Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 543 (2006) 166–173
www.elsevier.com/locate/ejphar

Functional expression of the $P2Y_{14}$ receptor in human neutrophils

Michelle Scrivens, John M. Dickenson *

School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK

Received 27 January 2006; received in revised form 16 May 2006; accepted 19 May 2006
Available online 7 June 2006

Abstract

Previous studies using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis have shown that the $P2Y_{14}$ receptor is expressed at high levels in human neutrophils. Therefore the primary aim of this study was to determine whether the $P2Y_{14}$ receptor is functionally expressed in human neutrophils. In agreement with previous studies RT-PCR analysis detected the expression of $P2Y_{14}$ receptor mRNA in human neutrophils. UDP-glucose (IC_{50} = 1 μM) induced a small but significant inhibition (*circa* 30%) of forskolin-stimulated cAMP accumulation suggesting functional coupling of endogenously expressed $P2Y_{14}$ receptors to the inhibition of adenylyl cyclase activity in human neutrophils. In contrast, the other putative $P2Y_{14}$ receptor agonists UDP-galactose and UDP-glucuronic acid (at concentrations up to 100 μM) had no significant effect, whereas 100 μM UDP-*N*-acetylglucosamine-induced a small but significant inhibition of forskolin-stimulated cAMP accumulation (20% inhibition). UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine behaved as partial agonists by blocking UDP-glucose mediated inhibition of forskolin-induced cAMP accumulation. Treatment of neutrophils with pertussis toxin ($G_{i/o}$ blocker) abolished the inhibitory effects of UDP-glucose on forskolin-stimulated cAMP accumulation. UDP-glucose (100 μM) also induced a modest increase in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, whereas the other sugar nucleotides had no effect on ERK1/2 activation. Finally, UDP-glucose and related sugar nucleotides had no significant effect on N-formyl-methionyl-leucyl-phenylalanine-induced elastase release from neutrophils. In summary, although we have shown that the $P2Y_{14}$ receptor is functionally expressed in human neutrophils (coupling to inhibition of forskolin-induced cAMP and ERK1/2 activation) it does not modulate neutrophil degranulation (assessed by monitoring elastase release). Clearly further studies are required in order to establish the functional role of the $P2Y_{14}$ receptor expressed in human neutrophils.

Keywords: $P2Y_{14}$ receptor; Human neutrophil; Adenylyl cyclase; Elastase release

1. Introduction

G-protein coupled receptors represent the largest group of membrane bound receptors and many neutrophil functions including chemotaxis, degranulation, adhesion to endothelial cells, phagocytosis and generation of toxic oxygen species are triggered directly (predominantly via chemokine and formyl peptide receptors; Wu et al., 2000; Le et al., 2002; Vines and Prossnitz, 2004) or can be modulated by them. G-protein coupled receptors associated with the modulation of neutrophil function include members of the P2Y receptor family, which are activated by the nucleotides ATP, ADP, UTP and UDP (Dawicki et al., 1995; Zhang et al., 1996) and members of the adenosine receptor family, which are activated by the nucleoside adenosine (Hasko and Cronstein, 2004). These important signalling molecules are released from damaged platelets and endothelial cells and thus their concentrations are elevated at sites of vascular injury (Ingerman et al., 1979; Pearson and Gordon, 1979).

The $P2Y_{14}$ receptor (a recent addition to the P2Y receptor family) is activated by uridine 5′-diphosphoglucose (UDP-glucose) and the related sugar nucleotides, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine (Chambers et al., 2000; Abbracchio et al., 2003). UDP-glucose stimulated GTPγS binding to membranes prepared from HEK 293 cells transfected with the human $P2Y_{14}$ receptor was blocked by pertussis toxin suggesting that the $P2Y_{14}$ receptor couples to $G_{i/o}$ proteins (Chambers et al., 2000). Recently we have reported pertussis toxin-sensitive coupling of endogenously expressed $P2Y_{14}$ receptors to inhibition of forskolin-stimulated cAMP accumulation in murine spleen-derived T-

* Corresponding author. Tel.: +44 115 948 6683; fax: +44 115 948 6636.
E-mail address: john.dickenson@ntu.ac.uk (J.M. Dickenson).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.05.037

lymphocytes (Scrivens and Dickenson, 2005). In addition, UDP-glucose has been shown to stimulate increases in intracellular Ca^{2+} concentration in rat cortical astrocytes, human immature monocyte-derived dendritic cells, N9 microglial cells and in the epithelial cell lines A549 and BEAS-2B (Fumagalli et al., 2003; Skelton et al., 2003; Bianco et al., 2005; Müller et al., 2005). Interestingly, UDP-glucose-mediated Ca^{2+} responses observed in immature monocyte-derived dendritic cells, A549 and BEAS-2B cells were sensitive to pertussis toxin again suggesting coupling of endogenous $P2Y_{14}$ receptors to $G_{i/o}$ proteins.

