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2,2'-Pyridylisatogen tosylate antagonizes P2Y₁ receptor signaling without affecting nucleotide binding

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Abstract

The effect of 2,2'-pyridylisatogen tosylate (PIT) on the human P2Y₁ receptor and on other recombinant P2Y receptors has been studied. We first examined the modulation by PIT of the agonist-induced accumulation of inositol phosphates. PIT blocked 2-methylthio-ADP (2-MeSADP)-induced accumulation of inositol phosphates in 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors in a non-competitive and concentration-dependent manner. The IC₅₀ for reduction of the maximal agonist effect was 0.14 μM. In contrast, MRS2179, a competitive P2Y₁ receptor antagonist, parallel-shifted the agonist concentration–response curve to the right. PIT also concentration-dependently blocked the P2Y₁ receptor signaling induced by the endogenous agonists, ADP and ATP. A simple structural analogue of PIT was synthesized and found to be inactive as a P2Y₁ receptor antagonist, suggesting that the nitroxyl group of PIT is a necessary structural component for P2Y₁ receptor antagonism. We next examined the possible modulation of the binding of the newly available antagonist radioligand for the P2Y₁ receptor, [³H] MRS2279. It was found that PIT (0.01–10 μM) did not inhibit [³H] MRS2279 binding to the human P2Y₁ receptor. PIT (10 μM) had no effect on the competition for [³H] MRS2279 binding by agonists, ADP and ATP, suggesting that its antagonism of the P2Y₁ receptor may be allosteric. PIT had no significant effect on agonist activation of other P2Y receptors, including P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ and P2Y₁₂ receptors. Thus, PIT selectively and non-competitively blocked P2Y₁ receptor signaling without affecting nucleotide binding.

Keywords

P2Y receptors; Allosteric modulation; ADP; ATP; PIT; GPCR; Purines; Nucleotides

1. Introduction

P2 receptors exist in most tissues [1–3]. Physiological responses to extracellular nucleotides occur via both ion channel-coupled P2X receptors and G protein-coupled P2Y receptors.

The P2Y₁ receptor was the first member in the P2Y receptor family to be identified through

cloning and is widely distributed. Its occurrence in mammalian brain, heart, vascular, kidney, liver, prostate, pulmonary and connective tissues has been described [1]. The activation of P2Y₁ receptors induces the activation of phospholipase C_β (PLC_β) leading to the formation of inositol trisphosphate and mobilization of intracellular Ca²⁺ as well as diacylglycerol and subsequent activation of protein kinase C (PKC). ADP and its more potent analogue 2-methylthio-ADP (2-MeSADP) are agonists at P2Y₁ receptors, while the degree of intrinsic efficacy of ATP and 2-MeSATP are controversial [4–6].

2,2'-Pyridylisatogen (PIT, as tosylate form) is one of the isatogen analogues that were initially designed to be antagonists for P2 receptors [7]. PIT had been used to differentiate P1 (adenosine) and P2 (nucleotide) receptors [8]. It was later demonstrated that PIT is an allosteric modulator of the chicken P2Y₁ receptor expressed in *Xenopus* oocytes [9]. Over a narrow concentration range (0.1–3 μM), PIT caused a potentiation (two to five-fold) of responses to ATP. However, PIT failed to potentiate inward currents induced by 2-MeSATP and it inhibited this agonist-induced response. PIT has diverse effects, including neuroprotective effects [10], and acts as a spin trapping agent [11].

Allosteric modulation of GPCRs is of increasing interest for possible therapeutic application [12]. In this study, we further evaluated the possible modulatory effects of PIT on agonist-induced accumulation of inositol phosphates in 1321N1 astrocytoma cells stably expressing P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors, in Chinese hamster ovary (CHO) cells expressing the P2Y₁₂ receptor, and on the binding of the newly available antagonist radioligand, [³H] MRS2279 [13], for the P2Y₁ receptor. We found that PIT selectively and non-competitively blocked P2Y₁ receptor signaling without affecting the binding of ADP and [³H] MRS2279. In contrast to previous findings with the avian subtype [9], PIT did not enhance human P2Y₁ receptor activity.

