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UDP-glucose acting at $P2Y_{14}$ receptors is a mediator of mast cell degranulation

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ABSTRACT

UDP-glucose (UDPG), a glycosyl donor in the biosynthesis of carbohydrates, is an endogenous agonist of the G protein-coupled $P2Y_{14}$ receptor. RBL-2H3 mast cells endogenously express a $P2Y_{14}$ receptor at which UDPG mediates degranulation as indicated by β-hexosaminidase (HEX) release. Both UDPG and a more potent, selective 2-thio-modified UDPG analog, MRS2690 (diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(2-thio)uridin-5″-yl] ester), caused a substantial calcium transient in RBL-2H3 cells, which was blocked by pertussis toxin, indicating the presence of the G_i-coupled $P2Y_{14}$ receptor, supported also by quantitative detection of abundant mRNA. Expression of the closely related $P2Y_6$ receptor was over 100 times lower than the $P2Y_{14}$ receptor, and the $P2Y_6$ agonist 3-phenacyl-UDP was inactive in RBL-2H3 cells. $P2Y_{14}$ receptor agonists also induced [^{35}S]GTPγS binding to RBL-2H3 cell membranes, and phosphorylation of ERK1/2, P38 and JNK. UDPG and MRS2690 concentration-dependently enhanced HEX release with EC_{50} values of 1150 ± 320 and 103 ± 18 nM, respectively. The enhancement was completely blocked by pertussis toxin and significantly diminished by $P2Y_{14}$ receptor-specific siRNA. Thus, mast cells express an endogenous $P2Y_{14}$ receptor, which mediates G_i-dependent degranulation and is therefore a potential novel therapeutic target for allergic conditions.

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1. Introduction

Mast cells play an important role in allergic diseases, such as bronchial asthma, rhinitis, anaphylaxis and urticaria [1,2]. Release of biogenic amines from the mast cells and lipid mediators result in bronchoconstriction, and released cytokines induce a late phase inflammatory response. Mast cell degranulation is known to be mediated via the high-affinity immunoglobulin E receptor, FcεRI [1–3]. However, there is increasing evidence that non-FcεRI mechanisms are also involved in the degranulation of mast cells in allergic reactions [3]. A growing number of G protein-coupled receptors (GPCRs), including those for adenosine [4], complement component C3a, macrophage inflammatory protein 1α, sphingosine-1-phosphate and prostaglandin E2 (PGE2), are demonstrated to affect mast cell degranulation [5–8]. The GPCRs expressed on mast cells may play an important role in human allergic diseases, and thus may serve as potential therapeutic targets. In fact, some GPCR ligands, such as agonists of the β_2 adrenergic receptor and antagonists of the $CysLT_1$ leukotriene receptor, have already been used in the clinic for asthma and a number of other allergic conditions.

UDP-glucose (UDPG) has a well-known intracellular role as a glycosyl donor in the biosynthesis of carbohydrates. However, recently UDPG was established as an endogenous extracellular agonist of the $P2Y_{14}$ receptor, the function of which is largely unclear [9,10]. In this study, we found that the $P2Y_{14}$ receptor is endogenously expressed on rat basophilic leukemia (RBL)-2H3 cells, which have been used as a mast cell model [4]. Both UDPG and its 2-thio analog, MRS2690 [11], can enhance antigen-induced mast cell degranulation in RBL-2H3 cells. The roles and mechanisms of the $P2Y_{14}$ receptor-mediated enhancement of mast cell degranulation were subsequently explored.

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DNP, 2,4-dinitrophenyl; EIA, enzyme immunometric assay; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-O-(γ-thio)triphosphate; HEX, β-hexosaminidase; LY294002, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one; MAPK, mitogen-activated protein kinase; MRS2690, diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(2-thio)uridin-5″-yl] ester disodium salt; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); PLC, phospholipase C; PTX, pertussis toxin; qRT-PCR, quantitative real-time polymerase chain reaction; RBL, rat basophilic leukemia; siRNA, small interfering RNA; TRIS, tris(hydroxymethyl)methylamine; U73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione; UDP, uridine 5′-diphosphate; UDPG, uridin 5′-diphosphoglucose disodium salt.

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2. Materials and methods

2.1. Materials

3-Phenacyl-UDP and MRS2690 (diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(4′-methylthio)uridin-5″-yl] ester disodium salt) were from Tocris (St. Louis, MO). UDP, UDPG (from Saccharomyces cerevisiae), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 2,4-dinitrophenyl bovine serum albumin (DNP-BSA), Triton X-100, LY294002, and anti-DNP antibody were obtained from Sigma (St. Louis, MO). Predesigned small interfering RNA (siRNA) for $P2Y_{14}$ receptors, negative control siRNA, Taqman Universal PCR master mix, Taqman (R) 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 3 dye kit was from Molecular Devices (Sunnydale, CA). MAP kinase assay kits were from Assay Designs (Ann Arbor, MI). All other reagents were from standard sources and are of analytical grade.

