, adenine; HPLC, high pressure liquid chromatography; PCA, perchloric acid; IP₃, inositol 1,4,5-trisphosphate.

Blood Collection and Platelet Preparation—Venous blood, freshly drawn from healthy volunteers, was collected in a 130 mM aqueous trisodium citrate anticoagulant solution (9:1). The donors declared not to have taken drugs known to interfere with platelet function during 2 weeks prior to blood collection. Washed platelets (WP) were prepared as described (8). Briefly, platelet-rich plasma, obtained by centrifugation of the whole blood at $100 \times g$ for 25 min, supplemented with 2 $\mu\text{g}/\text{ml}$ apyrase and 1 μM PGE₁, was centrifuged at $1000 \times g$ for 15 min. The platelet pellet was washed once with ACD solution (75 mM trisodium citrate, 42 mM citric acid, and 136 mM glucose, pH 4.8), centrifuged at $1000 \times g$ for 15 min, and then resuspended in Ca²⁺-free Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM glucose, 10 mM Hepes, pH 7.4) or in Ca²⁺-free Tyrode's-Hepes buffer (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 1 mM MgCl₂, 5 mM glucose, 5 mM Hepes, pH 7.4).

Cell Culture and Transfection—HeLa cells were grown on 24-well culture dishes and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine at 37 °C in a 5% CO₂ environment. Cells were transfected using FuGENE 6 (Roche Applied Science) transfection reagent 48 h prior to assay with either pcDNA 3.1 (empty vector), pcDNA3-hP2Y₁, or pcDNA3-hP2Y₁₂ together with pcDNA1-G $\alpha_{q/i}$. pcDNA1-G $\alpha_{q/i}$ directs expression of a chimera of G α_q containing the last five amino acids of G α_i , which couples G α_i -coupled receptors to activation of phospholipase C (9).

1321N1 cells expressing the human P2Y₁₁ receptor were generated by retroviral infection as described in Ref. 10 and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics.

Production and Purification of P18, P24, and Ap2A—P18 and P24, to be used for functional studies and as standards in the HPLC purification of the platelet dinucleotides, were produced with the purified ADP-ribosyl cyclase from *A. polyoides* and HPLC-purified as described by Basile *et al.* (4). ¹⁴C-Labeled P18, P24, and Ap2A were produced from [¹⁴C]NAD⁺ with the purified ADP-ribosyl cyclase from *A. polyoides* and HPLC-purified (4). Ap2A was synthesized and HPLC-purified as described by Rossi *et al.* (11).

HPLC Determination of P18, P24, and Ap2A Content in Human Platelets—The presence of the three adenylic dinucleotides was investigated both in resting and in thrombin-stimulated human platelets. In a typical experiment, 500- μl aliquots of WP suspension (5×10^8 platelets/ml) were incubated for 20 min at 37 °C without or with 0.05 units/ml thrombin. Thereafter, aliquots were centrifuged, each supernatant was recovered, and pellets were sonicated twice for 15 s with an interval of 1 min. Lysed platelets were centrifuged ($2600 \times g$ for 5 min), and supernatants and pellets were recovered and deproteinized with trichloroacetic acid (5% final concentration). Trace amounts of ¹⁴C-labeled P18, P24, and Ap2A were added to the acid extracts to calculate the percentages of recovery of the various dinucleotides at the end of the purification procedure. Excess trichloroacetic acid was removed by diethylether extraction, and samples were analyzed by HPLC for the presence of the adenylic dinucleotides. All HPLC analyses were performed on a Hewlett-Packard 1090 instrument, equipped with a diode array

spectrophotometric detector (HP 1040). Aliquots of 250 μl were subjected to the analytical phosphate HPLC (4). The column was a Delta Pak C18 column (150 \times 4.6 mm, 5 μm ; Waters). Solvent A was 0.1 M KH₂PO₄ containing 5 mM tetra-*n*-butylammonium, pH 5.0; solvent B was solvent A with 30% (v/v) methanol. The solvent program was a linear gradient starting at 100% A and increasing to 100% B in 30 min and then remaining at 100% B for 10 min. HPLC fractions containing P18, P24, or Ap2A were pooled, lyophilized, redissolved in deionized water, and injected into an analytical PLSAX column (50 \times 4.6 mm, 8 μm ; CPS). Solvent A was 20 mM ammonium bicarbonate, pH 7.6; solvent B was 500 mM ammonium bicarbonate, pH 7.6. The solvent program was the same as the one described above for reverse phase HPLC. The HPLC fractions containing the three dinucleotides were collected, dried, redissolved in deionized water, and reinjected into the analytical phosphate HPLC for final identification and quantitation (see Fig. 2 for representative chromatograms of the final purification step for each dinucleotide); P18, P24, and Ap2A were identified by comparison of their absorption spectra with computer-stored standards and were quantified by comparison of the computer-integrated peak areas with those of known amounts of the relevant dinucleotide standard. The percentage of recovery of the three adenylic dinucleotides at the end of the purification procedure was calculated from the yield of the corresponding radioactive tracer and was between 15 and 25% in the various purifications. Identification was confirmed by HPLC analysis of a nucleotide pyrophosphatase-digested parallel sample, showing conversion of the dinucleotide into the corresponding iso-AMP (or AMP for Ap2A) moiety (4). For final calculation of the intraplatelet concentration of the various dinucleotides, a platelet volume of 7 fl was considered.

P18, P24, and Ap2A Production and Degradation by Human Platelets—WP suspensions (1.0×10^9 platelets/ml) were sonicated and incubated at 37 °C with 0.65 mM NAD⁺ and 6.5 mM Ade in the presence of 2 mM MgCl₂, for dinucleotide production, or with 0.02 mM Ap2A, P18, or P24, for dinucleotide degradation. At various times, aliquots of the suspension were withdrawn and deproteinized with 5% trichloroacetic acid, excess trichloroacetic acid was removed by diethylether extraction, and samples were injected into the analytical phosphate HPLC (4).

Measurement of Platelet Aggregation—Platelet aggregation, performed on a Menarini Aggregometer PA-3210 aggregometer, was monitored according to Born's method (12) and quantified by light transmission for 3 min from the addition of the agonist. Platelet-rich plasma or WP (3.0×10^8 platelets/ml) were preincubated without (control) or with P18, P24, and Ap2A for 2 min at 37 °C before the addition of agonists (thrombin, ADP, or collagen).

