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Pharmacological Characterization of the Human $P2Y_{13}$ Receptor

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ABSTRACT

The $P2Y_{13}$ receptor has recently been identified as a new P2Y receptor sharing a high sequence homology with the $P2Y_{12}$ receptor as well as similar functional properties: coupling to G_i and responsiveness to ADP (Communi et al., 2001). In the present study, the pharmacology of the $P2Y_{13}$ receptor and its differences with that of the $P2Y_{12}$ receptor have been further characterized in 1321N1 cells (binding of [^{33}P]2-methylthio-ADP (2MeSADP) and of GTPγ[^{35}S]), 1321N1 cells coexpressing $G\alpha_{16}$ [AG32 cells: inositol trisphosphate (IP_3) measurement, binding of GTPγ[^{35}S]) and Chinese hamster ovary (CHO)-K1 cells (cAMP assay)]. 2MeSADP was more potent than ADP in displacing [^{33}P]2MeSADP bound to 1321N1 cells and increasing GTPγ[^{35}S] binding to membranes prepared from the same cells. Similarly, 2MeSADP was more potent than ADP in stimulating IP_3 accumulation after 10 min in AG32 cells and increasing cAMP in pertussis toxin-treated CHO-K1 cells stimulated by forskolin. On the other hand, ADP and 2MeSADP were equipotent at stimulating IP_3 formation in AG32 cells after 30 s and inhibiting forskolin-induced cAMP accumulation in CHO-K1 cells. These differences in potency cannot be explained by differences in degradation rate, which in AG32 cells was similar for the two nucleotides. When contaminating diphosphates were enzymatically removed and assay of IP_3 was performed after 30 s, ATP and 2MeSATP seemed to be weak partial agonists of the $P2Y_{13}$ receptor expressed in AG32 cells. The stimulatory effect of ADP on the $P2Y_{13}$ receptor in AG32 cells was antagonized by reactive blue 2, suramin, pyridoxal-phosphate-6-azophenyl-2′,4′disulfonic acid, diadenosine tetraphosphate, and 2-(propylthio)-5′-adenylic acid, monoanhydride with dichloromethylenebis (phosphonic acid) (AR-C67085MX), but not by N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate (MRS-2179) (up to 100 μM). The most potent antagonist was N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-5′-adenylic acid, monoanhydride with dichloromethylenebis (phosphonic acid) (AR-C69931MX) (IC_{50} = 4 nM), which behaved in a noncompetitive way. The active metabolite of clopidogrel was unable to displace bound 2MeSADP at concentrations up to 2 μM.

P2Y receptors are G protein-coupled receptors that are activated by extracellular nucleotides (Ralevic and Burnstock, 1998). A large variety of physiological functions is mediated by extracellular purine and pyrimidine nucleotides via P2Y receptors. For example, ADP activates platelet $P2Y_1$ and $P2Y_{12}$ receptors that play a crucial role in thrombus formation and stabilization (Fabre et al., 1999; Leon et al., 1999; Foster et al., 2001; Turner et al., 2001). ATP and UTP stimulate the airway mucociliary escalator via $P2Y_2$, and possibly $P2Y_4$ and $P2Y_6$, receptors that are expressed on epithelial cells (Communi et al., 1999) and represent potential targets for the therapy of cystic fibrosis and other pulmonary obstructive diseases (Cressman et al., 1999). ATP seems to play a role in the immune system through the $P2Y_{11}$ receptor activation that leads to granulocytic differentiation (Communi et al., 2000) and dendritic cell maturation (Wilkin et al., 2001).

The recent molecular cloning of the platelet ADP receptor $P2Y_{12}$ (Hollopeter et al., 2001) revealed the existence of two

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ABBREVIATIONS: AR-C67085MX, 2-(propylthio)-5′-adenylic acid, monoanhydride with dichloromethylenebis (phosphonic acid); 2MeS, 2-methylthio-; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); CHO, Chinese hamster ovary; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Ap_3A, diadenosine triphosphate; Ap_4A, diadenosine tetraphosphate; Ap_5A, diadenosine pentaphosphate; Ap_6A, diadenosine hexaphosphate; CP, creatine phosphate; CPK, creatine phosphokinase; TBAP, tetrabutylammonium dihydrogen phosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′disulfonic acid; 8-*p*-SPT, 8-(*p*-sulfophenyl)theophylline; AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-5′-adenylic acid, monoanhydride with dichloromethylenebis (phosphonic acid); MRS-2179, N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate; KRH, Krebs-Ringer-HEPES; HPLC, high-performance liquid chromatography; IP_3, inositol trisphosphate; RB-2, reactive blue-2.

structurally-distinct subfamilies of P2Y receptors. The first one is composed of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, all coupled to the phospholipase C pathway, except $P2Y_{11}$, which is also positively coupled to the cAMP pathway. The members of the second subfamily are $P2Y_{12}$, $P2Y_{13}$, and the UDP-glucose receptor recently renamed $P2Y_{14}$, which are all coupled to G_i. This group shows a low amino acid identity with the other P2Y receptors but shares some conserved positively charged amino acids in TM6 and TM7 (Erb et al., 1995). The $P2Y_{12}$ receptor is the target of antithrombotic agents: clopidogrel, which acts via the covalent binding of an active metabolite (Savi et al., 2001), and ATP derivatives (AR-C69931MX, etc.), which behave as competitive antagonists (Ingall et al., 1999).

The human $P2Y_{13}$ receptor was recently identified (Communi et al., 2001; Zhang et al., 2002). Its sequence displays 48% amino acid identity with the $P2Y_{12}$ receptor. It is coupled to G_i and responds to diphosphate adenine nucleotides (ADP, 2MeSADP, ADPβS). The abundance of $P2Y_{13}$ messengers is highest in the brain and the spleen, suggesting roles in nervous and immune systems. As a preliminary step to the evaluation of its potential physiological roles, we have now performed a detailed pharmacological characterization of the $P2Y_{13}$ receptor.

Materials and Methods

Cell Culture and Transfection. CHO-K1 and AG32 cells were transfected with the recombinant $P2Y_{13}$-pEFIN5 plasmid or the plasmid alone using FuGENE6 transfection reagent (Roche Applied Science, Mannheim, Germany) as described previously (Communi et al., 2001). AG32 are 1321N1 cells transfected with the recombinant $G\alpha_{16}$-pERAEQ2 plasmid.

Materials. ADP, ADPβS, 2MeSADP, ATP, 2MeSATP, ATPγS, Ap_3A, Ap_4A, Ap_5A, Ap_6A, 2MeSAMP, poly[A], poly[A].[G], creatine phosphate (CP), creatine phosphokinase (CPK), apyrase (grade I), TBAP, iodoacetamide, and pertussis toxin were from Sigma Chemicals (St. Louis, MO). Suramin, reactive blue 2, PPADS and 8-*p*-SPT were from RBI/Sigma (Natick, MA). AR-C67085MX and AR-C69931MX were generous gifts from Drs. J. D. Turner and D. Shah (AstraZeneca, Wilmington, DE). MRS-2179 was obtained from Tocris Chemicals (Ellisville, MO). Forskolin was purchased from Calbiochem (La Jolla, CA). Rolipram was a gift from the Laboratories Jacques Logeais (Trappes, France). Methanol SPECTRANAL was purchased from Riedel-de-Haen (Seelze, Germany). pEFIN5 and pERAEQ2 are expression vectors developed by Euroscreen (Brussels, Belgium). The radioactive products [*myo*-D-2-^{3}H]inositol (17.7 Ci/mmol) and ^{125}I were from Amersham Biosciences (Piscataway, NJ). Dowex AG1 × 8 (formate form) was from Bio-Rad Laboratories (Hercules, CA). The radioactive ligands [β-^{33}P]2MeSADP (2000 Ci/mmol) and [^{3}H]ADP (33.9 Ci/mmol) were purchased from PerkinElmer Life Sciences Inc. (Boston, MA). The radioactive ligand [^{3}H]2MeSADP (3 Ci/mmol) was purchased from Moravek Biochemicals Inc (Brea, CA). The active metabolite of clopidogrel was obtained as described previously (Savi et al., 2000).