2.2. Cell culture and detection of $P2Y_{14}$ receptor mRNA expression

RBL-2H3 cells (ATCC, Manassas, VA) 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 $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{14}$ 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 [12]. Values were normalized to GAPDH and were expressed as relative expression levels.

2.3. Measurement of release of β-hexosaminidase (HEX)

As an indicator of degranulation of RBL-2H3 cells, we measured the release of HEX (a granule-associated protein that parallels histamine release) using a method slightly modified from Ramkumar et al. [4]. RBL-2H3 cells were split to 24-well plates and incubated overnight with 0.5 μg/ml DNP-specific IgE antibody. Cells were washed twice with Siraganian buffer (pH 7.4; NaCl, 119 mM; KCl, 5 mM; PIPES, 25 mM; glucose, 5.6 mM; $CaCl_2$, 1 mM; $MgCl_2$ 0.4 mM; BSA, 0.1%). Cells were then incubated for 20 min with DNP-BSA in the absence or presence of $P2Y_{14}$ receptor agonists. HEX was measured in medium and 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, *p*-nitrophenate, 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. Results are reported as the percentage of intracellular HEX that was released into the medium.

2.4. $P2Y_{14}$ receptor siRNA inhibition assay

Predesigned siRNAs (Applied Biosystems, Foster City, CA) against rat $P2Y_{14}$ receptors (sequence of predesigned $P2Y_{14}$ specific siRNA (5′ → 3′): sense: GACCCUGCUCUAUACGAAAtt; antisense: UUUCGUAUAGAGCAGGGUCtc) and negative controls were used. Cells were transfected at about 50–60% confluency by addition of siRNA (1 μM) together with GeneJet transfection reagent (SignaGen Laboratories, Gaithersburg, MD) as instructed by the manufacturer. Cells were split to 24-well plates after 24 h of transfection and HEX release was measured after an additional 48 h incubation period.

2.5. 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, Sunnydale, 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 using a Flexstation I 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.

2.6. [^{35}S]GTPγS binding assay

The preparation of membranes from RBL-2H3 cells was as described previously [13]. [^{35}S]GTPγS binding was measured in 200 μl buffer containing 50 mM TRIS HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.2 nM [^{35}S]GTPγS, 0.004% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS), and 0.5% BSA. Incubations were initiated by addition of the membrane suspension (10 μg protein/tube) to the test tubes, and carried out in duplicate for 30 min at 25 °C. The reaction was stopped by rapid filtration through Whatman GF/B filters, pre-soaked in 50 mM TRIS HCl, 5 mM $MgCl_2$ (pH 7.4) containing 0.02% CHAPS. The filters were washed twice with 3 ml of the buffer mentioned before, and retained radioactivity was measured using liquid scintillation counting. Non-specific binding of [^{35}S]GTPγS was measured in the presence of 10 μM unlabelled GTPγS.

2.7. Assays of activation of mitogen-activated protein kinases (MAPKs)

The activation of ERK1/2, P38 and JNK was quantitatively determined using TiterZyme® enzyme immunometric assay (EIA) kit (Assay Designs Inc., Ann Arbor, MI). In brief, cells in 24-well tissue culture plates were treated with UDPG (10 μM) for 1–60 min followed by washing with ice-cold PBS and lysis in 0.2 ml RIPA cell lysis buffer. The cell lysates were diluted 1:10 with the assay buffer, and 0.1 ml of the diluted lysates were applied to the 96-well plate coated with monoclonal antibody to phosporylated ERK1/2, JNK, or P38 and incubated at room temperature for 60 min with shaking. The plate was washed four times with wash buffer and incubated with a rabbit polyclonal antibody to pERK or pJNK or pP38 (which binds to the captured antibody to pMAPKs in the plate) at room temperature for 60 min. After incubation and shaking for 60 min, the excess antibody was washed out, the plate was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (binding to the rabbit polyclonal antibody) for 30 min. After washing, 0.1 ml of substrate solution containing 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide was added to each well, and the plate was incubated for another 30 min. The reaction was stopped by 100 μl 1N HCl, and the OD values were measured at 450 nm.