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; Freeman et al., 2001). As detailed above functional $P2Y_{14}$ receptor expression has been reported in several cell types and established cell lines (Fumagalli et al., 2003; Lee et al., 2003; Skelton et al., 2003; Bianco et al., 2005; Müller et al., 2005; Scrivens and Dickenson, 2005). These results from these studies indicate that the $P2Y_{14}$ receptor plays a role in the regulation of immune responses since it stimulates the release of the proinflammatory cytokine Il-8 from A549 and BEAS-2B cells (Müller et al., 2005), possibly induces the maturation of dendritic cells (Skelton et al., 2003), inhibits T-lymphocyte proliferation (Scrivens and Dickenson, 2005) and mediates chemotaxis of bone-marrow hematopoietic stem cells (Lee et al., 2003). In addition, $P2Y_{14}$ receptor expression increases in rat brain and spleen following immunologic challenge with lipopolysaccharide (Moore et al., 2003; Charlton et al., 1997).

Recently, using Taq-Man quantitative RT-PCR, Moore et al. (2003) reported extremely high levels of $P2Y_{14}$ receptor expression in human neutrophils. Therefore, the primary aim of this study was to investigate whether the $P2Y_{14}$ receptor is functionally expressed on human neutrophils.

2. Materials and methods

2.1. Isolation of human neutrophils

Neutrophils were isolated essentially as described by Hallett et al. (1990). Briefly, 5 ml of dextran (60% 80 K) in balanced salt solution (BSS; 0.13 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.83 mM KH_2PO_4, pH 7.4) was added to 20 ml of heparinsed blood (1000 U/ml) taken from healthy volunteers and allowed to sediment for 40 min at room temperature. The middle layer containing the white cells was carefully removed using a sterile pasteur pipette, washed twice with 5 ml BSS (centrifugation 2000 rpm for 1 min) and red blood cells removed by hypotonic lysis. 1 ml of distilled water was added to the pellet of cells for 10 s before restoring osmolarity with 20 ml BSS. The polymorphonuclear leukocytes were separated from the other white blood cells using Ficoll–Paque density gradient centrifugation. Cells were overlaid on to 5 ml Ficoll–Paque and centrifuged for 1000 rpm for 20 min. Viable cells were counted using a haemocytometer and trypan blue exclusion method. The percentage of neutrophils present (87±3%) was determined using Leishman staining. The remaining 13% of cells are presumably other polymorphonuclear leukocytes such as basophils, mast cells and eosinophils.

2.2. RT-PCR analysis of $P2Y_{14}$ receptor mRNA expression

Total RNA was isolated from human neutrophil populations using RNA Bee according to the manufacturer's instructions. During the isolation procedure mRNA was routinely treated with RQ1 DNase (1 U/μl) 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 human $P2Y_{14}$ receptor gene specific primer sequences (318 bp product): forward 5′CGCAACATATTCAGCATCGTGT-3′ and reverse 5′-CAAAGTATCTGTGCTTTCAAGT-3′. GAPDH primers (501 bp product) were forward 5′-CTCATGACCACAGTCCATGC-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) 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.

2.3. cAMP accumulation assay

Human neutrophils (12×10^6 cells) 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). [^{3}H]-adenine-labelled cells 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 cyclic AMP phosphodiesterase inhibitor, rolipram (10 μM). Cells were then dispensed into 48-well culture plates (250 μl/well) and agonists were added (in 10 μl of medium) 5 min prior to incubation with 20 μM forskolin for 10 min. Incubations were terminated by the addition of 25 μl concentrated HCl and [^{3}H] cyclic AMP isolated by sequential Dowex-alumina chromatography as previously described (Donaldson et al., 1988). After elution, the levels of [^{3}H] cyclic AMP were determined by liquid scintillation counting.

2.4. Western blot analysis of ERK1/2 activation

Aliquots of human neutrophils (4×10^6 cells/90 μl serum free RPMI 1640 medium) were serum starved for 16 h at 37 °C in a humidified incubator (95% air/5% CO_2). Cells were then stimulated with agonists for 5 min (unless otherwise stated) and reactions terminated with 100 μl lysis buffer [150 mM NaCl, 50 mM Tris.HCl, 5 mM EDTA, 1% (v/v) IGEPAL CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin]. Cells were then incubated on ice for 5 min, after which the cell lysates were removed and placed into Eppendorf microcentrifuge tubes