Binding of [^{33}P]2MeSADP. Experiments on the specific binding of [^{33}P]2MeSADP to 1321N1 cells were performed with a filtration technique to separate the free from bound [^{33}P]2MeSADP, according to a previously published method (Savi et al., 1994). When the cultures were 80% confluent, the cells were harvested in phosphate-buffered saline-EDTA (5 mM), washed once, and resuspended in dialyzed fetal calf serum containing culture medium, supplemented with 1 mM EDTA, and used within 15 min. Incubations were carried out in 0.20 ml of culture medium that contained $P2Y_{13}$-expressing 1321N1 cells (5000 cells/well) and [^{33}P]2MeSADP (0 to 3 nM). Triplicate incubations were carried out at 20°C for 10 min and were stopped by the addition of 3 ml of ice-cold assay buffer followed by a rapid vacuum filtration over glass fiber filter (Filtermats 11734; Skatron Instruments Inc., Sterling, VA). The active metabolite of clopidogrel was incubated 1 h before radioligand addition.

Binding of GTPγ[^{35}S]. The measurement of nucleotide-stimulated GTPγ[^{35}S] binding to membranes of cells expressing $P2Y_{13}$ was performed as described previously (Kotani et al., 2001). Briefly, membranes (15 μg) from AG32-$P2Y_{13}$ or 1321N1-$P2Y_{13}$ cells were incubated for 15 min at room temperature in GTPγ[^{35}S] binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM GDP, and 10 μg/ml saponin) containing different concentrations of ADP and 2MeSADP in 96-well microplates (Basic FlashPlates; PerkinElmer). GTPγ[^{35}S] (0.1 nM) (Amersham Biosciences) was added, microplates were shaken for 1 min and further incubated at 30°C for 30 min. The incubation was stopped by centrifugation of the microplate for 10 min at 800*g* and 4°C, and the supernatant was aspirated. Microplates were counted in a TopCount scintillation and luminescence counter (PerkinElmer) for 1 min per well.

For the kinetic studies, incubation was initiated with the addition of ligand simultaneously with GTPγ[^{35}S] and was terminated after 1, 3, 10, or 30 min by the filtration through GF/B filters using a multiple membrane filter (Linca Lamon Instrumentation, Tel Aviv, Israel). Filters were washed three times with 4 ml of ice-cold binding buffer, dried, and bound GTPγ[^{35}S] was measured by liquid scintillation counting. In some case, AG32-$P2Y_{13}$ cells were treated overnight with 100 ng/ml pertussis toxin added to the culture medium.

Inositol Phosphate Measurement. AG32 cells were seeded (200,000 cells per dish) on 35-mm (diameter) Petri dishes and labeled for 24 h with 5 μCi/ml [*myo*-D-2-^{3}H]inositol in inositol-free Dulbecco's modified Eagle's medium containing 5% fetal calf serum, antibiotics, amphotericin, sodium pyruvate, and 400 μg/ml G418. Cells were incubated for 2 h in Krebs-Ringer-HEPES (KRH) buffer (124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, pH 7.4, and 8 mM D-glucose). The cells were then incubated in presence of the tested compounds for 30 s up to 10 min. The incubation was stopped by the addition of 1 ml of an ice-cold 3% perchloric acid solution. Inositol phosphates were extracted and separated on Dowex columns as described previously (Communi et al., 1995).

Cyclic AMP Assays. CHO-K1 Chinese hamster ovary cells were seeded (150,000 cells per dish) on 35-mm (diameter) Petri dishes in Ham's F-12 medium containing 10% fetal calf serum, antibiotics, amphotericin, sodium pyruvate, and 400 μg/ml G418. Cells were incubated for 2 h in KRH (see below) containing rolipram (25 μM) and incubated in the presence of the tested compound for 10 min with or without forskolin (3 μM). The incubation was stopped by the addition of 1 ml of HCl (0.1 M). The cellular lysate was dried and resuspended in an appropriate volume of water. Cyclic AMP was quantified by ^{125}I radioimmunoassay as described previously (Brooker et al., 1979).

Evaluation of Nucleotide Degradation. AG32 cells were seeded (200,000 cells per dish) on 35-mm (diameter) Petri dishes in inositol-free Dulbecco's modified Eagle's medium containing 5% fetal calf serum, antibiotics, amphotericin, sodium pyruvate, and 400 μg/ml G418. Cells were incubated for 2 h in KRH buffer. Cells were then incubated in the presence of 100 nM [^{3}H]ADP or 100 nM [^{3}H]2MeSADP for various times. Cell supernatants were filtered through a 0.4-μm filter (Millipore, Brussels, Belgium) and stored at −20°C. Radiolabeled nucleotides were separated by HPLC on a μBondapack C18 reverse phase 3.9 × 300 mm column (Millipore) and eluted at 1.5 ml/min with a linear methanol gradient between solution A (5 mM TBAP, 60 mM KH_2PO_4, and 5% methanol) and solution B (5 mM TBAP, 60 mM KH_2PO_4, and 35% methanol) at 2% per min. HPLC fractions were counted after addition of scintillation liquid, Insta-Gel II Plus (PerkinElmer). The column was calibrated using unlabeled nucleotides (AMP, ADP, ATP, 2MeSAMP, 2Me-

SADP, and 2MeSAMP) and radiolabeled nucleotides ([^{3}H]ADP and [^{3}H]2MeSADP).

Nucleotide Pretreatment. To eliminate contaminating diphosphate nucleotides, triphosphate nucleotides were pretreated as described previously (Hechler et al., 1998). In brief, ATP and 2MeSATP (1 mM solutions) were incubated for 90 min at room temperature with 20 units/ml CPK and 10 mM CP. The reaction was stopped by addition of 10 mM iodoacetamide. Apyrase (grade I) was also used as another way to remove the contaminating diphosphate nucleotides. In brief, poly[A] and ADP (10 mM solutions) were incubated for 5 min at 30°C in HEPES buffer, pH = 6.5, with 20 units/ml apyrase (grade I). The apyrase activity was checked by HPLC using the same conditions as described in the previous section.

Data Analysis. Graphs and data analysis were performed with Prism from GraphPad Software (San Diego, CA).

Results

Assay of [^{33}P]2MeSADP binding to 1321N1 cells. The binding assay of [^{33}P]2MeSADP was performed on 1321N1 cells transfected with the P2Y$_{13}$-pEFIN5 vector. As shown in Fig. 1A, binding of [^{33}P]2MeSADP to 1321N1 cells was concentration dependent, and the nonspecific binding measured in presence of unlabeled ADP (10 mM) was very low and depended linearly on the [^{33}P]2MeSADP concentration. A very low binding of [^{33}P]2MeSADP was found in nontransfected 1321N1 cells (Fig. 1A). The Scatchard plot (Fig. 1B) revealed the presence of one class of binding sites that exhibit high affinity with an apparent equilibrium dissociation constant (K_d) of 0.28 ± 0.12 nM. The total number of binding sites (B_{max}) corresponds to 72,240 ± 18,540 receptors per cell.

As shown in Fig. 1C and Table 1, the order of affinity for the receptor was 2MeSADP ≫ 2MeSATP > ADP = AR-C67085MX > ADPβS = ATPγS = ATP. The active metabolite of clopidogrel (Savi et al., 2000), a potent antagonist of the P2Y$_{12}$ receptor (Savi et al., 2001), did not inhibit the binding of [^{33}P]2MeSADP to P2Y$_{13}$-expressing 1321N1 cells.

Assay of GTPγ[^{35}S] Binding to Membranes. The effect of ADP and 2MeSADP on the binding of GTPγ[^{35}S] was assessed on membranes of both 1321N1 and AG32 cells transfected with P2Y$_{13}$-pEFIN5 vector. The same results were found with both cell lines. As shown in Fig. 2A, the binding of GTPγ[^{35}S] increased progressively after the addition of ADP or 2MeSADP. At all the times tested, the effect of 2MeSADP was greater than that of ADP. As shown in Fig. 2B, after 15 min of incubation, 2MeSADP was at least 30 times more potent than ADP. The binding of GTPγ[^{35}S] induced by 30 nM of ADP or 2MeSADP was abolished by a 20-h preincubation with pertussis toxin (100 ng/ml) (data not shown) or by coincubation with AR-C67085MX (5 μM) (Fig. 2C). In 1321N1 cells not transfected with the P2Y$_{13}$ receptor, neither ADP nor 2MeSADP had an effect on GTPγ[^{35}S] binding (data not shown).

