



Novel vasocontractile role of the P2Y14 receptor: characterisation of its signalling in porcine isolated pancreatic arteries

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Dear Editor,

We resubmit for consideration by the British Journal of Pharmacology our revised manuscript entitled “Novel vasocontractile role of the P2Y₁₄ receptor: characterisation of its signaling in porcine isolated pancreatic arteries” by Mouhamed Alsaqati, Liaque Latif, Susan L.F. Chan, Vera Ralevic.

We have revised the manuscript according to the suggestions provided by the Editor and the Reviewers, including the extra experimental data requested, and have responded to the comments raised point by point. We have also added new data (western blotting studying the level of phosphorylated MLC2 following activation with UDP-glucose and MRS2690 in the presence of PPTN) to make the manuscript stronger. In addition, we have changed the name of Compound 2 and replaced it by PPTN according to Barrett *et al.*, (2013) to prevent any confusion in the literature.

For all of these reasons we believe that the revised manuscript will be more convincing and informative about the novel role of P2Y₁₄ receptors in the vasculature and of interest to readers of the British Journal of Pharmacology.

Yours sincerely,

Authors

Novel vasocontractile role of the P2Y₁₄ receptor: characterisation of its signalling in porcine isolated pancreatic arteries

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Running title: Novel vasocontractile role of P2Y₁₄ receptor

Background and purpose: The P2Y₁₄ receptor is the newest member of the P2Y receptor family; it is G_{i/o} protein-coupled and is activated by UDP and selectively by UDP-glucose and MRS2690 (7-10 fold more potent than UDP-glucose). This study investigated whether the P2Y₁₄ receptor is functionally expressed in porcine isolated pancreatic arteries.

Experimental approach: Pancreatic arteries were prepared for isometric tension recording and UDP-glucose, UDP and MRS2690 applied cumulatively after precontraction with U46619, a thromboxane A₂ mimetic. Levels of phosphorylated MLC2 were assessed with Western blotting. Levels of cAMP were assessed using a competitive enzyme immunoassay kit.

Key results: Concentration-dependent contraction with a rank order of potency of MRS2690 (10-fold) > UDP-glucose ≥ UDP was observed; contraction was reduced by PPTN, a selective antagonist of P2Y₁₄ receptor, which did not affect the response to UTP. Contraction to UDP-glucose was not affected by MRS2578, a P2Y₆ receptor selective antagonist. In the presence of U46619 and forskolin, which increases cAMP levels, contraction to UDP-glucose was enhanced. In addition, UDP-glucose and MRS2690 inhibited forskolin-stimulated cAMP levels. Removal of the endothelium and inhibition of endothelium-derived contractile agents (thromboxane A₂, prostaglandin F_{2α} and endothelin-1) inhibited contractions. Y-27632, nifedipine and thapsigargin also reduced contraction to the agonists. UDP-glucose and MRS2690 increased MLC2 phosphorylation, which was blocked by PPTN.

Conclusions and implications: These data identify a novel vasocontractile role of P2Y₁₄ receptors in porcine pancreatic arteries. P2Y₁₄-mediated contraction involves cAMP-dependent mechanisms, an elevation of Ca²⁺ levels, activation of RhoA/ROCK signalling, and MLC2 in porcine pancreatic arteries. It also involves a release of thromboxane A₂, prostaglandin F_{2α} and endothelin-1.

Keywords: UDP-glucose, UDP, MRS2690, P2Y₁₄ receptor, vasoconstriction, endothelium.

Abbreviations: ATP, Adenosine-5'-triphosphate; HEK, human embryonic kidney; cAMP, 3'-5'-cyclic adenosine monophosphate; DAG, diacylglycerol; IP₃, inositol 1,4,5-triphosphate; MLC2, myosin light chain 2; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; PGF_{2α}, prostaglandin F_{2α}; PKC, protein kinase C; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulphonate); PPTN, 4-(4-(Piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid; RhoA, ras homolog gene family member A; ROCK, Rho-associated protein kinase; SNP, sodium nitroprusside; UDP, uridine diphosphate; UDP-glucose, uridine diphosphate glucose; UTP, uridine-5'-triphosphate.

Introduction

P2Y Receptors are members of the superfamily of G-protein coupled receptors (GPCRs); they are responsive to purine and pyrimidine nucleotides and nucleotide sugars (ADP, ATP, UTP, UDP and UDP-glucose). Eight mammalian subtypes of P2Y receptors have been identified: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors (Abbracchio *et al.*, 2006). The P2Y₁₄ receptor was the most recently identified (Chambers *et al.*, 2000). In contrast to other P2Y receptors, P2Y₁₄ receptors are activated by nucleotide sugars such as UDP-glucose, in addition to UDP-galactose and UDP-glucuronic acid which are less potent than UDP-glucose (Abbracchio *et al.*, 2003; Fricks *et al.*, 2008; Harden *et al.*, 2010). They are also activated by UDP and MRS2690 (2-thiouridine-5'-diphosphoglucose), which is more selective at P2Y₁₄ receptors and 7-10 fold more potent than UDP-glucose (Carter *et al.*, 2009; Jacobson *et al.*, 2009; Gao *et al.*, 2010). The P2Y₁₄ receptor is involved in G_i-protein-mediated signalling, leading to the inhibition of adenylyl cyclase activity and, accordingly, is pertussis toxin-sensitive (Jacobson *et al.*, 2009). G_i-protein-derived G_{βγ}-dimers can initiate phospholipase C_β (PLC_β) signalling pathways, which lead to the stimulation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) and subsequent activation of RhoA/ROCK signalling, protein kinase C (PKC) and myosin light chain (MLC) kinase, besides elevation of the intracellular calcium level (Amano *et al.*, 1996; Hartshorne & Gorecka, 2011; Sesma *et al.*, 2012).

Pyridoxalphosphate-6-azophenyl-2',4'-disulphonate (PPADS) and suramin are non-selective antagonists at most of the P2Y receptors, but some P2Y receptors are insensitive to these antagonists (Chootip *et al.*, 2005). There is currently no report of antagonist sensitivity of P2Y₁₄ receptors for suramin and PPADS. Recently, a novel antagonist at P2Y₁₄ receptors was

identified; this antagonist, named 4-(4-(piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid (PPTN) (Barrett *et al.*, 2013), was characterised in HEK cells through its ability to inhibit UDP-glucose-stimulated Ca^{2+} mobilization (Robichaud *et al.*, 2011). In addition, it showed good affinity for the P2Y_{14} receptor ($K_i = 1.9$ nM in a chimpanzee P2Y_{14} binding assay) (Robichaud *et al.*, 2011). When it was studied in human C6 glioma cells, PPTN showed selectivity for P2Y_{14} receptors with a K_B of 434 pM, with no agonist or antagonist affinity at P2Y_1 , P2Y_2 , P2Y_4 , P2Y_6 , P2Y_{11} , P2Y_{12} or P2Y_{13} receptors (Barrett *et al.*, 2013).

P2Y_{14} receptor mRNA and protein have a varied expression in the body; they have been found in the spleen, placenta, lung, heart, adipose tissue, gastrointestinal smooth muscle, endothelial cells, and immune cells (Chambers *et al.*, 2000; Scrivens & Dickenson, 2005; Umapathy *et al.*, 2010). Relatively little is known, however, about the functional expression of the P2Y_{14} receptor; best characterised is its role in regulation of the immune system, as a number of studies have described an involvement of P2Y_{14} receptors in modulation of the function of human neutrophils and T-lymphocytes, and in secretion of the pro-inflammatory cytokine IL-8 in airway epithelial cells (Scrivens & Dickenson, 2005, 2006; Jacobson *et al.*, 2009). UDP-glucose can be released in a constitutive manner, and during shear stress, to act as an extracellular signalling molecule (Lazarowski *et al.*, 2003); its release from multiple cell types suggests potentially broad role(s) of the P2Y_{14} receptor.

