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Functional expression of the $P2Y_{14}$ receptor in murine T-lymphocytes

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1 Quantitative reverse transcriptase polymerase chain reaction (RT–PCR) analysis has previously shown that the $P2Y_{14}$ receptor is expressed in peripheral immune cells including lymphocytes. Although in transfected cells the $P2Y_{14}$ receptor couples to pertussis toxin-sensitive $G_{i/o}$ protein, the functional coupling of endogenously expressed $P2Y_{14}$ receptors to the inhibition of adenylyl cyclase activity has not been reported. Therefore, the primary aim of this study was to determine whether the $P2Y_{14}$ receptor is functionally expressed in murine spleen-derived T- and B-lymphocyte-enriched populations.

2 RT–PCR analysis detected the expression of $P2Y_{14}$ receptor mRNA in whole spleen and isolated T- and B-lymphocytes.

3 In T cells, UDP-glucose ($EC_{50} = 335\,nM$) induced a small but significant inhibition (*circa* 20%) of forskolin-stimulated cAMP accumulation, suggesting functional coupling of endogenously expressed $P2Y_{14}$ receptors to the inhibition of adenylyl cyclase activity. In contrast, the other putative $P2Y_{14}$ receptor agonists UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine had no significant effect alone but behaved as partial agonists by blocking UDP-glucose responses. In B cells, UDP-glucose ($100\,\mu M$) had no significant effect on forskolin-stimulated cAMP accumulation.

4 Treatment of T cells with pertussis toxin ($G_{i/o}$ blocker) abolished the inhibitory effects of UDP-glucose on forskolin-stimulated cAMP accumulation.

5 T-cell proliferation in response to anti-CD3 monoclonal antibody ($1\,\mu g\,ml^{-1}$) was significantly inhibited by UDP-glucose (59% inhibition; $p[IC_{50}] = 5.9 \pm 0.3$), UDP-*N*-acetylglucosamine (37%; 6.1 ± 0.3), UDP-galactose (56%; 8.2 ± 0.2) and UDP-glucuronic acid (49%; 6.3 ± 0.2). Interleukin-2-($5\,ng\,ml^{-1}$) induced T-cell proliferation was also significantly inhibited by all four agonists.

6 In summary, we have shown that the $P2Y_{14}$ receptor appears to be functionally expressed in murine spleen-derived T-lymphocytes. These observations suggest that UDP-glucose and related sugar nucleotides presumably *via* the $P2Y_{14}$ receptor may play an important role in modulating immune function.
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Abbreviations: IL-2, interleukin-2; PTX, pertussis toxin

Introduction

Uridine 5′-diphosphoglucose (UDP-glucose) is widely known to function as a glycosyl donor in the biosynthesis of carbohydrates. However, it has recently been shown that UDP-glucose and related sugar nucleotides, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine are all potent agonists of the orphan human G-protein-coupled receptor (GPCR) KIAA0001 (Chambers *et al*., 2000). This orphan GPCR was originally cloned from the human immature myeloid cell line KG-1 (Nomura *et al*., 1994). More recently, the rat and mouse orthologues of the human KIAA0001 receptor have subsequently been cloned and show 80 and 83% amino-acid identity, respectively (Freeman *et al*., 2001). In line with GPCR nomenclature, KIAA0001 and the rodent orthologues were renamed GPR105 (Freeman *et al*., 2001). Since the UDP-glucose receptor shares approximately 45% amino-acid identity with the human $P2Y_{12}$ and $P2Y_{13}$ receptors, it has recently been included as a member of the P2Y receptor family and renamed the $P2Y_{14}$ receptor (Abbracchio *et al*., 2003).

The $P2Y_{14}$ receptor appears to couple $G_{i/o}$ proteins since pertussis toxin (PTX) treatment (which blocks $G_{i/o}$ protein coupling) completely inhibited UDP-glucose stimulated GTPγS binding to membranes prepared from transfected HEK 293 cells (Chambers *et al*., 2000). GPCRs that couple to $G_{i/o}$ proteins are classically associated with the inhibition of adenylyl cyclase activity; however, the functional coupling of endogenously expressed $P2Y_{14}$ receptors to inhibition of adenylyl cyclase activity has not been reported. However, UDP-glucose has been shown to stimulate increases in intracellular Ca^{2+} concentration in rat cortical astrocytes and human immature monocyte-derived dendritic cells (MDDC) (Fumagalli *et al*., 2003; Skelton *et al*., 2003). Interestingly, UDP-glucose-mediated Ca^{2+} responses observed in immature MDDCs were partially sensitive to PTX, suggesting coupling of the $P2Y_{14}$ receptor to $G_{i/o}$ proteins in an endogenous system.

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The physiological function of the $P2Y_{14}$ receptor is unknown at present, although it is widely expressed in human tissue, with highest expression levels in placenta, adipose tissue, stomach and intestine, and moderate levels in the brain, spleen, lung and heart (Chambers *et al*., 2000). Recently, using Taq-Man quantitative RT–PCR, Moore *et al*. (2003) reported the expression of the $P2Y_{14}$ receptor in peripheral immune cells such as lymphocytes and neutrophils. Therefore, the primary aim of this study was to investigate whether the $P2Y_{14}$ receptor is functionally expressed on murine spleen-derived T- and B-lymphocyte-enriched populations. We present clear evidence, which suggests that the $P2Y_{14}$ receptor is functionally expressed on murine spleen-derived T-lymphocytes.

Methods

Isolation of T- and B-lymphocyte-enriched populations

Balb/c mice were killed by cervical dislocation and spleens removed under aseptic conditions. Cells were obtained by flushing the spleen with 10 ml RPMI 1640 medium supplemented with 10% ($v\,v^{-1}$) foetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U ml^{-1})/streptomycin (100 $\mu g\,ml^{-1}$), fungizone (0.25 $\mu g\,ml^{-1}$), 0.1% ($v\,v^{-1}$) β-mercaptoethanol and 10 mM HEPES. Following centrifugation, cells were resuspended in RPMI 1640 medium at a density of $5 \times 10^6\,ml^{-1}$ and incubated for 1 h in a humidified incubator (95% air/5% CO_2 at 37°C) in 25 cm^2 culture flasks to allow B-cell adherence. After 1 h, the nonadherent-enriched T-cell population was removed, centrifuged and resuspended in serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin/streptomycin (100 U ml^{-1}), fungizone (0.25 $\mu g\,ml^{-1}$), 0.1% ($v\,v^{-1}$) β-mercaptoethanol and 10 mM HEPES. Adherent B cells were removed using trypsin (0.05% ($w\,v^{-1}$))/EDTA (0.02% ($w\,v^{-1}$)) and resuspended in serum-free RPMI 1640 at 2×10^6 cells ml^{-1}.

Cyclic AMP (cAMP) accumulation assay

T- or B-lymphocytes (12×10^6 cells 25 cm^{-2} culture flask) were incubated for 2 h at 37°C in a humidified incubator (95% air/5% CO_2) in 6 ml serum-free RPMI 1640 medium containing [^{3}H]-adenine (1.8 MBq $flask^{-1}$). [^{3}H]-adenine-labelled cells (B cells were briefly trypsinised) were washed once and then incubated for 15 min at 37°C in 6 ml Hanks/HEPES (H/H) buffer (pH 7.4) containing the cAMP phosphodiesterase inhibitor, rolipram (10 μM). Cells were then dispensed into 48-well culture plates (250 $\mu l\,well^{-1}$) and agonists were added (in 10 μl of medium) 5 min prior to incubation with 5 μM forskolin for 10 min. Incubations were terminated by the addition of 25 μl concentrated HCl. [^{3}H]-cAMP was isolated by sequential Dowex-alumina chromatography as described previously (Donaldson *et al*., 1988). After elution, the levels of [^{3}H]-cAMP were determined by liquid scintillation counting.

