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An Arginine/Glutamine Difference at the Juxtaposition of Transmembrane Domain 6 and the Third Extracellular Loop Contributes to the Markedly Different Nucleotide Selectivities of Human and Canine $P2Y_{11}$ Receptors

AI-DONG QI, ALEXANDER C. ZAMBON, PAUL A. INSEL, and ROBERT A. NICHOLAS

Department of Pharmacology (A.-D.Q., R.A.N.), University of North Carolina, Chapel Hill, North Carolina; and Departments of Pharmacology (A.C.Z., P.A.I.) and Medicine (P.A.I.), University of California at San Diego, La Jolla, California

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ABSTRACT

The recently cloned canine $P2Y_{11}$ receptor ($cP2Y_{11}$) and its human homolog ($hP2Y_{11}$) were stably expressed in Chinese hamster ovary cells (CHO-K1) and 1321N1 human astrocytoma cells, and their agonist selectivities and coupling efficiencies to phospholipase C and adenylyl cyclase were assessed. Adenosine triphosphate nucleotides were much more potent and efficacious at the $hP2Y_{11}$ receptor than their corresponding diphosphates in promoting both inositol phosphate and cyclic AMP accumulation. In contrast, adenosine diphosphate nucleotides were considerably more potent at the $cP2Y_{11}$ receptor than their corresponding triphosphate analogs. The tri- versus diphosphate specificity of the two receptors was further confirmed in studies using Ca^{2+} mobilization as a measure of receptor activation under conditions that minimized nucleotide degradation. Moreover, 2-methylthioadenosine-5′-triphosphate and 2-methylthioadenosine-5′-diphosphate were 58- and 75-fold more potent than ATP and ADP, respectively, at the $cP2Y_{11}$ receptor compared with only 2- to 3-fold more potent at the $hP2Y_{11}$ receptor. Mutational analysis revealed that the change of Arg-265, which is located at the juxtaposition of transmembrane domain 6 and the third extracellular loop in the $hP2Y_{11}$ receptor, to glutamine in the $cP2Y_{11}$ receptor is at least partly responsible for the diphosphate selectivity but not the increased sensitivity to 2-thioether-substituted adenine nucleotides at the canine receptor. These results imply a key role for a positively charged arginine residue in contributing to the recognition of extracellular nucleotides by the $P2Y_{11}$ receptor and perhaps other P2Y receptors.

Extracellular adenine and uridine nucleotides exert their physiological and pathophysiological responses through a large group of ligand-gated ion channel P2X receptors and G protein-coupled P2Y receptors (Fredholm et al., 1994; Ralevic and Burnstock, 1998). Molecular cloning and characterization studies have so far identified seven functional mammalian P2Y receptor subtypes ($P2Y_{1,2,4,6,11,12,13}$) (Harden, 1998; King et al., 1998) P2Y receptor subtypes 1, 2, 4, 6, and 11 couple to the stimulation of phospholipase C (PLC), which ultimately results in activation of protein kinase C and mobilization of calcium from intracellular stores. In addition to coupling to PLC, the $hP2Y_{11}$ receptor also couples to adenylyl cyclase to promote cyclic AMP accumulation (Communi et al., 1997, 1999; Qi et al., 2001). The $P2Y_{12}$ receptor (also known as $P2Y_{AC}$ or P2T) was recently cloned and shown to be the elusive platelet ADP receptor coupled to the inhibition of adenylyl cyclase (Foster et al., 2001; Hollopeter et al., 2001). The $P2Y_{13}$ receptor (6PR86), which is 48% identical to the $P2Y_{12}$ receptor, is also activated by ADP and coupled to inhibition of adenylyl cyclase (Communi et al., 2001).

Traditionally, G protein-coupled receptor subtypes have been classified by their agonist and antagonist profiles. However, pharmacological classification of G protein-coupled receptors is complicated by the observation that minor structural differences in species homologs can have profound consequences on receptor activity. For example,

Address correspondence to: Robert Nicholas, Department of Pharmacology, University of North Carolina, CB #7365 Mary Ellen Jones Building, Chapel Hill, NC 27599-7365.

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ABBREVIATIONS: PLC, phospholipase C; $hP2Y_{11}$, human $P2Y_{11}$; $cP2Y_{11}$, canine $P2Y_{11}$; CHO, Chinese hamster ovary; CHO-*x*$P2Y_{11}$, Chinese hamster ovary cells expressing the $P2Y_{11}$ receptor, where *x* is h (human) or c (canine); 1321N1-*x*$P2Y_{11}$, 1321N1 human astrocytoma cells expressing the $cP2Y_{11}$ receptor, where x is h (human) or c (canine); RB-2, reactive blue 2; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; 2MeSATP, 2-methylthioadenosine-5′-triphosphate; 2MeSADP, 2-methylthioadenosine-5′-diphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); TM, transmembrane domain; RIA, radioimmunoassay.

the rat 5-HT_{1B} receptor has 93% sequence identity with its human homolog but displays a markedly different rank order of agonist potencies (Voigt et al., 1991; Gerhardt and van Heerikhuizen, 1997; Hoyer and Martin, 1997). In the P2Y receptor family, the rat homolog of the $P2Y_4$ receptor has a markedly different nucleotide selectivity than its human homolog, even though the two receptors have 83% sequence identity (Bogdanov et al., 1998; Webb et al., 1998; Kennedy et al., 2000). Thus, species homologs, even those with high sequence identity overall, do not necessarily exhibit identical pharmacological properties. This potential for variance in the pharmacological properties of species homologs makes it important to characterize these receptors to determine whether species-specific differences exist, because erroneous conclusions can arise based on the false assumption that species homologs exhibit very similar or identical properties.

We recently cloned a P2Y receptor from Madin-Darby canine kidney epithelial cells that has approximately 70% amino acid identity to the $hP2Y_{11}$ receptor (Zambon et al., 2001). Initial characterization of this receptor in a canine thymocyte cell line revealed that it promoted both inositol lipid hydrolysis and cyclic AMP accumulation, strongly suggesting that this receptor is the canine homolog of the $P2Y_{11}$ receptor. In the current study, we stably expressed the canine and human $P2Y_{11}$ receptors in CHO-K1 and 1321N1 cells and characterized their nucleotide selectivities and second messenger coupling properties. These data demonstrate that the two receptors differ markedly in their nucleotide selectivity, their sensitivity to 2-thioether substitution of the adenine ring, and their coupling efficiency to adenylyl cyclase. We further show that at least part of the difference in nucleotide selectivity of the two receptors can be attributed to the amino acid at position 265 (numbering from the $hP2Y_{11}$ receptor) located at the juxtaposition of TM 6 and the third extracellular loop. These results thus identify a key residue involved in determining nucleotide binding and activation of this P2Y receptor.