Measurement of Intraplatelet Calcium Levels—WP (3.0×10^8 platelets/ml), resuspended in Hepes buffer, were incubated with 1 μM Fura-2/AM for 60 min at 37 °C. Prostaglandin E₁ (2 μM final concentration) and EGTA (1 mM final concentration) were added before centrifugation of the Fura-2-loaded platelets for 15 min at $1100 \times g$. The platelet pellet was resuspended at 2.0×10^8 platelets/ml in Hepes buffer and preincubated at 37 °C for 5 min with saline before the addition of the adenylic

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dinucleotides to be tested. When the effect of Ap2A, P18, and P24 was tested on platelets stimulated with thrombin, WP were preincubated with the adenylic dinucleotides for 5 min at 37 °C and then challenged with thrombin. Fluorescence of Fura-2-loaded platelets was measured at 37 °C in unstirred conditions in a PerkinElmer Life Sciences fluorescence spectrometer model LS50B with excitation at 340 and 380 nm and emission at 509 nm. The fluorescence of fully saturated Fura-2 (F_{\max}) was obtained by lysing platelets with 50 μM digitonin in the presence of 2 mM Ca^{2+} , whereas F_{\min} was determined by the subsequent addition of 20 mM EGTA. The fluorescence was fully quenched with 5 mM MnCl_2 to yield the autofluorescence value. Dedicated software converted data into cytosolic Ca^{2+} concentration applying a K_d value for Fura-2 and Ca^{2+} of 135 nM.

Inositol Phosphate Accumulation Assay in Transfected HeLa Cells—Cells were labeled overnight with 200 μl of serum-free, inositol-free Dulbecco's modified Eagle's medium containing 0.4 $\mu\text{Ci}/\text{well}$ ^3H -labeled *myo*-inositol (American Radiolabeled Chemicals, St. Louis, MO). Assays were initiated with the addition of 5 \times concentrations of the indicated compounds in 50 μl of 50 mM LiCl, 250 mM Hepes, pH 7.25. Following incubation for 15 min at 37 °C, the medium was removed by aspiration, and the reaction was terminated by the addition of 0.75 ml of boiling 10 mM EDTA, pH 8.0. [^3H]inositol phosphates were isolated by Dowex column chromatography as described previously (13).

Measurement of Nitric Oxide (NO) Production—WP (0.5 \times 10⁸ platelets/ml), resuspended in Hepes buffer containing 1 mM CaCl_2 and prewarmed at 37 °C for 10 min with saline or additions, were incubated with 40 μM L-arginine and the various dinucleoside diphosphates (10 μM), alone or with thrombin (0.05 units/ml), for 15 min at 37 °C under mild horizontal shaking. The incubation was stopped by sonicating samples on ice. To measure the NO content, 0.55-ml aliquots of supernatant were added to equal volumes of glycine buffer (15 g/liter glycine NaOH, pH 9.7) containing activated cadmium beds (Fluka AG), which catalyze the chemical reduction of nitrate to nitrite (14), and incubated overnight at room temperature under horizontal shaking. Cadmium beds were activated immediately before each experiment by three subsequent washings with 0.2 N H_2SO_4 , bidistilled water and glycine buffer, respectively. NO formation, determined by the Griess reagent (1% sulfanilamide in 2.5% H_3PO_4 , 0.1% naphthylendiamine dihydrochloride), was measured at 540 nm using a sodium nitrite calibration curve (15).

Determination of cAMP Levels in 1321N1 Cells and Human Platelets—Control and hP2Y₁₁-transfected 1321N1 cells were seeded in 35 \times 10-mm dishes (0.5 \times 10⁶ cells/dish). After 24 h, the medium was replaced with 0.6 ml of HBSS. P24, Ap2A, or buffer (control) were added at the indicated concentrations, and incubations were stopped after 0, 2.5, 5, or 15 min by removal of HBSS and the addition of 200 μl of ice-cold perchloric acid (PCA) (0.6 M). Cell extracts were collected and centrifuged to remove proteins; the cAMP content was measured on the neutralized cell extracts by radioimmunoassay ([^3H]cAMP assay system; GE Healthcare).

Aliquots (300 μl , 1 \times 10⁹ platelets/ml) of WP from different healthy donors were preincubated or not for 30 min at 37 °C with 1 μM NF157 prior to incubation with 10 μM P18, P24, or

Ap2A for 15 min. Incubations were stopped by the addition of 26 μl of 9 M PCA, and then each sample was sonicated in ice at 2.5 watts for 10 s and centrifuged at 2600 \times g for 3 min at 4 °C. The supernatant from each sample was collected, and the PCA was removed as described by Graeff and Lee (16). The cAMP content was measured on neutralized platelet extracts by radioimmunoassay ([^3H]cAMP assay system; GE Healthcare).

Determination of IP₃ Levels in 1321N1 Cells and Human Platelets—Control and hP2Y₁₁-transfected 1321N1 cells were seeded in 35 \times 10-mm dishes (0.5 \times 10⁶ cells/dish). After 24 h, the medium was replaced with 0.6 ml of HBSS. 10 μM P24 or Ap2A or buffer (control) were then added, and at 0, 0.5, 1, and 2.5 min after the addition, the incubation was stopped by the removal of HBSS, the addition of 300 μl of ice-cold 0.6 M PCA, and scraping of the cells. The IP₃ content was measured on the supernatants of the neutralized cell extracts by radioimmunoassay ([^3H]IP₃ Biotrak Assay System; Amersham Biosciences).

Aliquots (200 μl , 1 \times 10⁹ platelets/ml) of WP from different healthy donors resuspended in Hepes buffer were incubated at 37 °C for 30 s in the presence of 10 μM P24 or Ap2A or buffer (control). At the end of each incubation, the suspensions were withdrawn, and the reaction was stopped by adding 13 μl of 9 M PCA at 4 °C. PCA was removed as described (16). The intracellular IP₃ concentration was determined as described above.

Statistical Analyses—All parameters were tested by paired *t* test. *p* values of <0.05 were considered significant.

RESULTS

Production of P18, P24, and Ap2A by Human Platelets—Human CD38, along with the ADP-ribosyl cyclases from the sponge *A. polypoides* and from the mollusc *A. californica*, has been shown to produce P18, P24, and Ap2A from cyclic ADP-ribose (or NAD⁺) and Ade (Fig. 1) (4). CD38 is expressed in human platelets (5, 6), and its activity increases during platelet aggregation (7). Thus, we preliminarily tested whether platelets also produced these adenylic dinucleotides. Indeed, P18, P24, and Ap2A production was observed upon incubation of sonicated WP with NAD⁺ (precursor of cyclic ADP-ribose) and Ade (Table 1). Interestingly, dinucleotide production was higher in platelets stimulated with thrombin (0.05 units/ml) as compared with resting platelets (Table 1). In the presence of 20 mM nicotinamide, an inhibitor of ADP-ribosyl cyclase activity, production of the three adenylic dinucleotides was abrogated (Table 1). No degradation of P24 and Ap2A was detectable in lysed platelets, either resting or activated; conversely, P18 was hydrolyzed to ADPR and Ade, similarly to what was observed for recombinant human CD38 (4), and the P18-hydrolyzing activity decreased in thrombin-stimulated platelets (Table 1).