2. Materials and methods

2.1. Materials

myo-[³H] Inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). ADP, ATP, 2-MeSADP and MRS2179 (*N*⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate), amiloride (3,5-diamino-*N*-(aminoiminomethyl)-6-chloro-pyrazinecarboxamide) hydrochloride, amiloride analogues, and agmatine (*N*-(4-aminobutyl)guanidine) sulfate were purchased from Sigma (St. Louis, MO, USA). PIT (2-(2-pyridinyl)-(3*H*)-indol-3-one-1-oxide 4-methylbenzenesulfonate, >99% purity by HPLC), PPADS (pyridoxal phosphate-6-azobenzene-2,4-disulfonic acid), suramin, and SCH-202676 were from Tocris (Ellisville, MO, USA). Pharmacological substances were stored as DMSO stock solutions at 4 °C. The radioligand [³H] MRS2279 (2-chloro-*N*⁶-methyl(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate) was prepared as described [13].

2.2. Cell culture and membrane preparation

Human astrocytoma cells stably expressing human P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ and rat P2Y₆ receptors were cultured in Dulbecco's modified Eagle's medium (DMEM, JRH Biosciences Inc., Lenexa, KS, USA) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units penicillin per ml, 100 μg streptomycin/ml, 2 μmol glutamine per ml,

and 500 µg geneticin per ml. After harvesting, the cells were homogenized and suspended and then centrifuged at $100 \times g$ for 5 min at room temperature. The pellet was resuspended in 50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 7.4). The suspension was homogenized with a Polytron electric homogenizer (Brinkmann, NY, USA) for 10 s and was then re-centrifuged at $20,000 \times g$ for 20 min at 4 °C. The resultant pellets were resuspended in buffer, and the suspension was stored at –80 °C until the binding experiments. The protein concentration was measured with the Bradford assay [14].

2.3. Radioligand binding assays

P2Y₁ receptor binding experiments were performed as previously described [13]. Briefly, membranes (40 µg protein per tube) from astrocytoma cells stably expressing human P2Y₁ receptors were incubated with [³H] MRS2279 (8 nM) for 30 min at 4 °C in a total assay volume of 200 µl. The radiolabeled ligand concentration used in the assay approximated the K_d value in binding to the receptor. Binding reactions were terminated by filtration through Whatman GF/B glass-fiber filters under reduced pressure with a MT-24 cell harvester (Brandel, Gaithersburg, MD, USA), and radioactivity was determined with a Packard liquid scintillation counter (Perkin-Elmer, Downers Grove, IL, USA).

2.4. Functional assays of receptor activation

The assay of PLC activation was carried out as previously described [15]. 1321N1 astrocytoma cells stably expressing human P2Y receptors were harvested by trypsinization and grown in six-well plates ($\sim 10^6$ cells per well; Costar, Cambridge, MA) in DMEM culture medium supplemented with 2 µCi/ml of *myo*-[³H] inositol. After a 24-h labeling period, cells were pre-incubated with 10 mM LiCl and for 20 min at room temperature. Because IP₃, the initial product of PLC activity and the relevant second messenger downstream of the P2Y receptors, is rapidly converted to the di- and monophosphate, which is later metabolized to *myo*-inositol, lithium chloride was added to inhibit *myo*-inositol 1-phosphatase [16]. Thus, measurement of *myo*-inositol 1-phosphate (IP) as end product of this cascade represented PLC activity. The mixtures were swirled to ensure uniformity. Following the addition of agonists, the cells were incubated for 30 min at 37° and 5% CO₂. The supernatants were removed by aspiration, and 800 µl of cold 20 mM formic acid was added to each well. Cell extracts were collected after a 30 min incubation at 4 °C and neutralized with 300 µl of 60 mM NH₄OH. The inositol monophosphate fraction was then isolated by anion exchange chromatography. The content of each well was applied to a small anion exchange column (AG-1-X8; Bio-Rad, Hercules, CA) that had been pretreated with 15 ml of 0.1 M formic acid/3 M ammonium formate, followed by 15 ml of water. The columns were then washed with 15 ml of a solution containing 5 mM sodium borate and 60 mM sodium formate. [³H] Inositol phosphates were eluted twice with 5 ml of 0.1 M formic acid/ 0.2 M ammonium formate, and radioactivity was quantified by liquid scintillation counting (LKB Wallac 1215 Rackbeta scintillation counter).

