Diadenosine 5',5''-P1,P2-diphosphate (Ap2A) is one of the adenylic dinucleotides stored in platelet granules. Along with pro-aggregant 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 HPLC 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 HPLC through the 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, while 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 anti-aggregant. 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), adenosine guanosine diphosphate (Ap2G) and diadenosine 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 U/ml thrombin is approximately 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 (ADPRC) from Axinella polypoides, (Porifera, Demospongiae), Aplysia californica (Molluscs) and human CD38 can synthesize three adenylic dinucleotides from cADPR and adenine (Ado): 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 (Milan, Italy) and were of the highest purity grade available.

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 two 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 100xg for 25 min, supplemented with 2 µg/ml apyrase and 1 µM PGE$_{12}$, was centrifuged at 1000xg 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 1000xg for 15 min and then resuspended in Ca$^{2+}$-free Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 10 mM glucose, 10 mM HEPES, pH 7.4) or in Ca$^{2+}$-free Tyrode’s-Hepes buffer (134 mM NaCl, 12 mM NaHCO$_3$, 2.9 mM KCl, 1 mM MgCl$_2$, 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 DMEM supplemented with 10% FBS and 4 mM L-glutamine at 37°C in a 5% CO$_2$ environment. Cells were transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN) transfection reagent 48 h prior to assay with either pcDNA 3.1 (empty vector), pcDNA3-hP2Y$_1$, or pcDNA3-hP2Y$_{12}$ together with pcDNA1-Gaq/i. pcDNA1-Gaq/i directs expression of a chimera of Gaq containing the last five amino acids of Ga$i$, which couples Ga$i$-coupled receptors to activation of phospholipase C (9).

1321N1 cells expressing the human P2Y11 receptor were generated by retroviral infection as described in (10) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) 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 ADPRC from A. polypoides and HPLC-purified as described by Basile et al. (4). $[^{14}C]$-labelled P18, P24 and Ap2A were produced from $[^{14}C]$-NAD$^+$ with the purified ADPRC from A. polypoides 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 - 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 x 10$^8$ plts/ml) were incubated for 20 min at 37°C without or with 0.05 U/ml thrombin. Thereafter, aliquots were centrifuged, each supernatant was recovered and pellets were sonicated twice for 15 sec with an interval of 1 min. Lysed platelets were centrifuged (2600xg for 5 min), supernatants and pellets were recovered and deproteinized with trichloroacetic acid (TCA) (5% final concentration). Trace amounts of $[^{14}C]$-labelled 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 TCA was removed by diethyl ether extraction and samples were analyzed by HPLC for 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 x 4.6 mm, 5 µm, Waters); solvent A was 0.1 M KH$_2$PO$_4$ containing 5 mM tetra-n-butylammonium, pH 5.0; solvent B was solvent A with 30% (vol/vol) methanol. The solvent program was a linear gradient starting at 100% A and increasing to 100% B in 30 min, 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 x 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 re-injected 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 an NPP-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 x 10⁸ plts/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, deproteinized with 5% TCA, excess TCA 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 Aggrecooder PA-3210 aggregometer, was monitored according to Born’s method (12) and quantified by light transmission for 3 min from addition of the agonist. Platelet-rich plasma or WP (3.0 x 10⁸ plts/ml) were pre-incubated without (control) or with P18, P24 and Ap2A for 2 min at 37°C before addition of agonists (thrombin, ADP or collagen).