Although the exact mechanisms remain to be established, an increase in pancreatic endocrine cell activity during hormone secretion corresponds with an increase in blood flow, to meet metabolic demand. Thus, alterations in blood flow can influence pancreatic function. The role of exogenous purine and pyrimidine di- and triphosphate nucleotides in controlling the

functions of endocrine and exocrine components of the pancreas are well described (Novak, 2008; Burnstock & Novak, 2012). Within the pancreatic vasculature, P2X receptors were suggested to mediate vasoconstriction, and P2Y receptors vasodilatation, in response to ATP (Hillaire-Buys *et al.*, 1991). P2X₁, P2Y₂, P2Y₄ and P2Y₆ receptors, with different sensitivities to ATP, UDP and UTP, mediate vasoconstriction in a number of blood vessels (Ralevic and Burnstock, 1998), and there is some evidence for a vasocontractile function of the P2Y₁₂ receptor (Wihlborg *et al.*, 2004; Högberg *et al.*, 2010). In the current study, we describe the pharmacological characterisation of P2Y₁₄ receptor-mediated contractile responses of porcine isolated pancreatic artery preparations. A preliminary account of some of these data has previously been presented to the British Pharmacological Society (Alsaqati *et al.*, 2010).

Materials and methods

Tissue Preparation

Pancreases from pigs (either sex, age less than 6 mo, wt ~50 kg) was obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield). A crude dissection was conducted to isolate the pancreatic arteries (greater pancreatic artery) which were located in the body of the pancreas. The vessels were dissected out and placed in Krebs'-Henseleit buffer containing 2% (w/v) Ficoll (type 70) and refrigerated overnight at 4°C. The next day, fine dissection was performed, and the arteries cut into rings of 0.5 cm in length and suspended in Krebs'-Henseleit buffer (gassed, 95% O₂, 5% CO₂).

The endothelium of some arteries was removed by gently rubbing their innermost surface with forceps before attaching it to the setup (Rayment *et al.*, 2007a). Successful removal of endothelium was tested using substance P (10 nM). Endothelium-denuded arteries relaxed in

response to substance P to less than 10% of the U46619 (11 α ,9 α -epoxymethano-PGH₂) induced contraction, while in endothelium-intact arteries the relaxation to substance P was 36% \pm 8 (n=7, data not shown).

Responses in the porcine isolated pancreatic artery

Arterial rings were mounted onto wires in tissue baths (20 ml) containing warm (37°C), oxygenated Krebs'-Henseleit solution and were connected via isometric force transducers (ADInstruments, Sydney, Australia), to a PC running the computer program LabChart (ADInstruments, Sydney, Australia). Rings were put under tension (15 g) and allowed to equilibrate for 60 min before assessing viability with two challenges of 75 mM potassium chloride (KCl). The tissues were then allowed to equilibrate for 60 min, after which U46619 (10 - 100 nM), a thromboxane A₂ mimetic, was used to contract the tissues to between 40-80% of the second KCl response. This ensured that if there was a vasodilator component to the response, for example due to activation of multiple P2 receptor subtypes, this could be detected. Once an appropriate level of U46619 response had been achieved, cumulative addition of UDP-glucose, UDP or MRS2690 were applied. Antagonists or inhibitors were applied 10 min prior to the addition of U46619, allowing incubation with the tissues for a minimum of 30 min prior to the application of agonists. Desensitisation of the contraction to UDP-glucose was generated by exposing the arteries to UDP or UDP-glucose 10 min prior the addition of U46619. An exception to precontraction with U46619, were experiments with L-655,240 (1-[(4-Chlorophenyl)methyl]-5-fluoro-- α , α ,3-trimethyl-1H-indole-2-propanoic acid), in which arteries were precontracted with endothelin-1. In experiments using dimethyl sulfoxide (DMSO) as the solvent (see reagents and drugs section), DMSO was added to the arteries (vehicle control).

Effect of forskolin on subsequent UDP-glucose or UTP responses

Tissues were exposed to U46619 (10 - 100 nM) and relaxed with forskolin (1 μ M), involving elevation of cAMP, back to the baseline; cumulative concentration-response curves were then constructed for UDP-glucose (1 μ M – 1 mM) or UTP (10 μ M – 1 mM). Responses to UDP-glucose or UTP obtained under these conditions were compared to control responses in which drugs were added at basal tone without exposing to either U46619 or forskolin. The tissue was allowed to recover for 20 min before a concentration-response curve to UDP-glucose or UTP was constructed.

Western blotting

Segments of porcine pancreatic arteries were set up in the organ baths and tensioned to 15 g, then left for approximately 60 min to reach a new baseline of resting tension. Tissues were incubated with 100 μ M UDP-glucose for 30, 60, 120, 180, 240 and 300 s. In addition, in other experiments, the tissues were incubated with UDP-glucose (100 μ M) or MRS2690 (10 μ M) for 30 s in the presence or absence of PPTN. Segments were quickly removed from the organ baths and immediately frozen on dry ice. Control tissues were not exposed to any compound (basal conditions). Segments were then homogenised in lysis buffer (20 mM Tris, 1 mM EGTA, 0.1% (v/v) Triton X100, 1 mM NaF, 10 mM beta glycerophosphate, pH 7.6), containing protease inhibitor cocktail tablets, EDTA free. Samples with solubilisation buffer 6 \times SB: (24% (w/v) sodium dodecyl sulphate, 30% (v/v) glycerol, 5% (v/v) beta mercaptoethanol, 2.5% (v/v) bromophenol blue, 1.5M Tris HCl, pH 6.8) were heated at 95°C for 5 min. Subsequently, electrophoresis was carried out on 4-20% Tri-Glycine (PAGE) Gold Precast Gels (Bio-Rad, Hercules, CA, U.S.A.), 15 μ g protein per lane. Samples were transferred to nitrocellulose membranes. Next, blots were incubated in blocking solution (5% (w/v) powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween 20) for 60 min, at

room temperature. Blots were incubated overnight at 4°C with primary antibody against phosphorylated myosin light chain kinase 2 (MLC2, 1:500) or total (MLC2, 1:1000) diluted in blocking solution. After washing in Tris buffered saline containing 0.1% (v/v) Tween 20, the blots were incubated with an appropriate IRDye[®]-conjugated secondary antibody (LI-COR). Proteins were visualised using the Licor/Odyssey infrared imaging system (Biosciences, Biotechnology). Bands were analyzed by densitometry using the Odyssey application software and expressed as phosphorylated MLC2 normalised to total MLC2. For investigating the existence of P2Y₁₄ receptor in pancreatic arteries, untreated samples were homogenised and examined as explained above.

cAMP measurement in porcine pancreatic arteries

Pancreatic artery rings were stimulated with 75 mM KCl followed by U46619 (10 nM) and forskolin (1 µM), and then the arteries were challenged for 3 min with UDP-glucose (1 mM), MRS2690 (10 µM), UTP (1 mM) or distilled water (control group). Pancreatic rings were collected and immediately frozen on dry ice and then stored in vials at -80 C until their study. The tissues were homogenised in 5% (w/v) trichloroacetic acid (TCA) in water with a borosilicate glass, homogeniser, and then centrifuged for 10 min at 1500 g. TCA was extracted from the supernatant samples using water-saturated ether, and evaporated for 5 min to remove the residual ether from the aqueous fractions. Samples were diluted (1:2) in ether-extracted 5% (w/v) TCA. cAMP concentration was measured using a competitive enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). The working range of the cAMP assay was 0.1–1000 pmol/ml. cAMP concentration was expressed as a percentage of forskolin-induced [cAMP] elevation.

Reagents and Drugs

Krebs'-Henseleit buffer was composed of the following (mM); NaCl 118, KCl 4.8, CaCl₂.H₂O 1.3, NaHCO₃ 25.0, KH₂PO₄ 1.2, MgSO₄.7H₂O 1.2 and glucose 11.1. Suramin, NDGA, nifedipine, thapsigargin, UTP, U46619, sodium nitroprusside (SNP), zafirlukast, BQ788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-gmethylleucyl-D-1-methoxycarboyl-D-norleucine), UDP-glucose and UDP were purchased from Sigma (Poole, Dorset, UK), while DUP 697, MRS2578 (*N,N''*-1,4-butanediyl bis(*N'*-[3-isothiocynatophenyl]) thiourea), PPADS and substance P, MRS2690, L-655,240, endothelin-1, BQ123 (*cyc*(DTrp-DAsp-Pro-D-Val-Leu)), Y-27632 (*trans*-4-[(1R)-1-aminoethyl]-N-4-pyridinyl-cyclohexane carboxamide) and forskolin were from Tocris Biosciences Ltd. (Bristol, UK). PPTN, a selective high affinity antagonist of P2Y₁₄ receptor, was kindly gifted from Merck Frosst Centre for Therapeutic Research. Primary antibodies for Western blotting were purchased from Cell Signalling Technology for phosphorylated MLC2 antibody, (cat no. 3674S) and total MLC2 antibody, (cat no. 3672). Cyclic AMP EIA kit (cat No. 581001) was purchased from Cayman Chemical Company, (Ann Arbor, MI). U46619 was dissolved in ethanol at 10 mM stock concentration. DUP 697, PPTN, BQ788, MRS2578, L-655,240, nifedipine, thapsigargin, zafirlukast and forskolin were dissolved in DMSO. All other drugs were dissolved in distilled water. All reagents and drugs nomenclature conformed to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Data Analysis

Data were expressed as log concentration-response plots. The contraction to all agonists was expressed in g, and measured from the stabilised U46619 response. Values for all figures refer to mean ± S.E.M with 95% confidence. Results were compared by two-way ANOVA or

Student's unpaired *t*-test (Prism, GraphPad, San Diego, CA, USA). Differences were considered to be significant when the P value was < 0.05 . N expresses the number of animals.