Presence of P18, P24, and Ap2A in Resting and in Thrombin-stimulated Platelets—It has been recently demonstrated that secretory granules of human platelets contain Ap2A, which is released during platelet aggregation evoked by thrombin (1). This observation, together with the ability of platelet CD38 to synthesize the Ap2A isomers P18 and P24, in addition to Ap2A (Table 1), prompted us to investigate whether P18 and P24 were also present in human platelets and whether they were released during aggregation. HPLC analysis of acid extracts of resting platelets confirmed the presence of Ap2A at a concentration

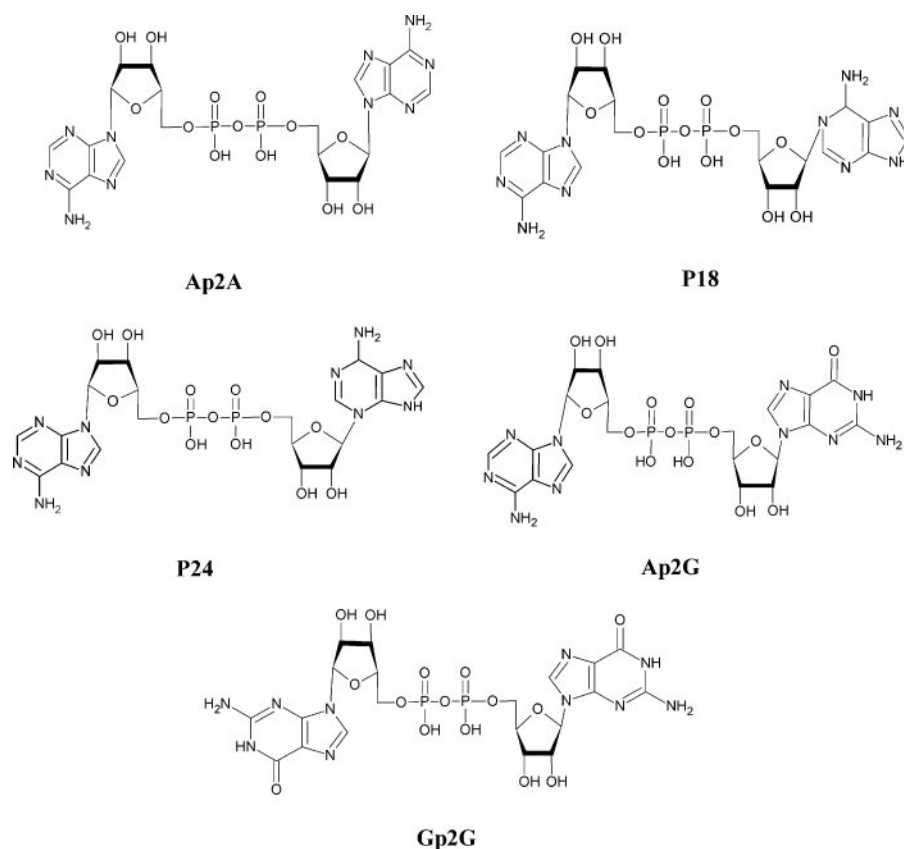


FIGURE 1. Structures of the dinucleotides Ap2A, P18, P24, Ap2G, and Gp2G. Ap2A and its isomers P18 and P24 differ in the position of the *N*-glycosidic bond linking one adenine to the ribose: C1'-N9 in Ap2A, C1'-N1 in P18, and C1'-N3 in P24. The structures of Ap2G and Gp2G, two dinucleotides known to be present in platelet granules, are shown for comparison.

TABLE 1
P18, P24, and Ap2A production by human platelets

WP suspensions (1×10^9 platelets/ml), preincubated or not with 0.05 units/ml thrombin in the presence or absence of 20 mM nicotinamide, were sonicated and incubated at 37 °C with 0.65 mM NAD⁺ and 6.5 mM Ade for dinucleotide production or with 0.02 mM Ap2A, P18, or P24 for dinucleotide degradation. At various times, aliquots of the suspensions were withdrawn, deproteinized, and analyzed by HPLC. Production and degradation of Ap2A, P18, and P24 by human platelets are expressed as pmol/min/ 10^9 platelets. Results are the mean of three experiments, performed on platelets from different healthy donors. ND, not detectable.

	Dinucleotide production			Dinucleotide hydrolysis	
	No additions	With thrombin	With thrombin and nicotinamide	No additions	With thrombin
	pmol/min/ 10^9 platelets			pmol/min/ 10^9 platelets	
P18	7.1 ± 0.3	12.2 ± 0.2	ND	6.3 ± 0.1	3.3 ± 0.1
P24	18.3 ± 1.1	26.4 ± 1.3	ND	ND	ND
Ap2A	9.1 ± 0.5	75.3 ± 2.8	ND	ND	ND

(~20 μM) similar to that already reported (1). Both Ap2A isomers were indeed detected at a similar intraplatelet concentration (~40 and 60 μM for P18 and P24, respectively). Neither Ap2A nor its isomers were detectable in the supernatant from resting platelets (Table 2). The intraplatelet concentration of all three adenylic dinucleotides increased in thrombin-stimulated, as compared with resting, platelets (1.5-, 1.3-, and 3-fold for P18, P24, and Ap2A, respectively; see Fig. 2 for representative chromatograms of the final purification step). All adenylic dinucleotides were released into the supernatant, where they were present at ~40, 50, and 15 μM for P18, P24, and Ap2A,

respectively (Table 2), when platelets were suspended at a concentration similar to that present in whole blood.