Measurement of intraplatelet calcium levels - WP (3.0 x 10⁸ plts/ml), resuspended in Hepes buffer, were incubated with 1 µM FURA 2 AM, for 60 min at 37°C. PGE₁ (2 µM final concentration) and EGTA (1 mM final concentration) were added before centrifugation of the FURA 2-loaded platelets for 15 min at 1100xg. The platelet pellet was resuspended at 2.0 x 10⁸ plts/ml in Hepes buffer and pre-incubated at 37°C for 5 min with saline before addition of the adenylic dinucleotides to be tested. When the effect of Ap2A, P18 and P24 was tested on platelets stimulated with thrombin, WP were pre-incubated 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 Perkin-Elmer Fluorescence Spectrometer model LS50B with excitation at 340 nm and 380 nm and emission at 509 nm. The fluorescence of fully saturated FURA 2 (Fₘₐₓ) was obtained by lysing platelets with 50 µM digitonin in the presence of 2 mM Ca²⁺, while Fₘᵢₙ was determined by the subsequent addition of 20 mM EGTA. The fluorescence was fully quenched with 5 mM MnCl₂ to yield the autofluorescence value. A dedicated software converted data into cytosolic Ca²⁺ concentration applying a K_d value for FURA 2 and Ca²⁺ of 135 nM.

Inositol Phosphates Accumulation Assay in transfected HeLa cells - Cells were labeled overnight with 200 µl serum-free, inositol-free DMEM containing 0.4 µCi/ well [³H]-myo-inositol (American Radiolabeled Chemicals, St. Louis, MO). Assays were initiated with the addition of 5x 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 terminated by addition of 0.75 ml boiling 10 mM EDTA, pH 8.0. [³H]Inositol phosphates were isolated by Dowex column chromatography as described previously (13).

Measurement of NO production - WP (0.5 x 10⁸ plts/ml), resuspended in Hepes buffer containing 1 mM CaCl₂ and pre-warmed 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 U/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/L 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 HSO₄, bidistilled water and glycine buffer, respectively. NO formation, determined by the Griess reagent (1% sulphanylamide 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 35x10 mm dishes (0.5x10⁶ cells/dish). After 24 h, the medium was replaced with 0.6 ml 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 addition of 200 µl ice-cold
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 ([^3]H)cAMP assay system, Amersham Bioscience AB, Uppsala, Sweden).

Aliquots (300 µl, 1 x 10^9 plt/ml) of WP from different healthy donors were pre-incubated 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 addition of 26 µl of 9 M PCA, then each sample was sonicated in ice at 2.5 W for 10 sec and centrifuged at 2600xg 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 ([^3]H)cAMP assay system, Ge-Healthcare, Milan, Italy).

Determination of IP3 levels in 1321N1 cells and human platelets - Control and hP2Y11-transfected 1321N1 cells were seeded in 35x10mm dishes (0.5x10^6 cells/dish). After 24 h, the medium was replaced with 0.6 ml HBSS. 10 µM P24 or Ap2A or buffer (control) were then added and at 0, 0.5, 1, 2.5 min after addition the incubation was stopped by removal of HBSS, addition of 300 µl ice-cold 0.6 M perchloric acid (PCA) and scraping of the cells. The IP3 content was measured on the supernatants of the neutralized cell extracts by radioimmunoassay ([^3]H[IP3] Biotrak Assay System, Amersham Bioscience AB, Uppsala, Sweden).

Aliquots (200 µl, 1 x 10^9 plt/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 9M 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 <0.05 were considered significant.