Experimental Procedures

Materials. All cell culture reagents were supplied by the Lineberger Comprehensive Cancer Center tissue culture facility (University of North Carolina, Chapel Hill, NC). Tunicamycin and hexokinase were from Roche Biomedical (Indianapolis, IN). ITP, XTP, diadenosine tetraphosphate, 3-isobutyl-1-methylxanthine, suramin, RB-2, PPADS, and apyrase were purchased from Sigma (St. Louis, MO). ATP, UTP, and GTP were from Pharmacia/Upjohn (Piscataway, NJ). ADP, AMP, UDP, 2MeSATP, 2MeSADP, ATPγS, and ADPβS were obtained from Calbiochem (La Jolla, CA). Stock solutions of ADP and 2MeSADP in Dulbecco's modified Eagle's high-glucose medium were treated for 2 h with 50 U/ml hexokinase (Kennedy et al., 2000). ATPγS and ADPβS stock solutions were treated for 1 h with 3 U/ml apyrase, and 2MeSATP was purified by high-performance liquid chromatography.

Expression of $hP2Y_{11}$ and $cP2Y_{11}$ Receptors in CHO-K1 and 1321N1 Cells. CHO-K1 cells have been used by us and others to characterize the $hP2Y_{11}$ receptor (Communi et al., 1997, 1999; Qi et al., 2001). Although CHO-K1 cells express a $P2Y_2$ receptor that responds to ATP and UTP (Iredale and Hill, 1993), exogenous expression of either the $cP2Y_{11}$ or $hP2Y_{11}$ receptor in these cells gave rise to ATP-promoted increases in inositol phosphate accumulation ≥20-fold higher than those in vector-infected cells (Qi et al., 2001). This allowed us to carry out pharmacological studies of the $P2Y_{11}$ receptor in CHO-K1 cells. In some experiments, $P2Y_{11}$ receptors were expressed in 1321N1 cells. Expression in CHO-K1 cells resulted in higher receptor levels than in 1321N1 human astrocytoma cells, as indicated by the lower EC_{50} values of all nucleotides in CHO-K1 cells for promotion of inositol phosphate and cyclic AMP accumulation. No differences in the rank order of potency of nucleotides in the two cell lines were observed (Qi et al., 2001), but the higher levels of expression in CHO-K1 cells allowed full concentration-effect curves to be generated for many of the lower potency agonists (especially in cyclic AMP accumulation experiments with the $hP2Y_{11}$ receptor). In some experiments (Figs. 6, 7, and 8), receptors were HA-tagged to estimate cell surface expression by RIA. Inclusion of an HA-tag at the N terminus of the $P2Y_{11}$ receptor had no effect on the nucleotide selectivity or signaling properties (data not shown).

Recombinant retrovirus particles were produced by calcium phosphate-mediated transfection of PA317 cells with the pLXSN vector containing either $hP2Y_{11}$ or $cP2Y_{11}$ cDNA as described previously (Comstock et al., 1997). Both CHO-K1 and 1321N1 cells were grown in monolayer culture at 37°C in 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (5% for 1321N1 cells), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were infected with retrovirus harboring the $hP2Y_{11}$ or $cP2Y_{11}$ receptor coding sequence or with control retrovirus. CHO-K1 cells were pretreated with the glycosylation inhibitor tunicamycin (0.3 μg/ml) for 19 h before infection to inhibit the production of endogenous factors that suppress infection of hamster cells (Miller and Miller, 1992, 1993). Geneticin-resistant cells were selected after 7 (1321N1 cells) or 14 (CHO-K1 cells) days in the presence of 1 mg/ml G418 and maintained in medium containing 0.4 mg/ml active G418.

Assays of Inositol Phosphate and Cyclic AMP Accumulation. Cells stably expressing the $hP2Y_{11}$ or $cP2Y_{11}$ receptor were seeded in 24-well plates at 5×10^4 cells/well (1×10^5 cells/well for 1321N1 cells) and assayed 3 days later when confluent. Inositol lipids were radiolabeled by overnight incubation of the cells with 200 μl of inositol-free Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and 0.4 μCi of *myo*-[^{3}H]inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. Agonists or antagonists were added at 5× concentration in 50 μl of 50 mM LiCl and 250 mM HEPES, pH 7.25. After a 5-min incubation at 37°C, the medium was aspirated, and the assay was terminated by adding 0.75 ml of boiling EDTA, pH 8.0. [^{3}H]Inositol phosphates were resolved by Dowex AG1-X8 columns as described previously (Lazarowski et al., 1995).

To monitor cyclic AMP accumulation, cells were incubated with 0.8 μCi of [^{3}H]adenine for 2 h. Before the assay, cells were supplemented with 40 mM HEPES, pH 7.4, and 200 μM 3-isobutyl-1-methylxanthine (final concentrations in the assay). Drugs were added at 6× concentration in 50 μl of Hanks' balanced salt solution without calcium and magnesium. Drug challenges typically were carried out for 10 min at 37°C. The reactions were terminated by aspiration of the drug-containing medium and addition of 1 ml of 5% ice-cold trichloroacetic acid. [^{3}H]Cyclic AMP was purified using Dowex and alumina chromatography as described previously (Harden et al., 1982).

Intracellular [Ca^{2+}] Measurements. Agonist-promoted increases in intracellular [Ca^{2+}] were quantified under constant superfusion as described previously (Kennedy et al., 2000). These studies were carried out in 1321N1 cells expressing the human or canine $P2Y_{11}$ receptor, because the endogenous $P2Y_2$ receptor in CHO-K1 cells, although not a problem when measuring inositol phosphate or cyclic AMP accumulation of exogenously expressed $P2Y_{11}$ receptors, interferes with Ca^{2+} mobilization studies. 1321N1 cells do not express endogenous P2Y receptors and thus are well suited for nucleotide-promoted [Ca^{2+}] measurements. Agonists were applied for 30 s in the superfusate (1.4 ml/min) and the change in intracellular [Ca^{2+}] was measured in 7 to 16 individual cells per coverslip and averaged. To generate concentration-effect curves, each concentra-

tion of nucleotide was applied only once to each individual coverslip (to avoid receptor desensitization) and the average response from 7 to 16 cells per coverslip was measured from four to six coverslips. Data were recorded and processed using an InCyt IM2 digital imaging system (Intracellular Imaging Inc., Cincinnati, OH).