RT–PCR analysis of $P2Y_{14}$ receptor mRNA expression

Total RNA was isolated from murine spleen- and spleen-derived T- and B-lymphocytes cells using the Promega SV total RNA isolation kit according to the manufacturer's instructions. During the isolation procedure, mRNA was routinely treated with RQ1 DNase (1 U μl^{-1}) in order to remove genomic DNA. First-strand complementary DNA (cDNA) was synthesised utilising random primers and M-MLV reverse transcriptase. PCR was performed using the following $P2Y_{14}$ receptor gene-specific primer sequences (270 bp product): forward 5′-TTCTGGGTCGTGTTTCTTCTG-3′ and reverse 5′-CGAGAGTAGCAGAGTGAATTC-3′. GAPDH primers (370 bp product) were forward 5′-CTCATGACCACAGTC CATGC-3′ and reverse 5′-GGTCCAGGGGTCTTACTCC-3′. PCR conditions for the $P2Y_{14}$ receptor were 40 cycles of 94°C for 1 min, 50°C for 1.5 min and 72°C for 2 min. PCR conditions for GAPDH were 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. PCR products were subjected to 1% ($w\,v^{-1}$) agarose gel electrophoresis and visualised by ethidium bromide staining. Running PCR reactions prior to cDNA synthesis using GAPDH primers controlled for potential contamination by genomic DNA.

T-cell proliferation assay

T cells (2×10^5 cells $well^{-1}$) in RPMI 1640 medium supplemented with 10% ($v\,v^{-1}$) FCS, 2 mM L-glutamine, penicillin/streptomycin (100 U ml^{-1}), fungizone (0.25 $\mu g\,ml^{-1}$), 0.1% ($v\,v^{-1}$) β-mercaptoethanol and 10 mM HEPES were seeded in 96-well round-bottomed plates. T cells were activated by the addition of either murine interleukin-2 (IL-2; 5 ng ml^{-1}) or anti-CD3 monoclonal antibody (1 $\mu g\,ml^{-1}$) followed by the addition of RPMI 1640 medium alone or containing the appropriate concentration of agonist (UDP-glucose, CGS 21680 (4-[2-[[-6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl] benzenepropanoic acid hydrochloride), Cl-IB-MECA) to achieve a final volume of 200 μl. Cells were incubated for 72 h at 37°C in a humidified incubator (95% air/5% CO_2) after which [^{3}H]-thymidine (0.037 MBq $well^{-1}$) was added and incubated for a further 18 h. Cells were harvested into 96-well UniFilter® plates bonded with GF/C filters (Perkin-Elmer, Wellesley, Massachusetts, U.S.A.) using a Packard FilterMate™ harvester. Plates were then dried for 1 h at 37°C and sealed before the addition of 40 $\mu l\,well^{-1}$ of TopCount® microplate scintillant. Levels of [^{3}H]-thymidine incorporation were determined by liquid scintillation counting (TopCount NXT™, Packard).

Data analysis

Agonist pEC_{50} and pIC_{50} values (concentrations of drug producing 50% of the maximal stimulation or inhibition) were obtained by computer-assisted curve fitting by use of the computer programme Prism (GraphPAD, San Diego, California, U.S.A.). Statistical significance was determined by Student's unpaired *t*-test ($P<0.05$ was considered statistically significant). All data are presented as mean ± s.e.m. The *n* in the text refers to the number of separate experiments.

Materials

[2,8-^{3}H]-adenine and [^{3}H]-thymidine were from Amersham International (Aylesbury, Bucks, U.K.). Forskolin, PTX, rolipram, UDP, UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All molecular biology reagents including RQ1 RNase-free DNase, M-MLV

reverse transcriptase, SV total RNA isolation kit and random primers were obtained from Promega. Primers for RT–PCR analysis were synthesised by Sigma-Genosys (Pampisford, Cambridgeshire, U.K.). CGS 21680, 2-Cl-IB-MECA (1-[2-chloro-6[[(3-iodophenyl)-methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*β*-D-ribofuranuron-amide) and HU 210 were from Tocris (Bristol, U.K.). Murine IL-2 and anti-CD3 monoclonal antibody were from Peprotech EC Ltd and Serotec Ltd (Kidlington, Oxford, U.K.) respectively. RPMI 1640 medium, FCS, trypsin (10 ×), L-glutamine (200 mM), penicillin (10,000 U ml^{-1})/streptomycin (10,000 $\mu g\,ml^{-1}$) and fungizone (250 $\mu g\,ml^{-1}$) were purchased from BioWhittaker U.K. Ltd (Nottingham, U.K.). All other chemicals were of analytical grade.

Results

RT–PCR analysis of $P2Y_{14}$ expression in murine spleen and T- and B-lymphocytes

Previous studies using quantitative RT–PCR indicated moderate levels of $P2Y_{14}$ receptor expression in human spleen (Chambers *et al*., 2000). Similarly, Northern blot analysis revealed that the $P2Y_{14}$ receptor is also present at high levels in rat spleen (Charlton *et al*., 1997). More recently, $P2Y_{14}$ receptor expression has been detected in peripheral immune cells such as lymphocytes and neutrophils (Moore *et al*., 2003). In this study, we investigated $P2Y_{14}$ receptor expression in murine spleen and isolated T- and B-lymphocytes using RT–PCR. As shown in Figure 1a, using $P2Y_{14}$ receptor-specific primers, we detected the expression of the $P2Y_{14}$ receptor in murine spleen. Subsequently, RT–PCR analysis was performed using spleen-derived T- and B-lymphocyte-enriched populations. As shown in Figure 1b, the $P2Y_{14}$ receptor appears to be expressed predominantly in T-lymphocytes since only a weak signal was detected in B-lymphocytes (Figure 1c). Importantly, PCR reactions (using GAPDH primers) performed prior to cDNA synthesis indicated that the mRNA was not contaminated with genomic DNA. Overall, these data suggest that the $P2Y_{14}$ receptor is predominantly expressed in T-lymphocytes.