RESULTS

Production of P18, P24, and Ap2A by human platelets - Human CD38, along with the ADPRCs from the sponge A. polypoides and from the mollusc A. californica, has been shown to produce P18, P24 and Ap2A from cADPR (or NAD+) and adenine (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 adenyllic dinucleotides. Indeed, P18, P24 and Ap2A production was observed upon incubation of sonicated WP with NAD+ (precursor of cADPR) and Ade (Table 1). Interestingly, dinucleotide production was higher in platelets stimulated with thrombin (0.05 U/ml) as compared to resting platelets (Table 1). In the presence of 20 mM nicotinamide, an inhibitor of ADPRC activity, production of the three adenyllic dinucleotides was abrogated (Table 1). No degradation of P24 and Ap2A was detectable in lysed platelets, either resting or activated; conversely, P18 was hydrolysed to ADPR and Ade, similarly to what 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 presence of Ap2A at a concentration (approximately 20 µM) similar to that already reported (1). Both Ap2A isomers were indeed detected at a similar intraplatelet concentration (approximately 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 adenyllic dinucleotides increased in thrombin-stimulated, as compared to resting, platelets (1.5- to 3-fold for P18 and P24, respectively, see Fig. 2 for representative chromatograms of the final purification step). All adenyllic dinucleotides were released into the supernatant, where they were present at approximately 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, 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. Pre-treatment 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 minutes at 37°C under stirring. Conversely, down-regulation of platelet aggregation was observed when platelets were pre-treated 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 \( I_{50} \) values of 7.2, 7.9 and 6.3 µM for P18, P24 and Ap2A, respectively (Table 3). In platelets treated with sub-optimal concentrations of thrombin (0.05 U/ml) or collagen (1.5 µg/ml), aggregation was also inhibited in a concentration-dependent way by each of the three dinucleotides, although \( I_{50} \) values were slightly higher compared to those observed on ADP-induced aggregation (Table 3).

**P18, P24 and Ap2A reduce the thrombin-induced \([Ca^{2+}]_i\), rise in intact platelets** - During platelet aggregation, an increase of the intracellular calcium concentration ([\(Ca^{2+}\)]) 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 to investigate the effect of these dinucleotides on the [\(Ca^{2+}\)]. To this purpose, platelets were pre-incubated with Ap2A, P18 or P24 and then stimulated with thrombin. In platelets challenged with thrombin (0.05 U/ml), the [\(Ca^{2+}\)] increased rapidly from a resting value of 65±11 nM to a maximal value of 172±35 nM. At the same time point, the [\(Ca^{2+}\)] increase observed in platelets pre-treated for 5 min with the adenylic dinucleotides (10 µM) before addition of thrombin was significantly reduced (Table 4). P18 exerted the highest percentage of inhibition (54%), in line with the lower \( I_{50} \) value observed on thrombin-induced aggregation (Table 3). In the absence of thrombin, all three adenylic dinucleotides evoked a very limited increase of the [\(Ca^{2+}\)], (≈8% of the basal value), which was apparently insufficient to induce platelet aggregation, as neither dinucleotide per se had any pro-aggregant effect (see above).

**Are P18, P24 and Ap2A ADP antagonists on P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors?** - Platelet granules are known to contain high concentrations of adenylic nucleotides, most notably ADP and ATP, which are released during platelet aggregation and contribute 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 P2Y<sub>1</sub> and P2Y<sub>12</sub> 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 P2Y<sub>1</sub> or P2Y<sub>12</sub> receptors. HeLa cells were transfected with human P2Y<sub>1</sub> or co-transfected with human P2Y<sub>12</sub> and Goα/ι to generate a cell system responsive to ADP with an increase of the inositol phosphates concentration. Incubation of P2Y<sub>1</sub>- or P2Y<sub>12</sub>/Goα/ι-transfected cells with 30 µM P24 did not increase the inositol phosphates concentration in either cell type (Fig. 4). Conversely, 1 µM 2MeSADP, a stable analogue of ADP, strongly increased the inositol phosphates concentration both in P2Y<sub>1</sub>- and P2Y<sub>12</sub>/Goα/ι-transfected cells and this increase was not inhibited by the simultaneous addition of P24 (Fig. 4). The inability of P24 to bind to P2Y<sub>1</sub> receptors was also confirmed by scatchard analysis. P24 failed to displace [\(^{32}P\)]MRS2500, a specific P2Y<sub>1</sub> receptor radioligand (22), from the P2Y<sub>1</sub> receptor (not shown). The fact that P24 did not interact with P2Y<sub>1</sub> nor with P2Y<sub>12</sub> suggested to 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** - Nitric oxide (NO), a potent antagonist of platelet aggregation (23-25), is synthesized in human platelets by a constitutive NO synthase (cNOS) (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 x 10⁶ plts/ml) resuspended in Hepes buffer containing 1 mM CaCl₂ and pre-warmed at 37°C for 10 min, were incubated with 40 µM L-arginine and the various dinucleoside diphosphates (10 µM) alone or with thrombin (0.05 U/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 sub-optimal thrombin concentration used for platelet activation (29). 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).