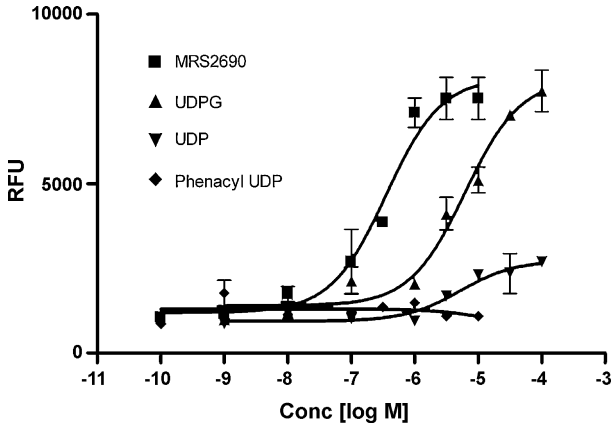

Fig. 1. Agonist-induced intracellular calcium mobilization in RBL-2H3 cells. Results are expressed as mean ± S.E. from a triplicate determination representative of 3–4 separate experiments performed in duplicate or triplicate. The mean EC_{50} values of agonists are listed in the text.

2.8. Statistical analysis

EC_{50} values were calculated with Prism 4 (GraphPad, San Diego, CA). Data were analyzed by analysis of variance (ANOVA) (followed by post-hoc analysis) or via Student's *t*-test to check the statistical difference among groups with P value less than 0.05 being considered significant. Results were expressed as mean ± S.E.

3. Results

3.1. Intracellular calcium mobilization assay

Both the native agonist of the $P2Y_{14}$ receptor, UDPG, and its more potent synthetic analogue MRS2690 [11] induced intracellular calcium mobilization in a concentration-dependent manner with EC_{50} values of 5980 ± 1140 and 538 ± 106 nM, respectively (Fig. 1), and with equal maximal efficacy. UDP was also shown to induce intracellular calcium mobilization, but it was less efficacious than UDPG (the maximum effect of UDP was 28% of that of UDPG) or MRS2690. The EC_{50} of UDP was calculated to be 5230 ± 1280 nM. A selective $P2Y_6$ receptor agonist, 3-phenacyl-UDP [14], was shown to be inactive at concentrations up to 10 μM (Fig. 1). Calcium transients induced by UDPG and MRS2690 were blocked by pretreatment with pertussis toxin (PTX) or an inhibitor of phospholipase C (PLC), U73122 (Fig. 2).

3.2. Measurement of the expression level of $P2Y_{14}$ receptor mRNA

We measured the expression level of the $P2Y_{14}$ receptor and compared it with those of three other P2Y receptors for uridine nucleotides ($P2Y_2$, $P2Y_4$ and $P2Y_6$) using real-time RT-PCR. mRNA expression of these four P2Y receptors was normalized to the GAPDH level. The gene expression levels are expressed as fold in comparison with that of the $P2Y_6$ receptor (set as 1), which showed the lowest expression. Fig. 3 shows that the expression levels of $P2Y_2$, $P2Y_4$ and $P2Y_{14}$ receptors (fold in comparison with that of the $P2Y_6$) were 6.13 ± 0.54, 1.58 ± 0.47 and 175 ± 52, respectively (n = 3).

3.3. Agonist-stimulated [^{35}S]GTPγS binding assay

As $P2Y_{14}$ receptors couple to $G_{i/o}$ proteins, we then determined the capability of UDPG and MRS2690 to stimulate the binding of [^{35}S]GTPγS to membranes prepared from RBL-2H3 cells. Fig. 4 shows that UDPG and MRS2690 stimulated [^{35}S]GTPγS binding in a concentration-dependent manner corresponding to EC_{50} values of 189 ± 29 and 8.1 ± 1.6 nM, respectively, with similar maximum agonist effects.

3.4. UDPG-induced activation of ERK1/2, JNK and P38 MAP kinases

Exposure of RBL-2H3 cells to the $P2Y_{14}$ agonist UDPG (10 μM) induced phosphorylation of all three MAPKs measured over a 30 or