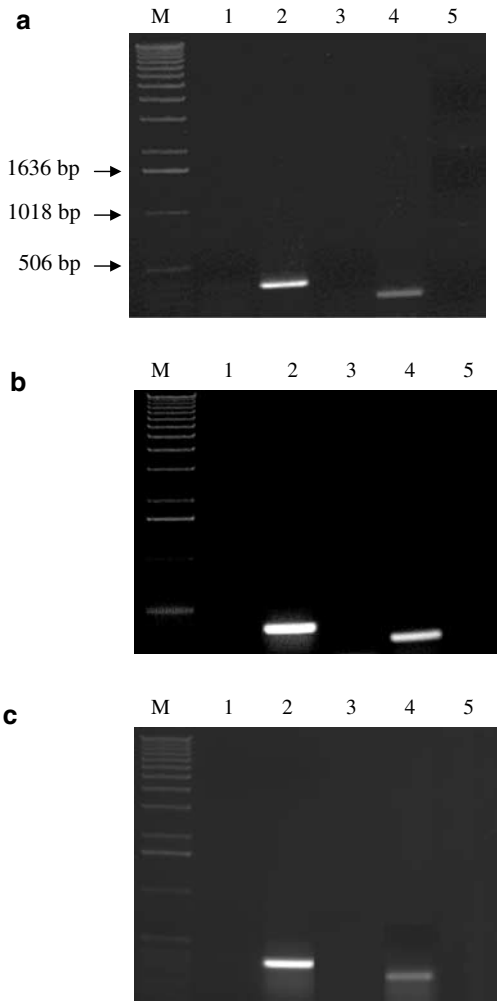

Figure 1 $P2Y_{14}$ receptor mRNA expression in murine spleen and isolated T- and B-lymphocyte-enriched populations. mRNA isolated from (a) whole murine spleen, (b) T-lymphocytes and (c) B-lymphocytes was subjected to RT–PCR using $P2Y_{14}$ receptor gene-specific primers. M: 1-Kb DNA standard; lane 1: no DNA control; lane 2: GAPDH primers (370 bp product); lanes 3: no DNA control; lane 4: $P2Y_{14}$ receptor primers (270 bp product); and lane 5: RNA control with GAPDH primers. The results presented are representative of three separate experiments.

Effect of UDP-glucose and related sugar nucleotides on forskolin-stimulated cAMP accumulation

In transfected HEK 293 cells, the $P2Y_{14}$ receptor reportedly couples $G_{i/o}$ proteins since PTX treatment, which blocks $G_{i/o}$ protein coupling, completely inhibited UDP-glucose-stimulated GTPγS binding to membranes prepared from these cells (Chambers *et al*., 2000). However, the functional coupling of endogenously expressed $P2Y_{14}$ receptors to inhibition of adenylyl cyclase activity has not been reported. Hence, in this study, we determined the effects of UDP-glucose on forskolin-stimulated cAMP accumulation in spleen-derived T- and B-lymphocytes. As shown in Figure 2a, UDP-glucose induced a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in T-lymphocytes ($p[IC_{50}] = 6.5 \pm 0.6$; $n = 5$). UDP-glucose (100 μM) inhibited $22 \pm 4\%$ ($n = 5$) of the cAMP response induced by 5 μM forskolin. In contrast, UDP-glucose had no significant effect on forskolin-stimulated cAMP accumulation in B cells. Previous studies have shown that T cells express A_3 adenosine and CB_1 cannabinoid receptors, both of which are G_i protein coupled (Noe *et al*., 2000; Hoskin *et al*., 2002; Gessi *et al*., 2004). Hence, as a comparison, we determined the effects of the A_3 adenosine and CB_1/CB_2 receptor agonists Cl-IB-MECA and HU 210, respectively, on forskolin-stimulated cAMP accumulation. As shown in Figure 2b, Cl-IB-MECA induced a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in T cells ($p[IC_{50}] = 8.5 \pm 0.4$; $n = 4$) with maximal inhibition ($30 \pm 7\%$) occurring at 10 nM Cl-IB-MECA. It is notable that the concentration–response curve for Cl-IB-MECA appears biphasic. This may reflect A_3 adenosine receptor-mediated stimulation of cAMP (at concentrations of Cl-IB-MECA above 10 nM) as previously reported in human eosinophils (Ezeamuzie & Philips, 2003) and rat cardiomyocytes (Germack & Dickenson, 2004). Finally, the cannabinoid HU 210 also inhibited forskolin-induced cAMP accumulation ($p[IC_{50}] = 8.9 \pm 0.2$; $n = 4$) with maximal inhibition ($39 \pm 7\%$) occurring at 1 μM HU 210 (Figure 2c). As an alternative to

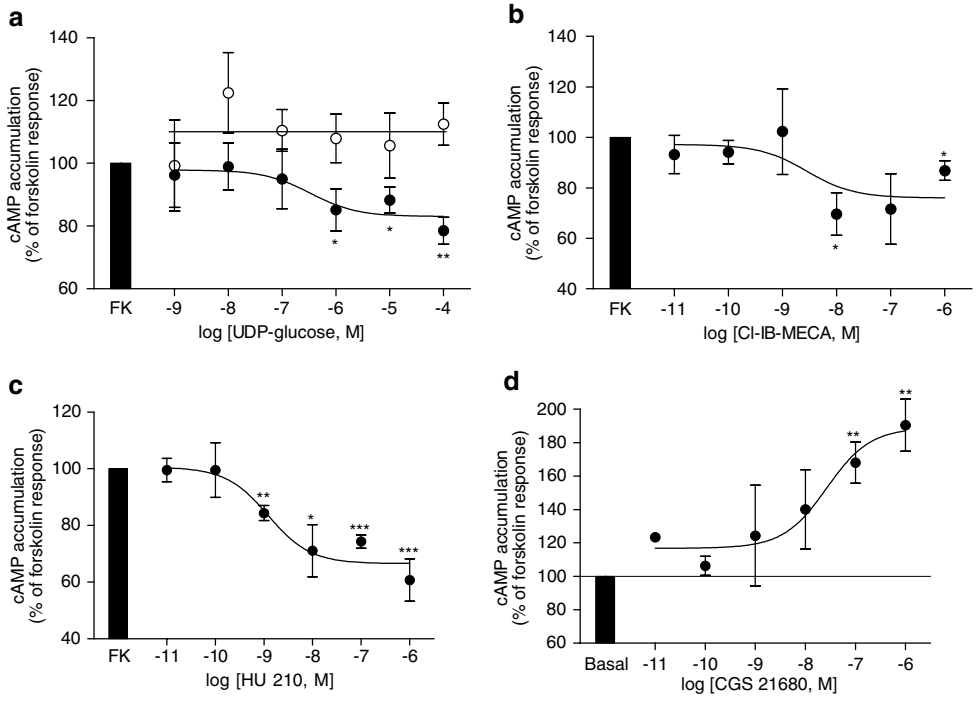

Figure 2 Effect of UDP-glucose, Cl-IB-MECA, HU 210 and CGS 21680 on cAMP accumulation in T- and B-lymphocytes. In panel (a), T-lymphocytes (closed circles) and B-lymphocytes (open circles) cells were pretreated for 5 min with the indicated concentrations of UDP-glucose prior to stimulation with 5 μM forskolin (FK) for 10 min. In (b) and (c), T-lymphocytes were pretreated with Cl-IB-MECA and HU 210 prior to stimulation with 5 μM FK for 10 min. Data are expressed as the percentage of the forskolin response in the absence of agonist (100%). In panel (d), T-lymphocytes were stimulated with the indicated concentrations of CGS 21680 for 15 min. Data are expressed as the percentage of the basal cAMP accumulation (100%). The results represent mean $\pm$ s.e.m. of four to six independent experiments each performed in triplicate. $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ *versus* forskolin alone response.

using forskolin (which is a direct activator of adenylyl cyclase), we also explored the effects of UDP-glucose on G_s-coupled A_{2A} adenosine receptor-mediated cAMP accumulation. In agreement with previous studies, the selective A_{2A} adenosine receptor agonist CGS 21680 induced a concentration-dependent ($p[EC_{50}] = 7.6 \pm 0.3$; $n = 3$) increase in cAMP in T-lymphocytes (Figure 2d). Unfortunately, due to the relatively small response obtained with CGS 21680 (1 μM; 90 $\pm$ 15%; $n = 3$; above basal cAMP levels) compared to forskolin (5 μM; 350 $\pm$ 55%; $n = 6$; above basal cAMP levels), the results obtained with CGS 21680 in the presence of UDP-glucose were inconclusive (data not shown). Finally, UDP (Figure 3a) had no significant effects on forskolin-stimulated cAMP accumulation, indicating that the inhibition of cAMP accumulation observed with UDP-glucose is not due to UDP derived from the breakdown of UDP-glucose. This is important since T cells reportedly express the $P2Y_6$ receptor, which may inhibit cAMP accumulation *via* a G_q-protein-dependent pathway (Somers *et al*., 1998; Wong *et al*., 2000).