Mutagenesis. Mutations were incorporated into the human and canine $P2Y_{11}$ receptor by four-primer polymerase chain reaction (Ho et al., 1989). To account for differences in receptor expression, wild-type and mutant receptor cDNA containing an HA epitope tag immediately after the start codon served as template in the polymerase chain reactions. After amplification, the mutant receptors were cloned into the *Eco*RI and *Xho*I sites of pLXSN, and the presence of the mutations was verified by sequencing.

RIA for Detection of HA-Tagged Receptors. 1321N1 cells expressing HA-tagged wild-type and mutated $P2Y_{11}$ receptors were seeded at 1×10^5 cells/well in a 24-well plate. Assays to quantitate the expression of HA-tagged receptors were performed on confluent cells 3 days after plating essentially as described previously (Brinson and Harden, 2001). Briefly, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After the washing and blocking steps, cells were incubated for 1 h at 37°C with a 1:1000 dilution of mouse anti-HA monoclonal antibody (clone HA.11; Covance Research Products, Denver, PA). Cells were washed twice with Hanks' balanced salt solution (20 mM HEPES, pH 7.4, and 150 mM NaCl) containing Ca^{2+} and Mg^{2+}, followed by addition (typically 1×10^5 cpm/well) of ^{125}I-labeled rabbit anti-mouse antibody. After a 2-h incubation at room temperature, the cells were washed twice with Hanks' balanced salt solution containing Ca^{2+} and Mg^{2+}. Cells were then solubilized with 1 M NaOH and transferred to glass tubes for quantitation of radioactivity by γ-counting.

Statistics. Data are expressed as the mean ± S.D. or geometric mean. Concentration-response curves were fitted to the data by logistic (Hill equation), nonlinear regression analysis with DeltaGraph software (SPSS, Chicago, IL). Data were compared using one-way analysis of variance and Tukey's comparison or by Student's t test with $P < 0.05$ considered to be statistically significant.

Results

Adenine Nucleotide Selectivities of $hP2Y_{11}$ and $cP2Y_{11}$ Receptors. Both the $cP2Y_{11}$ and $hP2Y_{11}$ receptor were stably expressed in CHO-K1 cells, and their nucleotide selectivities and second messenger signaling properties were determined. As was observed previously with the $hP2Y_{11}$ receptor, ATP promoted both inositol phosphate and cyclic AMP accumulation in CHO-K1 cells expressing the $cP2Y_{11}$ receptor.[1] Adenosine triphosphate nucleotides were considerably more potent and efficacious as agonists at the $hP2Y_{11}$ receptor than their corresponding diphosphate nucleotides for promotion of both inositol phosphate and cyclic AMP accumulation (Fig. 1; Table 1). The potency order of these nucleotides was ATPγS ≥ 2MeSATP ≥ ATP ≈ ADPβS > 2MeSADP > ADP. In addition, ADP, ADPβS, and 2MeSADP were partial agonists at the $hP2Y_{11}$ receptor, with apparent efficacies that were 60 to 80% of maximal response to ATP (Table 1). As reported previously in CHO-$hP2Y_{11}$ cells (Qi et al., 2001), cyclic AMP responses to ADP and 2MeSADP (both up to 300 μM) did not reach a clear maximum and an EC_{50} value could not be determined (Fig. 1B).

In marked contrast to the results with the $hP2Y_{11}$ receptor,

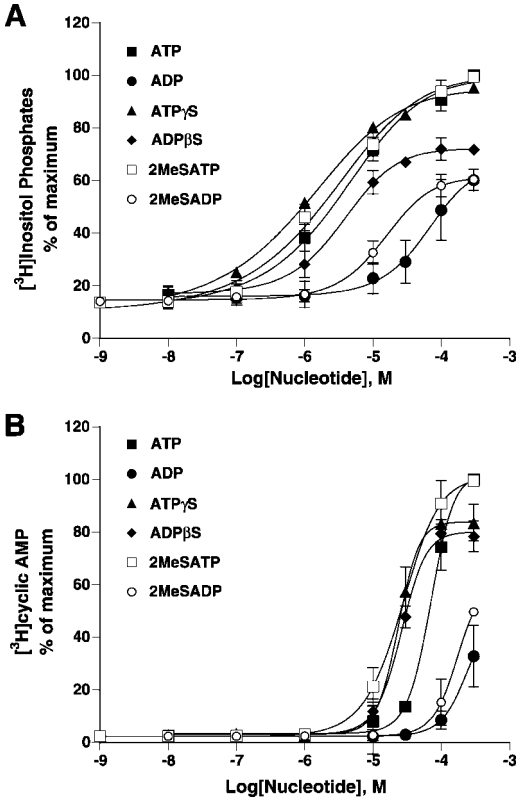

Fig. 1. The capacity of adenine nucleotides to promote inositol phosphate and cyclic AMP accumulation in CHO-$hP2Y_{11}$ cells. Nucleotide-promoted increases in inositol phosphate (A) or cyclic AMP (B) accumulation were measured in CHO-$hP2Y_{11}$ cells. Responses were normalized to the maximal response obtained with ATP (300 μM). Data shown are the mean of triplicate assays from three to six separate experiments.

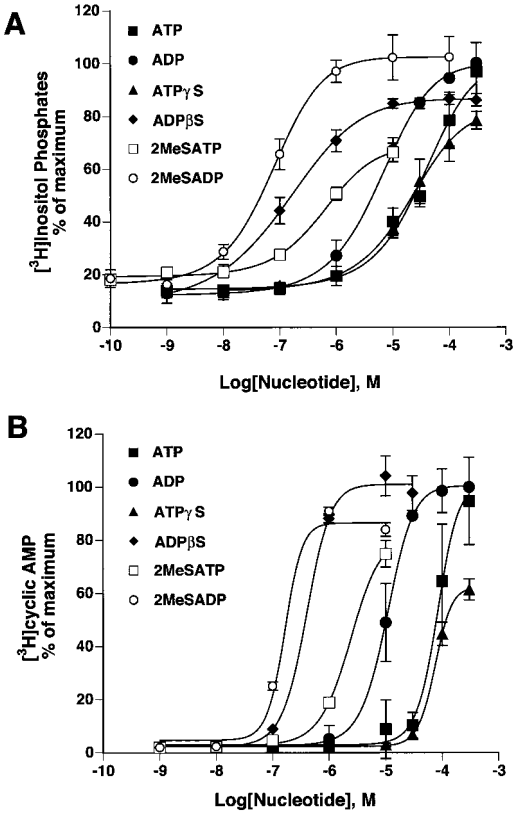

Fig. 2. The capacity of adenine nucleotides to promote inositol phosphate and cyclic AMP accumulation in CHO-$cP2Y_{11}$ cells. Nucleotide-promoted increases in inositol phosphate (A) or cyclic AMP (B) accumulation were measured in CHO-$cP2Y_{11}$ cells. Responses were normalized to the maximal response obtained with ADP (100 μM). Data shown are the mean of triplicate assays from three to six separate experiments.