Results

Effect of UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

To investigate the possible functional expression of P2Y₁₄ receptors and their role in porcine pancreatic arteries, agonists for this receptor were applied as cumulative concentrations. MRS2690, a selective P2Y₁₄ receptor agonist (0.1 μ M to 30 μ M), UDP-glucose (1 μ M to 1 mM) and UDP (1 μ M to 1 mM) were added after precontraction with U46619. All of the agonists induced a concentration-dependent contraction with a rank order of potency of MRS2690 $>$ UDP-glucose \geq UDP. MRS2690 was significantly more potent, by approximately 10-fold, than UDP-glucose ($P < 0.01$, two-way ANOVA, Figure 1A), while UDP-glucose and UDP responses were equipotent (Figure 1A). Both UDP-glucose and UDP (P2Y₁₄ receptor agonists) separately induced significant attenuation of the contraction to UDP-glucose; for instance, the response to 100 μ M UDP-glucose was decreased by $55 \pm 7\%$ in the presence of 100 μ M UDP-glucose, and by $53 \pm 7\%$ in the presence of 100 μ M UDP ($P < 0.001$, $n=10-13$, Figure 1B).

Effect of PPADS and suramin on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Responses to UDP-glucose, UDP and MRS2690 were characterised using the non-selective P2 receptor antagonists PPADS (10 μ M) and suramin (100 μ M) (Rayment *et al.*, 2007b). Both PPADS and suramin significantly enhanced the contractions evoked by UDP-glucose and UDP (Figure 2A, 2B). The contraction to 100 μ M UDP-glucose was enhanced by $121 \pm$

38% ($P < 0.001$, $n=7$) and by $100 \pm 23\%$ ($P < 0.001$, $n=8$) in the presence of PPADS and suramin respectively (Figure 2A). The contraction to 1 mM UDP was enhanced by $180 \pm 46\%$ ($P < 0.001$, $n=8$), and by $154 \pm 30\%$ ($P < 0.001$, $n=8$) in the presence of PPADS and suramin respectively (Figure 2B). Suramin and PPADS failed to alter the contraction to MRS2690 (Figure 2C). In contrast, suramin and PPADS blocked the contraction to UTP, a P2Y₂ and P2Y₄ receptors agonist (von Kügelgen, 2006) (unpublished observations). UDP is a ligand at P2Y₆ receptors as well as P2Y₁₄ receptors. Therefore, the effect of UDP was examined in the presence of MRS2578 (10 μ M), a P2Y₆ receptor selective antagonist (Mamedova *et al.*, 2004). The contraction evoked by UDP was unaffected at lower concentrations but was augmented at higher concentrations of UDP (supplementary information), while MRS2578 did not alter the responses to UDP-glucose or MRS2690 (supplementary information).

Effect of PPTN, a selective high affinity antagonist of P2Y₁₄ receptor, on responses to UDP-glucose and MRS2690 in porcine isolated pancreatic arteries

The responses to UDP-glucose and MRS2690 were examined in the presence of PPTN (1 μ M), a selective high affinity antagonist of P2Y₁₄ receptor (Robichaud *et al.*, 2011; Barrett *et al.*, 2013). This compound significantly reduced the contraction evoked by UDP-glucose at basal tone (Supplementary information) and that by UDP-glucose and MRS2690 at raised tone (Figure 3A, 3B). PPTN inhibited the contraction to 100 μ M UDP-glucose and to 10 μ M MRS2690 by $55 \pm 10\%$ ($P < 0.05$, $n=7$) and by $46 \pm 9\%$ ($P < 0.01$, $n=9$) respectively (Figure 3A, 3B). The contraction to UTP was not altered in the presence of PPTN (Figure 3C). Typical traces, showing the effect of MRS2690 in the absence and in the presence of PPTN, are shown in Figure 3D.

Effect of endothelium removal on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

The responses of UDP-glucose, UDP and MRS2690 were studied after the endothelium had been removed. The contractions induced by UDP-glucose, UDP and MRS2690 were significantly attenuated in the endothelium-denuded arteries (Figure 4). Removal of endothelium reduced the contractions to 1 mM UDP-glucose by $50 \pm 7\%$ ($P < 0.001$, $n=12$), that to 1 mM UDP by $61 \pm 11\%$ ($P < 0.001$, $n=15$) and that to 30 μM MRS2690 by $41 \pm 5\%$ ($P < 0.01$, $n=5$) (Figure 4).

Effect of DUP 697 on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Because the contractions to P2Y_{14} receptor agonists were mainly endothelium-dependent, these were studied in the presence of DUP 697, a cyclooxygenase-2 (COX-2) inhibitor, since COX-2 facilitates the release of agents which are responsible for endothelium-dependent contraction. DUP 697 (3 μM) diminished the responses to UDP-glucose, UDP and MRS2690 to a similar extent as removal of the endothelium (Figure 5), while DUP 697 did not alter the contraction to U46619 (the precontraction agent) or the contraction to ATP (data not shown). DUP 697 reduced the contraction to 1 mM UDP-glucose by $34 \pm 10\%$ ($P < 0.001$, $n=11$), that to 1 mM UDP by $38 \pm 7.5\%$ ($P < 0.001$, $n=15$) and that to 30 μM MRS2690 by $22.5 \pm 13\%$ ($P < 0.01$, $n=4$), (Figure 5).

The role of endothelium-derived contracting factors in the response to UDP-glucose in porcine isolated pancreatic arteries

The following experiments were carried out using mainly UDP-glucose or UDP due to the cost considerations involved with use of MRS2690. The possible involvement of thromboxane A_2 , leukotrienes, endothelin-1 and $PGF_{2\alpha}$, which can be released from endothelial cells (Mombouli & Vanhoutte, 1993; Kurahashi *et al.*, 2003; Wong *et al.*, 2009), in endothelially-mediated contraction to UDP-glucose was investigated. The contraction to UDP-glucose was not altered in the presence of nordihydroguaiaretic acid (NDGA) (10 μ M), a lipoxygenase inhibitor (Figure 6A) or zafirlukast (10 μ M), a leukotriene receptor inhibitor (Figure 6B). The contraction to UDP-glucose was reduced in the presence of BQ123 (1 μ M), a selective ET_A receptor antagonist; the response to 100 μ M UDP-glucose was attenuated by $10 \pm 3\%$ ($P < 0.05$, $n=14$) in the presence of BQ123 (Figure 6C), which was only effective with intact endothelium (Supplementary information). The contraction to UDP-glucose was unaltered in the presence of BQ788 (1 μ M), a selective ET_B receptor antagonist (Supplementary information). In addition, UDP-glucose induced-contraction was reduced in the presence of L-655,240 (1 μ M), a selective thromboxane A_2 /prostaglandin endoperoxide receptor antagonist. The response to 100 μ M UDP-glucose was inhibited by $38 \pm 5\%$ ($P < 0.001$, $n=9$) in the presence of L-655,240 (Figure 6D). These data indicate that UDP-glucose mediated-contraction occurs mainly via thromboxane and prostaglandins, with a lesser involvement of endothelin-1.