Kinetics of IP$_3$ Formation in AG32 Cells. We studied the time course of IP$_3$ accumulation in AG32 cells stably expressing the P2Y$_{13}$ receptor using two distinct P2Y$_{13}$ agonists: ADP and 2MeSADP. As shown in Fig. 3A, the 2MeSADP-induced IP$_3$ accumulation had a longer duration than the response to ADP. The peak of IP$_3$ accumulation was at 1 min and 30 s, respectively, for 2MeSADP and ADP. The concentration-action curves characterizing the effects of ADP, 2MeSADP, and ADPβS on IP$_3$ formation after 30 s have been reported previously (Communi et al., 2001): they were almost superimposable for ADP and 2MeSADP; ADPβS was slightly less potent. We have now obtained the corresponding concentration-action curves after 10 min, in experiments performed in the presence of LiCl (10 mM) and 8-*p*-SPT (100 μM), to prevent an effect of adenosine formed by ADP degradation on the A$_2$ receptor endogenously expressed

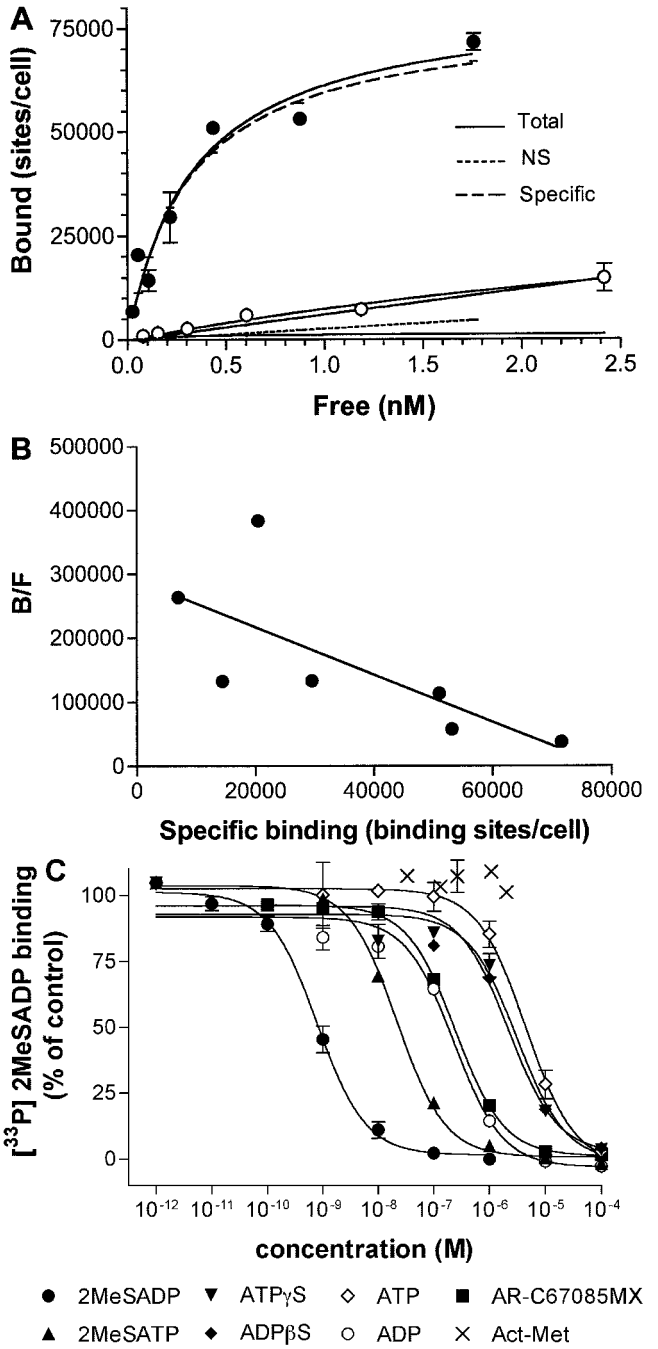

Fig. 1. Binding of [^{33}P]2MeSADP to P2Y$_{13}$-1321N1 cells. A, saturation. P2Y$_{13}$-1321N1 cells (●) and 1321N1 cells (○) were incubated (5000 cells/sample) for 10 min with [^{33}P]2MeSADP at concentrations ranging from 0.01 to 1.8 nM. Nonspecific binding was evaluated in P2Y$_{13}$-1321N1 cells by adding 10 mM unlabeled ADP in the binding medium. B, Scatchard representation of the specific binding. C, effect of different compounds on the binding of [^{33}P]2MeSADP to P2Y$_{13}$-1321N1 cells. ADP, ADPβS, ATP, ATPγS, 2MeSADP, 2MeSATP, and AR-C67085MX were incubated for 10 min with P2Y$_{13}$-1321N1 cells. The active metabolite of clopidogrel was incubated 1 h before radioligand addition.

by 1321N1 cells. As shown in Fig. 3B, after 10 min of stimulation, the 2MeSADP concentration-action curve was shifted to the left compared with the 30-s stimulation, whereas the ADP and ADPβS curves were shifted to the right. In these 10-min experiments, the following EC_{50} values were computed for ADP, 2MeSADP, and ADPβS, respectively: 194 ± 26, 1.2 ± 0.3, and 280 ± 63 nM (mean ± S.D. of three independent experiments). For 30-s experiments, the following EC_{50} values were computed for ADP, 2MeSADP, and ADPβS, respectively: 45 ± 4.7, 57 ± 19, and 82 ± 13 nM (mean ± S.D. of three independent experiments). The time-dependent variation of the relative potencies of ADP and 2MeSADP cannot be explained by differences in the degradation rate, which was very similar for the two nucleotides (Fig. 4).

ATP and 2MeSATP Effect on IP$_3$ in AG32 Cells. ATP and 2MeSATP were tested on the hP2Y$_{13}$-expressing AG32 cells. Commercial powders are contaminated by degradation products: contamination by the respective diphosphate derivative is around 1% for ATP and 10% for 2MeSATP (Hechler et al., 1998). Therefore, the diphosphate contaminants were enzymatically removed using CP/CPK as described under *Materials and Methods*. As shown in Fig. 5, after such treatment, ATP and 2MeSATP behaved as partial agonists with EC_{50} values of 4.2 ± 0.8 and 1.5 ± 0.2 μM, respectively (mean ± S.D. of three independent experiments).

Effect of Other Nucleotide Derivatives on IP$_3$ in AG32 Cells. The effect of diadenosine polyphosphates was also tested on AG32 cells stably expressing the P2Y$_{13}$ receptor (Fig. 6). After 30 s of incubation, the effect of Ap_3A on IP_3 accumulation was almost equal to that of ADP. In contrast, Ap_4A, Ap_5A, and Ap_6A were inactive. In view of a recent report showing that extracellular mRNA can activate a P2Y receptor via its poly[A] tail in a pertussis toxin-sensitive manner (Ni et al., 2002), we tested the effect of poly[A] on the P2Y$_{13}$ receptor in AG32 cells. As shown in Fig. 7A, poly[A] and poly[A].[G] also induce an increase of IP_3 in AG32 transfected cells. As previously discussed, contamination with ADP derivatives can be avoided by CP/CPK treatment. This treatment totally abolished poly[A].[G] activity and partially abolished that of poly[A]. After such treatment, the EC_{50} value expressed in AMP equivalents, was 205 ± 60 μM. However pretreatment with apyrase, which degrades ADP into AMP instead of converting it into ATP, a partial agonist of P2Y$_{13}$, totally abolished the effect of poly[A] (100 μM) (Fig. 7B).