TABLE 1
Activities of adenine nucleotides in CHO-hP2Y$_{11}$ and CHO-cP2Y$_{11}$ cells
Data shown are mean ± S.D. from three to six separate experiments.

Nucleotide	IP Accumulation		Cyclic AMP Accumulation		Ratio[b]
	EC_{50}	IA[a]	EC_{50}	IA[a]	
Human P2Y$_{11}$ Receptor	μM		μM	%	
ATP	3.6 ± 1.3	100	62.4 ± 15.6	100	17.3
ADP	50.1 ± 20.6	60.2 ± 4.3	NM	NM	NM
ATPγS	1.2 ± 0.4	95.3 ± 4.3	23.2 ± 6.5	83.5 ± 7.0	19.3
ADPβS	3.7 ± 0.7	71.8 ± 1.1	25.9 ± 0.4	78.4 ± 5.8	7
2MeSATP	2.4 ± 0.4	99.4 ± 8.2	26.5 ± 3.7	99.4 ± 5.5	11
2MeSADP	14.6 ± 3.2	60.5 ± 6.0	NM	NM	NM
Canine P2Y$_{11}$ Receptor					
ATP	32.8 ± 13.4	96.8 ± 9.3	76.4 ± 29.2	94.8 ± 9.3	2.3
ADP	6.1 ± 1.4	100	10.9 ± 3.7	100	1.8
ATPγS	19.3 ± 4.9	78.4 ± 3.4	74.0 ± 3.6	61.4 ± 4.0	3.8
ADPβS	0.14 ± 0.07	86.0 ± 2.4	0.40 ± 0.06	97.7 ± 6.5	2.9
2MeSATP	0.57 ± 0.11	66.2 ± 3.5	2.2 ± 0.4	74.9 ± 5.0	3.9
2MeSADP	0.08 ± 0.01	102 ± 6.8	0.16 ± 0.03	84.0 ± 2.5	2.0

[a] IA, intrinsic activity, normalized to the maximal response of 300 μM ATP (hP2Y$_{11}$) or 100 μM ADP (cP2Y$_{11}$).
[b] Ratio of the EC_{50} for cyclic AMP accumulation to the EC_{50} for inositol phosphate (IP) accumulation.
NM, response did not reach a maximum.

adenosine diphosphates were much more potent at the cP2Y$_{11}$[1] receptor than their corresponding triphosphates, with a potency order of 2MeSADP > ADPβS > 2MeSATP > ADP > ATPγS ≥ ATP (Fig. 2; Table 2). ADP, ADPβS and 2MeSADP were between 5- and 180-fold more potent than their corresponding triphosphates (ATP, ATPγS, and 2MeSATP, respectively) for promotion of both inositol phosphate and cyclic AMP accumulation in CHO-cP2Y$_{11}$ cells. Whereas all three diphosphate nucleotides tested (ADP, ADPβS, and 2MeSADP) were potent, full agonists at the cP2Y$_{11}$ receptor, ATPγS and 2MeSATP behaved as partial agonists and promoted inositol phosphate and cAMP accumulation to levels 60 to 85% of the maximal response to ADP (Fig. 2; Table 1). Analysis of these two receptor homologs expressed in 1321N1 cells gave very similar results to those in CHO-K1 cells, except that nucleotide potencies for both inositol phosphate and cyclic AMP accumulation were lower in 1321N1 cells (data not shown). The difference in nucleotide potencies in the two cell lines was observed previously (Qi et al., 2001).

These data also show that at the cP2Y$_{11}$ receptor, 2-thioether substitution of ATP and ADP preferentially increases agonist potencies relative to ATP and ADP, respectively. Thus, 2MeSATP and 2MeSADP were 58- and 76-fold more potent at the cP2Y$_{11}$ receptor than ATP and ADP, respectively, whereas the 2-thioether-substituted nucleotides were only 1.5- to 3.5-fold more potent than their corresponding natural nucleotides at the hP2Y$_{11}$ receptor (Table 1; Figs. 1 and 2).

Although much information can be gleaned from studies of inositol phosphate and cyclic AMP accumulation, an inherent disadvantage of these assays is that they are carried out under conditions, i.e., at high cell densities and with prolonged incubations with nucleotides, that accentuate issues of nucleotide release, breakdown, and bioconversion. For example, unlike other triphosphates, ATP shows full efficacy as an agonist at the cP2Y$_{11}$ receptor, but this may be due to breakdown of ATP to ADP during the assay. The ADP thus generated may then account for much of the effect of ATP at the ADP-sensitive cP2Y$_{11}$ receptor. To define unambiguously the triphosphate versus diphosphate nucleotide selectivities of these receptors, we measured receptor activation by monitoring intracellular Ca^{2+} mobilization of 1321N1-hP2Y$_{11}$ cells plated at low density and under constant superfusion. We have shown previously that these conditions minimize the metabolism of nucleotides (Palmer et al., 1998; Kennedy