\( \text{P18, P24 and Ap2A increase the [cAMP], and the} \ [IP_3], \text{in human platelets through P2Y}_{11} \text{ receptor stimulation} \) – Stimulation of NO production by P18, P24 and Ap2A suggested to explore the effect of the dinucleotides on the [cAMP], as NOS activity is known to be stimulated by PKA (30). Moreover, an increase of [cAMP] is known to induce a complete inhibition of platelet function (31, 32). Platelet incubation for 15 min at 37°C with 10 μM P18, P24 or Ap2A indeed increased the [cAMP] 2.1-, 1.8- or 1.5-fold respectively over values measured in untreated platelets (Fig. 6 panel A; control, n = 5, p < 0.05). The only purinergic receptor known to stimulate adenyl cyclase (AC) is P2Y\(_{11}\) (10, 33), which is also expressed in human platelets (34). Pre-incubation of platelets for 30 min with 1 μM NF157, a specific inhibitor of P2Y\(_{11}\) (35), reduced cAMP overproduction induced by P18, P24 and Ap2A by approximately 70, 75 and 100%, respectively (Fig. 6, panel A).

As P2Y\(_{11}\) is also coupled to a phospholipase C (PLC), we investigated whether the dinucleotides induced intraplatelet inositol-1,4,5-trisphosphate ([IP_3]) rise. As shown in Fig. 6 (panel B), platelet stimulation with 10 μM P24 or Ap2A evoked an [IP_3] rise with the highest values (1.45 ± 0.21 and 1.58 ± 0.26 relative to control, respectively) being recorded after 30 sec incubation. The [IP_3] rise produced by the two adenylic dinucleotides was significantly less than that observed with 0.1 U/ml Thrombin, in agreement with the inability of the three adenylic nucleotide to evoke aggregation. Finally, we investigated whether stimulation of NO production by Ap2A, P18 and P24 was downstream of P2Y\(_{11}\) activation. Pre-treatment 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).

\( \text{P24 and Ap2A induce a [cAMP], and [IP}_3, \text{increase in control and hP2Y}_{11}-\text{transfected 1321N1} \) – To confirm that the observed increase of the [cAMP] and of the [IP_3], in platelets was a consequence of binding of the adenylic dinucleotides to the P2Y\(_{11}\) receptor, we measured the concentration of the two intracellular second messengers in control and hP2Y\(_{11}\)-transfected 1321N1. Cells stimulation with 10 μM P24 or Ap2A showed a time-dependent increase in [cAMP] with the maximum value reached for both adenylic dinucleotides after 15 minutes of incubation (Fig. 7, panel A). Conversely, none of the two adenylic dinucleotides generated a [cAMP] rise in control 1321N1 (not shown).

Similarly to what observed for [cAMP], the [IP_3], was also enhanced by 10 μM P24 or Ap2A only in P2Y\(_{11}\)-transfected cells with the highest value reached for both adenylic dinucleotides at 30 sec (Fig. 7, panel B).

**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.

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 ADPRC 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 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 on 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 anti-aggregant 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. nitric oxide (NO) (25, 23, 37). Although it was recently reported that sub-threshold concentrations of thrombin (< 0.02 U/ml) induce release of very low (nM) concentrations of platelet-derived NO, which behaves as a pro-aggregant (38), there is general agreement that pro-aggregant agonist concentrations induce release of higher (µM) NO concentrations, which then behaves as a potent endogenous anti-thrombotic (30).