Effect of inhibition of calcium release and calcium entry on the response to UDP-glucose and UDP in porcine isolated pancreatic arteries

Binding of agonist to the recombinant $P2Y_{14}$ receptor leads to increase Ca^{2+} flux in some cells (Skelton *et al.*, 2003; Gao *et al.*, 2010). To test this in porcine pancreatic arteries, responses to UDP-glucose and UDP were examined in the presence and absence of nifedipine (1 μ M), an L-type voltage-gated calcium channel blocker, and thapsigargin (100 nM), a

potent inhibitor of sarco-endoplasmic reticulum Ca^{2+} -ATPases which leads to depletion of intracellular calcium. Both of these inhibitors reduced the contraction evoked by 100 μM UDP-glucose, by $39 \pm 5\%$ ($P < 0.001$, $n=15$) in the presence of nifedipine, and by $25 \pm 8\%$ ($P < 0.05$, $n=12-15$) in the presence of thapsigargin (Figure 7A, 7B). Responses to 100 μM UDP were inhibited by $53 \pm 2\%$ ($P < 0.001$, $n=9$) in the presence of nifedipine, and by $36 \pm 6\%$ ($P < 0.001$, $n=9$) in the presence of thapsigargin (data not shown). Typical traces showing the effect of UDP-glucose in the absence and in the presence of nifedipine are shown in Figure 7C.

Effect of inhibition of the Rho-kinase pathway on the responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Following the activation of P2Y_{14} receptors, MLC may be phosphorylated because of upstream stimulatory RhoA/ROCK signalling (Amano *et al.*, 1996; Hartshorne & Gorecka, 2011). To test the possible involvement of this pathway, experiments were conducted to study the contractions to UDP-glucose, UDP and MRS2690 in the presence of Y-27632 (5 μM), a selective inhibitor of the Rho-associated protein kinase p160ROCK. Y-27632 significantly inhibited the contraction evoked by UDP (Figure 8A), MRS2690 (Figure 8B) and UDP-glucose (data not shown). For instance, the response to 300 μM UDP was reduced by $50 \pm 4\%$ ($P < 0.001$, $n=15$, Figure 8A), that to 30 μM MRS2690 by $50 \pm 15\%$ ($P < 0.001$, $n=9$, Figure 8B) and that to 1 mM UDP-glucose by $52 \pm 8\%$ ($P < 0.001$, $n=9$, data not shown). The response to UDP-glucose was associated with an increase in the level of MLC2 phosphorylation, after 30–60 s of treatment with 100 μM UDP-glucose, and returned to the basal level of phosphorylated MLC2 from 120–300s (Figure 8C), which suggested an involvement of MLC2 activation in the response to UDP-glucose P2Y_{14} receptor signalling pathway. The ability of UDP-glucose (100 μM) or MRS2690 (10 μM) to elevate the MLC2

phosphorylation (after 30 s of treatment) was abolished in the presence of PPTN (1 μ M), which shows that the ability of UDP-glucose or MRS2690 to elevate MLC2 phosphorylation in porcine pancreatic arteries is mediated by P2Y₁₄ receptors (Figure 9).

Effect of pre-constriction with U46619, and relaxation with forskolin, on the response to UDP-glucose in porcine isolated pancreatic arteries

Agonist-promoted activation of G_i and subsequent inhibition of adenylyl cyclase is one of the signalling responses of P2Y₁₄ receptors. Therefore, the response to UDP-glucose was tested after exposure to U46619 and subsequent relaxation by forskolin (back to the baseline), involving elevation of intracellular cyclic AMP. UDP-glucose induced a greater contraction compared with the control, in which UDP-glucose was applied at basal tone without the tissues being exposed to U46619 or forskolin (Figure 10). The contraction to 100 μ M UDP-glucose was enhanced by $930 \pm 108\%$ ($P < 0.001$, $n=11$) after the exposure to U46619 and forskolin (Figure 10A). In contrast, when responses to UTP, an agonist at P2Y₂ and P2Y₄ receptors (G_q protein coupled receptors) were investigated in vessels exposed to U46619 and forskolin, there was no change in the contractions (Figure 10B). In addition, the response to UDP-glucose was not altered after contraction with U46619 and relaxation with SNP (100 μ M), which elevates intracellular cyclic GMP (Roberts *et al.*, 1999). Typical traces showing the effect of UDP-glucose on basal tone and after the tissues had been contracted by U46619 and then relaxed with forskolin are shown in Figure 10C.

Effect of UDP-glucose and MRS2690 on the cAMP level in porcine isolated pancreatic arteries

On the basis that cAMP is involved in the contraction to P2Y₁₄ receptor agonists, we measured the cellular levels of this second messenger in pancreatic artery rings. We investigated the effects of UDP-glucose, MRS2690 and UTP (as a control, since it is coupled to G_q protein) on cAMP level in the presence of U46619 + forskolin (to mimic the raised tone condition of the pharmacology experiments). UDP-glucose (1 mM) and MRS2690 (10 μM) induced a significant decrease in cAMP level in relative to the control (*P < 0.05, n=4), while UTP had no significant effect on cAMP levels (Figure 11).

Discussion

The current study presents evidence for the functional expression of contractile P2Y₁₄ receptors, sensitive to the endogenous nucleotides UDP-glucose and UDP in arteries of pig pancreas. Evidence from the contractile studies and the cAMP immunoassay is consistent with the ability of this receptor to inhibit adenylyl cyclase, while immunoblotting indicates the downstream involvement of MLC2. The contractile response was mediated largely by the endothelium with an involvement of endothelin, thromboxane A₂ and PGF_{2α}.

Contractile studies showed that contractions to UDP-glucose and UDP were almost equipotent, whereas the contraction to MRS2690, a selective P2Y₁₄ receptor agonist, was approximately 10-fold more potent than that of UDP-glucose at the P2Y₁₄ receptor (Figure 1A). This is consistent with previous reports which suggest a 7-10 fold greater potency of MRS2690 over UDP-glucose (Jacobson *et al.*, 2009; Gao *et al.*, 2010). MRS2690 activity was observed at ≤ 10 μM; at 10 μM MRS2690 has been reported to be inactive at P2Y₂ receptors (Ko *et al.*, 2009).

PPTN is a non-nucleotide, high affinity competitive antagonist at P2Y₁₄ receptors. It was assessed in HEK cells using a calcium mobilization assay and it inhibited UDP-glucose mediated signalling, and showed no effect on other P2Y receptors at concentrations up to 10 μM (Robichaud *et al.*, 2011; Barrett *et al.*, 2013). The responses to the P2Y₁₄ receptor agonists were examined in the presence of this antagonist. PPTN blocked the contractions induced by UDP-glucose and MRS2690 which confirms the involvement of P2Y₁₄ receptors in our arteries (Figure 3A, 3B).

The responses to the P2Y₁₄ receptor agonists were examined in the presence of the non-selective P2 receptor antagonists, suramin and PPADS. The non-selective antagonists induced a small increase in the contractions to UDP and UDP-glucose (Figure 2A, 2B). However, they failed to change the response to MRS2690 (Figure 2C). The lack of effect of suramin and PPADS appears to rule out an involvement of P2Y₂ and/or P2Y₄ receptors, since we have shown that they blocked the responses to UTP in porcine pancreatic arteries (unpublished observations) and other tissues (Rayment *et al.*, 2007b). It is unclear why these antagonists enhanced the effects of P2Y₁₄ receptor agonists. However, it is clear that suramin and PPADS had different effect on UTP responses versus responses of MRS2690 and UDP-glucose indicating actions at different receptors.

Contractile responses to UDP-glucose, UDP and MRS2690 were significantly inhibited after the endothelium was removed (Figure 4), which indicated that the main expression of P2Y₁₄ receptor may be on the endothelial layer of porcine pancreatic arteries. Similarly, the P2Y₁₄ receptor has been reported to be expressed in endothelial cells of porcine coronary artery, human lung microvascular endothelial cells, and pulmonary artery vasa vasorum endothelial cells (Umapathy *et al.*, 2010; Abbas *et al.*, 2011; Lyubchenko *et al.*, 2011). P2Y₁₄ receptor expression was barely detectable in mouse thoracic aorta (Kauffenstein *et al.*, 2010); in

contrast, rat aortic smooth muscle cells show robust expression of the receptor, as observed in freshly isolated and cultured cells (Govindan *et al.*, 2010).

To investigate the mechanism underlying the contraction mediated by P2Y₁₄ receptors in pancreatic arteries, responses to UDP-glucose, UDP and MRS2690 were examined in the presence of DUP 697. As seen in Figure 5, the endothelium-dependent contractions were attenuated in the presence of the selective COX-2 inhibitor. Endothelial cells can release contractile factors (EDCFs), which may include thromboxane A₂, PGF_{2α}, leukotrienes and endothelin-1. Thromboxane A₂ and PGF_{2α} are released from the endothelium due to the activity of cyclooxygenase-2 (Mombouli & Vanhoutte, 1993; Wong *et al.*, 2009). To characterise EDCFs involved in the contraction to UDP-glucose, experiments were conducted to study the responses to UDP-glucose in the presence of zafirlukast, BQ123, BQ788 and L-655,240. Only BQ123 and L-655,240 were able to inhibit the contraction evoked by UDP-glucose which indicated an involvement of thromboxane A₂, prostaglandins and endothelin-1 (Figure 6). These agents, after being released from the endothelium, may act on their receptors on vascular smooth muscle cells (Wong *et al.*, 2009).