A functional assay of stimulation of adenylate cyclase via the hP2Y₁₂ receptor stably expressed in CHO cells was carried out in the presence of 10 µM forskolin by methods previously described [17].

2.5. Synthesis of MRS3461

N-Pyridin-2-yl-phthalimide (MRS3461) was synthesized from equimolar amounts of pyridine-2-yl-amine and phthaloyl dichloride in *N,N*-dimethylformamide at room temperature [18]. The solution was concentrated and poured into water, which was then extracted with diethyl ether. The ether extracts were concentrated, and the product was purified on preparative thin layer chromatography, eluting with chloroform/methanol (98/2, by volume), and re-crystallized from ethyl acetate. Mass spectra and nuclear magnetic resonance spectra confirmed the white crystals to be the desired compound. ¹H NMR (CDCl₃, 300 MHz) δ 8.75–8.65 (m, 1H), 7.99–7.79 (m, 4H), 7.46–7.43 (m, 1H), 7.38–7.35 (m, 1H), 7.26 (s, 1H). MS (fast atom bombardment, positive mode) *m/z* = 225 (*M* + 1).

2.6. Statistical analysis

Binding and functional parameters were calculated using the Prism software (GraphPAD, San Diego, CA, USA). IC₅₀ values obtained from competition curves were converted to *K_i* values using the Cheng–Prusoff equation [19]. Data were expressed as mean ± standard error.

3. Results

3.1. Effects of PIT on agonist-induced accumulation of inositol phosphates in 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors

The P2Y₁ receptor agonist 2-MeSADP-induced accumulation of inositol phosphates with an EC₅₀ value of 36 ± 14 nM (*n* = 3). PIT (0.1–10 μM) diminished human P2Y₁ receptor signaling in a non-competitive, concentration-dependent manner. The maximal agonist effect was reduced progressively by increasing concentrations of PIT, with an IC₅₀ of 0.14 μM. PIT (10 μM) completely blocked the agonist activity of 2-MeSADP (Fig. 1A). As a control, MRS2179, a known competitive P2Y₁ receptor antagonist [20], right-shifted the agonist concentration–response curve in parallel (Fig. 1B). PIT itself (0.1–10 μM) failed to induce accumulation of inositol phosphates directly in 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors (data not shown); thus, it was not an agonist at this receptor.

Based on the finding that PIT antagonized activation of the P2Y₁ receptor, we prepared a structural analogue MRS3461, which contained a carbonyl group in place of the nitroxyl group of PIT (Fig. 2). In contrast to PIT, which concentration-dependently inhibited accumulation of inositol phosphates induced by the agonist 2-MeSADP, MRS3461 had no significant effect (Fig. 3a). PIT also blocked the P2Y₁ receptor signaling induced by the endogenous agonist ADP in a concentration-dependent manner (Fig. 3b).

3.2. Effects of PIT on other P2Y receptors

PIT (10 μM) had no significant effects on UTP-induced P2Y₂ and P2Y₄ receptor signaling, ATP-induced P2Y₁₁ receptor signaling, or 2-MeSADP-induced P2Y₁₂ receptor signaling (Fig. 4). PIT also had no effect on rat P2Y₆ receptor signaling at concentrations up to 10 μM. At concentrations > 1 μM, PIT had no effect on the activation of the human P2Y₆

receptor, and at a higher concentration (10 μ M) PIT induced only a modest decrease of the signaling (Fig. 4).