P18, P24, and Ap2A Inhibit Agonist-induced Platelet Aggregation—Production of P18, P24, and Ap2A from NAD⁺ and Ade by human platelet lysates, the presence of the dinucleotides in resting platelets, and their release upon platelet activation by thrombin prompted us to explore the possibility that P18, P24, and Ap2A may affect platelet aggregation. Pretreatment of platelets for 2 min at 37 °C with increasing concentrations of P18, P24, or Ap2A (1, 5, and 10 μM) had no effect on platelet aggregation, as observed during the following 3 min at 37 °C under stirring. Conversely, down-regulation of platelet aggregation was observed when platelets were pretreated with the adenylic dinucleotides and then challenged with ADP, thrombin, or collagen. As shown in Fig. 3, ADP-induced platelet aggregation was significantly reduced by all three adenylic dinucleotides, with IC₅₀ values of 7.2, 7.9, and 6.3 μM for P18, P24, and Ap2A, respectively (Table 3). In platelets treated with suboptimal concentrations of thrombin (0.05 units/ml) or collagen (1.5 μg/ml), aggregation was also inhibited in a concentration-dependent way by each of the three dinucleotides, although IC₅₀ values were slightly higher compared with those observed upon ADP-induced aggregation (Table 3).

P18, P24, and Ap2A Reduce the Thrombin-induced [Ca²⁺]_i Rise in Intact Platelets—During platelet aggregation, an increase of the intracellular calcium concentration ([Ca²⁺]_i) is known to occur downstream of the activation by ADP-, thrombin-, or collagen-specific receptors (17, 18). The inhibitory effect of P18, P24, and Ap2A on agonist-induced platelet aggregation suggested that we should investigate the effect of these dinucleotides on the [Ca²⁺]_i. To this purpose, platelets were preincubated with Ap2A, P18, or P24 and then stimulated with thrombin. In platelets challenged with thrombin (0.05 units/ml), the [Ca²⁺]_i increased rapidly from a resting value of 65 ± 11 nM to a maximal value of 172 ± 35 nM. At the same time point, the [Ca²⁺]_i increase observed in platelets pretreated for 5 min with the adenylic dinucleotides (10 μM) before the addition of thrombin was significantly reduced (Table 4). P18 exerted the highest percentage of inhibition (54%), in line with the lower IC₅₀ value observed upon thrombin-induced aggregation (Table 3). In the absence of thrombin, all three adenylic dinucleotides evoked a very limited increase of the [Ca²⁺]_i (≤8% of the basal value), which was apparently insufficient to

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induce platelet aggregation, since neither dinucleotide *per se* had any proaggregant effect (see above).

Are P18, P24, and Ap2A ADP Antagonists on P2Y₁ and P2Y₁₂ Receptors?—Platelet granules are known to contain high concentrations of adenylic nucleotides, most notably ADP and ATP, which are released during platelet aggregation and con-

tribute to a positive feedback mechanism, by acting through specific purinergic receptors on the platelet membrane (19–21). Thus, platelet aggregation induced by collagen, thrombin, or endothelial injury induces release of ADP, which in turn binds to P2Y₁ and P2Y₁₂ receptors, further stimulating aggregation (20). The structural homology between the adenylic dinucleotides (Ap2A, P18, and P24) and ADP prompted us to investigate whether they behaved as ADP antagonists on P2Y₁ or P2Y₁₂ receptors. HeLa cells were transfected with human P2Y₁ or co-transfected with human P2Y₁₂ and G $\alpha_{q/i}$ to generate a cell system responsive to ADP with an increase of the inositol phosphate concentration. Incubation of P2Y₁- or P2Y₁₂/G $\alpha_{q/i}$ -transfected cells with 30 μ M P24 did not increase the inositol phosphate concentration in either cell type (Fig. 4). Conversely, 1 μ M 2MeSADP, a stable analogue of ADP, strongly increased the inositol phosphate concentration both in P2Y₁- and P2Y₁₂/G $\alpha_{q/i}$ -transfected cells, and this increase was not inhibited by the simultaneous addition of P24 (Fig. 4). The inability of P24 to bind to P2Y₁ receptors was also confirmed by

TABLE 2

Presence of P18, P24, and Ap2A in platelets and supernatants from thrombin-stimulated platelets

P18, P24, and Ap2A were detected and quantified in acid extracts of platelets (Pellet) and in the supernatants (Sup) by HPLC (see details under "Experimental Procedures"). Mean results from at least three experiments are shown. ND, not detectable.

	Resting platelets		Thrombin-activated platelets	
	Pellet	Sup	Pellet	Sup
	μ M		μ M	
P18	41.1 \pm 3.2	ND	60.4 \pm 4.7 ^a	37.3 \pm 2.5
P24	63.5 \pm 5.8	ND	82.2 \pm 6.3 ^a	52.1 \pm 4.4
Ap2A	18.3 \pm 2.4	ND	55.1 \pm 3.6 ^a	15.6 \pm 1.1

^a $p < 0.01$ compared with the dinucleotide concentration in resting platelets.

