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$P2Y_{13}$ receptor is responsible for ADP-mediated degranulation in RBL-2H3 rat mast cells

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ABSTRACT

Extracellular ADP is known to play many important physiological roles. In this study, we identified the $P2Y_{13}$ receptor in a rat mast cell line (RBL-2H3) and explored the functional role of ADP, its endogenous agonist. ADP induced both intracellular calcium mobilization and release of hexosaminidase (Hex). In an assay of intracellular calcium, ADP was 100-fold less potent than and equally efficacious as the $P2Y_1$ receptor-selective agonist MRS2365. However, ADP was more potent and efficacious than MRS2365 in inducing Hex release and in enhancing antigen-induced Hex release. ADP-induced intracellular calcium mobilization was blocked by phospholipase C inhibitor U73122 and by $P2Y_1$ receptor-selective antagonist MRS2500, but not by pertussis toxin (PTX), suggesting a mechanism mediated by the G_q-coupled $P2Y_1$ receptor, but not $P2Y_{13}$ (G_i-coupled) or P2X receptors. ADP-induced Hex release was blocked by PTX and a selective $P2Y_{13}$ receptor antagonist MRS2211, but not by MRS2500 or $P2Y_1$ receptor-specific siRNA, suggesting a G_i-coupled $P2Y_{13}$ receptor-related mechanism. Measurement of gene expression confirmed high expression of both $P2Y_1$ and $P2Y_{13}$ receptors (in comparison to a previously reported $P2Y_{14}$ receptor) in RBL-2H3 cells. Thus, we demonstrated that ADP-mediated intracellular calcium mobilization and Hex release in RBL-2H3 cells are via $P2Y_1$ and $P2Y_{13}$ receptors, respectively. Selective antagonists of the $P2Y_{13}$ receptor might be novel therapeutic agents for various allergic conditions.

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1. Introduction

Mast cell degranulation plays a crucial role in the development of allergic diseases, which has traditionally been thought to be mediated via the FcεRI receptor [1–3]. However, recent evidence suggests that a growing number of G protein-coupled receptors (GPCRs), such as sphingosine-1-phosphate, C3a, C5a, adenosine, and UDP-glucose ($P2Y_{14}$) receptors, are also involved in this process [4–6]. Understanding the regulatory mechanisms of mast cell degranulation would help reduce unwanted allergic reactions in the development of novel therapeutic agents.

The role of extracellular nucleotides acting at P2X receptors (ion channels) and at P2Y receptors (GPCRs) in mast cells has been explored [7]. The P2Y receptors, which are activated by a range of extracellular mono- and di-nucleotides, are widespread in the immune system [8] and are an increasingly important focus of preclinical research [9]. In a recent study, we have shown that an extracellular nucleotide-sugar, UDP-glucose, can enhance antigen-induced β-hexosaminidase (Hex) release in rat basophilic leukemia (RBL)-2H3 cells via the $P2Y_{14}$ receptor [6]. Previously, some other nucleotides, including ATP and ADP, have also been indicated to be involved in mast cell function, although the P2Y receptor subtypes responsible for degranulation in various cases have not been fully identified partly due to the lack of proper pharmacological and biochemical tools [10–12]. For example, ADP has been shown to enhance IgE-mediated secretion of [^{3}H]-5-hydroxytryptamine by IL-3-dependent bone marrow-derived mast cells (BMMC) in a pertussis toxin-sensitive manner, but the receptor subtype involved could not be demonstrated [12]. ATP-induced histamine release in rat peritoneal mast cells has been

Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate; DMEM, Dulbecco's modified Eagle's medium; DNP, 2,4-dinitrophenyl; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; Hex, β-hexosaminidase; MRS2211, 2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde; PLC, phospholipase C; PTX, pertussis toxin; qRT-PCR, quantitative real-time polymerase chain reaction; RBL, rat basophilic leukemia; U73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione.

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ascribed to a mechanism mediated by phospholipase A2 or a P2X receptor [10].

ADP has been identified as the endogenous agonist of both human [13] and rat [14] $P2Y_{13}$ receptors and also activates $P2Y_1$ and $P2Y_{12}$ receptors. Cord blood-derived human mast cells were found to express mRNA encoding all three of these ADP-responsive receptors [11]. High expression levels of the $P2Y_{13}$ receptor in hematopoietic cells and the immune system, such as spleen, lymph nodes, and bone marrow, suggest a role for this subtype in inflammation-related disorders [13] and potentially in mast cell degranulation.

In the present study, we compared the expression levels of P2Y receptor subtypes using quantitative real-time PCR and found that $P2Y_{13}$ and $P2Y_1$ receptors are equally highly expressed in RBL-2H3 cells. We found that the $P2Y_{13}$ receptor is responsible for ADP-mediated Hex release, whereas the $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization.