We also determined the effects of the related sugar nucleotides, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine, which are also putative agonists of the $P2Y_{14}$ receptor, on forskolin-stimulated cAMP responses. However, as shown in Figure 3, all three agonists had no significant effect on forskolin-stimulated cAMP accumulation in T-lymphocytes. However, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine appear to be partial agonists of the $P2Y_{14}$ receptor expressed in murine T cells since all three blocked UDP-glucose-mediated inhibition of forskloin-induced cAMP responses (Figure 4).

Since the $P2Y_{14}$ receptor reportedly couples $G_{i/o}$ proteins, we determined the effect of PTX ($G_{i/o}$ blocker) on UDP-glucose-mediated inhibition of forskolin-induced cAMP responses. As shown in Figure 5, pretreatment with PTX (100 ng ml^{-1}, 16 h) completely abolished the inhibitory effects of 100 μM UDP-glucose and 1 μM HU 210 on forskolin-induced cAMP accumulation. Overall, these data indicate that the murine T-lymphocytes express functional $G_{i/o}$-protein-coupled $P2Y_{14}$ receptors.

Effect of UDP-glucose and related sugar nucleotides on T-cell proliferation

Previous studies have shown that activation of G_s-coupled A_{2A} adenosine and $G_{i/o}$-protein-coupled A_3 adenosine receptors inhibit T-cell proliferation (Huang *et al*., 1997; Hoskin *et al*., 2002). Thus, we initially investigated the effect of a maximal concentration of UDP-glucose on T-cell proliferation induced by IL-2 (5 ng ml^{-1}) and anti-CD3 monoclonal antibody (1 μg ml^{-1}). As shown in Figure 6a, UDP-glucose (100 μM) significantly inhibited T-cell proliferation induced by IL-2. The selective A_{2A} adenosine receptor agonist CGS 21680 (1 μM) also significantly inhibited IL-2-triggered T-cell proliferation, whereas the selective A_3 adenosine receptor agonist Cl-IB-MECA had no significant effect. Given that the $P2Y_{14}$ receptor is $G_{i/o}$ protein linked, we also determined whether UDP-

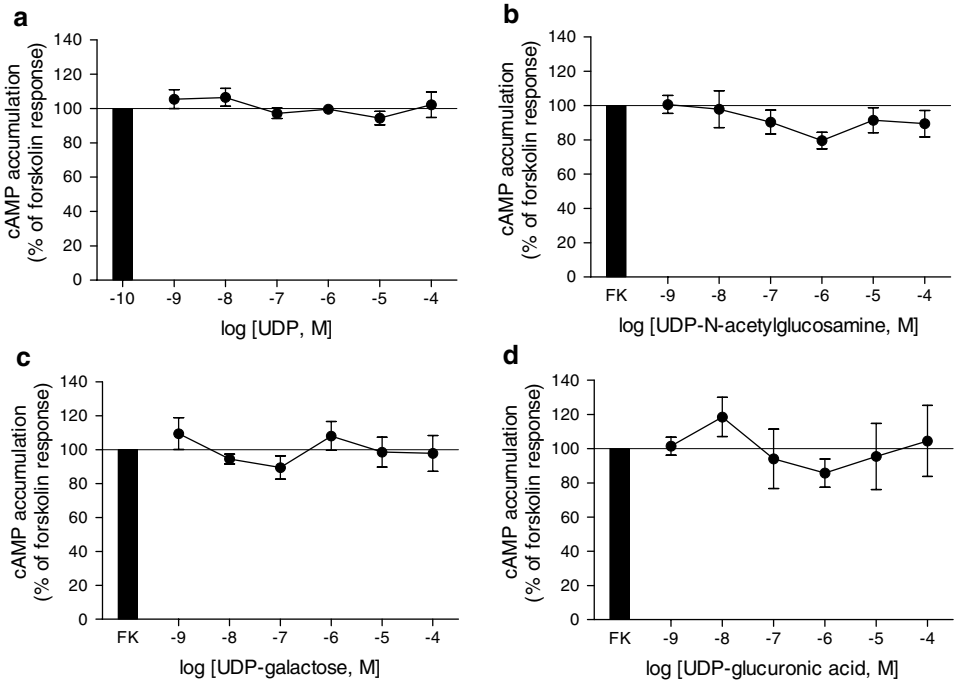

Figure 3 Effect of UDP, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine on forskolin-stimulated cAMP accumulation in T-lymphocytes. Enriched T cells were initially pretreated for 5 min with the indicated concentrations of (a) UDP, (b) UDP-*N*-acetylglucosamine, (c) UDP-galactose and (d) UDP-glucuronic acid prior to stimulation with 5 μM forskolin (FK) for 10 min. Data are expressed as the percentage of the forskolin response in the absence of agonist (100%). The results represent mean $\pm$ s.e.m. of four independent experiments each performed in triplicate.

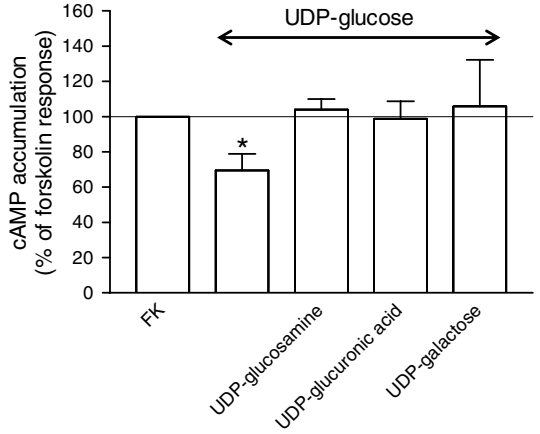

Figure 4 Partial agonist properties of UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine in T-lymphocytes. Enriched T cells were initially pretreated for 5 min with UDP-glucose alone (100 μM) or UDP-glucose (100 μM) in the presence of 100 μM UDP-*N*-acetylglucosamine, UDP-galactose or UDP-glucuronic acid prior to stimulation with 5 μM forskolin (FK) for 10 min. Data are expressed as the percentage of the forskolin response in the absence of agonist (100%). The results represent mean $\pm$ s.e.m. of four independent experiments each performed in triplicate. $^*P<0.05$ *versus* forskolin alone response.