In nucleated cells, the biological activity is markedly different among Ap2A and its isomers. On human hemopoietic progenitors (CD34+ cells), Ap2A stimulates proliferation, while P18 and, particularly, P24, induce apoptosis, with LD50 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 (ΔΨm), neither P18 nor Ap2A show any effect on respiration or on ΔΨm (39). In contrast to the diverse effects observed on nucleated cells, all three adenylc dinucleotides share the same inhibitory effect on agonist-induced platelet aggregation, with P18 showing the lowest IC50 values (Table 3). As 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. P2Y1 and P2Y12). However, the following experimental findings rule out the possibility that the adenylc dinucleotides behave as ADP antagonists on P2Y1 and on P2Y12: P24 neither induced an increase of the [IP3]i by itself nor antagonized the increase of the [IP3]i induced by the synthetic purinergic agonist 2MeSADP on P2Y1 and on P2Y12/Gq-transfected HeLa cells (Fig. 4). Moreover, P24 failed to displace [3H]PMPRS2500, a specific P2Y1 receptor radioligand (22), from the P2Y1 receptor (not shown), ruling out a direct effect of this dinucleotide on P2Y1.

The mechanism through which Ap2A and its isomers inhibit agonist-induced platelet aggregation seems to depend on: i) reduction of the agonist-triggered [Ca2+]i rise (Table 4), which is causally related to platelet aggregation (7, 18); ii) increased platelet production of NO (Fig. 5) and iii) increased intraplatelet concentration of cAMP ([cAMP]) (Fig. 6). The following results indicate that the receptor mediating the anti-aggregant effects of Ap2A, P18 and P24 is the purinergic receptor P2Y11: i) the P2Y11-specific inhibitor NF157 (35) prevents the increase of the [cAMP] and of NO release induced in platelets by the adenylc dinucleotides (Figs. 5 and 6); ii) 1321N1 cells transfected with hP2Y11 respond to the adenylc dinucleotides with an increase of the [cAMP] and of the [IP3]i (Fig. 7), in agreement with the fact that P2Y11 activates both AC and PLC (10, 33). The increase of the intraplatelet [IP3]i induced by the adenylc dinucleotides is significantly less than that observed with thrombin (Fig. 6B), in agreement with the absence of a significant [Ca2+]i rise and of a pro-aggregant effect by the adenylc nucleotides per se.

P2Y11 mRNA levels in platelets were reported to be much lower than those of P2Y1, P2Y12 and P2X1, 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, P2Y11 mRNA is present at lower levels compared to mRNA of other purinergic receptors (40). In fact, expression of the P2Y11 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 P2Y11 ligation (41-43). At present, binding of Ap2A, P18 and P24 to other purinergic and/or adenosine receptors, in addition to P2Y11, cannot be ruled out. Adenosine is known to induce an increase of the [cAMP], and to exert anti-aggregant 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 P2Y11-specific antagonist NF157 inhibits by 100%, 70% and 75% the [cAMP] 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] increase, and only for P18 and P24.

Both endogenous NO production and an intraplatelet [cAMP] 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]. Moreover, PKA is known to phosphorylate and activate platelet NOS (30). Thus, a positive feed-back mechanism maintains elevated levels of [cAMP] via NO. A high [cAMP] inhibits IP3-induced Ca2+ release in human platelets and megakaryocytes (47, 48). The fact that Ap2A, P18 and P24 induce an increase of the [cAMP], could result in both activation of NOS via
PKA and in a reduction of the \([\text{Ca}^{2+}]\) 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 U/ml) which 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., who reported that the antiaggregant effect of L-arginine on platelets stimulated with thrombin (0.03 U/ml) became evident only upon addition of sub-threshold concentrations of anti-aggregant prostacyclin (a known stimulator of [cAMP], increase) (49).