Effect of Selective and Nonselective Antagonists on IP$_3$ in AG32 Cells. The classic nonselective antagonists of P2 receptors, RB-2, suramin, and PPADS (von Kugelgen and Wetter, 2000), were tested on the hP2Y$_{13}$ receptor in the presence of 100 nM of ADP. As shown in Table 2 and Fig. 8A, all three agents were inhibitory: RB-2 was slightly more potent than suramin, whereas PPADS was significantly less potent. We also tested three selective P2Y antagonists cur-

TABLE 1
Affinities of various purinergic ligands for human P2Y$_{13}$-1321N1 cells
Values represent the means ± S.D. of three independent experiments.

Compound	IC_{50}
	nM
2MeSADP	0.98 ± 0.51
2MeSATP	20.03 ± 3.48
AR-C67085MX	212.7 ± 34.8
ADP	308 ± 1.
ADPβS	1871 ± 570[a]
ATPγS	3429 ± 304[a]
ATP	4289 ± 175
Act-Met	>2100[a]

[a] n = 2.

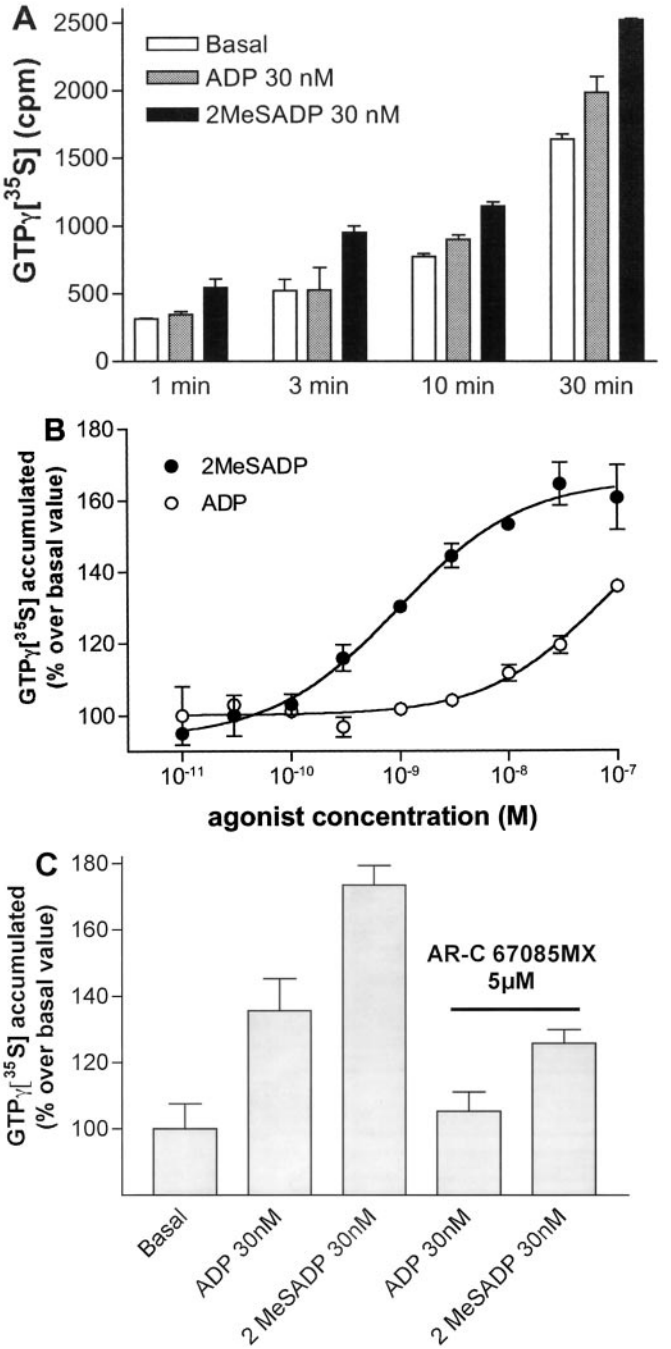

Fig. 2. Binding of GTPγ[^{35}S] A, kinetic study of binding to 1321N1-P2Y$_{13}$ cells. ADP and 2MeSADP were incubated for various times. The data represent the mean ± S.D. of triplicate experimental points obtained in one experiment that was representative of five. B, concentration-action curves for ADP and 2MeSADP incubated for 15 min with AG-32-P2Y$_{13}$. The data represent the mean ± S.D. of triplicate experimental points obtained from one experiment that was representative of five. C, ADP (30 nM) and 2MeSADP (30 nM) stimulation is reversed by AR-C67085MX (5 μM). The data represent the mean ± S.D. of triplicate experimental points obtained in one experiment that was representative of three.

rently available: AR-C67085MX and AR-C69931MX, reported to be selective for $P2Y_{12}$ (Ingall et al., 1999), and MRS-2179, selective for $P2Y_1$ (Boyer et al., 1998). As shown in Fig. 8B, AR-C67085MX and AR-C69931MX were able to completely inhibit the response to 100 nM ADP with IC_{50} values of 0.63 and 4.6 nM, respectively. The action of AR-C69931MX was noncompetitive (Fig. 8C). At 100 μM, MRS-2179 had almost no effect on IP_3 accumulation induced by 100 nM ADP (data not shown). As shown in Fig. 8D, MRS-2179 and PPADS were unable to displace [^{33}P]2MeSADP bound to 1321N1 cells expressing $P2Y_{13}$, whereas suramin and RB-2 could partially displace [^{33}P]2MeSADP at high concentration. These results suggest that PPADS, suramin, and RB-2 act, at least in part, by a noncompetitive mechanism. Furthermore, RB-2, suramin, and PPADS (100 μM) had no specificity for the $P2Y_{13}$ receptor, because they also inhibited the IP_3 response to adenosine that in AG32 cells results from the coupling to $G\alpha_{16}$ of an endogenous adenosine receptor normally coupled to G_s (Communi et al., 1999) (Fig. 8E). As shown in Fig. 8F, two other nucleotides that are antagonists of $P2Y_{12}$ receptor were tested: Ap_4A (Zamecnik et al., 1992) and 2MeSAMP (Hollopeter et al., 2001). Ap_4A was a complete antagonist of $hP2Y_{13}$ with an IC_{50} of 216 nM. In contrast, 2MeSAMP behaved as a partial agonist, producing a maximal effect that was 38% of that of ADP. Consistent with that partial agonist property, increasing amounts of 2MeSAMP decreased ADP-promoted IP_3 level by 62%, with an IC_{50} of 2.4 μM.

Effect on cAMP in CHO-K1 Cells. As shown in Fig. 9A, ADP and 2MeSADP produced a comparable inhibition of forskolin (3 μM)-induced accumulation of cAMP in CHO-K1 cells stably expressing the $P2Y_{13}$ receptor. The IC_{50} values of ADP and 2MeSADP were 0.07 ± 0.14 and 0.1 ± 0.3 nM, respectively (mean ± S.D. of three independent experiments). The maximal inhibition was of 49 ± 7.6% at 10 nM for ADP and of 54 ± 9.7% at 1 nM for 2MeSADP (mean ± S.D. of three independent experiments). At higher concentrations, the inhibition was reversed. The EC_{50} values charac-