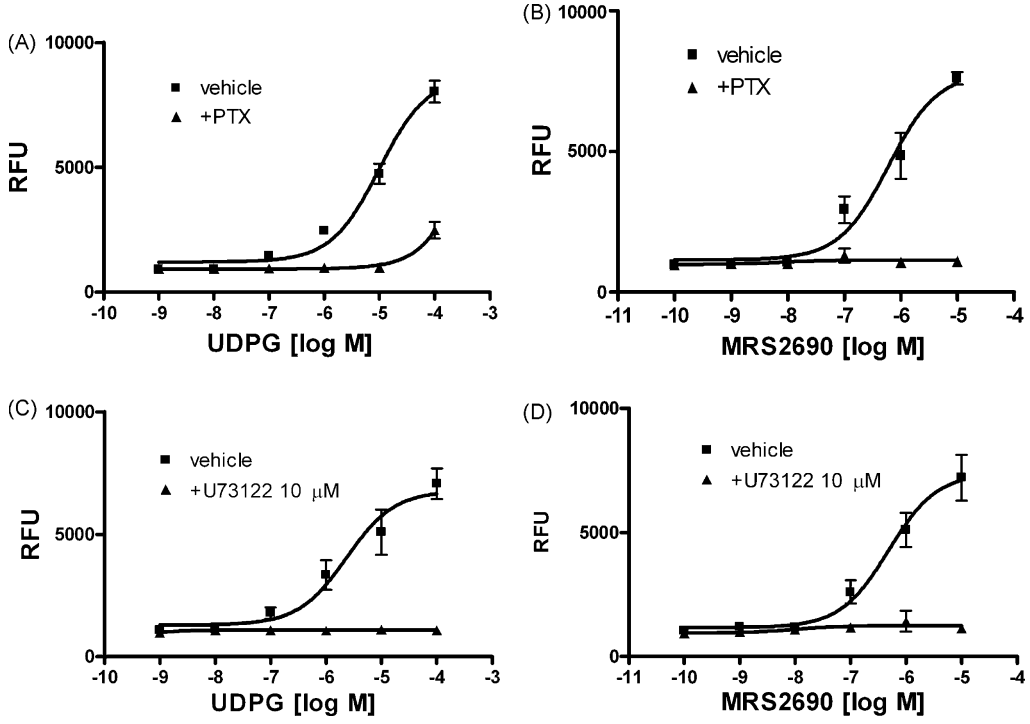

Fig. 2. (A–D) Effects of pretreatment with PTX and a phospholipase C inhibitor U73122 on agonist-induced calcium mobilization in RBL-2H3 cells. Cells were pretreated with PTX (100 ng/ml) for 24 h and with U73122 (10 μM) for 20 min before the measurements. Data are from one experiment performed in triplicate representative of three separate experiments performed in triplicate. Results are expressed as mean ± S.E.

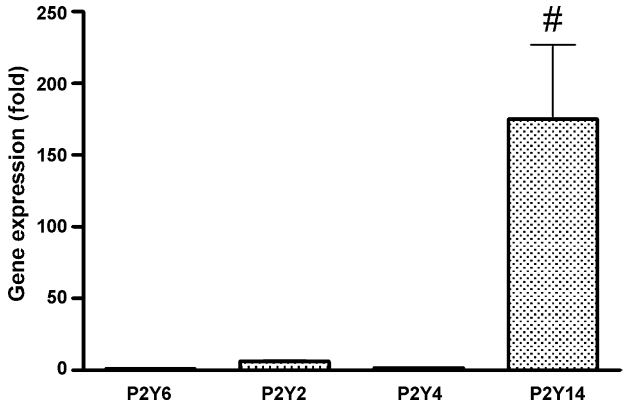

Fig. 3. mRNA expression of $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors in RBL-2H3 mast cells measured by real-time RT-PCR. mRNA expression of P2Y receptors was normalized to the GAPDH level. Results are expressed as fold in comparison with that of the $P2Y_6$ receptor (which showed the lowest expression and was set as 1) and are expressed as mean ± S.E. from three independent experiments. The expression levels of $P2Y_2$, $P2Y_4$ and $P2Y_{14}$ receptors (fold in comparion with that of the $P2Y_6$) are 6.13 ± 0.54, 1.58 ± 0.47 and 175 ± 52, respectively ($n = 3$). #Significantly different from the expression level of the $P2Y_6$ receptor ($P < 0.01$).

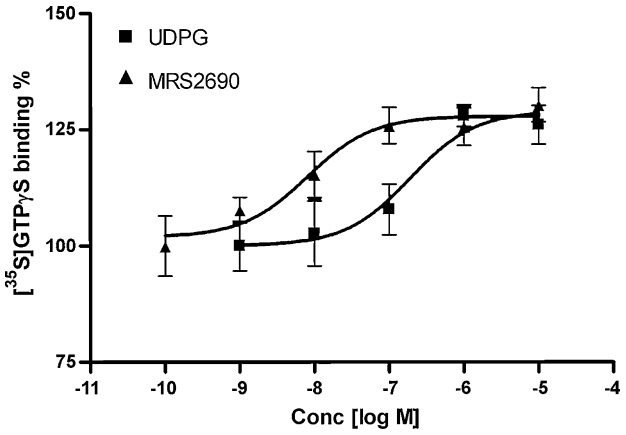

Fig. 4. UDPG and MRS2690-induced [^{35}S]GTPγS binding to membranes prepared from RBL-2H3 cells. Results are expressed as mean ± S.E. and are from three independent experiments performed in duplicate.

60 min period, although to a different extent and with different kinetics (Fig. 5). UDPG produced an approximately 2-fold increase in the activation of ERK1/2 (Fig. 5A). The ERK1/2 activation was transient, returning to baseline in 10 min. Maximal activation was observed in approximately 3 min, and then the activity slowly declined to basal levels. UDPG-induced ERK1/2 phosphorylation was shown to be PTX-sensitive. In contrast to the activation of ERK1/2, the phosporylation of JNK was sustained, but with less than 2-fold of basal value (Fig. 5C). UDPG was more efficacious in inducing P38 activation (>3-fold of basal value) compared with its effect on ERK1/2 and JNK (Fig. 5B). The activation of P38 reached a maximum at 10 min, and the activity returned to its basal level in about 60 min. MRS2690 (1 μM) was shown to activate P38 in a manner similar to that of UDPG (Fig. 5D).