2. Materials and methods

2.1. Materials

MRS2211 (2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde disodium salt) [15]; MRS2365 ([[(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl]diphosphoric acid mono ester trisodium salt) and MRS2500 ((1R,2S,4S,5S)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt) were from Tocris (St. Louis, MO). ADP, ATP, 2-MeSADP, U73122, pertussis toxin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, and 2,4-dinitrophenyl-bovine serum albumin (DNP-BSA), Triton X-100 and anti-DNP antibody were obtained from Sigma (St. Louis, MO). Taqman Universal PCR master mix, Taqman Gene Expression Assays, High Capacity cDNA Reverse Transcription Kit and Taqman Rodent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control were purchased from Applied Biosystems (Foster City, CA). Calcium assay kit was from Molecular Devices (Sunnyvale, CA). All other reagents were from standard sources and are of analytical grade.

2.2. Cell culture and detection of gene expression of P2Y receptors

Cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 3 μmol/ml glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. Total cellular RNA was isolated from RBL-2H3 cells using an RNA isolation kit (RNeasy, Qiagen, Valencia, CA) and was reversed-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Real-time PCR detection of the expression of the rat P2Y receptor gene and the endogenous reference GAPDH mRNA was performed using the 7900HT Fast System (Applied Biosystems, Foster City, CA). Quantitative analysis of data was performed by using the $2^{-\Delta\Delta Ct}$ method [16]. Values were normalized to GAPDH and were expressed as relative expression levels.

2.3. Measurement of release of Hex

We measured the release of Hex, a granule-associated protein that parallels histamine release and is an indicator of degranulation of RBL-2H3 cells, as previously described [6]. In brief, RBL-2H3 cells were split to 24-well plates (10^5 cells/ml) and incubated overnight with 0.1 μg/ml DNP-specific IgE antibody. Cells were washed twice and then stimulated with DNP-BSA, ADP, or both for 30 min. Hex was measured in the medium and in cell lysates (in 0.1% Triton X-100) by a colorimetric assay. Aliquots (20 μl) of samples were incubated with 20 μl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide at 37 °C in 0.1 M sodium citrate buffer (pH 4.5) for 1 h. The product, *p*-nitrophenol, was converted to the chromophore, 4-nitrophenoxide, by addition of 200 μl of a 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ buffer. Absorbance was read at 405 nm using a spectrophotometer. The $P2Y_1$ receptor-specific siRNA assay was performed as previously described [6]. Results are reported as the percentage of intracellular Hex that was released into the medium.

2.4. Intracellular calcium mobilization

RBL-2H3 cells were grown overnight in 100 μl media in 96-well flat bottom plates at 37 °C at 5% CO_2 or until 80–90% confluency. The calcium assay kit (Molecular Devices, Sunnyvale, CA) was used as directed without washing cells, and with probenecid added to the loading dye at a final concentration of 2.5 mM to increase dye retention. Cells were loaded with 50 μl dye with probenecid to each well and incubated for 60 min at room temperature. The compound plate was prepared using dilutions of various compounds in Hanks Buffer (pH 7.4). Samples were run in duplicate at room temperature. Cell fluorescence was measured (excitation at 485 nm; emission at 525 nm) following exposure to agonists. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure.

2.5. Statistical analysis

EC_{50} values were calculated with Prism 5 (GraphPad, San Diego, CA). Data were analyzed by analysis of variance (ANOVA) (followed by post hoc analysis) to check the statistical difference among groups with *P*-value less than 0.05 being considered significant. Results were expressed as mean ± SE.

3. Results

3.1. ADP-induced intracellular calcium mobilization in RBL-2H3 cells

In an initial experiment, it was found that ADP induced intracellular calcium mobilization in a concentration-dependent manner with an EC_{50} value of 278 ± 42 nM (Fig. 1). ATP was also shown to induce intracellular calcium increase, albeit less potently (EC_{50} = 1170 ± 220 nM). As ADP is the endogenous agonist for three subtypes of P2Y receptors ($P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$),

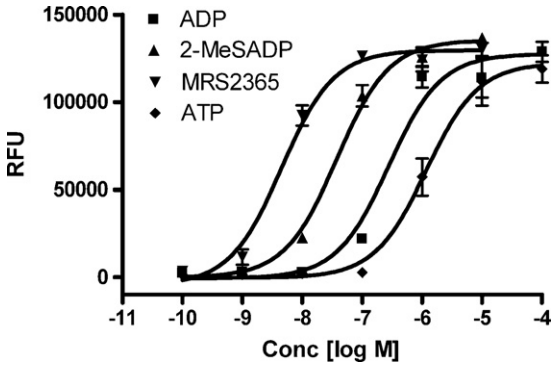

Fig. 1. $P2Y_1$ receptor agonist-induced intracellular calcium mobilization in RBL-2H3 cells. Results are expressed as mean ± SE from a triplicate determination representative of three independent experiments performed in duplicate or triplicate. The calcium mobilization was measured 20 s after addition of agonists and lasted for 120 s. The protocol used was similar to previously described [29]. The mean EC_{50} values of agonists are listed in the text.