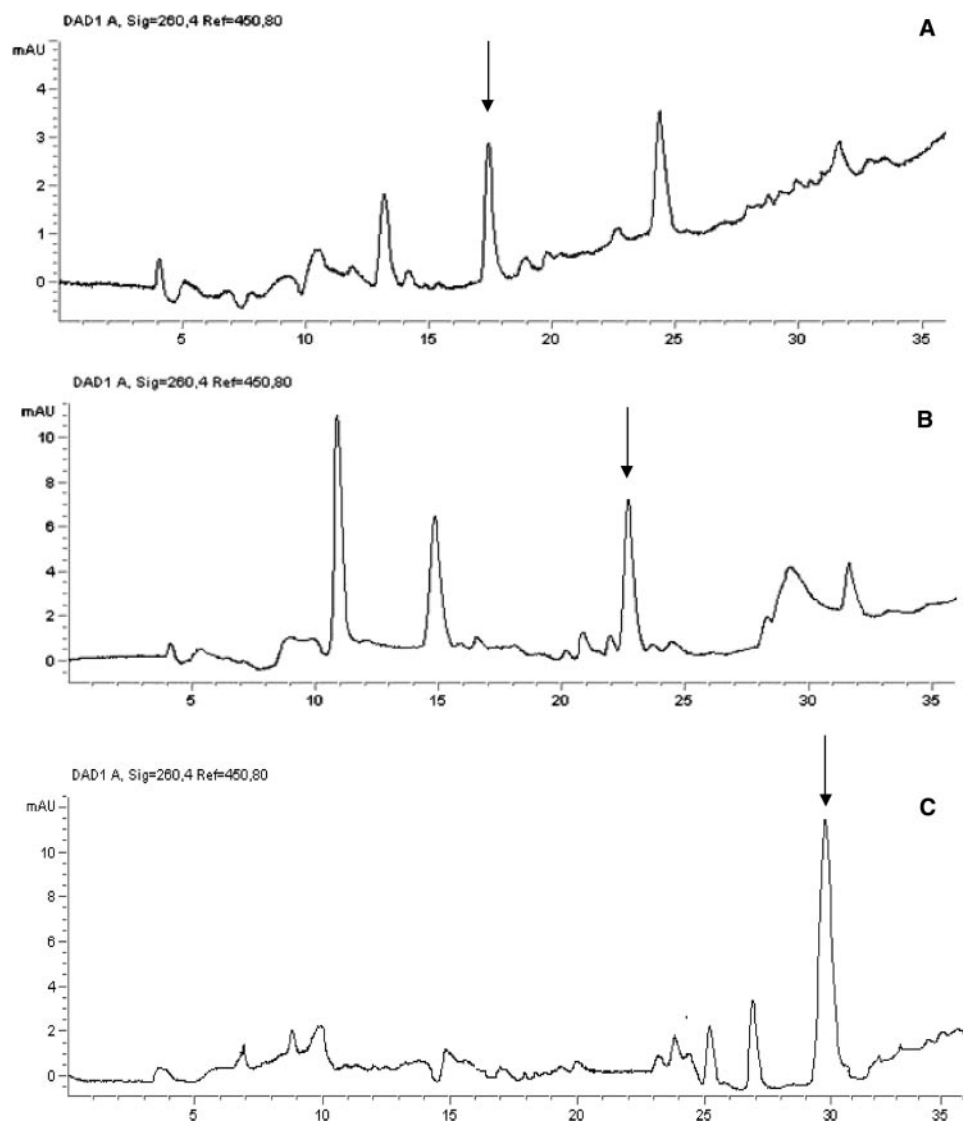


FIGURE 2. Representative chromatograms of the final purification step of P18 (A), P24 (B), and Ap2A (C) from thrombin-activated platelets.

Scatchard analysis. P24 failed to displace [³²P]MRS2500, a specific P2Y₁ receptor radioligand (22), from the P2Y₁ receptor (not shown). The fact that P24 did not interact with P2Y₁ or with P2Y₁₂ suggested that we should explore other mechanisms of action, resulting in an inhibition of platelet aggregation by Ap2A, P18, and P24.

P18, P24, and Ap2A Stimulate Platelet Nitric Oxide Production in the Presence of Thrombin—NO, a potent antagonist of platelet aggregation (23–25), is synthesized in human platelets by a constitutive NO synthase (26–28). The inhibitory effect of Ap2A, P18, and P24 on platelet aggregation induced by the physiological agonists ADP, thrombin, or collagen prompted us to investigate whether these adenylic dinucleotides could stimulate platelet NO production. WP (0.5 \times 10⁸ platelets/ml) resuspended in HEPES buffer containing 1 mM CaCl₂ and prewarmed at 37 °C for 10 min were incubated with 40 μ M L-arginine and the various dinucleoside diphosphates (10 μ M) alone or with thrombin (0.05 units/ml) for 15 min at 37 °C under mild shaking. In platelets treated with thrombin alone, NO production was not increased over control values, measured in untreated platelets (Fig. 5, white bars), as expected due to the suboptimal thrombin concentration used for platelet activation (29).

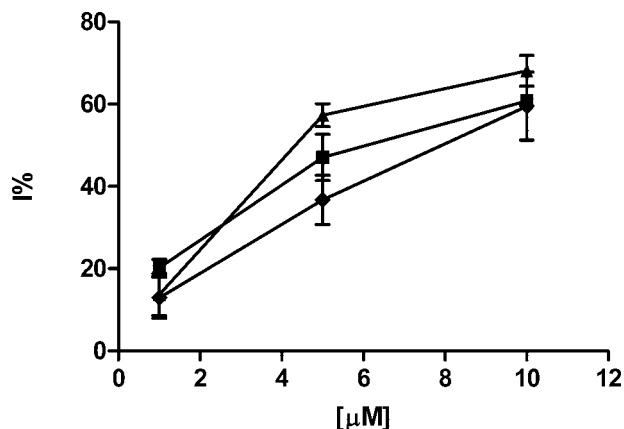


FIGURE 3. Down-regulation of ADP-induced platelet aggregation by P18, P24, and Ap2A. WP (3×10^8 platelets/ml) were preincubated for 2 min in the absence (control) or in the presence of increasing concentrations (1, 5, and 10 μM) of P18 (black squares), P24 (black rhombuses), or Ap2A (black triangles) and then incubated for 3 min with 2.5 μM ADP. Results are expressed as percentage of inhibition relative to controls and are the mean \pm S.D. of at least three different experiments, performed on platelets from different donors.

TABLE 3

IC₅₀ values of P18, P24, and Ap2A on platelet aggregation induced by ADP, thrombin, or collagen

WP (3×10^8 platelets/ml) were preincubated for 2 min in the presence or absence (control) of increasing concentrations of P18, P24, and Ap2A and then incubated for 3 min with 2.5 μM ADP, 0.05 units/ml thrombin, or 1.5 $\mu\text{g/ml}$ collagen. IC₅₀ values were calculated from dose-response curves (see Fig. 1) of the percentage of inhibition relative to controls. Results are the mean \pm S.D. of at least three different experiments, performed on platelets from different donors.

	IC ₅₀		
	ADP	Thrombin μM	Collagen
P18	7.2 \pm 0.6	7.3 \pm 0.7	12.8 \pm 1.2
P24	7.9 \pm 0.9	9.8 \pm 1.1	16.8 \pm 1.8
Ap2A	6.3 \pm 0.7	18.9 \pm 1.7	11.1 \pm 0.8

TABLE 4

Effect of P18, P24, and Ap2A on the thrombin-induced [Ca²⁺]_i rise in human platelets

FURA2-loaded WP (2×10^8 platelets/ml) were preincubated in the absence (control) or in the presence of the adenylic dinucleotides for 5 min at 37 °C and then challenged with 0.05 units/ml thrombin. The percentage of inhibition (*I*) on thrombin-induced [Ca²⁺]_i rise is reported. Results are the mean \pm S.D. of three different experiments performed on platelets from different healthy donors.

	<i>I</i>
	%
P18	54 \pm 5
P24	33 \pm 2
Ap2A	36 \pm 3

Neither adenylic dinucleotide, in the absence of thrombin, modified basal NO production (Fig. 5, striped bars). Conversely, in platelets incubated with thrombin and either of the three adenylic dinucleotides, NO production increased over control values, 3.1-, 2.1-, and 1.3-fold in P18-, P24-, and Ap2A-treated-platelets, respectively (Fig. 5, black bars).