3.5. Agonists of the $P2Y_{14}$ receptor enhance antigen-induced HEX release

Antigen (DNP-BSA, 10 ng/ml) induced the release of HEX from RBL-2H3 cells (Fig. 6A). The percentage of HEX release in the presence of 10 ng/ml antigen was significantly different from those in the control, UDPG-treated, or MRS2690-treated groups ($P < 0.01$). The $P2Y_{14}$ receptor agonists UDPG and MRS2690 alone were unable to induce HEX release (Fig. 6A; $P > 0.05$ compared with the control group). Although UDPG and MRS2690 alone were inactive, they concentration-dependently enhanced antigen (10 ng/ml)-induced HEX release (Fig. 6B), with EC_{50} values of 1150 ± 320 and 103 ± 18 nM, respectively. UDPG-induced enhancement of HEX release was completely blocked by PTX pretreatment (Fig. 6C). Pretreatment of RBL-2H3 cells with a PLC inhibitor U73122 (10 μM) blocked both antigen-induced and UDPG-enhanced HEX release (Fig. 6D).

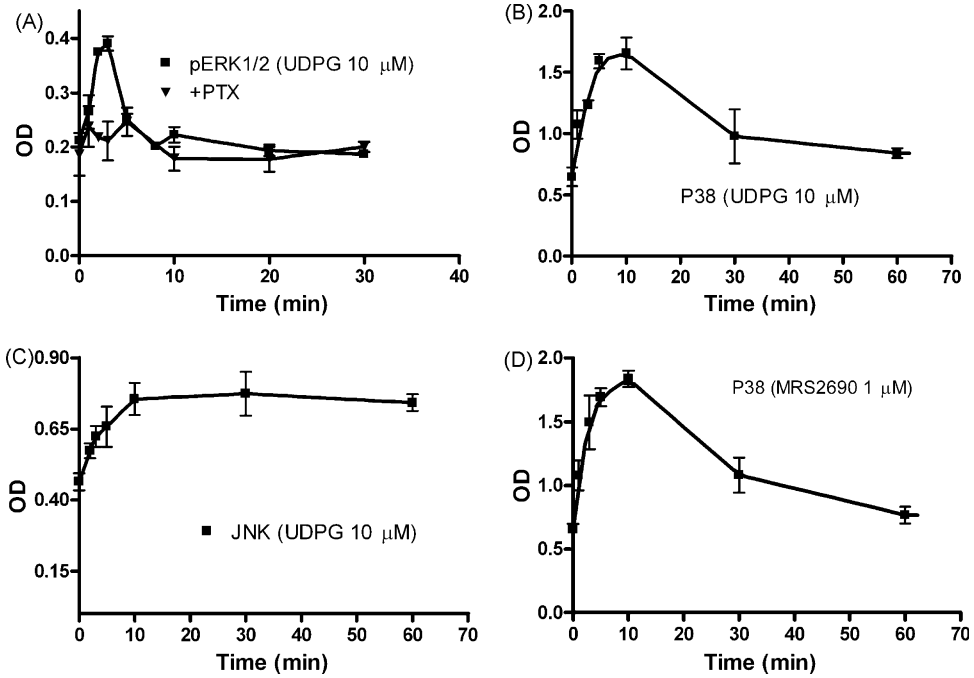

Fig. 5. Effect of UDPG (10 μM) and MRS2690 (1 μM) on phosphorylation of ERK1/2 (A), P38 (B and D) and JNK (C) in RBL-2H3 cells. In (A), cells were pretreated with PTX (100 ng/ml) for 24 h. Results are expressed as mean ± S.E. ($n = 3$).