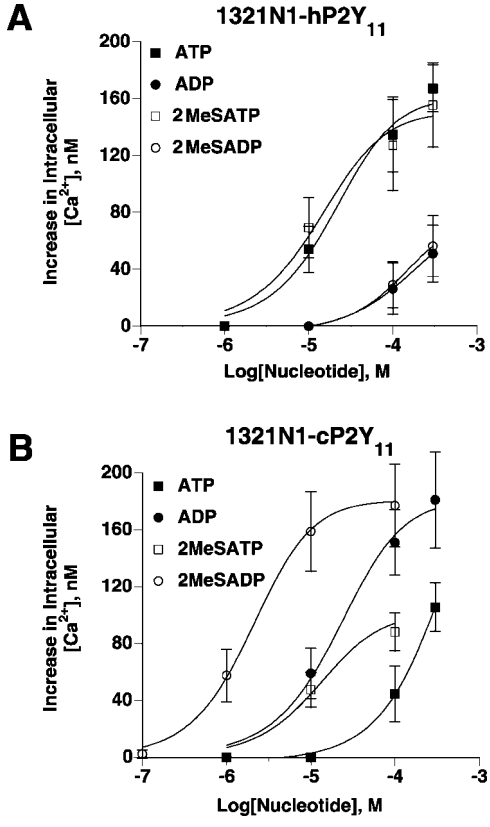

Fig. 3. The capacity of nucleotides to increase intracellular [Ca^{2+}] in 1321N1 cells expressing either the hP2Y$_{11}$ or cP2Y$_{11}$ receptor. Intracellular [Ca^{2+}] in response to the indicated nucleotides was measured under constant superfusion in 1321N1-hP2Y$_{11}$ cells (A) and 1321N1-cP2Y$_{11}$ cells (B). Each point was determined from a minimum of four coverslips, with 9 to 16 cells monitored per coverslip, as described under *Experimental Procedures*.

[1] The curves for cyclic AMP accumulation were steep, with Hill coefficients in the range of 2.5 to 3. The reasons for this deviation from mass-action are unknown and have not been pursued further.

et al., 2000). As shown in Fig. 3, ATP and 2MeSATP increased intracellular [Ca^{2+}] in 1321N1-hP2Y$_{11}$ cells with nearly equal potency and efficacy, whereas ADP and 2MeSADP were much less potent and efficacious than their triphosphate counterparts. In contrast, 2MeSADP and ADP were potent, full agonists in 1321N1-cP2Y$_{11}$ cells, whereas 2MeSATP and ATP were partial agonists. These data also highlight the sensitivity of the cP2Y$_{11}$ receptor to 2-thioether substitution of the adenine ring. Thus, the potencies of the 2-methylthio derivatives increased by at least 10-fold relative to their corresponding unsubstituted nucleotides. In contrast, little or no difference between the natural nucleotides and their 2-methylthio derivatives were observed at the hP2Y$_{11}$ receptor.

Agonist Activities of Other Nucleotides. To investigate whether the cP2Y$_{11}$ receptor is activated by a broader range of natural nucleotides than the hP2Y$_{11}$ receptor, we tested the capacities of UTP, UDP, GTP, ITP, XTP, and AMP to promote inositol phosphate accumulation in CHO-hP2Y$_{11}$ and CHO-cP2Y$_{11}$ cells. None of these nucleotides exhibited significant agonist activity at either the hP2Y$_{11}$ or cP2Y$_{11}$ receptor (data not shown). At a concentration of 300 μM, apyrase-treated Ap_4A promoted inositol phosphate accumulation at 34 ± 4.6% of the ATP response at the hP2Y$_{11}$ receptor, and 24 ± 3.0% of the ADP response at the cP2Y$_{11}$ receptor (basal subtracted, n = 3, data not shown). These data indicate that both the cP2Y$_{11}$ and hP2Y$_{11}$ receptors are highly adenine nucleotide-specific.

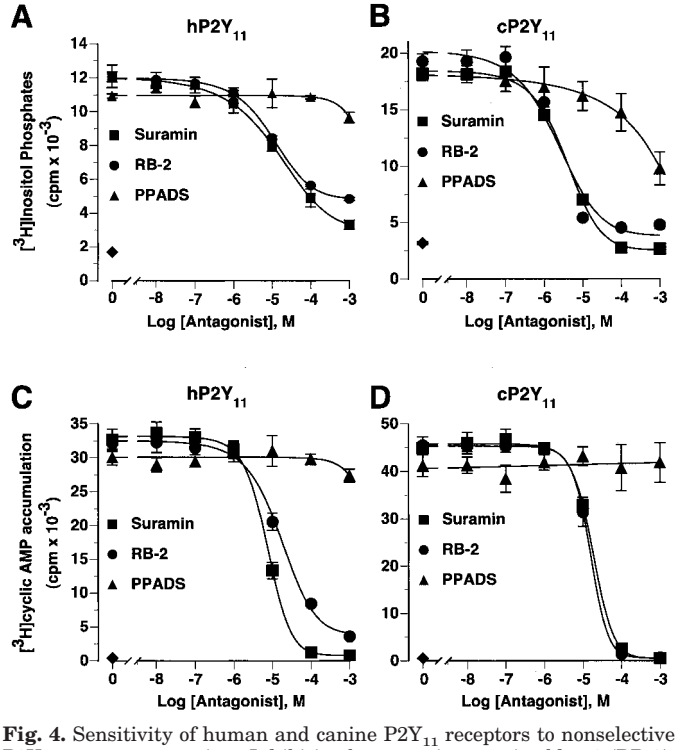

Fig. 4. Sensitivity of human and canine P2Y$_{11}$ receptors to nonselective P2Y receptor antagonists. Inhibition by suramin, reactive blue 2 (RB-2), and PPADS of inositol phosphate accumulation (A and B) or cyclic AMP accumulation (C and D) promoted by either 300 μM ATP in CHO-hP2Y$_{11}$ cells (A and C) or 100 μM ADP in CHO-cP2Y$_{11}$ cells (B and D). The diamonds in each graph represent the basal [^{3}H]inositol phosphate or cyclic AMP accumulation obtained in the absence of nucleotides. Data shown are the mean ± standard deviation of triplicate assays from three separate experiments.

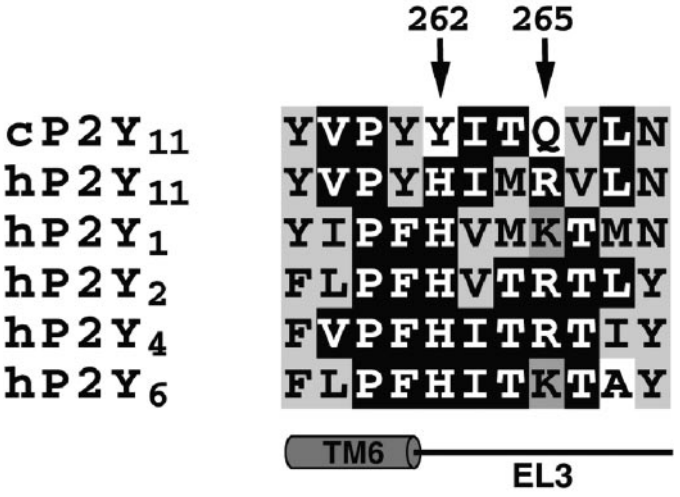

Fig. 5. Alignment of the amino acid sequences of P2Y receptors at the juxtaposition of TM 6 and the third extracellular loop. The arrows denote the two residues of the hP2Y$_{11}$ receptor (His-262 and Arg-265) that are different in the cP2Y$_{11}$ receptor. See text for more details.