3.3. Affinity of PIT and other potential allosteric modulators, and known P2Y₁ ligands for human P2Y₁ receptors stably expressed in human astrocytoma cells

Fig. 5 shows that the competitive antagonist MRS2179 displaced the binding of [³H] MRS2279 with a K_i value of 103 ± 37 nM ($n = 3$). PIT at concentrations ranging from 0.1 to 100 μ M had no effect on [³H] MRS2279 binding; thus, it appeared to antagonize the P2Y₁ receptor through an allosteric mechanism. Similarly various broad spectrum GPCR modulators such as the thiadiazole derivative SCH-202676, amiloride and its analogues, and agmatine [21–27] also did not compete for the binding of [³H] MRS2279. The potency of known agonists and antagonists and potential allosteric modulators were summarized in Table 1.

3.4. Effect of PIT on agonist-competition curves for [³H] MRS2279

Both ADP and ATP concentration-dependently inhibited [³H] MRS2279 binding to P2Y₁ receptors (Fig. 6). Since PIT blocked agonist-induced receptor activation in a concentration-dependent manner, we examined the possibility of PIT to shift the agonist competition curves using the antagonist radioligand [³H] MRS2279. However, PIT (10 μ M) failed to influence the competition of ADP and ATP for the binding of [³H] MRS2279 (Fig. 6).

3.5. ADP and ATP-induced-accumulation of inositol phosphates in 1321N1 astrocytoma cells stably expressing P2Y₁ receptors and the effects of PIT

The effects of ADP and ATP as agonists were compared. It was found that both ADP and ATP were fully efficacious at the human P2Y₁ receptor in the current assay, with ADP being more potent (Fig. 7A). As King et al. [9] reported, PIT might enhance the effect of ATP on the chicken P2Y₁ receptor expressed in *Xenopus* oocytes over a narrow range of concentrations; here we further examined if PIT may enhance the functional effect of ATP on the human P2Y₁ receptor stably expressed in 1321N1 astrocytoma cells. However, contrary to previous findings, PIT did not enhance the ATP effect (Fig. 7B).

3.6. Effect of PIT on the kinetics of dissociation of [³H] MRS2279 from P2Y₁ receptors

The binding of 8 nM [³H] MRS2279 to membranes from 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors was rapid, within 5 min reaching an apparent steady state that was maintained for at least 60 min (data not shown). The dissociation was initiated by addition of 10 μ M MRS2179 in the absence and presence of 10 μ M PIT. As shown in Fig. 8, PIT had no effect on [³H] MRS2279 dissociation. The k_{-1} values in the absence and presence of 10 μ M of PIT were 0.55 ± 0.13 and 0.49 ± 0.04 min⁻¹, respectively, which were not significantly different.

4. Discussion

Here we demonstrated that PIT selectively, non-competitively blocked human P2Y₁ receptor activation in 1321N1 astrocytoma cells. PIT did not affect the binding of the antagonist [³H] MRS2279 or agonists ADP/ATP to the human P2Y₁ receptor. It has been reported

previously that PIT might enhance the effect of ATP but not 2-MeSATP on the chicken P2Y₁ receptor expressed in *Xenopus* oocytes over a narrow range of concentrations (0.1–3 μM) [9]. Also, PIT enhanced purinergic vasoconstriction in the rabbit splenic artery [28]. However, in the present study PIT failed to potentiate the response induced by any of the agonists at the human P2Y₁ receptor; on the contrary, it blocked the agonist-induced response. The apparent difference between the results from the present study and those previously reported [9] might be due to a number of factors, for example, species difference, different cell lines, different assay systems and differences in receptor reserve. By analogy, differences in receptor reserve may account for the observed variation in the intrinsic efficacy of ATP at P2Y₁ receptors. It has been reported that ATP is an antagonist for human P2Y₁ receptors in Jurkat cells and endothelial cells [4]. However, Palmer et al. [5] reported that ATP is an agonist in 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors. In the present study, consistent with the report by previous findings [5], ATP was as efficacious as the full agonist ADP.