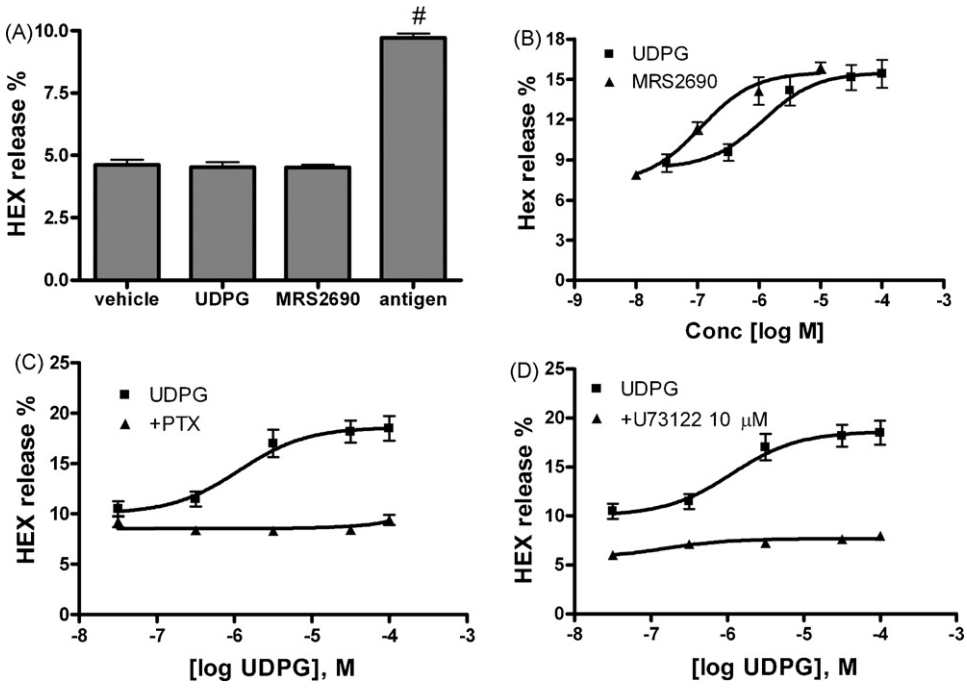

Fig. 6. HEX release in RBL-2H3 cells. Cells were primed with anti-DNP-BSA antibody for 24 h before testing for the release of HEX. (A) Comparison of HEX release induced by UDPG (10 μM), MRS2690 (1 μM) and antigen (10 nM). (B) UDPG and MRS2690 enhanced HEX release in the presence of 10 nM antigen. (C) Effect of PTX (100 ng/ml) pretreatment for 24 h. (D) Effect of pretreatment of a PLC inhibitor U73122 (10 μM) for 20 min.

3.6. siRNA attenuated $P2Y_{14}$ receptor induced HEX release

We next studied whether the enhancement of HEX release by UDPG was specifically mediated via the $P2Y_{14}$ receptor. In the absence of an antagonist for the $P2Y_{14}$ receptor, we used siRNA specific for the $P2Y_{14}$ receptor. As shown in Fig. 7, the $P2Y_{14}$ siRNA significantly diminished the maximum effect of UDPG-induced HEX release ($P < 0.05$ compared with control). The siRNA specific for GAPDH did not show any effect on HEX release induced by UDPG.

3.7. Comparison of the effects of PTX and enzyme inhibitors on antigen-induced and MRS2690-induced HEX release

As Fig. 6 shows that PTX and U73122 may have different effects on the release of HEX induced by antigen and antigen plus UDPG, we further tested this difference using the synthetic agonist MRS2690. Fig. 8A shows that PTX significantly affected neither basal HEX release nor antigen-induced HEX release ($P > 0.05$), but it significantly diminished MRS2690-induced enhancement of HEX release. The degree of HEX release in the group treated with antigen plus MRS2690 was $20.7 \pm 0.9\%$, which was significantly different from that in the PTX-treated group ($8.26 \pm 0.36\%$) ($P < 0.01$). The PLC inhibitor U73122 induced a larger inhibition compared with PTX, and so did an inhibitor of phosphoinositide 3-kinase (PI3K), LY294002. The percentages of HEX release in U73122- and LY294002-treated groups were found to be significantly lower than that in the PTX-treated group ($P < 0.05$).

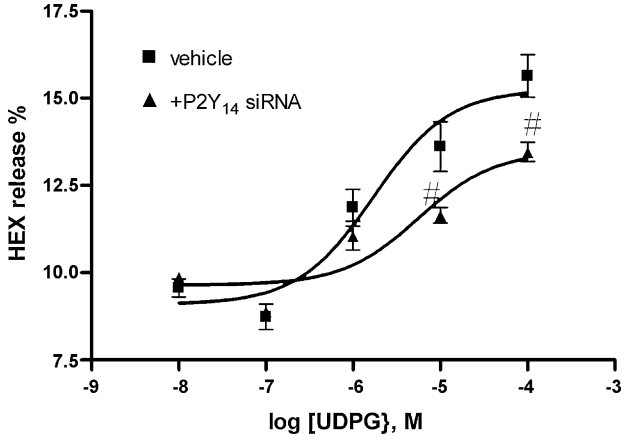

Fig. 7. HEX release from RBL-2H3 cell in the presence and absence of $P2Y_{14}$ receptor siRNA. Results are expressed as mean ± S.E. and were from 3 to 4 separate experiments performed in duplicate. $^{\#}P < 0.05$, compared with control.