Coupling Efficiencies of Human and Canine P2Y$_{11}$ Receptors to Inositol Lipid Hydrolysis and Cyclic AMP Accumulation. We have previously demonstrated that although the hP2Y$_{11}$ receptor promotes both inositol phosphate and cyclic AMP accumulation, it promotes inositol lipid hydrolysis with much higher efficiency than cyclic AMP accumulation (Qi et al., 2001). In contrast to the hP2Y$_{11}$ receptor, the cP2Y$_{11}$ receptor activates both second messenger pathways with very similar potencies (Fig. 2). Thus, agonist potencies for promotion of inositol phosphate accumulation at the hP2Y$_{11}$ receptor were 7- to 19-fold greater than for promotion of cyclic AMP accumulation (Table 1), whereas agonist potencies for promotion of the two second messenger responses differed by only 2- to 4-fold at the cP2Y$_{11}$ receptor (Table 2). These data indicate that the cP2Y$_{11}$ receptor couples to cyclic AMP accumulation with higher efficiency than does the hP2Y$_{11}$ receptor.

Sensitivity to P2Y Receptor Antagonists. We also determined the sensitivities of the two species homologs to nonspecific P2Y antagonists. Three antagonists, suramin, RB-2, and PPADS, were examined for their ability to block ATP- or ADP-promoted inositol phosphate and cyclic AMP accumulation in CHO-hP2Y$_{11}$ and CHO-cP2Y$_{11}$ cells, respectively. Whereas PPADS had little to no effect at either receptor, both suramin and RB-2 behaved as antagonists with similar potencies (Fig. 4). These data suggest that there is very little difference between canine and human P2Y$_{11}$ receptors in their sensitivity to nonselective P2Y antagonists.

The Role of Arg-265 in Triphosphate versus Diphosphate Selectivity in the P2Y$_{11}$ Receptor. Comparison of the deduced human and canine P2Y$_{11}$ sequences reveals two changes in the cP2Y$_{11}$ receptor (His-262 to Tyr and Arg-265 to Gln) located at the juxtaposition of TM 6 and the third extracellular loop that potentially underlie the distinct nucleotide selectivities of the two receptors (Fig. 5). In all other P2Y receptors, there are basic residues at these two positions. These positively charged amino acids have been predicted to interact with the phosphate moiety of nucleotide agonists (Erb et al., 1995; Jiang et al., 1997). To further examine their roles in nucleotide selectivity of the P2Y$_{11}$

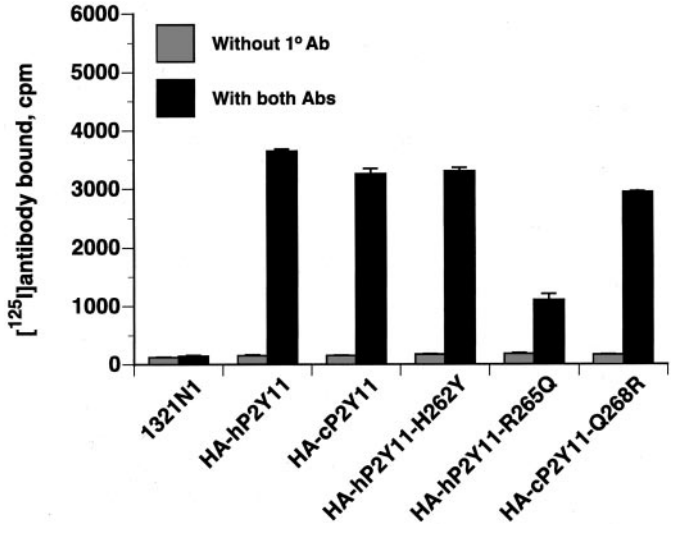

Fig. 6. Quantitation of the expression of mutant receptor constructs. Radioimmunoassay showing the relative expression of HA-tagged wild-type and mutant $P2Y_{11}$ receptors in 1321N1 cells.

receptor, we mutated His-262 and Arg-265 in the $hP2Y_{11}$ receptor, both individually and together, to their amino acid counterparts in the $cP2Y_{11}$ receptor. In addition, both wild-type and mutant receptors were HA-tagged to give an indication of the relative levels of expression.

Wild-type and mutant receptors were expressed in 1321N1 cells and their relative expression levels were assessed by an RIA assay (Fig. 6). These data indicated that all of the receptors were expressed at similar levels, with the exception of HA-$hP2Y_{11}$-R265Q, which was expressed at 30% of the level of the other receptors. Because the absolute values of agonist potencies are dependent on the level of receptor expression (Kenakin, 1997), it is not possible to compare directly the potencies from one cell line to the other. However, the relative potencies of ATP and ADP remain constant over a wide range of receptor levels and thus are indicative of differences in agonist selectivity.

Functional analyses of these receptors indicated that, whereas the H262Y mutation in the HA-$hP2Y_{11}$ receptor had no effect on the relative potencies of ATP and ADP, the R265Q mutation increased both the potency and efficacy of ADP relative to ATP, such that ATP and ADP had nearly equal potency and efficacy (Fig. 7). The receptor harboring both mutations was essentially identical to the receptor with only the single R265Q mutation (data not shown). We also tested the reverse mutation in the $cP2Y_{11}$ receptor, in which Gln-268 was mutated to arginine. Similar to the HA-$hP2Y_{11}$-R265Q receptor, the HA-$cP2Y_{11}$-Q268R receptor increased both efficacy and potency of ATP relative to ADP, such that ATP and ADP were essentially equipotent and equi-efficacious (Fig. 7). The identical efficacies of ADP and ATP at HA-$hP2Y_{11}$-R265Q and HA-$cP2Y_{11}$-Q268R receptors in inositol phosphate assays were verified by measuring the increases in intracellular $[Ca^{2+}]$ after challenge with 300 μM ADP or ATP (Fig. 8).