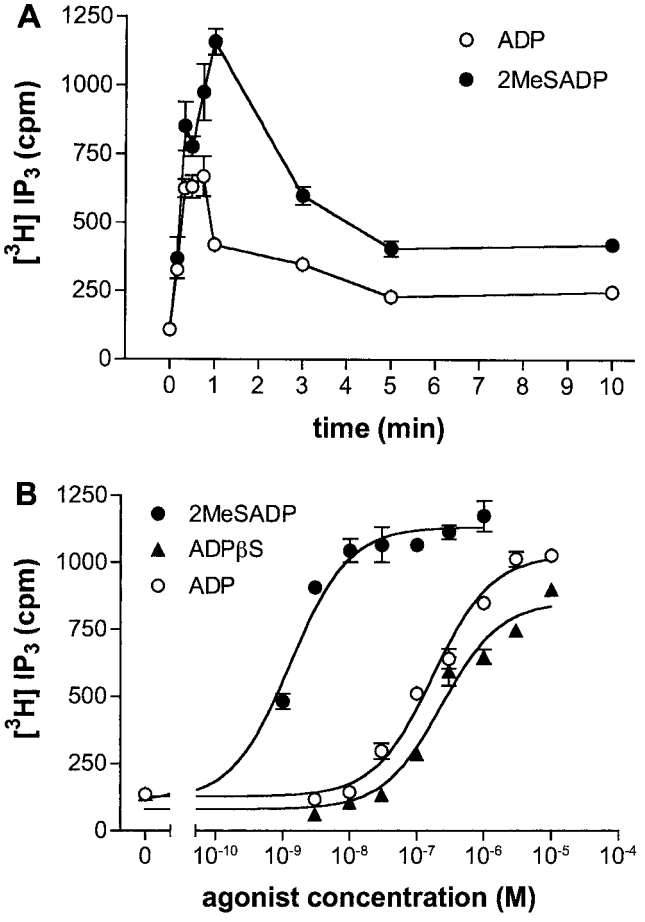

Fig. 3. A, time course of the IP_3 response of $hP2Y_{13}$-AG32 cells to ADP and 2MeSADP. 1321N1 cells expressing both $hP2Y_{13}$ receptor and $G\alpha_{16}$ protein (AG32) were incubated with 100 nM of ADP or 2MeSADP for different times. The data represent the mean ± S.D. of triplicate experimental points obtained in one experiment that was representative of three. B, concentration-action curves of ADP, 2MeSADP, and ADPβS on IP_3 accumulation in AG32 cells. AG32 cells were incubated with different agonist concentrations for 10 min. A preincubation with LiCl (10 mM) and 8-*p*-SPT (100 μM) was performed 10 min before stimulation with agonist. The data represent the mean ± S.D. of triplicate experimental points obtained in one experiment that was representative of three.

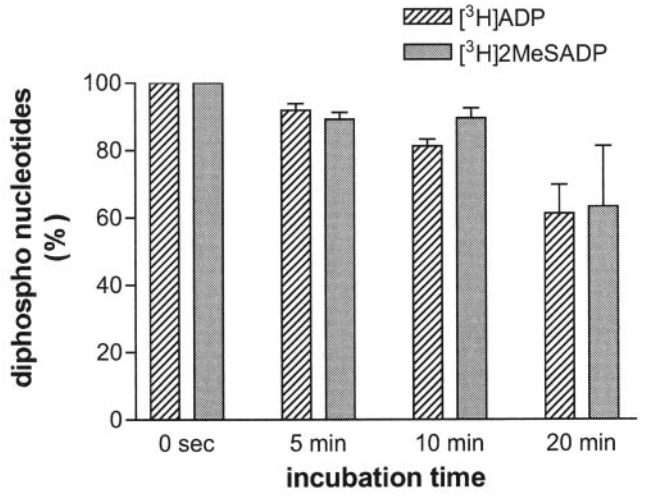

Fig. 4. Estimation of extracellular ADP and 2MeSADP degradation by AG32-$P2Y_{13}$. Cells were incubated with [^{3}H]ADP and [^{3}H]2MeSADP for various times. Cell supernatants were fractionated by HPLC and fractions were then counted for [^{3}H] radioactivity.

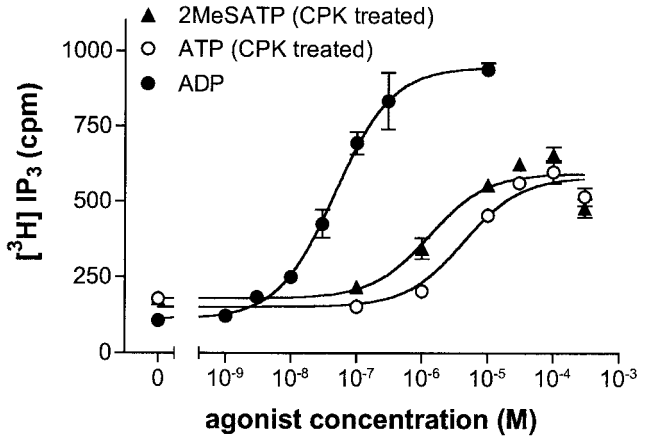

Fig. 5. Partial agonist action of ATP and 2MeSATP on human $P2Y_{13}$ receptor. 1321N1 cells transfected with both $hP2Y_{13}$ receptor and $G\alpha_{16}$ protein were incubated with various concentrations of ADP, ATP, and 2MeSATP for 30 s. ATP and 2MeSATP were treated with 10 mM CP and 20 units/ml CPK, as described under *Materials and Methods*, before the stimulation. The data represent the mean ± S.D. of triplicate experimental points obtained in one experiment that was representative of three.

terizing that reversal were strikingly different for 2MeSADP and ADP: 6.3 ± 2 and 397 ± 130 nM, respectively (mean ± S.D. of three independent experiments). After an 18-h pretreatment with 100 ng/ml pertussis toxin, ADP and 2MeSADP produced only enhancement of cAMP accumulation in response to forskolin (3 μM) (Fig. 9B). The EC_{50} values of 2MeSADP and ADP were 4.2 ± 1.6 and 158 ± 15 nM, respectively (mean ± S.D. of three independent experiments).

Discussion

The human $P2Y_{13}$ receptor was initially characterized after stable expression in 1321N1 cells coexpressing $G\alpha_{16}$ (AG32 cells) and CHO-K1 cells (Communi et al., 2001). In AG32 cells, but not in 1321N1 cells, ADP, 2MeSADP, and ADPβS stimulated inositol phosphate formation in a pertussis toxin-sensitive manner. In CHO-K1 cells, ADP inhibited forskolin-induced cAMP accumulation, an effect that was abolished by pertussis toxin. Therefore, the $P2Y_{13}$ receptor was defined as a receptor for diphosphate adenine nucleotides coupled to G_i (Communi et al., 2001). Using different expression systems, Zhang et al. (2002) reached essentially similar conclusions. In the present study, we have further characterized the pharmacology of the $P2Y_{13}$ receptor and its differences with that of the $P2Y_{12}$ receptor, with the use of additional assays (binding of [^{33}P]2MeSADP and GTPγ[^{35}S]) and a special emphasis on the following issues: activity of triphosphate adenine nucleotides, relative potency of ADP and 2MeSADP, sensitivity to $P2Y_{12}$ antagonists (clopidogrel active metabolite, AR-C67085MX, and AR-C69931MX).

When contaminating diphosphates were enzymatically removed and testing was performed over a short period (30 s), ATP and 2MeSATP seemed to be partial agonists of the $P2Y_{13}$ receptor with a weak potency. In the radioligand binding assay, the IC_{50} of 2MeSATP was 20-fold greater than that of 2MeSADP, but this difference is likely to have been underestimated because of ectonucleotidase-catalyzed conversion of 2MeSATP into 2MeSADP during the 10-min course of the assay. In contrast, 2MeSATP has been consistently reported by several groups to be almost equipotent to 2MeSADP at the $P2Y_{12}$ receptor (Savi et al., 2001; Takasaki et al., 2001; Zhang et al., 2001), although it is known that 2MeSATP behaves as an antagonist rather than an agonist of the $P2Y_{12}$ receptor natively expressed on platelets (Park and Hourani, 1999). Zhang et al. (2002) have reported that after CP/CPK treatment, 2MeSATP retains a high potency on the $P2Y_{13}$ receptor (EC_{50} = 83 nM): the reason for this discrepancy with our data are unclear but might be related to different levels of receptor expression. Palmer et al. (1998) have indeed shown that the capacity of ATP to activate the $P2Y_1$ receptor depends on the degree of receptor reserve.