3.8. Potential cooperative effects between the FcεRI and $P2Y_{14}$ receptor signaling pathways revealed in the absence of antibody priming

In order to explore the potential differences and interactions between the FcεRI and $P2Y_{14}$ receptor signaling pathways, we performed a study in the absence of antibody priming of cells before stimulation with antigen and/or $P2Y_{14}$ agonists (Fig. 8B). It was found that neither $P2Y_{14}$ receptor agonists nor antigen had an effect, but the combination of either UDPG or MRS2690 with antigen (1 nM) produced a modest but significant increase in HEX release. The degrees of HEX release in the antigen plus UDPG and antigen plus MRS2690 groups were $6.80 \pm 0.44\%$ ($n = 3$) and $6.78 \pm 0.24\%$ ($n = 3$), respectively, which were significantly different from the control group with $4.78 \pm 0.19\%$ ($P < 0.01$). However, the groups with antigen, UDPG, or MRS2690 alone were not significantly different from the control group ($P > 0.05$).

4. Discussion

The presence of a functional $P2Y_{14}$ receptor in RBL-2H3 cells from rat has been demonstrated pharmacologically and genetically. Our observations that the abundant gene expression and the involvement in degranulation in mast cells of the $P2Y_{14}$ receptor may imply a possible role for this receptor in the control of allergic

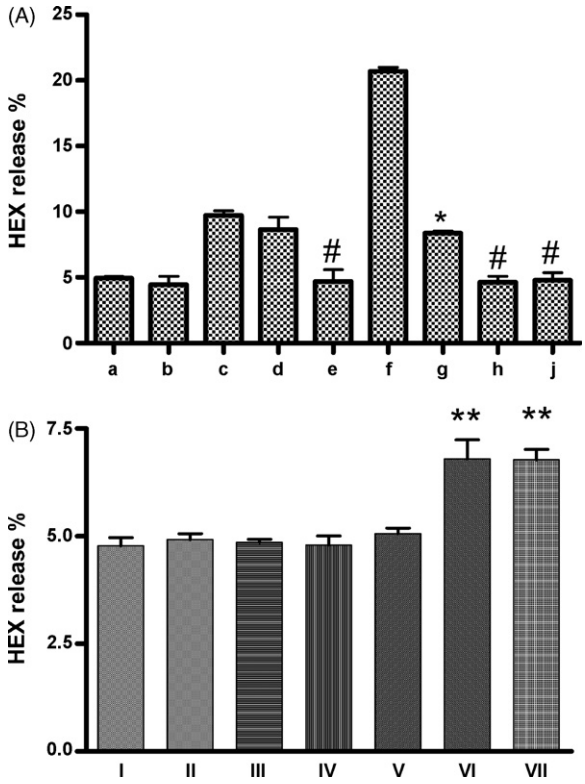

Fig. 8. (A) Effects of PTX (100 ng/ml) and U73122 (10 μM) on HEX release induced by antigen or antigen plus MRS2690. (a) Control; (b) PTX; (c) antigen (1 μM); (d) antigen + PTX; (e) antigen + U73122; (f) antigen + MRS2690 (1 μM); (g) antigen + MRS2690 + PTX; (h) antigen + MRS2690 + U73122; (j) antigen + MRS2690 + LY294002 (10 μM). $^{*}P < 0.01$ compared with group (f). $^{\#}P < 0.05$ compared with groups (d) and (h). (B) Cooperative effect in HEX release between antigen and agonist (either UDPG or MRS2690) in the absence of antibody priming. Cells were grown in the absence of anti-DNP-BSA antibody for 24 h before testing for the release of HEX. (I) Control; (II) UDPG (10 μM); (III) MRS2690 (1 μM); (IV) antigen (1 nM); (V) antigen (1 μM); (VI) antigen (1 μM) + UDPG; (VII) antigen (1 μM) + MRS2690.

conditions. Mechanistically, the effect is dependent on G_i protein. Activation of the $P2Y_{14}$ receptor in RBL-2H3 cells also raises intracellular calcium and activates kinases.

$P2Y_{14}$ receptors have previously been found in several cell types and tissues, with high expression in those related to immune function [16,17], such as spleen. Effects of UDPG in the immune system have been reported to be both $P2Y_{14}$ receptor-dependent and independent. For example, the receptor-dependent effect has been reported in human lung epithelial cells [18], neutrophils [19] and dendritic cells [20,21]. Functional expression has also been detected in murine spleen-derived T-lymphocytes [17] and in bone-marrow derived hematopoietic cells with stem cell characteristics [22]. A PTX-sensitive effect of UDPG has also been reported in differentiated HL-60 cells [23].

A number of $P2Y_{14}$ receptor-independent effects of UDPG have also been reported. In murine N9 microglia cells the pro-inflammatory effect of UDPG was insensitive to PTX [24]. UDPG was able to inhibit cyclic AMP production in human astrocytoma U373 MG cells and with sensitivity to PTX, however, the $P2Y_{14}$ receptor was not detected in these cells [17]. UDPG modulates gastric function through both $P2Y_{14}$-dependent and independent effects, as demonstrated using $P2Y_{14}$ receptor-knockout mice [25]. Thus, previous studies of the physiological role of the $P2Y_{14}$ receptor have led to inconsistent conclusions.