The involvement of extracellular calcium influx and calcium released from sarcoplasmic reticulum (SR) as a part of the response to P2Y₁₄ receptors activation has been reported (Verin *et al.*, 2011). In addition, calcium-induced release of calcium from SR and influx of external Ca²⁺ in excitation-contraction in response to some activated receptors have been also reported (Fabiato, 1983; Li *et al.*, 2003). In porcine pancreatic arteries, contractions to UDP-glucose (Figure 7A, B) and UDP (data not shown) were significantly decreased in the presence of nifedipine or thapsigargin, which identified an involvement of elevated intracellular calcium level. Collectively, our results suggest that when UDP-glucose, UDP and MRS2690 act at the P2Y₁₄ receptors, which are expressed mainly on the endothelium, the intracellular Ca²⁺ levels may be elevated; this may then activate endothelin-1 and

phospholipase A₂ which liberates arachidonic acid, which is then converted by the activity of COX-2 to produce the contractile agents, thromboxane A₂ and prostaglandin, which bind to receptors on vascular smooth muscle cells to induce contraction.

A number of studies have considered the signalling mechanism underlying the functional response of P2Y₁₄ receptors (Harden *et al.*, 2010; Sesma *et al.*, 2012). Recent reports claimed that UDP-glucose promotes concentration-dependent activation of RhoA in isolated human neutrophils (Sesma *et al.*, 2012). This was tested in porcine pancreatic arteries when responses were investigated in the presence of Y-27632; this compound inhibited the contraction evoked by UDP-glucose, UDP and MRS2690 (Figure 8A, 8B), which showed an involvement of RhoA in the response to P2Y₁₄ receptor agonists. Activation of RhoA leads to phosphorylation of MLC and subsequently contraction of arteries in a calcium-independent manner (Amano *et al.*, 1996; Hartshorne & Gorecka, 2011). Accordingly, when UDP-glucose or MRS2690 were incubated with the tissues, the level of phosphorylated MLC2 was elevated, but this elevation was abolished in the presence of PPTN, confirming that it is happening through the activation of P2Y₁₄ receptors (Figure 8C, 9).

It is well established that the P2Y₁₄ receptor is coupled to G_i protein, leading to the inhibition of forskolin-stimulated adenylyl cyclase activity, and hence inhibition of the cyclic AMP level. Accordingly, the P2Y₁₄ receptor is pertussis toxin-sensitive (PTX) (Jacobson *et al.*, 2009). It is notoriously difficult to successfully block G_i coupled receptors with PTX in isolated blood vessels, as we have also found. However, in tissues which had been precontracted with U46619 and relaxed with forskolin (to elevate the cAMP levels), subsequent addition of UDP-glucose produced a significantly greater contraction compared with the controls (UDP-glucose added at basal tone, or UTP applied after precontraction with U46619 and relaxation with forskolin) (Figure 10), consistent with an involvement of G_i

coupled receptors. Other purine receptors, namely P2Y₁, P2Y₂, P2Y₄, P2Y₆ receptors, are generally G_q protein-coupled. Furthermore, relaxation with SNP (to elevate cGMP levels) after precontraction with U46619 had no effect on the contractile response to UDP-glucose (data not shown). These findings together with the data obtained from cAMP assay (Figure 11) indicate that the enhanced contraction to UDP-glucose is mainly dependent on the agonist's ability to lower cyclic AMP levels.

Reduction in pancreatic blood flow has been observed in acute and chronic pancreatitis and some other pancreatic diseases (Satoh *et al.*, 2000; Nguyen *et al.*, 2010), implicating pancreatic tissue perfusion as an important factor in pathogenesis of pancreatic diseases and symptoms. There is increasing evidence for the role of purinergic signalling in the pathophysiology of the pancreas (Burnstock & Novak, 2012). Drugs designed to target specific component of purinergic system may be of relevance to the management of pancreatitis, cystic fibrosis, pancreatic cancer and diabetes.

In conclusion, this study has shown a novel vasocontractile action of UDP-glucose which appears to be mediated by the P2Y₁₄ receptor in porcine isolated pancreatic arteries. P2Y₁₄ receptors induce contraction via an involvement of endothelin-1, thromboxane A₂ and prostaglandins released from the endothelium. In addition, P2Y₁₄-mediated contraction involves the activation of Rho kinase, and the subsequent phosphorylation of MLC2. The ability of P2Y₁₄-receptor agonist to inhibit cAMP level indicates P2Y₁₄ receptor coupling to G_i proteins. This study identifies a novel role for the P2Y₁₄ receptor as a vasocontractile P2 receptor.

Acknowledgments

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Figure Legends

Figure 1. (A) Concentration-dependent contractions evoked by UDP-glucose, UDP and MRS2690 in U46619-precontracted porcine pancreatic arteries (**P < 0.01, two-way ANOVA, MRS2690 response vs UDP-glucose and UDP responses, F=13.74, 16.03; n=9-12). (B). Attenuation of UDP-glucose-induced contraction (the control) in the presence of UDP-glucose (100 μ M) and UDP (100 μ M) (added 10 min prior to U46619 addition). Both UDP-glucose and UDP significantly attenuated the contraction evoked by UDP-glucose (***P < 0.001, two-way ANOVA, UDP-glucose contraction in the absence or presence of UDP-glucose or UDP, F=63.11, 56.48; n=10-13). Data are presented as mean \pm SEM.

Figure 2. Effect of PPADS (10 μ M) and suramin (100 μ M) on responses to (A) UDP-glucose, (B) UDP, and (C) MRS2690 in U46619-precontracted porcine pancreatic arteries. (A) Suramin and PPADS enhanced the effects of UDP-glucose (***P < 0.001, two-way ANOVA, UDP-glucose with suramin or PPADS vs UDP-glucose alone, F=19.85, 23.07; n=6-10). (B) Suramin and PPADS enhanced the effects of UDP (***P < 0.001, two-way ANOVA, UDP with suramin or PPADS vs UDP alone, F=16.83, 45.24; n=8-16). (C) Suramin and PPADS had no effect on the contraction to MRS2690 (n=5-9). Data are presented as mean \pm SEM.

Figure 3. Effect of PPTN (1 μ M), a P2Y₁₄ receptor antagonist, on responses to (A) UDP-glucose, (B) MRS2690, (C) UTP in U46619-precontracted porcine pancreatic arteries. (A) PPTN inhibited the effect of UDP-glucose (*P < 0.05, two-way ANOVA, F=6.56; n=7). (B) PPTN inhibited the effect of MRS2690 (**P < 0.01, two-way ANOVA, F=12.85; n=9). (C) PPTN had no effect on the response to UTP (n=8-10). Data are presented as mean \pm SEM. (D) Typical traces showing the effect of MRS2690 in the absence and in the presence of PPTN.

Figure 4. Effect of removal of the endothelium on responses to (A) UDP-glucose, (B) UDP, (C) MRS2690, in U46619-precontracted porcine pancreatic arteries. The removal of endothelium reduced the contractions evoked by (A) UDP-glucose, (B) UDP, (C) MRS2690 (**P < .01, ***P < 0.001, two-way ANOVA, F=8.15, 51.24, 9.48; n=4-15). Data are presented as mean \pm SEM.

Figure 5. Effect of DUP 697 (3 μ M) on responses to (A) UDP-glucose, (B) UDP, (C) MRS2690 in U46619-precontracted porcine pancreatic arteries. DUP 697 inhibited the contractions evoked by (A) UDP-glucose, (B) UDP, (C) MRS2690 (** P < 0.01, *** P < 0.001, two-way ANOVA, F=7.85, 35.31, 4.95; n=4-15). Data are presented as mean \pm SEM.

Figure 6. The contraction evoked by UDP-glucose in the presence of (A) NDGA (10 μ M), (B) BQ123 (1 μ M), (C) zafirlukast (10 μ M) in U46619-precontracted porcine pancreatic arteries, (D) L-655,240 (1 μ M) in endothelin-1-precontracted porcine pancreatic arteries. (A), (C) NDGA and zafirlukast did not alter the response to UDP-glucose (n=7-10). (B), (D) BQ-123 and L-655,240 inhibited the response evoked by UDP-glucose (*P < 0.05, ***P < 0.001, two-way ANOVA, F=4.97, 19.03; n=9-14). Data are presented as mean \pm SEM.