P18, P24, and Ap2A Increase the [cAMP]_i and the [IP₃]_i in Human Platelets through P2Y₁₁ Receptor Stimulation—Stimulation of NO production by P18, P24, and Ap2A suggested that we should explore the effect of the dinucleotides on the [cAMP]_i, since NOS activity is known to be stimulated by PKA (30). Moreover, an increase of [cAMP]_i is known to induce a complete inhibition of platelet function (31, 32). Platelet incu-

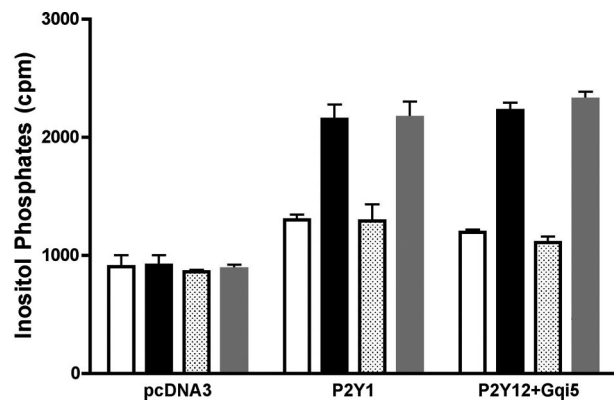


FIGURE 4. Inositol phosphate accumulation in HeLa cells transiently transfected with P2Y₁ or with P2Y₁₂/G_{q/i}. Cells transfected with pcDNA3 (empty vector) or P2Y₁ or P2Y₁₂/G_{q/i} were stimulated for 15 min with buffer (white bars) or 1 μM 2MeSADP (black bars) or 30 μM P24 alone (dotted bars) or with 1 μM 2MeSADP (gray bars). Inositol phosphate production was determined as described under "Experimental Procedures." Results are the mean \pm S.D. of at least three experiments.

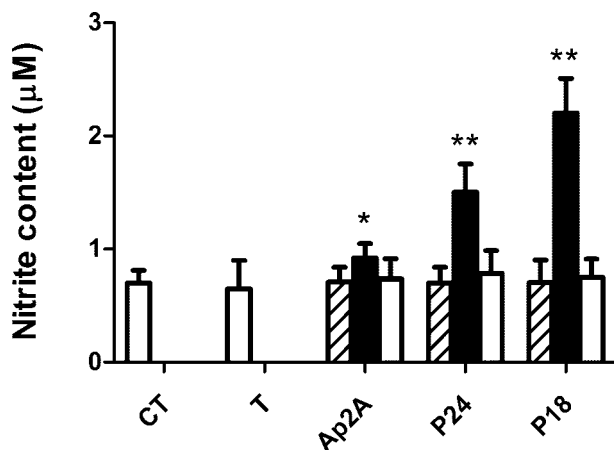


FIGURE 5. Effect of P18, P24, and Ap2A on NO production. WP (0.5×10^8 platelets/ml) were incubated for 15 min with 10 μM P18, P24, or Ap2A alone (striped bars) or in the presence of 0.05 units/ml thrombin without (black bars) or with 1 μM NF157 (gray bars). NO production was measured as detailed under "Experimental Procedures." CT, control, untreated platelets (white bars); T, platelets incubated with thrombin, in the absence of dinucleotides. Results are the mean \pm S.D. of five experiments, performed on platelets from different healthy donors. *, $p < 0.05$; **, $p < 0.001$ compared with control.

bation for 15 min at 37 °C with 10 μM P18, P24, or Ap2A indeed increased the [cAMP]_i 2.1-, 1.8-, or 1.5-fold, respectively, over values measured in untreated platelets (Fig. 6A; control, $n = 5$, $p < 0.05$). The only purinergic receptor known to stimulate adenylic cyclase is P2Y₁₁ (10, 33), which is also expressed in human platelets (34). Preincubation of platelets for 30 min with 1 μM NF157, a specific inhibitor of P2Y₁₁ (35), reduced cAMP overproduction induced by P18, P24, and Ap2A by ~70, 75, and 100%, respectively (Fig. 6A).

Since P2Y₁₁ is also coupled to a phospholipase C, we investigated whether the dinucleotides induced intraplatelet inositol 1,4,5-trisphosphate ([IP₃]_i) rise. As shown in Fig. 6B, platelet stimulation with 10 μM P24 or Ap2A evoked an [IP₃]_i increase with the highest values (1.45 \pm 0.21 and 1.58 \pm 0.26 relative to control, respectively) being recorded after 30 s of incubation. The [IP₃]_i rise produced by the two adenylic dinucleotides was significantly less than that observed with 0.1 units/ml thrombin, in agreement with the inability of the three adenylic nucleotides to