We also examined whether the amino acids at these two positions play a role in the differential sensitivity of the two receptors to 2-thioether substitution of the adenine ring (Fig. 7). Unlike the changes observed in diphosphate versus triphosphate selectivity, each mutant receptor displayed a similar enhancement of potency of 2MeSADP relative to ADP as the parent wild-type receptor. That is, the ADP to 2MeSADP potency ratios of HA-$hP2Y_{11}$-H262Y and HA-$hP2Y_{11}$-R265Q receptors were 3.8 and 2.8, respectively, compared with 1.6 for the wild-type $hP2Y_{11}$ receptor. Likewise, the ADP to 2MeSADP potency ratio of the HA-$cP2Y_{11}$-Q268R receptor was 33 compared with 67 for the $cP2Y_{11}$ receptor. Although the efficacy of 2MeSADP in the HA-$hP2Y_{11}$-R265Q mutant receptor increased relative to the $hP2Y_{11}$ receptor, no change in efficacy of 2MeSADP was observed in the HA-$cP2Y_{11}$-Q268R receptor relative to wild-type. These data indicate that the Arg-265/Gln-268 site contributes to diphosphate versus triphosphate selectivity of the two receptors but not to 2-thioether sensitivity.

Discussion

We demonstrate in this study that the $cP2Y_{11}$ receptor is an adenosine diphosphate-preferring receptor, in contrast to its adenosine triphosphate-preferring human homolog. The triphosphate versus diphosphate preferences of the two receptors were confirmed in studies monitoring nucleotide-promoted increases in intracellular $[Ca^{2+}]$ under conditions that minimized nucleotide breakdown and interconversion. Although differences in nucleotide selectivity between species

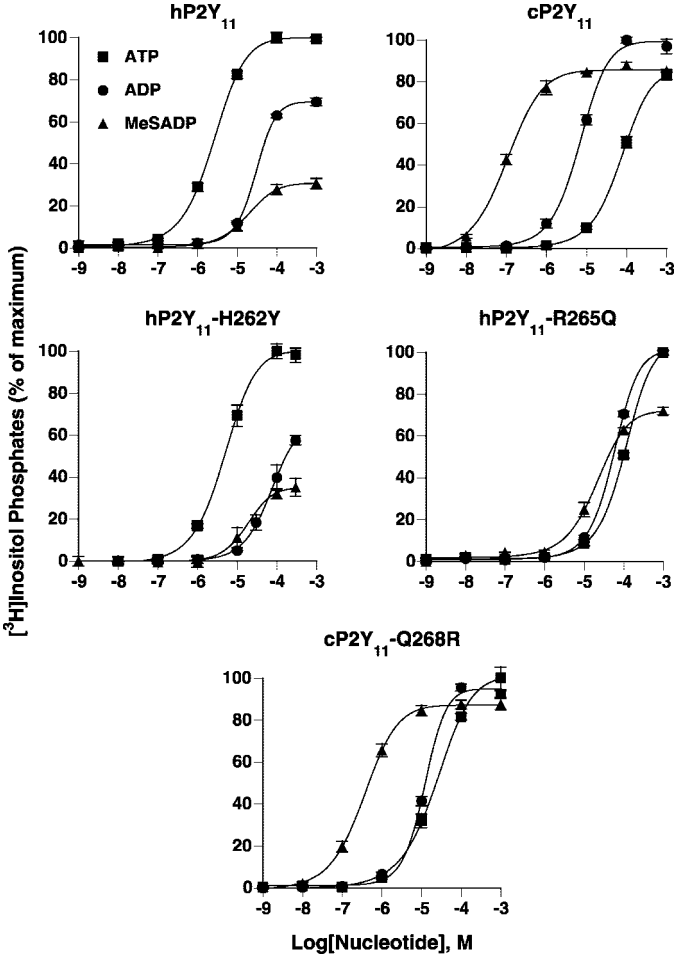

Fig. 7. The capacity of ATP, ADP, and 2MeSADP to promote inositol phosphate accumulation in 1321N1 cells expressing wild-type and mutant $P2Y_{11}$ receptors. The data shown are the mean of triplicate assays from one experiment repeated at least three times with similar results.

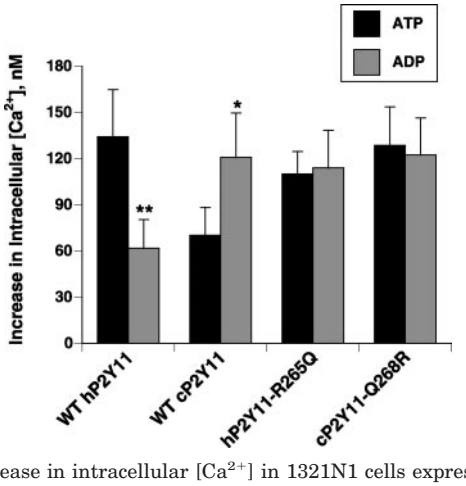

Fig. 8. Increase in intracellular $[Ca^{2+}]$ in 1321N1 cells expressing wild-type and mutant $P2Y_{11}$ receptors in response to 300 μM ADP and ATP. Each bar represents the mean increase in intracellular $[Ca^{2+}]$ from five coverslips, with 7 to 14 cells monitored per coverslip, as described under *Experimental Procedures*. *, $P < 0.05$, **, $P < 0.01$, relative to the response to ATP.

homologs of P2Y receptors have been noted (Li et al., 1998; Kennedy et al., 2000), this is the first example of species homologs that change their preference for diphosphate versus triphosphate nucleotides.

We also show that the $cP2Y_{11}$ receptor is markedly more sensitive to 2-methylthioether substitution of the adenine ring than the $hP2Y_{11}$ receptor and that the $cP2Y_{11}$ receptor couples to cyclic AMP accumulation with greater efficiency than the $hP2Y_{11}$ receptor. The increased coupling of the $cP2Y_{11}$ receptor to cyclic AMP accumulation relative to inositol lipid hydrolysis may be due to either changes in the primary structure of the $cP2Y_{11}$ receptor that facilitate a better coupling efficiency to Gs [there are multiple amino acid differences in the putative intracellular regions of the two receptors (Zambon et al., 2001)] or perhaps reflects the ability of the $cP2Y_{11}$ receptor to adopt a conformation upon binding of nucleotides that is more efficient at activating Gs than the one adopted by the agonist-occupied $hP2Y_{11}$ receptor. It should be noted that interaction of the $P2Y_{11}$ receptor with Gs is inferred, because there is no unequivocal evidence that the receptor directly activates Gs.