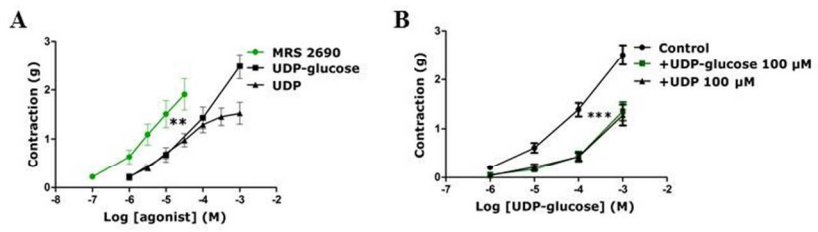
Figure 7. Effect of (A) nifedipine (1 μ M) and (B) thapsigargin (100 nM) on the response to UDP-glucose in U46619-precontracted porcine pancreatic arteries. Both inhibitors, nifedipine and thapsigargin, inhibited the contraction evoked by UDP-glucose (*P < 0.05, ***P < 0.001, two-way ANOVA, F=32.5, 5.84; n= 9-13). Data are presented as mean \pm SEM. (C) Typical traces showing the responses of UDP-glucose in the absence and in the presence of nifedipine.

Figure 8. Effect of Y-27632 (5 μ M), a selective inhibitor of the Rho-associated protein kinase on responses to (A) UDP, (B) MRS2690 in U46619-precontracted porcine pancreatic arteries. Y-27632 reduced the contraction to (A) UDP, (B) MRS2690 (***P < 0.001, two-way ANOVA, F=53.07, 10.32; n=9-15). Data are presented as mean \pm SEM. (C) MLC2 phosphorylation induced by 100 μ M UDP-glucose in porcine pancreatic arteries. A time-course (above) and a representative blot (below) of phospho- (green) and total- (red) MLC2 (*P < 0.05, one-way ANOVA, n=4).

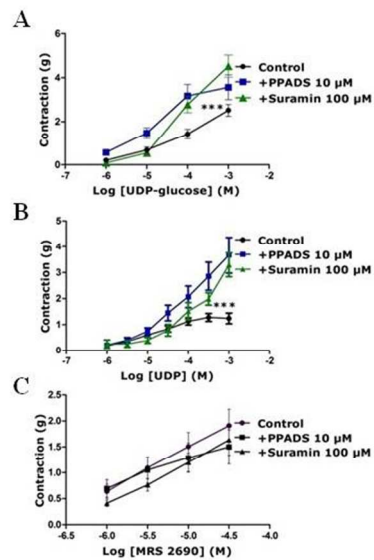
Figure 9. PPTN, a selective high affinity antagonist of P2Y₁₄ receptor, abolished the ability of UDP-glucose and MRS2690 to elevate the level of MLC phosphorylation (*P < 0.05, Student's unpaired *t*-test, n=3). Data are presented as mean ± SEM. Representative immunoblots of phospho- (green) and total- (red) MLC2 from three separate experiments in the absence or presence of PPTN.

Figure 10. Effect of precontraction with U46619 followed by relaxation with forskolin (1 μM) on the responses to (A) UDP-glucose, (B) UTP in porcine pancreatic arteries. (A) Exposing the tissues to U46619 followed by forskolin significantly enhanced the contraction evoked by UDP-glucose (***P < 0.001, two-way ANOVA, F=54.34; n=8-13). Data are presented as mean ± SEM. (B) Exposing the tissues to U46619 followed by forskolin failed to affect the response to UTP. (C) Typical traces showing the effect of UDP-glucose on basal tone (inset) and after the tissues were precontracted with U46619 and then relaxed with forskolin (main).

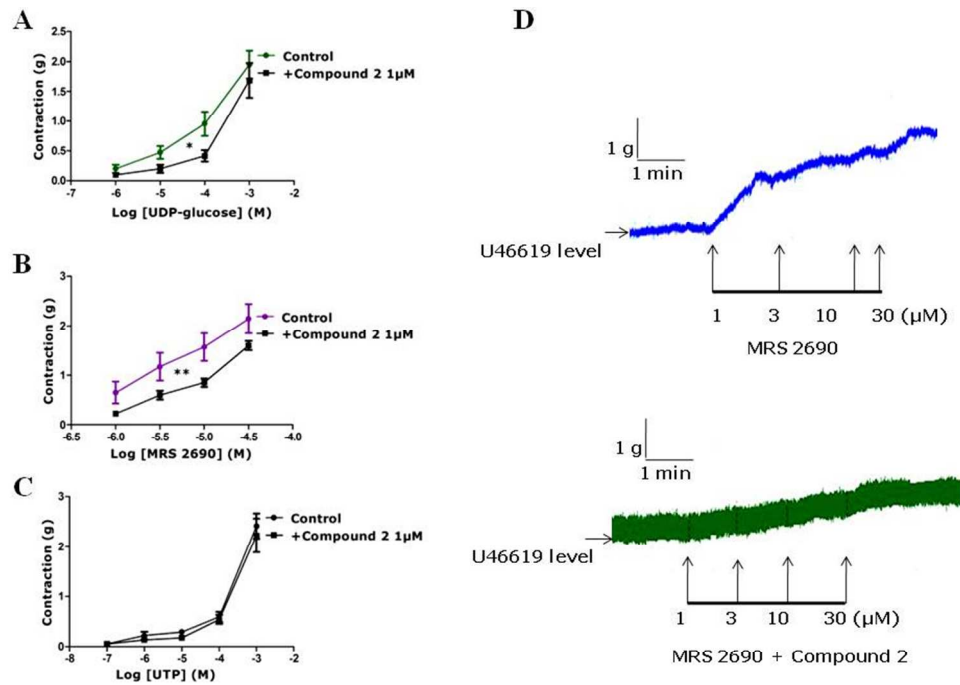
Figure 11. Effect of UTP (1 mM), UDP-glucose 1 (mM) and MRS2690 (10 μM) on the cAMP concentrations in porcine pancreatic arteries exposed to U46619 followed by forskolin. UTP had no significant effect on cAMP level, while UDP-glucose and MRS2690 reduced significantly the cAMP level (*P < 0.05, Student's unpaired *t*-test, the response to UTP or UDP-glucose or MRS2690 vs their respective controls, n=4). Basal cyclic AMP level represents the level of cAMP in the absence of forskolin which was also significantly different to that in the presence of forskolin (*P < 0.05, Student's unpaired *t*-test, n=4). Data are presented as mean ± SEM.