In the present study, we have compared the action of several agonist ligands, but in the absence of a selective antagonist for the $P2Y_{14}$ receptor, we used $P2Y_{14}$ specific siRNA to help confirm the involvement of this receptor in mast cell granulation. The inhibition suggests a $P2Y_{14}$ receptor-dependent effect of UDPG in the inhibition of HEX release. In addition, PTX-sensitive effect of $P2Y_{14}$ receptor agonists was also demonstrated in assays of calcium mobilization, ERK1/2 and HEX release, further supporting a $P2Y_{14}$ receptor-dependent effect. UDP, which is also a native modulator of the $P2Y_{14}$ receptor [15] that seems to be an agonist, partial agonist, or antagonist depending on the cell system studied, induced calcium mobilization in RBL-2H3 cells with similar potency but lower efficacy than UDPG. The synthetic agonist MRS2690 is both more potent and more selective than UDPG for the $P2Y_{14}$ receptor in comparison to $P2Y_2$ and $P2Y_6$ receptors [26]. It was consistently more potent than UDPG in RBL-2H3 cells and fully efficacious in these $P2Y_{14}$ receptor-mediated effects. In addition to the use of this selective agonist, we have used real-time RT-PCR and a potent $P2Y_6$ receptor agonist to eliminate the possibility of involvement of the closely related $P2Y_6$ receptor.

Activation of MAPKs by both the FcεRI and GPCRs has been reported in RBL-2H3 cells and in other cells, although via different mechanisms of action. ERK1/2 and P38 were shown to be phosphorylated in RBL-2H3 cells upon ionomycin treatment [27]. Ionomycin-induced degranulation was substantially diminished by a MEK1/2 inhibitor PD98059 but not by a P38 inhibitor SB203580. The antigen-induced activation of ERK1/2 in RBL-2H3 cells, and the inhibition of PKC or tyrosine kinase markedly inhibited ERK1/2 activation in parallel with serotonin release [28] in differentiated HL-60 cells. In neutrophils, UDPG-induced ERK1/2 but not P38 and JNK [23]. However, UDPG failed to modulate neutrophil degranulation, although it was able to inhibit cyclic AMP production in a PTX-sensitive manner and to induce ERK1/2 activation [19]. In the present study of RBL-2H3 mast cells, activation of the $P2Y_{14}$ receptor induced phosphorylation of all three MAPKs, although to different extents and with different kinetic patterns.

Crosstalk between the FcεRI and GPCRs has already been reported, but the exact points of mechanistic interaction are still largely unclear. One mechanism of the enhancement of antigen-induced HEX release by a GPCR is the synergistic activation of PLCβ and PLCγ, and subsequently the mobilization of calcium [3]. In the present study, a PLC inhibitor blocked both antigen-induced and UDPG-enhanced HEX release, whereas PTX only diminished UDPG-enhanced HEX release, suggesting that the mechanism activated by the $P2Y_{14}$ receptor is distinct from that of FcεRI. It is also interesting to note that, in the absence of antibody priming, neither antigen nor UDPG had an effect on HEX release. However, their combination produced a modest but significant increase in HEX release. The mechanisms underlying the cooperative effect between UDPG and antigen remain to be characterized. Previous studies suggested that the synergistic effect of adenosine was mediated via a PI3K-dependent pathway [29], whereas the enhancement of antigen-mediated mast cell activation by PGE2 was independent of PI3K but was more probably a consequence of the synergistic activation of PLCβ by PGE2 and PLCγ by antigen [6]. This synergistic effect may also involve inositol-1,4,5-trisphosphate, calcium ions, and activation of PKC, all of which are critical for degranulation.

Cellular UDPG is concentrated in the lumen of the endoplasmic reticulum, and its constitutive release has been demonstrated suggesting that UDPG acts as an autocrine activator of the $P2Y_{14}$ receptor [30]. The observed activity of a uracil nucleotide warrants further studies using primary human mast cells. Mast cell responses to various other extracellular nucleotides have been ascribed to P2Y receptors [31]. The facilitation of mast cell degranulation by adenosine involves several receptor subtypes [4,32,33].

In summary, our results demonstrate a novel function of the $P2Y_{14}$ receptor in facilitating mast cell degranulation that is G protein-dependent. Thus, inhibition of the $P2Y_{14}$ receptor may be a

novel target for therapeutic intervention of asthma and allergic conditions. Selective antagonists of this receptor are needed to test this concept. The ongoing study of structure–activity relationships at this receptor [15,11,26] might lead to the identification of new pharmacological probes for the study of this receptor and possibly pharmaceutical leads.

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