Because the canine receptor has only 70% sequence identity to the $hP2Y_{11}$ receptor and displays a markedly different nucleotide selectivity, it might be argued that the canine receptor is a distinct P2Y receptor subtype and not the species homolog of the $hP2Y_{11}$ receptor. However, several properties of this receptor make this possibility unlikely. First, both the human and canine receptors couple simultaneously to inositol lipid hydrolysis and cyclic AMP synthesis. The $P2Y_{11}$ receptor is the only P2Y receptor and one of only a few G protein-coupled receptors that are dually coupled to both of these signaling pathways (Qi et al., 2001). Secondly, although the receptors have distinct selectivity profiles, both receptors are highly adenine nucleotide-selective and have similar sensitivities to nonselective P2Y receptor antagonists. Taken together, these data strongly suggest that the $cP2Y_{11}$ receptor is the canine homolog of the $hP2Y_{11}$ receptor. Similar arguments have been made that the avian p2y3 receptor is the species homolog of the mammalian $P2Y_6$ receptor, even though the two receptors share only 65% amino acid identity and show differences in their nucleotide selectivities (Li et al., 1998).

The marked differences in nucleotide potencies of the human and canine homologs of the $P2Y_{11}$ receptor have important ramifications on physiological and pharmacological studies compared across species. Studies of native $P2Y_{11}$ receptors in cells and tissues derived from other mammalian species must now be conducted with caution. Until the $P2Y_{11}$ receptors from these species are cloned and characterized, their nucleotide selectivities and signaling properties cannot be inferred simply from data on the two molecularly identified $P2Y_{11}$ receptors. Thus, it will be important to clone the $P2Y_{11}$ receptors from rat, mouse and other mammalian species and to determine their pharmacological properties. In addition, these studies may also help to understand more clearly the structural and functional aspects of receptor-nucleotide interaction.

The residues involved in nucleotide recognition in the $P2Y_{11}$ receptor have not been delineated. In other P2Y receptors, studies have demonstrated a marked decrease in nucleotide potencies after mutation of both charged and uncharged residues located between TMs 3 and 7 of $P2Y_1$ (Jiang et al., 1997) and $P2Y_2$ (Erb et al., 1995) receptors. A subsequent study also showed that several residues in the putative second and third extracellular loops of the $P2Y_1$ receptor are involved in nucleotide binding and/or receptor activation (Hoffmann et al., 1999). However, outside of a few highly conserved charged amino acids within the TM regions, data from these studies are hard to apply to the $P2Y_{11}$ receptor due to its low sequence identity to other P2Y receptors. Because of the marked differences in nucleotide selectivity of $hP2Y_{11}$ and $cP2Y_{11}$ receptors, we compared the two sequences to identify amino acid changes that might account for these differences.

Inspection of the sequences identified two basic amino acids, His-262 and Arg-265, located at the juxtaposition of TM6 and the third extracellular loop in the $hP2Y_{11}$ receptor, that are changed to tyrosine and glutamine, respectively, in the $cP2Y_{11}$ receptor. These residues have been proposed to form part of the nucleotide binding pocket in other P2Y receptors and to form an electrostatic interaction with the phosphate moiety of the nucleotide (Erb et al., 1995; Jiang et al., 1997). Our data indicate that, whereas mutation of His-262 in the $hP2Y_{11}$ receptor to tyrosine had no effect on nucleotide selectivity, mutation of Arg-265/Gln-268 had marked effects on the relative potencies and efficacies of ATP and ADP in the two receptors. Thus, mutation of Arg-265 to glutamine in the $hP2Y_{11}$ receptor increased both the relative efficacy and potency of ADP, which is a lower potency partial agonist at the wild-type receptor, to the same as ATP. Likewise, mutation of Gln-268 to arginine in the $cP2Y_{11}$ receptor increased the relative potency and efficacy of ATP to the same levels as ADP. These data are consistent with the idea that Arg-265 in the $hP2Y_{11}$ residue is involved in nucleotide binding and functions in part to discriminate between triphosphate and diphosphate nucleotides. Unfortunately, in the absence of a reliable radioligand binding assay for the $P2Y_{11}$ receptor, it is not possible to demonstrate directly whether Arg-265 is involved in nucleotide binding. Even with this limitation, however, it is clear that the Arg-265/Gln-268 residue plays a significant role in defining whether the receptor prefers triphosphate or diphosphate nucleotides.

Previous studies by Jiang et al. (1997) showed that mutation of His-277 and Lys-280 in the $P2Y_1$ receptor (the "equivalent" residues to His-262 and Arg-265 in the $hP2Y_{11}$ receptor; Fig. 5) to alanine resulted in a marked decrease in potency of both 2MeSATP and 2MeSADP, although the effects of mutating Lys-280 were more dramatic than those for mutation of His-277. No changes in the triphosphate versus diphosphate selectivities of the mutant receptors were observed. Similarly, mutation of the residues in the $P2Y_2$ receptor equivalent to His-262 and Arg-265 to leucine (Erb et al., 1995) markedly decreased the potency of both UTP and ATP, also with no change in their selectivities for triphosphate and diphosphate nucleotides. Our results are very different from these earlier studies and suggest that in the $P2Y_{11}$ receptor, the identity of the residue at position 265 plays a major role in determining whether the receptor is more readily activated by ATP or ADP nucleotides. These data further suggest that the $P2Y_{11}$ receptor binds nucleotides in a subtly different manner than either the $P2Y_1$ or $P2Y_2$ receptor.

Importantly, neither of these mutations was sufficient to convert completely the selectivity of one homolog to the other, which suggests that other regions of the receptor must also contribute to defining diphosphate versus triphosphate selectivity. Likewise, these mutations had no effect on the sensitivity to 2-thioether substitution of the adenine base (Fig. 7), again suggesting that the amino acid(s) conferring this property must reside in other regions. Interestingly, the two receptors differ most noticeably in their putative extracellular regions, especially in the N terminus and the second and third extracellular loops. These regions may be involved in binding the adenine base and thus may mediate the differential sensitivity to 2-thioether substitution. The reasonably high sequence identity between the two receptors should facilitate a chimeric receptor approach to pinpoint the important regions and residues involved in these differences. These studies are in progress.

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