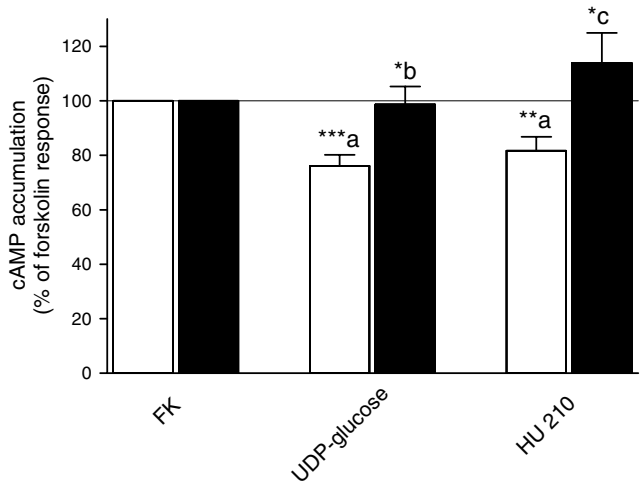

Figure 5 Effect of pertussis toxin (PTX) on UDP-glucose- and HU 210-mediated inhibition of forskolin-stimulated cAMP accumulation in T-lymphocytes. Control- (open bars) and PTX- (100 ng ml^{-1} for 16 h) treated T cells (filled bars) were stimulated with UDP-glucose (100 μM) or HU 210 (1 μM) for 5 min prior to stimulation with 5 μM forskolin (FK) for 10 min. Data are expressed as the percentage of the forskolin response in the absence of agonist (100%). The results represent mean $\pm$ s.e.m. of four independent experiments each performed in triplicate. $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ (a) *versus* forskolin alone, (b) *versus* UDP-glucose in the absence of PTX and (c) *versus* HU 210 in the absence of PTX.

glucose would attenuate the effects of CGS 21680 since the effects of the A_{2A} adenosine receptor on T-cell proliferation are presumably mediated *via* increases in cAMP. However, the effects of combining UDP-glucose and CGS 21680 on T-cell proliferation were comparable to CGS 21680 alone. We subsequently investigated the effect of a maximal concentration of UDP-glucose (100 μM) on anti-CD3 monoclonal antibody-induced T-cell proliferation. As shown in Figure 6b, 100 μM UDP-glucose significantly inhibited anti-CD3 antibody-induced T-cell proliferation. For comparison,

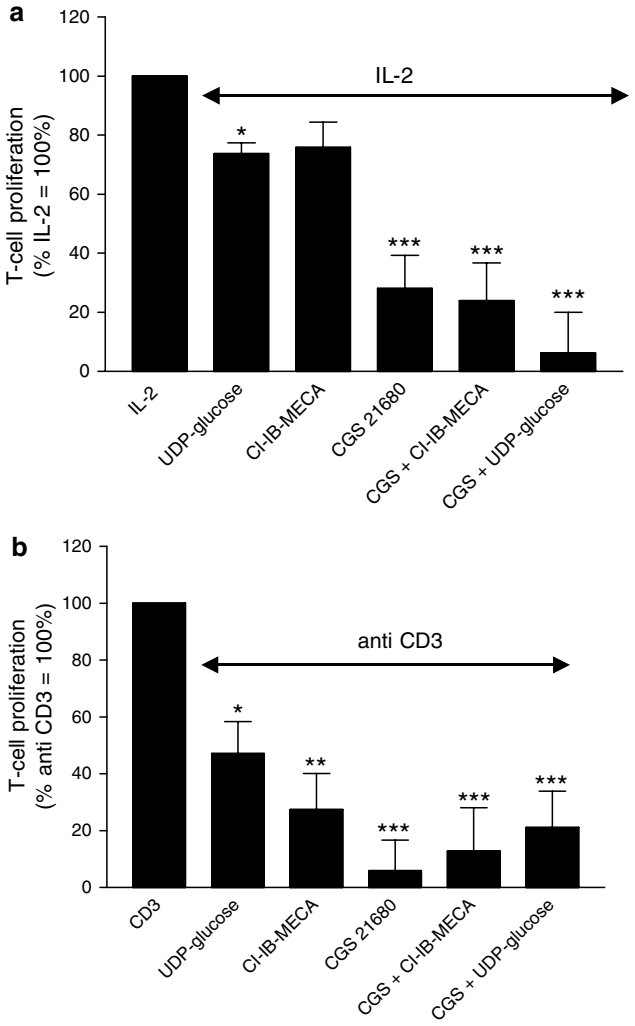

Figure 6 Effect of UDP-glucose on T-cell proliferation induced by interleukin-2 (IL-2) and anti-CD3 monoclonal antibody. T-lymphocytes were stimulated with (a) IL-2 ($5\,ng\,ml^{-1}$) or (b) anti-CD3 monoclonal antibody ($1\,\mu g\,ml^{-1}$) for 72 h in the absence (control cells) or presence of UDP-glucose (100 μM), Cl-IB-MECA (100 nM) and CGS 21680 (1 μM) alone or CGS 21680 (1 μM) in the presence of Cl-IB-MECA (100 nM) or UDP-glucose (100 μM). After 72 h, [^{3}H]-thymidine was added and the cells incubated for a further 18 h. Data are expressed as the percentage of T-cell proliferation induced by IL-2 or anti-CD3 monoclonal antibody (100%). The results represent mean$\pm$s.e.m. of five independent experiments each performed in replicates of 12. $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ *versus* control proliferation induced by IL-2 or anti-CD3 antibody.

Cl-IB-MECA and CGS 21680 (the absence and presence of UDP-glucose) also significantly inhibited anti-CD3 antibody-induced T-cell proliferation. Finally, we determined the concentration-dependent effects of UDP-glucose and related sugar nucleotides (UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine) on IL-2- and anti-CD3 antibody-induced T-cell proliferation. As shown in Figure 7, UDP-glucose ($p[IC_{50}]=5.1\pm0.3$; $n=4$) and UDP-galactose ($p[IC_{50}]=5.5\pm0.4$; $n=4$) inhibited IL-2-induced T-cell proliferation in a concentration-dependent manner with maximal inhibition (25 ± 1 and 25 ± 2%, respectively; $n=4$) occurring at 100 μM. UDP-glucuronic acid (100 μM) and UDP-*N*-acetylglucosamine (100 μM) also induced small but significant inhibition of IL-2-induced T-cell proliferation by 11 ± 2 and 14 ± 4%, respectively. All four agonists significantly inhibited anti-CD3 antibody-induced T-cell proliferation in a concentration-dependent manner (Figure 8). $p[IC_{50}]$ values and maximal inhibition (at 100 μM) are as follows: UDP-glucose (59 ± 3%; 5.9 ± 0.3; $n=4$), UDP-*N*-acetylglucosamine (37 ± 3%; 6.1 ± 0.3; $n=4$), UDP-galactose (56 ± 3%; 8.2 ± 0.2; $n=4$) and UDP-glucuronic acid (49 ± 5%; 6.3 ± 0.2; $n=4$). It is notable that the marked effects of UDP-glucose and related sugar nucleotides on T-cell proliferation contrast with the small attenuation of forskolin-induced cAMP responses observed only for UDP-glucose. Since these differences may reflect changes in $P2Y_{14}$ receptor expression during T-cell activation, we determined (*via* RT–PCR analysis) $P2Y_{14}$ receptor expression in T cells activated by anti-CD3 monoclonal antibody ($1\,\mu g\,ml^{-1}$; 72 h). As shown in Figure 9, there was no significant alteration in $P2Y_{14}$ receptor expression in activated T cells compared to freshly isolated cells.