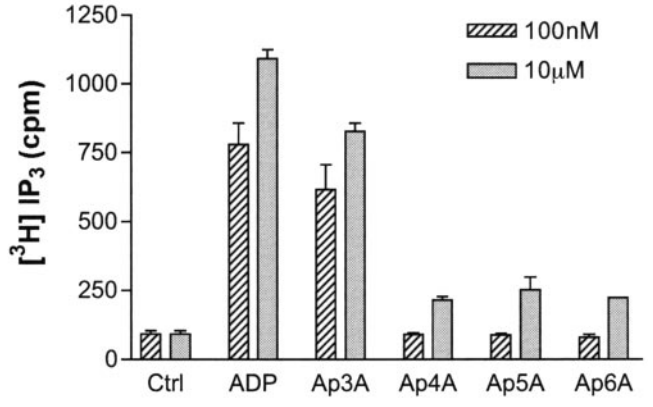

Fig. 6. Effect of different diadenosine polyphosphates on $hP2Y_{13}$. 1321N1 cells transfected with both $hP2Y_{13}$ receptor and $G\alpha_{16}$ protein were challenged for 30 s with two different concentrations (100 nM and 10 μM) of ADP or ApnA (n = 3 to 6). The data represent the mean ± S.D. of triplicate experimental points obtained from one experiment that was representative of three.

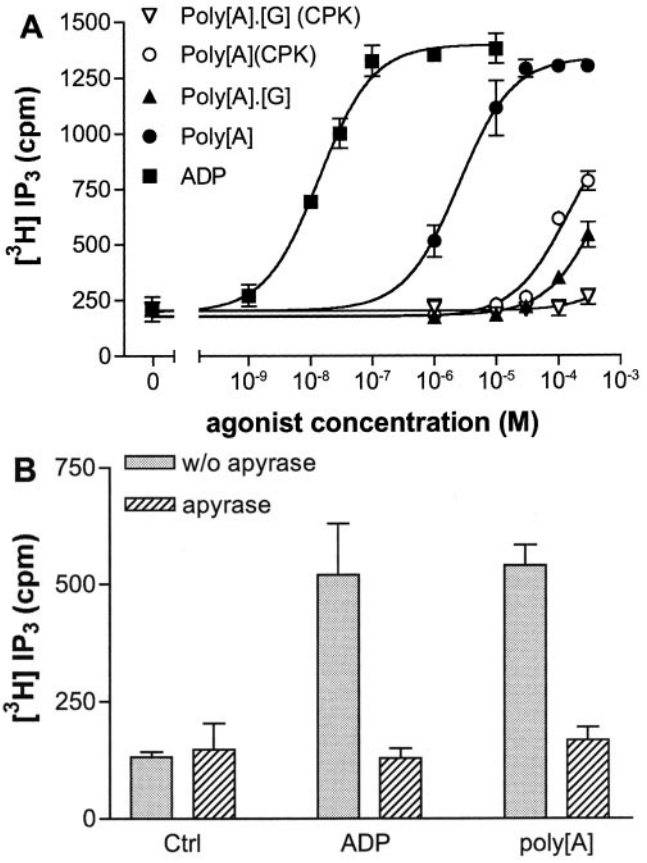

Fig. 7. Concentration-response curves of different nucleotide derivatives at $hP2Y_{13}$ receptor. A, poly[A] and poly[A].[G] were treated with 10 mM CP and 20 units/ml CPK, as described under *Materials and Methods*, before the stimulation. Both treated and untreated poly[A] and poly[A].[G] were added for 30 s on 1321N1 cells transfected with both $hP2Y_{13}$ receptor and $G\alpha_{16}$ protein. ADP was used as reference compound. B, poly[A] was treated with 20 units/ml apyrase, as described under *Materials and Methods*, before the stimulation. Poly[A] was added for 30 s on 1321N1 cells transfected with both $hP2Y_{13}$ and $G\alpha_{16}$ protein. ADP was used as reference compound. For A and B, the data represent the mean ± S.D. of triplicate experimental points obtained from one experiment that was representative of three.

TABLE 2

Potency of antagonists in human $P2Y_{13}$-AG32 cells.

Values represent the means ± S.D. of three independent experiments.

Antagonist	IC_{50}
AR-C69931MX	4.6 ± 1.6nM
Ap_4A	216 ± 66 nM
AR-C67085MX	630 ± 0.1nM
Reactive blue 2	1.9 ± 0.1μM
Suramin	2.3 ± 0.4μM
2MeSAMP	2.5 ± 1.4μM
PPADS	11.7 ± 0.9μM
MRS-2179	>100 μM

2MeSADP is 1 to 2 orders of magnitude more potent than ADP at the human $P2Y_{12}$ receptor (Hollopeter et al., 2001; Savi et al., 2001; Takasaki et al., 2001; Zhang et al., 2001). The situation seems more complex in the case of the $P2Y_{13}$ receptor. Binding of [^{33}P]2MeSADP to intact 1321N1 cells revealed a greater affinity of 2MeSADP compared with ADP. 2MeSADP was also more potent than ADP in stimulating the binding of GTPγ[^{35}S] to 1321N1 or AG32 membranes, and