In addition to PIT, which did not affect nucleotide binding, other substances known to act as general GPCR modulators also did not show any effect on [³H] MRS2279 binding to the P2Y₁ receptor. These modulators include SCH-202676 [21–23], amiloride analogues [24–26], agmatine [27]. The results suggested that for the P2Y₁ receptor, modes of interaction of orthosteric and allosteric binding sites might be different from that of other GPCRs.

The non-competitive antagonism of the accumulation of inositol phosphates induced by a P2Y₁ receptor agonist in 1321N1 astrocytoma cells suggested that PIT might shift agonist competition curves in binding of the antagonist radioligand, [³H] MRS2279. However, this was not demonstrated in the present study. Therefore, one possible explanation of the antagonism by PIT is that it is allosteric, i.e. by acting at a site on the receptor distinct from the agonist binding site. Another explanation would be action at a protein that associates with the P2Y₁ receptor, such as G_{αq}. The antagonism could not be purely at the level of the G protein, since other P2Y receptors, which act through the same effector system remained functional in the presence of PIT. It would be helpful to demonstrate if PIT could influence the dissociation kinetics of the agonist radioligand. However, the P2Y₁ receptor agonist radioligand has not been commercially available. Also, we did not measure the possible action (or lack of effect) of PIT on another ADP-preferring nucleotide receptor, the P2Y₁₃ subtype [17].

Although this study did not investigate in detail the structure activity relationship of PIT and its analogues [10], a simple structural analogue MRS 3461 was synthesized and found to be inactive as a P2Y₁ receptor antagonist. Thus, it appears that the nitroxyl group of PIT is a necessary structural component for antagonism of the P2Y₁ receptor.

In conclusion, our data demonstrated that PIT subtype-selectively and non-competitively blocked P2Y₁ receptor signaling without affecting the nucleotide binding. We found no evidence for allosteric enhancement by PIT of the recombinant human P2Y₁ receptor expressed in astrocytoma cells.

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Abbreviations

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
IP	<i>myo</i> -inositol 1-phosphate
MRS2179	<i>N</i> ⁶ -methyl-2-deoxyadenosine-3',5'-bisphosphate
MRS2279	2-chloro- <i>N</i> ⁶ -methyl-(<i>N</i>)-methanocarpa-2'-deoxyadenosine-3',5'-bisphosphate
MRS3461	<i>N</i> -pyridin-2-yl-phthalimide
2-MeSADP	2-methylthio-adenosine 5'-diphosphate
PIT	2,2'-pyridylisatogen tosylate
PLC	phospholipase C
SCH-202676	<i>N</i> -(2,3-diphenyl-1,2,4-thiadiazol-5-(2H)-ylidene)methanamine
Tris	tris(hydroxymethyl)aminomethane

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Fig. 1.

Effects of PIT and MRS2179, a competitive P2Y₁ receptor antagonist, on agonist-induced accumulation of IP, as a measure of inositol phosphates in 1321N1 astrocytoma cells stably expressing the human P2Y₁ receptor. Data were from one experiment performed in duplicate, which represents at least three independent experiments of similar results. The EC₅₀ value of 2-MeSADP listed in the text was calculated from three independent experiments performed in duplicate.

Fig. 2.
Chemical structures of pyridylisatogen and MRS3461.

Fig. 3.

(A) Effects of PIT and a structural analogue MRS3461 on agonist 2-MeSADP (100 nM)-induced accumulation of IP, as a measure of inositol phosphates in 1321N1 astrocytoma cells stably expressing P2Y₁ receptors ($n = 3$). (B) Effect of PIT on agonist ADP (1 μ M)-induced accumulation of inositol phosphates.