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 anti-thrombotic and vasodilating activities are being developed for treatment of arterial thrombosis (52). The discovery of new endogenous negative modulators of platelet aggregation, which act through stimulation of platelet NO production could possibly lead to the development of a new family of anti-thrombotic drugs.
REFERENCES


FOOTNOTES
We are indebted to Prof. Antonio De Flora for critical reviewing of the manuscript. This work was supported in part by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), from the Italian Ministry of Education, University and Scientific Research (MIUR-PRIN 2003, MIUR FIRB RBAU019A3C, MIUR FIRB RBNE01ERXR and MIUR FIRB RBLA039LSF), the University of Genova and Fondazione Cassa di Risparmio di Genova e Imperia.

1 The abbreviations used are: Ap2A, Diadenosine 5′,5″-P1,P2-diphosphate; ADPRC, ADP-ribosyl cyclase; NO, nitric oxide; WP, washed platelet; NPP, Nucleotide pyrophosphatase; [Ca2+], intracellular calcium concentration; 2MeSADP, 2-methylthioadenosine-5′diphosphate; AC, Adenylyl cyclase; Ade, Adenine.
FIGURE LEGENDS

Fig. 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.

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

Fig. 3 Down-regulation of ADP-induced platelet aggregation by P18, P24 and Ap2A.
WP (3x10^8 plts/ml) were pre-incubated for 2 min in the absence (control) or in the presence of increasing concentrations (1, 5, 10 µM) of P18 (black squares), P24 (black rhombus), or Ap2A (black triangles) and then incubated for 3 min with 2.5µM ADP. Results are expressed as percentage of inhibition relative to controls and are the mean ± SD of at least three different experiments, performed on platelets from different donors.

Fig. 4 Inositol phosphates accumulation in Hela cells transiently transfected with P2Y_1 or with P2Y_{12}/Gq_i.
Cells transfected with pCDNA3 (empty vector) or P2Y_1 or P2Y_{12}/Gq_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 (grey bars). Inositol phosphates production was determined as described in Material and Methods. Results are the mean ± SD of at least three experiments.

Fig. 5 Effect of P18, P24 and Ap2A on NO production.
WP (0.5x10^8 plts/ml) were incubated for 15 min with 10 µM P18, P24 or Ap2A alone (striped bars) or in the presence of 0.05 U/ml thrombin without (black bars) or with 1 µM NF157 (grey bars). NO production was measured as detailed in Materials and Methods. CT, control, untreated platelets (white bars); T, platelets incubated with thrombin, in the absence of dinucleotides. Results are the mean ± SD of at least three experiments.

Fig. 6 Increase of [cAMP]_i and [IP_3]_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 alone or pre-treated with NF157 and then challenged with 10 µM of each one of the three adenylic dinucleotides. cAMP production was measured as detailed in Materials and Methods. The basal intraplatelet concentration of cAMP was 2.49±0.77 pmoles/10^8 platelets. B: [IP_3]_i, in extracts from human platelets after 30 sec incubation at 37°C with 1 µM thrombin or 10 µM P24 or Ap2A. The [IP_3]_i was measured as detailed in Materials and Methods. The basal intraplatelet concentration of IP_3 was 1.2±0.2 pmoles/10^8 platelets. Results are the mean ± SD of at least three experiments for each assay, performed on platelets from different healthy donors. *, p<0.05; **, p<0.01 compared to each dinucleotide in the presence of NF157.