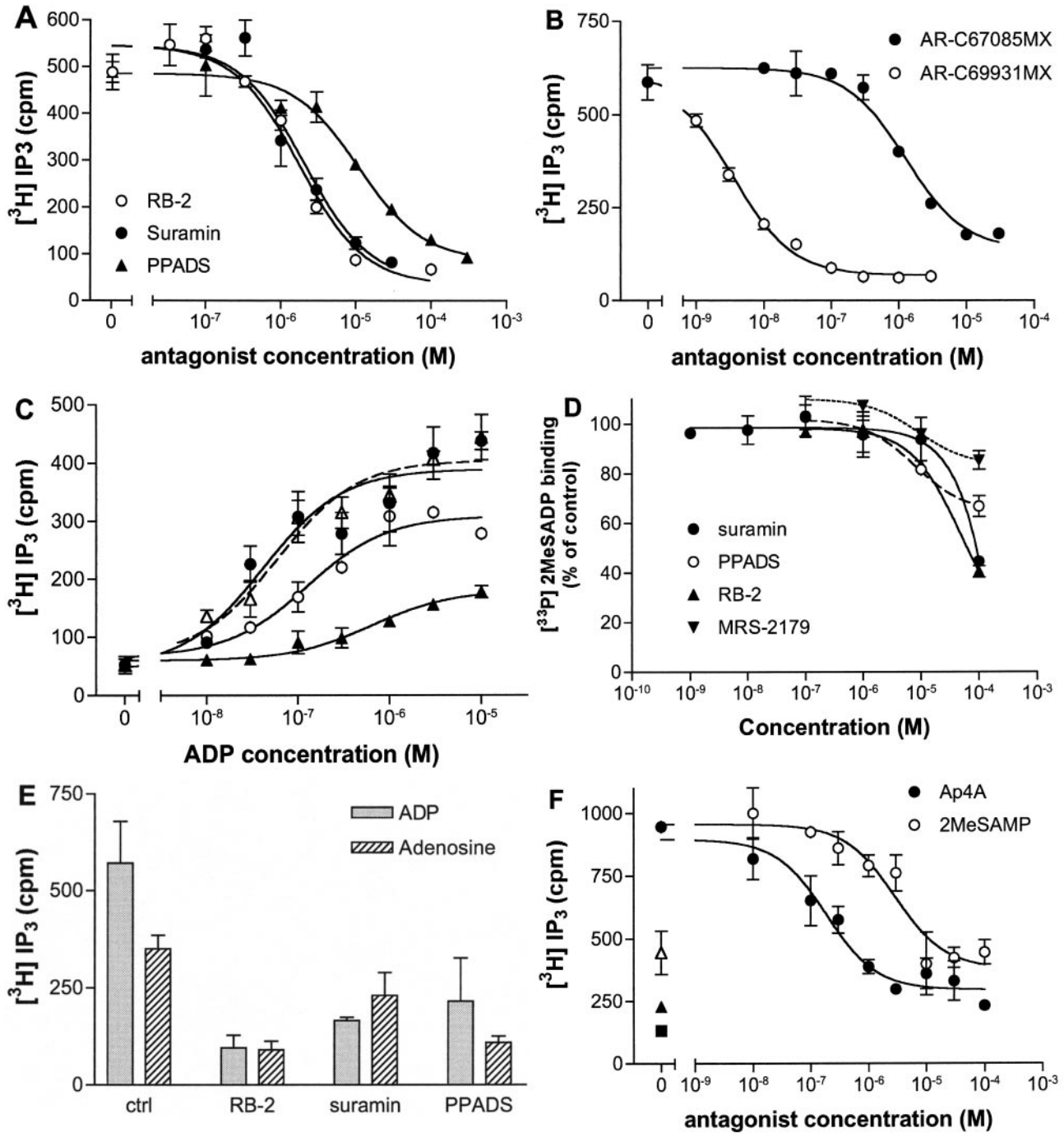

Fig. 8. A, effect of nonselective P2 antagonists on $hP2Y_{13}$ receptor. 1321N1 cells transfected with both $hP2Y_{13}$ receptor and $G\alpha_{16}$ protein (AG32) were preincubated with different concentrations of RB-2, suramin, or PPADS for 10 min. After 10 min, the cells were stimulated with 100 nM ADP for 30 s. B, effect of selective P2 antagonists on $hP2Y_{13}$ receptor. The same protocol was applied for AR-C67085MX and AR-C69931MX. C, characterization of the antagonism by AR-C69931MX. 1321N1 cells transfected with both $hP2Y_{13}$ receptor and $G\alpha_{16}$ protein were preincubated with different concentrations of AR-C69931MX (●, Ctrl, △, 0.1 nM; ○, 1 nM; ▲, 10 nM) for 10 min. After 10 min, cells were stimulated with different concentration of ADP for 30 s. D, binding of [^{33}P]2MeSADP to 1321N1 cells. Displacement of [^{33}P]2MeSADP by different antagonists of $P2Y_{13}$ (suramin, RB-2, PPADS) and a $P2Y_1$ antagonist, MRS-2179. E, nonselective effect of suramin, RB-2, and PPADS. AG32 cells were preincubated with 100 μM RB-2, suramin, or PPADS for 10 min. After 10 min, the cells were stimulated with 100 nM ADP or 30 μM of adenosine for 30 s. F, effect of two selective $P2Y_{12}$ antagonists: Ap_4A (●) and 2MeSAMP (○). The protocol is identical to the one followed for other antagonists. ▲ and △, cells preincubated 10 min with Ap_4A and 2MeSAMP, respectively, without addition of ADP (100 nM). ■, control cells. For A to F, the data represent the mean ± S.D. of triplicate experimental points obtained in one experiment that was representative of three.

this difference was observed at all the times tested. However, in AG32 cells, the EC_{50} values characterizing the effects of 2MeSADP and ADP on IP_3 measured after 30 s have been reported to be almost equal (Communi et al., 2001). Using a different expression system, Zhang et al. (2002) reported EC_{50} values of 19 and 60 nM, respectively. We have now shown that the relative potencies of 2MeSADP and ADP are critically dependent on the experimental conditions. In AG32 cells, the ADP and 2MeSADP EC_{50} values for IP_3 accumulation were almost equal after 30 s. However, a 200-fold difference was noticed after 10 min, because of decreased potency of ADP and increased potency of 2MeSADP. Degradation by ectonucleotidases cannot explain these discrepancies because the degradation rates of ADP and 2MeSADP were comparable and relatively slow. Neither can they be considered an artifact resulting from the use of $G\alpha_{16}$-mediated activation of phospholipase C as a readout of receptor activation. Indeed, using the GTPγ[^{35}S] binding assay, 2MeSADP was more potent than ADP in both plain 1321N1 cells and 1321N1 cells cotransfected with $G\alpha_{16}$(AG32 cells). Furthermore, discrepancies were also found in CHO-K1 cells where $G\alpha_{16}$ was not coexpressed. In CHO-K1 cells incubated for 10 min, ADP and 2MeSADP produced an equipotent inhibition of cAMP. However, the reversal of that inhibition at higher concentration, as well as the enhancement of cAMP accumulation observed after pertussis toxin pretreatment, were characterized by a greater potency of 2MeSADP. These effects are explained by a promiscuous coupling to G_s, which has been observed with several other G_i-coupled receptors (Kukkonen et al., 2001). Although the binding assays indicate that 2MeSADP is intrinsically more potent than ADP at the P2Y$_{13}$ receptor, as it is on the P2Y$_{12}$ receptor, the other results, taken together, suggest that the P2Y$_{13}$ receptor could exist in multiple active conformations, characterized by differences in affinity for 2MeSADP versus ADP, kinetics, and preference for G proteins (Leff et al., 1997).

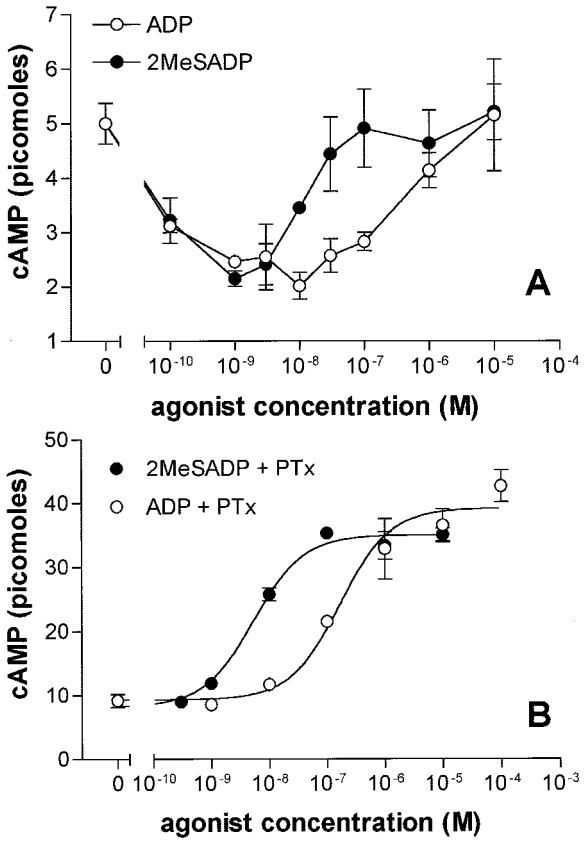

Fig. 9. A, CHO-K1 transfected cells were incubated with ADP or 2MeSADP for 10 min in presence of 3 μM forskolin. The data represent the mean ± S.D. of triplicate experimental points obtained from one experiment that was representative of three. B, CHO-K1 transfected cells were preincubated with 100 ng/ml pertussis toxin for 18 h and then incubated with ADP or 2MeSADP for 10 min in presence of 3 μM forskolin. The data represent the mean ± S.D. of triplicate experimental points obtained from one experiment that was representative of three.

The antiplatelet and antithrombotic action of clopidogrel is mediated by an active metabolite (Savi et al., 2000). That active metabolite has been shown to prevent the binding of [^{33}P]2MeSADP to the P2Y$_{12}$ receptor with an IC_{50} of 100 nM (Savi et al., 2001). When the same radioligand binding assay was performed on the P2Y$_{13}$ receptor, no displacement of the ligand was observed at concentrations up to 2 μM. On the other hand, AR-C67085MX and AR-C69931MX belong to a group of ATP derivatives that are specific antagonists of the P2Y$_{12}$ receptor and inhibit platelet aggregation at nanomolar concentrations (Ingall et al., 1999). At the recombinant P2Y$_{12}$ receptor, AR-C69931MX had an IC_{50} of 2.4 nM (Takasaki et al., 2001). In AG32 cells expressing the P2Y$_{13}$ receptor, the IC_{50} of AR-C67085MX was 0.6 μM. This lower potency on the P2Y$_{13}$ receptor is consistent with the low affinity of triphosphates (ATP, 2MeSATP) mentioned earlier. However, AR-C69931MX was a very potent antagonist of the P2Y$_{13}$ receptor, with an IC_{50} comparable with that obtained at the P2Y$_{12}$ receptor. Interestingly, this action of AR-C69931MX seems to be noncompetitive. MRS-2179 had no significant inhibitory effect on the P2Y$_{13}$ receptor at concentrations up to 100 μM. This observation further strengthens the claim that it is a selective antagonist of the P2Y$_1$ receptor (Boyer et al., 1998).