Discussion

Previous studies using transfected cells suggest that the $P2Y_{14}$ receptor couples to members of the $G_{i/o}$ protein family since PTX treatment (which blocks $G_{i/o}$ protein coupling) completely inhibited UDP-glucose-stimulated GTPγS binding to membranes prepared from HEK 293 cells (Chambers *et al.*, 2000). Similarly, Ca^{2+} measurements using HEK 293 cells cotransfected with $P2Y_{14}$ and either $G\alpha_{qo5}$, $G\alpha_{qi5}$ or $G\alpha_{qs5}$ have revealed that the $P2Y_{14}$ receptor couples to $G_{i/o}$ proteins and not G_s or endogenous $G_{q/11}$ proteins (Moore *et al.*, 2003). The chimeric Gα subunits used by Moore *et al.* (2003) were constructed by replacing the five C-terminal amino acids of human $G\alpha_q$ with the corresponding residues from $G\alpha_s$, $G\alpha_o$ or $G\alpha_i$. Overall, the above studies using transfected cell lines indicate that the $P2Y_{14}$ receptor couples to members of the $G_{i/o}$ protein family. However, the functional coupling of endogenously expressed $P2Y_{14}$ receptors to inhibition of adenylyl cyclase activity (which represents classical $G_{i/o}$-protein-mediated signaling pathway) has not been reported. Therefore, one of the main aims of this study was to investigate the effect of UDP-glucose on forskolin-stimulated cAMP accumulation in cells that endogenously express the $P2Y_{14}$ receptor. As detailed in the Introduction, moderate levels of $P2Y_{14}$ receptor expression have been detected in human spleen (Chambers *et al.*, 2000) and, more recently, in peripheral immune cells such as lymphocytes and neutrophils (Moore *et al.*, 2003). Therefore, based on these studies, we have determined whether the $P2Y_{14}$ receptor is functionally expressed on murine spleen-derived T- and B-lymphocyte-enriched populations. Using RT–PCR analysis, we identified $P2Y_{14}$ receptor mRNA expression in murine spleen. These data are in agreement with previous studies, which have reported $P2Y_{14}$ receptor expression in rat and human spleen tissue (Charlton *et al.*, 1997; Chambers *et al.*, 2000). Subsequent RT–PCR analysis using enriched populations of T- and B-lymphocytes isolated from murine spleen revealed that the $P2Y_{14}$ receptor is predominantly expressed in T cells (see Figure 2b). During the isolation procedure, adherent cells were classified as B cells, whereas nonadherent cells represent predominantly T-lymphocytes.

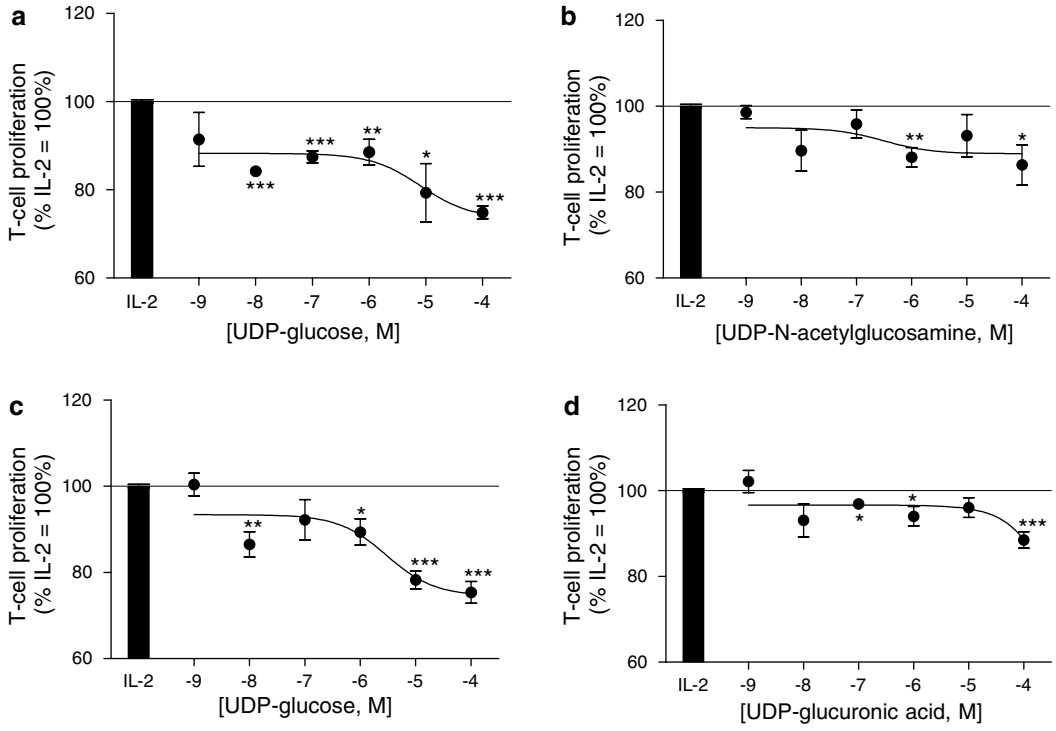

Figure 7 Concentration-dependent effects of UDP-glucose, UDP-*N*-acetylglucosamine, UDP-galactose and UDP-glucuronic acid on interleukin-2 (IL-2)-induced T-cell proliferation. T-lymphocytes were stimulated with IL-2 (5 ng ml^{-1}) for 72 h in the absence (control cells) or presence of the indicated concentrations of (a) UDP-glucose, (b) UDP-*N*-acetylglucosamine, (c) UDP-galactose and (d) UDP-glucuronic acid. After 72 h, [^{3}H]-thymidine was added and the cells incubated for a further 18 h. Data are expressed as the percentage of T-cell proliferation induced by IL-2 (100%). The results represent mean ± s.e.m. of four independent experiments each performed in replicates of 12. *$P<0.05$, **$P<0.005$, ***$P<0.001$ *versus* control proliferation induced by IL-2.

Having established that spleen-derived T-lymphocytes express P2Y$_{14}$ receptor mRNA, we then determined whether the P2Y$_{14}$ receptor is functional in these cells. The data presented in Figure 2a clearly shows that UDP-glucose inhibits, in a concentration-dependent manner, forskolin-stimulated cAMP accumulation in T-lymphocytes. To our knowledge, this is the first report describing the functional coupling of the G$_i$-coupled P2Y$_{14}$ receptor to the inhibition of adenylyl cyclase. In contrast, UDP-glucose had no significant effect on forskolin-stimulated cAMP response in B cells. The maximal inhibition of forskolin-stimulated cAMP responses achieved with UDP-glucose (100 μM; 22% inhibition) is similar to the inhibition observed with the A$_3$ adenosine receptor agonist Cl-IB-MECA (10 nM; 30%) and the CB$_1$/CB$_2$ cannabinoid receptor agonist HU 210 (10 nM; 29%). It is notable that the EC$_{50}$ value for UDP-glucose-mediated inhibition of forskolin-stimulated cAMP accumulation (*circa* 335 nM) is comparable to the EC$_{50}$ values reported for UDP-glucose induced Ca^{2+} responses (80 nM) in HEK 293 cells stably cotransfected with the human P2Y$_{14}$ receptor and G$_{\alpha 16}$ and also UDP-glucose-stimulated GTPγS binding (234 nM) to membranes prepared from transfected HEK 293 cells without recombinant G proteins (Chambers *et al*., 2000). Furthermore, in HEK 293 cells transiently cotransfected with the human P2Y$_{14}$ and either Gα_{qo5}, Gα_{qi5} or Gα_{16}, UDP-glucose stimulated intracellular Ca^{2+} release with EC$_{50}$ values of 29, 32 and 191 nM, respectively (Moore *et al*., 2003). It should be noted that in transfected HEK 293 cells, both rat and mouse P2Y$_{14}$ receptor orthologues are activated by UDP-glucose and related sugar nucleotides with similar affinities (Freeman *et al*., 2001).