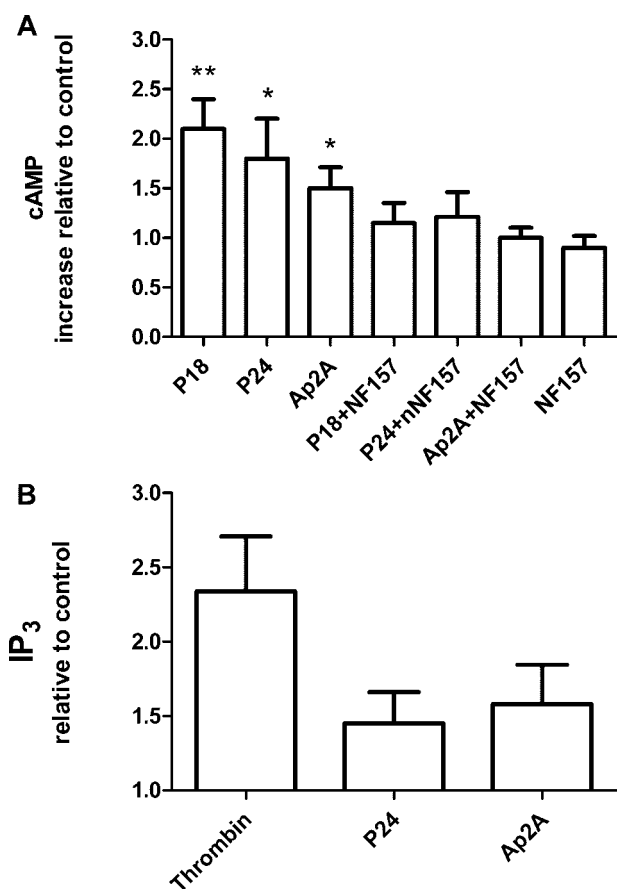


FIGURE 6. Increase of [cAMP]_i and [IP₃]_i in platelets stimulated with P18, P24, and Ap2A. A, [cAMP]_i in extracts from human platelets stimulated for 15 min at 37 °C with 10 μM P18, P24, or Ap2A or 5 μM NF157 or pretreated for 30 min with NF157 and then challenged with a 10 μM concentration of each of the three adenylic dinucleotides. cAMP production was measured as detailed under "Experimental Procedures." The basal intraplatelet concentration of cAMP was 2.49 ± 0.77 pmol/10⁸ platelets. B, [IP₃]_i in extracts from human platelets after a 30-s incubation at 37 °C with 0.1 units/ml thrombin or 10 μM P24 or Ap2A. The [IP₃]_i was measured as detailed under "Experimental Procedures." The basal intraplatelet concentration of IP₃ was 1.2 ± 0.2 pmol/10⁸ platelets. Results are the mean ± S.D. of at least three experiments for each assay, performed on platelets from different healthy donors. * p < 0.05; **, p < 0.01 compared with each dinucleotide in the presence of NF157.

evoke aggregation. Finally, we investigated whether stimulation of NO production by Ap2A, P18, and P24 was downstream of P2Y₁₁ activation. Pretreatment of platelets with 1 μM NF157 before stimulation with 10 μM P24 or Ap2A completely abrogated the release of NO by the two adenylic dinucleotides (Fig. 5).

P24 and Ap2A Induce a [cAMP]_i and [IP₃]_i Increase in Control and hP2Y₁₁-transfected 1321N1—To confirm that the observed increase of the [cAMP]_i and of the [IP₃]_i in platelets was a consequence of binding of the adenylic dinucleotides to the P2Y₁₁ receptor, we measured the concentration of these two intracellular second messengers in control and hP2Y₁₁-transfected 1321N1. Cells stimulated with 10 μM P24 or Ap2A showed a time-dependent increase in [cAMP]_i, with the maximum value reached for both adenylic dinucleotides after 15 min of incubation (Fig. 7A). Conversely, none of the two adenylic dinucleotides generated a [cAMP]_i rise in control 1321N1 (not shown).

Similar to what was observed for [cAMP]_i, the [IP₃]_i was also enhanced by 10 μM P24 or Ap2A only in P2Y₁₁-transfected

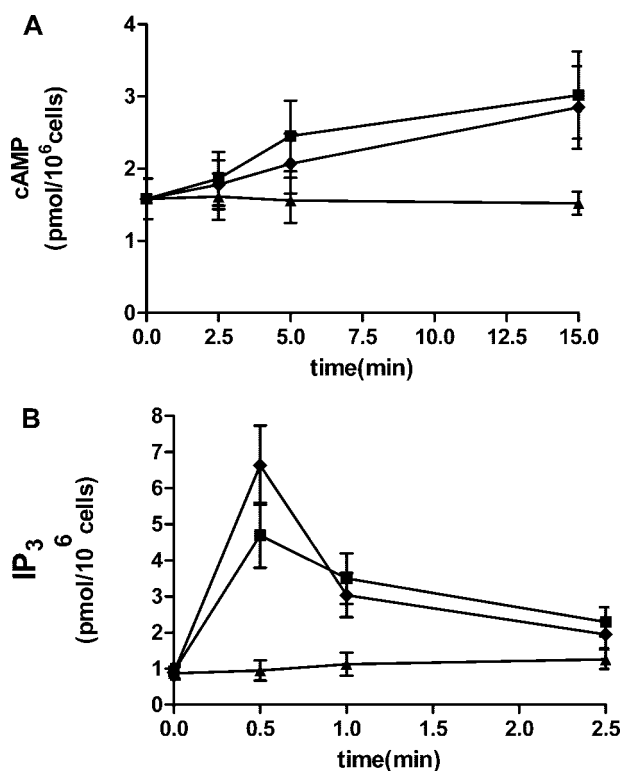


FIGURE 7. Increase of [cAMP]_i and [IP₃]_i in hP2Y₁₁-transfected 1321N1 cells. After the addition of 10 μM P24 (black rhombuses) or Ap2A (black squares) to control, untreated cells (black triangles), [cAMP]_i (A) or [IP₃]_i (B) levels were determined after 15 min and 30 s, respectively, as described under "Experimental Procedures." Results are the mean ± S.D. of at least three experiments for each assay.

cells, with the highest value reached for both adenylic dinucleotides at 30 s (Fig. 7B).

DISCUSSION

Here we show that platelets produce and release three adenylic dinucleotides, Ap2A and two isomers thereof, characterized by an unusual N-glycosidic bond between one of the adenines and the ribose: C1'-N1 and C1'-N3 in P18 and P24, respectively (Fig. 1) (4). Ap2A, P18, and P24 behave as negative modulators of platelet aggregation induced by the physiological agonists ADP, thrombin, or collagen.

The presence of Ap2A in human platelets was already reported, although the enzyme responsible for its synthesis and the effect of the dinucleotide on platelet aggregation were not investigated (1). Human CD38 has been recently demonstrated to synthesize Ap2A, P18, and P24 (Fig. 1) (4). The fact that synthesis of the adenylic dinucleotides by the platelet lysate is inhibited by nicotinamide (Table 1), a known inhibitor of ADP-ribosyl cyclase activity (36), strongly suggests that platelet CD38 is responsible for synthesis of Ap2A and of its isomers.

P18 and P24 are present in resting platelets at micromolar concentrations, similar to those already reported for Ap2A (1) (Table 2). Platelet activation by thrombin stimulates production of Ap2A, P18, and P24 (Table 2), suggesting the presence of a feedback mechanism, limiting platelet aggregation through generation of the three adenylic dinucleotides. Release of the adenylic dinucleotides from thrombin-activated platelets, at a

platelet density similar to that present in human plasma, results in extracellular concentrations of each dinucleotide equal to or higher than their IC_{50} value upon agonist-induced platelet aggregation (Table 3). These results suggest a role for Ap2A, P18, and P24 as negative endogenous modulators of platelet aggregation. Release of antiaggregant molecules by agonist-stimulated platelets is not unprecedented; platelet activation by thrombin, ADP, or collagen also results in the production of the most potent endogenous inhibitor of platelet activation, adhesion, and aggregation (*i.e.* NO) (25, 23, 37). Although it was recently reported that subthreshold concentrations of thrombin (<0.02 units/ml) induce release of very low (nanomolar) concentrations of platelet-derived NO, which behaves as a proaggregant (38), there is general agreement that proaggregant agonist concentrations induce release of higher (micromolar) concentrations of NO, which then behaves as a potent endogenous antithrombotic (30).