We have confirmed the observation by Zhang et al. that Ap_3A is a potent agonist of the P2Y$_{13}$ receptor. Furthermore, we have shown that Ap_4A, Ap_5A, and Ap_6A are completely inactive. The same profile has been observed with the P2Y$_1$ receptor (Patel et al., 2001), suggesting that a selective sensitivity to Ap_3A is a common feature of ADP receptors. Ap_4A, previously shown to inhibit platelet aggregation (Zamecnik et al., 1992) , was an antagonist of the P2Y$_{13}$ receptor. Recently Ni et al. (2002) have suggested that extracellular mRNA can activate dendritic cells via an interaction between the poly[A] tail and an unidentified P2Y receptor. This conclusion was based on the observation that commercial poly[A] increased cytosolic calcium in dendritic cells, a response blocked by suramin and pertussis toxin. We have now shown that poly[A] activates the P2Y$_{13}$ receptor. Because commercial poly[A] is prepared using ADP as starting material, contamination by residual ADP may complicate the interpretation of these results. Indeed pretreatment with CP/CPK abolished the effect of poly[A].[G] and shifted the poly[A] concentration-action curve to the right, whereas apyrase abolished its effect completely. Therefore, it is unlikely that the P2Y$_{13}$ receptor plays a role in the activation of dendritic cells by extracellular mRNA.

In conclusion, we have identified three properties that distinguish the recombinant human P2Y$_{13}$ receptor from the P2Y$_{12}$ receptor: it is less sensitive than the P2Y$_{12}$ receptor to activation by triphosphate nucleotides; the potency of 2MeSADP is superior or equal to that of ADP, depending on the

measured endpoint; and it is antagonized less potently by AR-C67085MX and not at all by the active metabolite of clopidogrel. However, these studies have also revealed that in several respects, the $P2Y_{13}$ receptor is even more similar to the $P2Y_{12}$ than initially believed: greater potency of 2MeSADP compared with ADP in radioligand binding assays ([^{33}P]2MeSADP, GTPγ[^{35}S]) that assess binding to the receptor or its interaction with G_i, and antagonism by 2MeSAMP, Ap_4A, and especially AR-C69931MX. These similarities between the pharmacological profiles of the $hP2Y_{12}$ and $hP2Y_{13}$ receptors seems logical when considering the high degree of amino acid identity (47.7%) and the short distance (10 kilobases) between these two syntenic genes located in tandem on 3q25 (Wittenberger et al., 2001).

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References

Boyer JL, Mohanram A, Camaioni E, Jacobson KA, and Harden TK (1998) Competitive and selective antagonism of P2Y1 receptors by N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate. *Br J Pharmacol* **124:**1–3.

Brooker G, Harper JF, Terasaki WL, and Moylan RD (1979) Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv Cyclic Nucleotide Res* **10:**1–33.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, and Boeynaems JM (2001) Identification of a novel human ADP receptor coupled to G_i. *J Biol Chem* **276:**41479–41485.

Communi D, Janssens R, Robaye B, Zeelis N, and Boeynaems JM (2000) Rapid up-regulation of P2Y messengers during granulocytic differentiation of HL-60 cells. *FEBS Lett* **475:**39–42.

Communi D, Paindavoine P, Place GA, Parmentier M, and Boeynaems JM (1999) Expression of P2Y receptors in cell lines derived from the human lung. *Br J Pharmacol* **127:**562–568.

Communi D, Pirotton S, Parmentier M, and Boeynaems JM (1995) Cloning and functional expression of a human uridine nucleotide receptor. *J Biol Chem* **270:** 30849–30852.

Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, and Grubb BR (1999) Effect of loss of $P2Y_2$ receptor gene expression on nucleotide regulation of murine epithelial Cl^- transport. *J Biol Chem* **274:**26461–26468.

Erb L, Garrad R, Wang Y, Quinn T, Turner JT, and Weisman GA (1995) Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. *J Biol Chem* **270,** 4185–4188.

Fabre JE, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM, and Koller BH (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* **5:**1199–1202**.**

Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ Jr, Wiekowski MT, et al. (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Investig* **107,** 1591–1598.

Hechler B, Vigne P, Leon C, Breittmayer JP, Gachet C, and Frelin C (1998) ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. *Mol Pharmacol* **53:**727–733.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature (Lond)* **409:**202–207.

Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, et al. (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. *J Med Chem* **42:**213–220.

Kotani M, Mollereau C, Detheux M, Le Poul E, Brezillon S, Vakili J, Mazarguil H, Vassart G, Zajac JM, and Parmentier M (2001) Functional characterization of a human receptor for neuropeptide FF and related peptides. *Br J Pharmacol* **133:** 138–144.

Kukkonen JP, Nasman J, and Akerman KE (2001) Modelling of promiscuous receptor-Gi/Gs-protein coupling and effector response. *Trends Pharmacol Sci* **22:**616–622.

Leff P, Scaramellini C, Law C, and McKechnie K (1997) A three-state receptor model of agonist action. *Trends Pharmacol Sci* **18:**355–362.

Leon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave JP, and Gachet C (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. *J Clin Investig* **104,** 1731–1737.

Ni H, Capodici J, Cannon G, Communi D, Boeynaems JM, Kariko K, and Weissman D (2002) Extracellular mRNA induces dendritic cell activation by stimulating tumor necrosis factor-α secretion and signaling through a nucleotide receptor. *J Biol Chem* **277:**12689–12696.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA, and Harden TK (1998) Agonist action of adenosine triphosphates at the human P2Y1 receptor. *Mol Pharmacol* **54:**1118–1123.

Park HS and Hourani SM (1999) Differential effects of adenine nucleotide analogues on shape change and aggregation induced by adenosine 5-diphosphate (ADP) in human platelets. *Br J Pharmacol* **127:**1359–1366.

Patel K, Barnes A, Camacho J, Paterson C, Boughtflower R, Cousens D, and Marshall F (2001) Activity of diadenosine polyphosphates at P2Y receptors stably expressed in 1321N1 cells. *Eur J Pharmacol* **430:**203–210.

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* **50:**413–492.

Savi P, Labouret C, Delesque N, Guette F, Lupker J, and Herbert JM (2001) $P2y_{12}$, a new platelet ADP receptor, target of clopidogrel. *Biochem Biophys Res Commun* **283:**379–383.

Savi P, Laplace MC, Maffrand JP, and Herbert JM (1994) Binding of [^{3}H]-2-methylthio ADP to rat platelets—effect of clopidogrel and ticlopidine. *J Pharmacol Exp Ther* **269:**772–777.

Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M, and Herbert JM (2000) Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* **84:**891–896.

Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, Soga T, Matsushime H, and Furuichi K (2001) Molecular cloning of the platelet $P2T_{AC}$ ADP receptor: pharmacological comparison with another ADP receptor, the $P2Y_1$ receptor. *Mol Pharmacol* **60:**432–439.

Turner NA, Moake JL, and McIntire LV (2001) Blockade of adenosine diphosphate receptors $P2Y_{12}$ and $P2Y_1$ is required to inhibit platelet aggregation in whole blood under flow. *Blood* **98,** 3340–3345.

von Kugelgen I and Wetter A (2000) Molecular pharmacology of P2Y-receptors. *Naunyn Schmiedeberg's Arch Pharmacol* **362:**310–323.

Wilkin F, Duhant X, Bruyns C, Suarez-Huerta N, Boeynaems JM, and Robaye B (2001) The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *J Immunol* **166,** 7172–7177.

Wittenberger T, Schaller HC, and Hellebrand S (2001) An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. *J Mol Biol* **307:**799–813.

Zamecnik PC, Kim B, Gao MJ, Taylor G, and Blackburn GM (1992) Analogues of diadenosine 5′, 5‴-P1, P4-tetraphosphate (Ap4A) as potential anti-platelet-aggregation agents. *Proc Natl Acad Sci USA* **89,** 2370–2373.

Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, Liu YH, Chen G, Pramanik B, Laz TM, et al. (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. *J Biol Chem* **276:**8608–8615.

Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, and Monsma F Jr (2002) $P2Y_{13}$: identification and characterization of a novel Gαi-coupled ADP receptor from human and mouse. *J Pharmacol Exp Ther* **301:** 705–713.

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