At present, very few studies have investigated signalling pathways activated by endogenously expressed P2Y$_{14}$ receptors. However, UDP-glucose has been shown to stimulate increases in [Ca^{2+}]$_i$ in rat cortical astrocytes and human immature MDDCs (Fumagalli *et al*., 2003; Skelton *et al*., 2003). Interestingly, the EC$_{50}$ for UDP-glucose-mediated Ca^{2+} responses in immature MDDCs was 11.5 μM (Skelton *et al*., 2003), which is significantly higher than the EC$_{50}$ value of 335 nM observed in this study for the inhibition of forskolin-stimulated cAMP accumulation. The difference in EC$_{50}$ values may reflect coupling of the P2Y$_{14}$ receptor to G$_{i/o}$ proteins for inhibition of adenylyl cyclase and G$_{q/11}$ proteins for the activation of phospholipase C and Ca^{2+} mobilisation. However, it is notable that UDP-glucose-mediated Ca^{2+} responses observed in immature MDDCs were partially sensitive to PTX, suggesting the involvement of G$_{i/o}$ proteins. In this study, we determined whether UDP-glucose-mediated inhibition of forskolin-stimulated cAMP accumulation involved coupling to G$_{i/o}$ proteins. As clearly shown in Figure 5, treatment with PTX completely blocked the effect of UDP-glucose, suggesting coupling to G$_{i/o}$ proteins. Overall, these data show for the first time the functional coupling of endogenously expressed P2Y$_{14}$ receptors to inhibition of adenylyl cyclase activity *via* G$_{i/o}$ protein coupling.

In addition to UDP-glucose, the related sugar nucleotides, UDP-galactose, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine, are also putative agonists of the P2Y$_{14}$ receptor

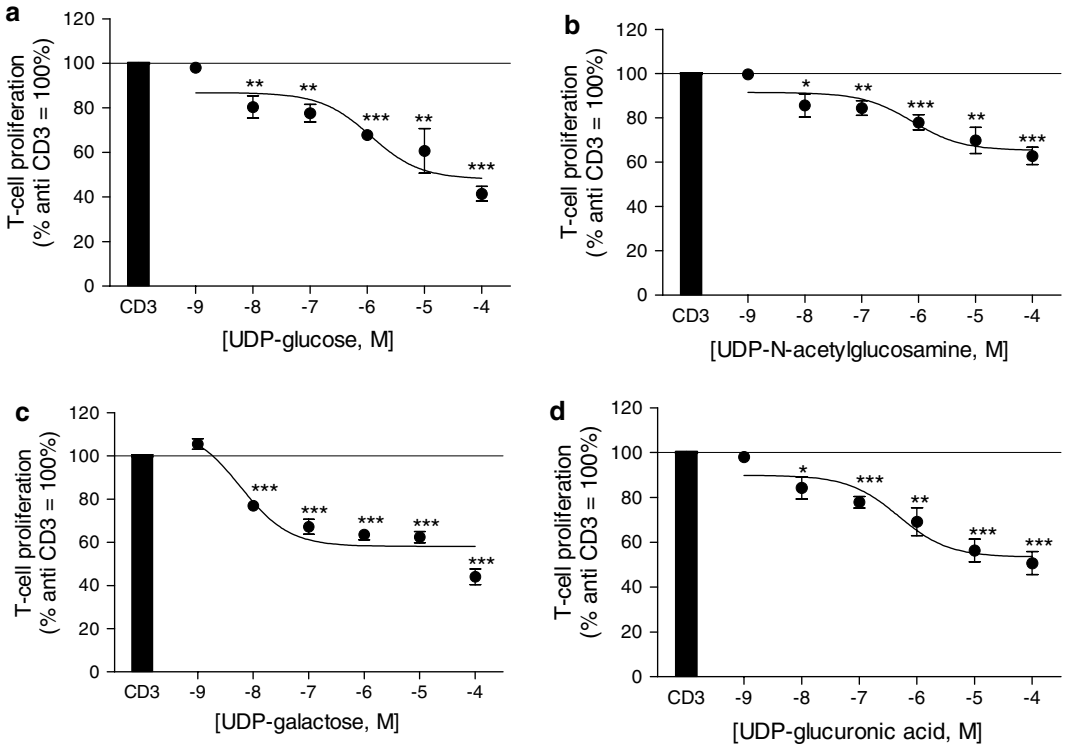

Figure 8 Concentration-dependent effects of UDP-glucose, UDP-*N*-acetylglucosamine, UDP-galactose and UDP-glucuronic acid on anti-CD3 monoclonal antibody induced T-cell proliferation. T-lymphocytes were stimulated with anti-CD3 monoclonal antibody (1 μg ml^{-1}) for 72 h in the absence (control cells) or presence of the indicated concentrations of (a) UDP-glucose, (b) UDP-*N*-acetylglucosamine, (c) UDP-galactose and (d) UDP-glucuronic acid. After 72 h, [^{3}H]-thymidine was added and the cells incubated for a further 18 h. Data are expressed as the percentage of T-cell proliferation induced by anti-CD3 monoclonal antibody (100%). The results represent mean $\pm$ s.e.m. of four independent experiments each performed in replicates of 12. *$P<0.05$, **$P<0.005$, ***$P<0.001$ *versus* control proliferation induced by anti-CD3 monoclonal antibody.

(Chambers *et al*., 2000). All four sugar nucleotides are agonists of cloned human, rat and mouse $P2Y_{14}$ receptors expressed in HEK 293 cells (Chambers *et al*., 2000; Freeman *et al*., 2001). However, at present only Fumagalli *et al*. (2003) have reported signalling responses to sugar nucleotides other than UDP-glucose in cells endogenously expressing the $P2Y_{14}$ receptor. In their study, using rat cortical astrocytes, they observed increases in $[Ca^{2+}]_i$ in response to 100 μM UDP-glucose and 500 μM UDP-galactose. In this study we, determined the ability of UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine to inhibit forskolin-stimulated cAMP responses in T-lymphocytes. However, unlike UDP-glucose, they had no significant effect on forskolin-induced cAMP accumulation. Indeed, it would appear that UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine behave as partial agonists of the murine $P2Y_{14}$ receptor (at least when measuring inhibition of adenylyl cyclase) since they blocked UDP-glucose-mediated inhibition of forskolin-induced cAMP responses (see Figure 4). These observations clearly highlight the problems of comparing the pharmacology of endogenously expressed receptors *versus* cloned receptors in transfected cells. The lack of effect of the other putative sugar nucleotide agonists may also reflect agonist trafficking. For example, stimulation of the $P2Y_{14}$ receptor with UDP-glucose may trigger coupling to $G_{i/o}$ proteins, whereas the other sugar nucleotides may promote coupling to other members of the heterotrimeric G protein family such as $G_{q/11}$. Clearly, further studies using T-lymphocytes are required in order to investigate the effects UDP-glucose and related sugar nucleotides on additional signalling pathways such as stimulation of inositol phospholipid hydrolysis (*via* $G_{q/11}$-mediated phospholipase C activation) and activation of various kinase cascades including extracellular signal-regulated kinase (ERK1/2) and protein kinase B (also known as Akt).