In nucleated cells, the biological activity is markedly different among Ap2A and its isomers. On human hemopoietic progenitors ($CD34^+$ cells), Ap2A stimulates proliferation, whereas P18 and, particularly, P24 induce apoptosis, with LD_{50} values on colony growth of 1.0 and 0.18 μM , respectively (4). P24 cytotoxicity, demonstrated on a wide range of different cell types (4), is due to its mitochondrial effects, which include inhibition of complex I of the respiratory chain and dissipation of the proton gradient ($\Delta\Psi_m$); neither P18 nor Ap2A show any effect on respiration or on $\Delta\Psi_m$ (39). In contrast to the diverse effects observed on nucleated cells, all three adenylic dinucleotides share the same inhibitory effect on agonist-induced platelet aggregation, with P18 showing the lowest IC_{50} values (Table 3). Since Ap2A, P18, and P24 all share a common ADP moiety, inhibition of platelet aggregation could be due to binding of these dinucleotides to the purinergic receptors involved in endogenous ADP-induced aggregation (*i.e.* $P2Y_1$ and $P2Y_{12}$). However, the following experimental findings rule out the possibility that the adenylic dinucleotides behave as ADP antagonists on $P2Y_1$ and on $P2Y_{12}$. P24 neither induced an increase of the $[IP_3]_i$ by itself nor antagonized the increase of the $[IP_3]_i$ induced by the synthetic purinergic agonist 2MeSADP on $P2Y_1$ -transfected and on $P2Y_{12}/G_i$ -transfected HeLa cells (Fig. 4). Moreover, P24 failed to displace $[^{32}P]MRS2500$, a specific $P2Y_1$ receptor radioligand (22), from the $P2Y_1$ receptor (not shown), ruling out a direct effect of this dinucleotide on $P2Y_1$.

The mechanism through which Ap2A and its isomers inhibit agonist-induced platelet aggregation seems to depend on (i) reduction of the agonist-triggered $[Ca^{2+}]_i$ rise (Table 4), which is causally related to platelet aggregation (17, 18); (ii) increased platelet production of NO (Fig. 5); and (iii) increased intraplatelet concentration of cAMP ($[cAMP]_i$) (Fig. 6). The following results indicate that the receptor mediating the antiaggregant effects of Ap2A, P18, and P24 is the purinergic receptor $P2Y_{11}$: (i) the $P2Y_{11}$ -specific inhibitor NF157 (35) prevents the increase of the $[cAMP]_i$ and of NO release induced in platelets by the adenylic dinucleotides (Figs. 5 and 6); (ii) 1321N1 cells transfected with hP2Y₁₁ respond to the adenylic dinucleotides with an increase of the $[cAMP]_i$ and of the $[IP_3]_i$ (Fig. 7), in agreement with the fact that $P2Y_{11}$ activates both adenylyl cyclase and phospholipase C (10, 33). The increase of the

intraplatelet $[IP_3]_i$ induced by the adenylic dinucleotides is significantly less than that observed with thrombin (Fig. 6B), in agreement with the absence of a significant $[Ca^{2+}]_i$ rise and of a proaggregant effect by the adenylic nucleotides *per se*.

$P2Y_{11}$ mRNA levels in platelets were reported to be much lower than those of $P2Y_1$, $P2Y_{12}$, and $P2X_1$, although the level of mRNA depends on both its synthesis and degradation rates and may not linearly correlate with its protein expression (34). Also in human granulocytes, $P2Y_{11}$ mRNA is present at lower levels compared with mRNA of other purinergic receptors (40). In fact, expression of the $P2Y_{11}$ protein in granulocytes was found to be even lower than that observed in platelets (41). Nonetheless, ATP, NAD^+ , and $NAADP^+$ have been shown to induce significant biochemical and functional effects in granulocytes through $P2Y_{11}$ ligation (41–43). At present, binding of Ap2A, P18, and P24 to other purinergic and/or adenosine receptors, in addition to $P2Y_{11}$, cannot be ruled out. Adenosine is known to induce an increase of the $[cAMP]_i$ and to exert antiaggregant effects on platelets by binding to A-type receptors (44), and Ap2A has been shown to be an agonist of A1 and A2 adenosine receptors in rat kidney vasculature (45). However, the fact that the $P2Y_{11}$ -specific antagonist NF157 inhibits by 100, 70, and 75% the $[cAMP]_i$ increase induced by Ap2A, P18, and P24, respectively, suggests that an effect of these dinucleotides on adenosine receptors may account for ≤ 20 –25% of the $[cAMP]_i$ increase and only for P18 and P24.

Both endogenous NO production and an intraplatelet $[cAMP]_i$ rise are known to inhibit platelet activation (25, 31, 32). NO increases intraplatelet cGMP, which inhibits cAMP phosphodiesterase (46), contributing to the elevation of the $[cAMP]_i$. Moreover, PKA is known to phosphorylate and activate platelet NOS (30). Thus, a positive feedback mechanism maintains elevated levels of $[cAMP]_i$ via NO. A high $[cAMP]_i$ inhibits IP_3 -induced Ca^{2+} release in human platelets and megakaryocytes (47, 48). The fact that Ap2A, P18, and P24 induce an increase of the $[cAMP]_i$ could result both in activation of NOS via PKA and in a reduction of the $[Ca^{2+}]_i$ in thrombin-stimulated platelets. NO release from platelets incubated with the adenylic dinucleotides occurred only in the presence of thrombin (Fig. 5), at a concentration (0.05 units/ml) that did not induce NO release *per se* (Fig. 5), as already reported (29, 49). A possible synergism between the pathways triggered by thrombin and by $P2Y_{11}$ and leading to NOS activation could explain this observation. A similar interpretation of their results was proposed by Radomski *et al.* (49), who reported that the antiaggregant effect of L-arginine on platelets stimulated with thrombin (0.03 units/ml) became evident only upon the addition of subthreshold concentrations of antiaggregant prostacyclin (a known stimulator of $[cAMP]_i$ increase).

A deficiency of bioactive NO is known to be associated with arterial thrombosis both in animal models and in humans (50, 51), and new NO donors with antithrombotic and vasodilating activities are being developed for treatment of arterial thrombosis (52). The discovery of new endogenous negative modulators of platelet aggregation that act through stimulation of platelet NO production could possibly lead to the development of a new family of antithrombotic drugs.

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