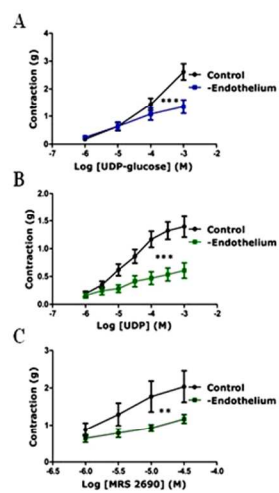
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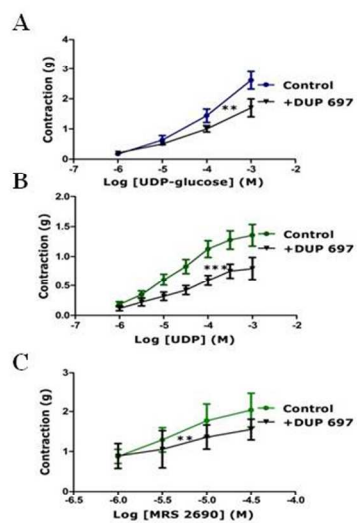
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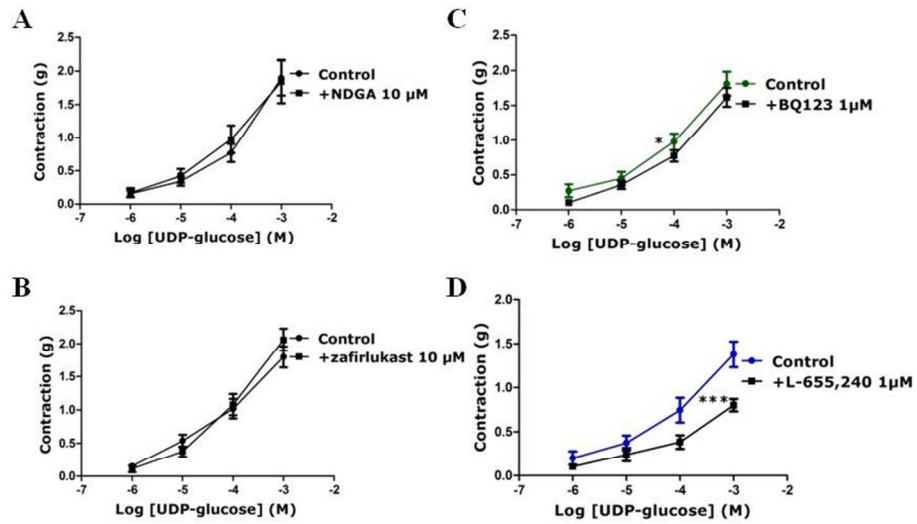
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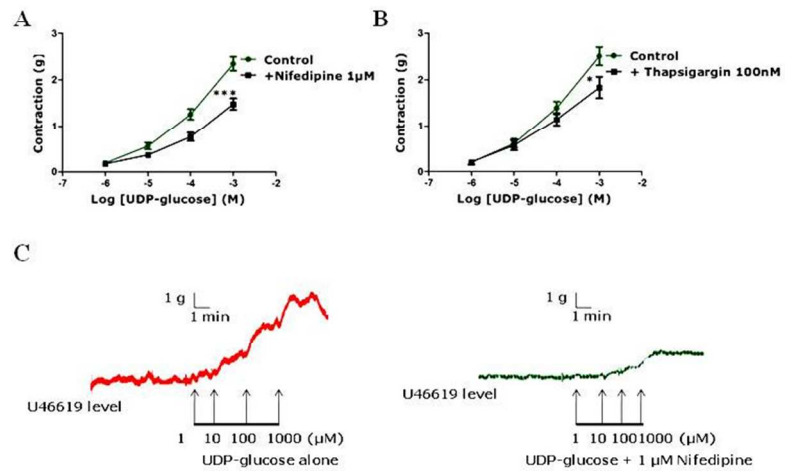
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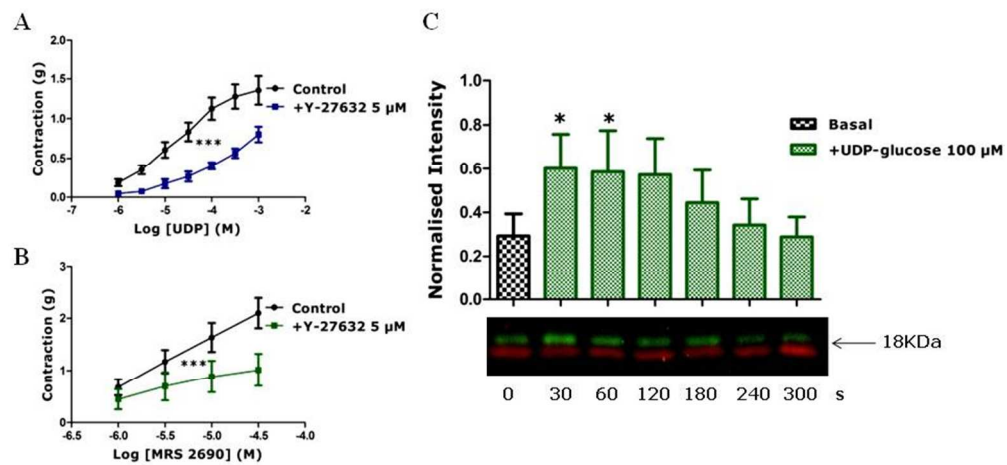
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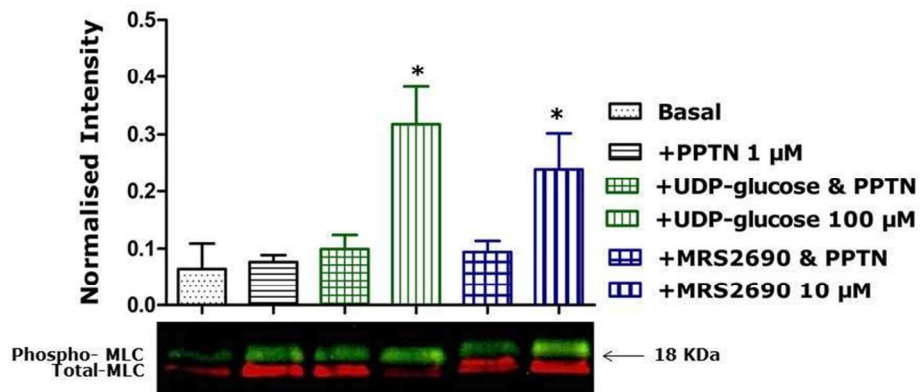
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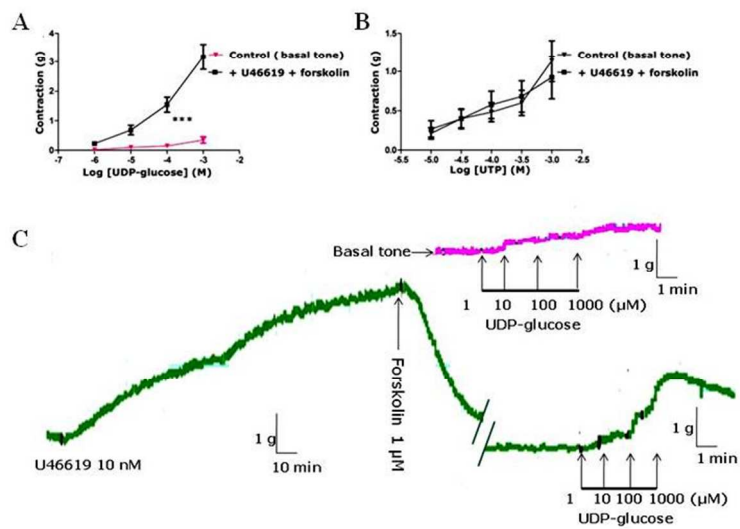


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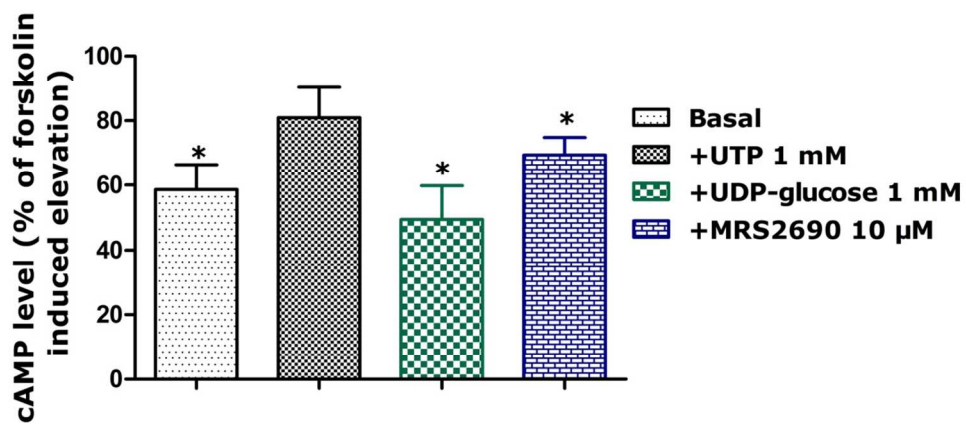


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Review



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Supplementary information for the manuscript**Effect of endothelium removal and BQ123, an ET_A receptor antagonist on contraction to UDP-glucose in porcine isolated pancreatic arteries**

The contraction to UDP-glucose was reduced in endothelium-denuded arteries, but BQ123 (1 μ M) did not induce any further reduction in endothelium-denuded arteries (Figure 1), although it reduced UDP-glucose contraction in endothelium-intact arteries. This showed that the contribution of endothelin-1, acting at ET_A receptors, in UDP-glucose induced contraction is solely endothelium-dependent.

Effect of BQ788, an ET_B receptor antagonist, on contraction to UDP-glucose in porcine isolated pancreatic arteries

To find out whether endothelin-1 (released as a result of UDP-glucose activating P2Y₁₄ receptors) acts at ET_B receptor, the response to UDP-glucose was examined in the presence of BQ788 (1 μ M). BQ788 failed to alter the contraction to UDP-glucose which showed that ET_B receptor is not involved in the contraction evoked by UDP-glucose (Figure 2).