Since it is known that other members of the GPCR superfamily modulate T-lymphocyte proliferation including the A_{2A} and A_3 adenosine receptors (Huang *et al*., 1997; Hoskin *et al*., 2002; Erdmann *et al*., 2005), we determined the effect of UDP-glucose and related sugar nucleotides on T-cell proliferation induced by IL-2 and anti-CD3 antibody. As shown in Figure 6, a maximally effective concentration of UDP-glucose (100 μM) significantly inhibited T-cell proliferation induced by IL-2 and anti-CD3 antibody. In agreement with previous studies, A_{2A} and A_3 adenosine receptor activation also inhibited T-cell proliferation (Huang *et al*., 1997; Hoskin *et al*., 2002). It is important to note that A_{2A} and A_3 adenosine receptors generally couple to G_s and G_i proteins, respectively, and thus inhibition of T-cell proliferation *via* GPCRs may involve cAMP-dependent (G_s) and -independent (G_i) pathways. However, G_i-PCRs including the A_3 adenosine receptor also stimulate increases in $[Ca^{2+}]_i$, which may be involved in modulating T-cell proliferation (Englert *et al*., 2002; Fossetta *et al*., 2003; Shneyvays *et al*., 2004). UDP-glucose and related sugar nucleotides UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine all induced significant and concentration-dependent inhibition of anti-

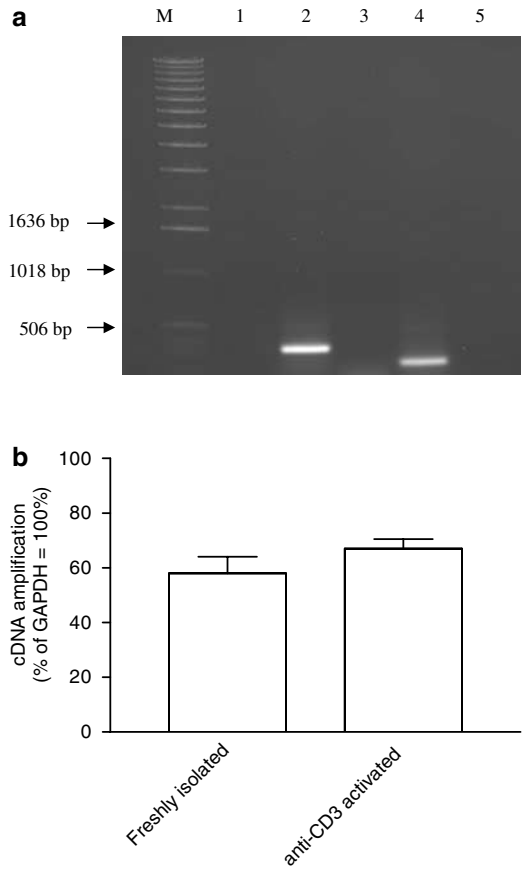

Figure 9 Effect of anti-CD3 antibody treatment on $P2Y_{14}$ receptor expression and coupling to inhibition of adenylyl cyclase in T-lymphocytes. (a) mRNA isolated from activated T-lymphocytes (treated with 1 $\mu g\,ml^{-1}$ anti-CD3 monoclonal antibody for 72 h) was subjected to RT–PCR using $P2Y_{14}$ receptor gene-specific primers. M: 1-Kb DNA standard; lane 1: no DNA control; lane 2: GAPDH primers (370 bp product); lanes 3: no DNA control; lane 4: $P2Y_{14}$ receptor primers (270 bp product); and lane 5: RNA control with GAPDH primers. The results presented are representative of three separate experiments. (b) Quantitative analysis of $P2Y_{14}$ receptor expression in freshly isolated and anti-CD3 antibody-treated T-lymphocytes. Levels of $P2Y_{14}$ receptor are expressed as a percentage of GAPDH expression (100%) in freshly isolated and anti-CD3-treated T cells. The results represent mean ± s.e.m. of three independent experiments.

CD3 antibody-induced T-cell proliferation (see Figure 8). Although all four agonists reduced IL-2-induced T-cell proliferation, it is notable that the maximal inhibition was markedly less than that observed using anti-CD3 antibody (see Figure 7). This presumably reflects the different signalling pathways involved in IL-2- and anti-CD3 antibody-induced T-cell proliferation. IL-2-mediated signalling predominantly involves activation of JAK/STAT pathways, whereas activation of the T-cell antigen receptor (*via* anti-CD3 antibody) triggers NFAT and PKC (Szamel & Resch, 1995; Liu *et al*., 1998). It is notable that although all four agonists reduced T-cell proliferation, only UDP-glucose inhibited forskolin-induced adenylyl cyclase activity, suggesting that the attenuation of T-cell proliferation is independent of changes in intracellular cAMP levels. These observations compare to the inhibition of T-cell proliferation induced by G_i-coupled A_3 adenosine receptor. It is conceivable that $P2Y_{14}$ and A_3 adenosine receptor-mediated inhibition of T-cell proliferation involves changes in intracellular calcium levels, especially given the effect of UDP-glucose on $[Ca^{2+}]_i$ (Fumagalli *et al*., 2003; Skelton *et al*., 2003). Finally, the difference between the ability of UDP-glucose and related sugar nucleotides to inhibit T-cell proliferation *versus* inhibition of adenylyl cyclase may reflect changes in $P2Y_{14}$ receptor expression during T-cell activation. However, levels of $P2Y_{14}$ receptor expression did not change in T cells activated by anti-CD3 monoclonal antibody (see Figure 9). Overall, these observations represent the first reported modulation of T-cell function by the $P2Y_{14}$ receptor. Clearly, further studies are required to investigate the effect of $P2Y_{14}$ receptor activation on IL-2- and anti-CD3 antibody-induced cell signaling.

In conclusion, this study has shown for the first time that the $P2Y_{14}$ receptor is functionally expressed on murine spleen-derived T-lymphocytes. UDP-glucose, but not the related sugar nucleotides, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine, induced a small but significant and PTX-sensitive inhibition of forskolin-stimulated cAMP accumulation. To our knowledge, this is the first report describing the coupling of the $P2Y_{14}$ receptor to the inhibition of adenylyl cyclase in cells that endogenously express the receptor. Furthermore, UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine induced significant inhibition of anti-CD3 antibody- and IL-2-induced T-cell proliferation suggesting for the first time a role for the $P2Y_{14}$ receptor in modulating immunological responses involving T-lymphocytes. Further studies are required in order to further determine the role of the $P2Y_{14}$ receptor in T-lymphocyte function.

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