Fig. 7 Increase of [cAMP]_i and [IP_3]_i in hP2Y_{11}-transfected 1321N1 cells.
After addition of 10 µM P24 (black rhombus) or Ap2A (black squares) to control, untreated cells (black triangles), [cAMP]_i or [IP_3]_i (B) levels were determined after 15 min and 30 sec, respectively, as described in Materials and Methods. Results are the mean ± SD of at least three experiments for each assay.
TABLE 1
P18, P24 and Ap2A production by human platelets.
WP suspensions (1x10^9 plts/ml), pre-incubated or not with 0.05 U/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 plt. Results are the mean of three experiments, performed on platelets from different healthy donors. nd, not detectable

<table>
<thead>
<tr>
<th></th>
<th>dinucleotide production (pmol/min/10^9 plt)</th>
<th>dinucleotide hydrolysis (pmol/min/10^9 plt)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+ Thrombin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P18</td>
<td>7.1±0.3</td>
<td>12.2±0.2</td>
</tr>
<tr>
<td>P24</td>
<td>18.3±1.1</td>
<td>26.4±1.3</td>
</tr>
<tr>
<td>Ap2A</td>
<td>9.1±0.5</td>
<td>75.3±2.8</td>
</tr>
</tbody>
</table>
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 and in the supernatants (sup) by HPLC (see details in Materials and Methods). Mean results from at least three experiments are shown. nd, not detectable.

<table>
<thead>
<tr>
<th></th>
<th>Resting platelets (µM)</th>
<th>Thrombin-activated platelets (µM)</th>
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<tr>
<td></td>
<td>pellet</td>
<td>sup</td>
</tr>
<tr>
<td>P18</td>
<td>41.1±3.2</td>
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</tr>
<tr>
<td>P24</td>
<td>63.5±5.8</td>
<td>nd</td>
</tr>
<tr>
<td>Ap2A</td>
<td>18.3±2.4</td>
<td>nd</td>
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</table>

* p<0.01 compared to the dinucleotide concentration in resting platelets
TABLE 3
IC$_{50}$ values of P18, P24 and Ap2A on platelet aggregation induced by ADP, thrombin or collagen.
WP (3x10$^8$ plts/ml) were pre-incubated 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 U/ml thrombin or 1.5 µg/ml collagen. IC$_{50}$ values were calculated from dose-response curves (see Fig. 1) of the percentage of inhibition relative to controls. Results are the mean ± SD of at least three different experiments, performed on platelets from different donors.

<table>
<thead>
<tr>
<th></th>
<th>ADP (µM)</th>
<th>Thrombin (µM)</th>
<th>Collagen (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P18</td>
<td>7.2±0.6</td>
<td>7.3±0.7</td>
<td>12.8±1.2</td>
</tr>
<tr>
<td>P24</td>
<td>7.9±0.9</td>
<td>9.8±1.1</td>
<td>16.8±1.8</td>
</tr>
<tr>
<td>Ap2A</td>
<td>6.3±0.7</td>
<td>18.9±1.7</td>
<td>11.1±0.8</td>
</tr>
</tbody>
</table>
**TABLE 4**  
Effect of P18, P24 and Ap2A on the thrombin-induced $[Ca^{2+}]_i$ rise in human platelets.  
FURA2-loaded WP (2x10$^8$ plt/ml) were pre-incubated in the absence (control) or in the presence of the adenylc dinucleotides for 5 min at 37°C and then challenged with 0.05 U/ml thrombin. The percentage of inhibition on thrombin-induced $[Ca^{2+}]_i$ rise is reported. Results are the mean ± SD of three different experiments performed on platelets from different healthy donors.

<table>
<thead>
<tr>
<th></th>
<th>% I</th>
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<tbody>
<tr>
<td>P18</td>
<td>54±5</td>
</tr>
<tr>
<td>P24</td>
<td>33±2</td>
</tr>
<tr>
<td>Ap2A</td>
<td>36±3</td>
</tr>
</tbody>
</table>
Fig. 1

Ap2A

P18

P24

Ap2G

Gp2G
Fig. 5

![Bar graph showing nitrite content (µM)](image-url)
Fig. 6

A

![Graph showing cAMP increase relative to control for different treatments.](image)

B

![Graph showing IP3 relative to control for different treatments.](image)
Fig. 7

A

B
Adenylic dinucleotides produced by CD38 are negative endogenous modulators of platelet aggregation

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