Fig. 4.

Effects of PIT on activation of human P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ and P2Y₁₂ receptors and rat P2Y₆ receptors. Inhibition by 2-MeSADP of forskolin (10 μ M)-stimulated activation of adenylate cyclase was the indicator of activity at the P2Y₁₂ receptor (receptor stably expressed in CHO cells). At all of the other receptors, stimulation of PLC was measured (receptors expressed in 1321N1 astrocytoma cells), using as agonists: UTP (P2Y₂, P2Y₄), UDP (P2Y₆), and ATP (P2Y₁₁). Data were from one experiment performed in duplicate, which represents three independent experiments of similar results.

Fig. 5. Effect of PIT and MRS2179 on the binding of [^3H] MRS2279 to membranes from astrocytoma cells stably expressing the human P2Y_1 receptor. Membranes (40 μg protein per tube) from 1321N1 astrocytoma cells stably expressing the human P2Y_1 receptor were incubated with [^3H] MRS2279 (8 nM) for 30 min at 4 $^{\circ}\text{C}$. Results were expressed as mean $\pm$ S.E.M. from three independent experiments performed in duplicate. The potencies of PIT and MRS2179 are listed in Table 1.

Fig. 6. Effect of PIT on the competition curves of ADP and ATP for binding of the antagonist radioligand [³H] MRS2279. Membranes (40 μg protein per tube) from 1321N1 astrocytoma cells stably expressing the human P2Y₁ receptor were incubated with [³H] MRS2279 (8 nM) for 30 min at 4 °C. Data were expressed as mean ± S.E.M. from three independent experiments performed in duplicate.

Fig. 7.

(A) ADP- and ATP-induced accumulation of IP, as a measure of inositol phosphates in 1321N1 astrocytoma cells stably expressing the human P2Y₁ receptor. (B) Effect of PIT on ATP-induced accumulation of inositol phosphates.

Fig. 8.

Effect of PIT on the dissociation of the antagonist radioligand [^3H] MRS2279 from P2Y_1 receptors. Membranes (40 μg protein per tube) from 1321N1 astrocytoma cells stably expressing the human P2Y_1 receptor were incubated with [^3H] MRS2279 (8 nM) for 30 min at 4 $^\circ\text{C}$. The dissociation was initiated by addition of 10 μM MRS2179 in the absence and presence of PIT. Data were from a representative experiment performed in duplicate. The k_{-1} values listed in the text were calculated from three independent experiments performed in duplicate.

Table 1

Effects on radioligand binding at the human P2Y₁ receptor of PIT and various known, broad-spectrum allosteric modulators of GPCRs and a series of P2Y₁ receptor agonists and antagonists^a

Allosteric modulators, % inhibition (concentration)		P2Y receptor ligands, K_i (nM)	
PIT ^b (μM)	0 (100)	ADP	897 ± 163
SCH-202676 (μM)	0 (10)	ATP	1246 ± 331
Agmatine (mM)	0 (1)	2-MeSADP	57 ± 12
Amiloride (mM)	0 (1)	2-MeSATP	66 ± 20
DMA ^c (μM)	0 (100)	Suramin	3265 ± 976
HMA ^c (μM)	0 (100)	PPADS	5320 ± 1238
MIBA ^c (μM)	0 (100)	MRS2179	103 ± 37

^a Membranes (40 μg protein) from 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors were incubated with [³H] MRS2279 (8 nM) for 30 min at 4 °C. Results were expressed as mean ± S.E.M. from three independent experiments performed in duplicate.

^b PIT was found to compete for binding of [³H] *N*⁶-*R*-(phenylisopropyl)adenosine to human A₁ adenosine receptors with a pK_i of 5.3 [9].

^c Abbreviations: DMA, 5-(*N,N*-dimethyl)amiloride; HMA, 5-(*N,N*-hexamethylene)amiloride; MIBA, 5-(*N*-methyl-*N*-isobutyl)amiloride.