Effect of MRS2578, a P2Y₆ receptor selective antagonist, on responses to UDP, UDP-glucose and MRS2690 in porcine isolated pancreatic arteries

Responses to UDP-glucose, UDP and MRS2690 were characterised using a P2Y₆ receptor selective antagonist MRS2578 (10 μ M). The contraction evoked by UDP was unaffected at lower concentrations but was augmented at higher concentrations of UDP (**P < 0.01, n=8-13) (Figure 3A), while MRS2578 did not alter the responses to UDP-glucose or MRS2690

(n=6-13) (Figure 3B, C). MRS2578 (10 μ M) partly blocked contraction to UTP in porcine coronary arteries, confirming its effect in porcine vessels at this concentration (data not shown).

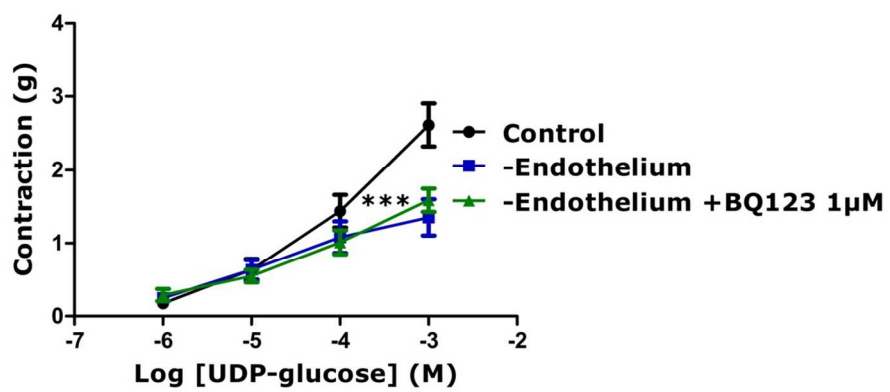
Figure Legends

Figure 1. Effect of BQ123 (1 μ M) in endothelium-denuded arteries and the effect of endothelium removal on responses to UDP-glucose in U46619-precontracted porcine pancreatic arteries. BQ123 in endothelium-denuded arteries did not induce further reduction of the contraction to UDP-glucose (**P < 0.001, two-way ANOVA, F= 8.149, 9.294; n=14). Data are presented as mean \pm SEM.

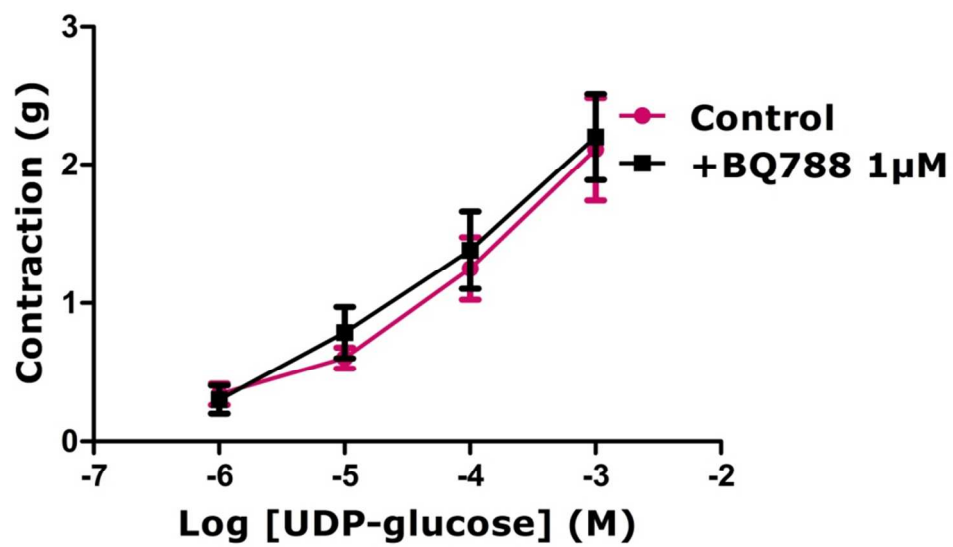
Figure 2. Effect of BQ788 (1 μ M) on response to UDP-glucose in U46619-precontracted porcine pancreatic arteries. BQ788 had no effect on the contraction to UDP-glucose (n=7-8). Data are presented as mean \pm SEM.

Figure 3. Effect of MRS2578 (10 μ M) on responses to UDP, UDP-glucose and MRS2690 in U46619-precontracted porcine pancreatic arteries. (A) MRS2578 enhanced significantly the contraction evoked by UDP (**P < 0.01, two-way ANOVA, F= 9.953; n=8-13). (B), (C) MRS2578 failed to alter the contraction to UDP-glucose and MRS2690 (n=6-13). Data are presented as mean \pm SEM.

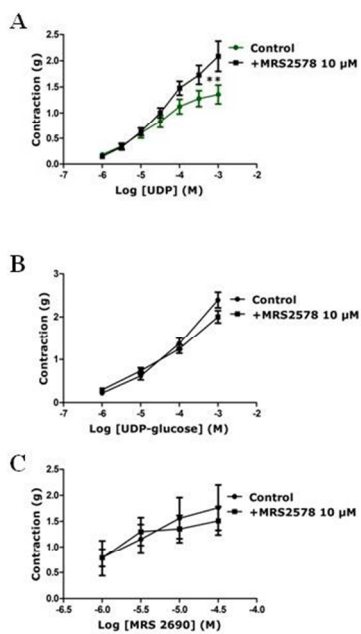
Figure 4. Concentration-dependent contraction evoked by UDP-glucose, in porcine pancreatic arteries in the presence and absence of PPTN (1 μ M) (**P < 0.001, two-way ANOVA, F= 24.37; n=12).



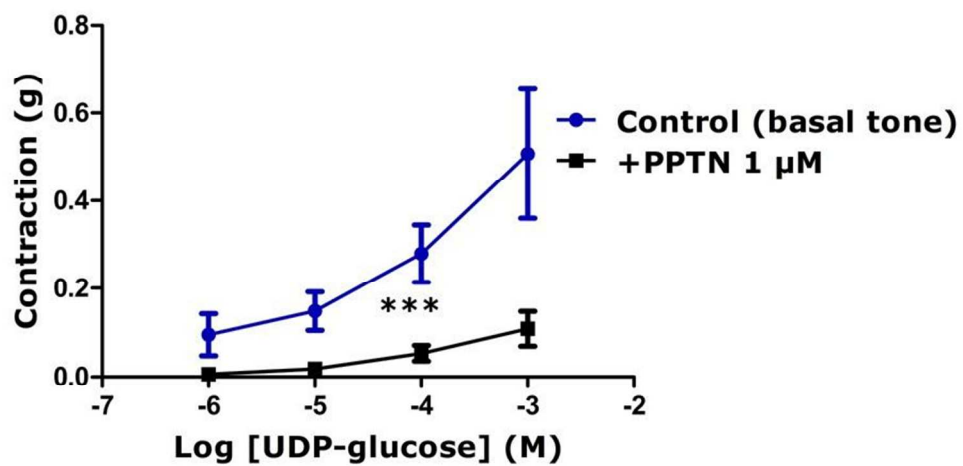
159x87mm (220 x 220 DPI)



121x73mm (220 x 220 DPI)



254x190mm (96 x 96 DPI)



108x55mm (220 x 220 DPI)

Editor

We are grateful to the Editor and Reviewers for constructive comments on the manuscript. We have addressed all of the comments through further experimentation and through changes to the manuscript as detailed below.

In view of these comments, we have carried out a number of major changes to the manuscript that we believe further improve the robustness of the data. In addition we have now added data showing the effect of UDP-glucose and MRS2690 on MLC2 phosphorylation in the presence and absence of PPTN, a selective high affinity antagonist of the P2Y₁₄ receptor, to confirm that the ability of UDP-glucose and MRS2690 to elevate level of phosphorylated MLC2 occurred through P2Y₁₄ receptors (Figure 9). As recommended by both Reviewers the immunostaining plus the western blotting experiments have been removed from the manuscript as well as from the supplementary information due to the lack of a negative control for the P2Y₁₄ receptor (despite our best attempts to identify one); further details are below.

Comments to the Author (REQUIRED):

The authors answered most queries raised in a convincing fashion. The two expert referees however, point out that some valid concerns were not fully addressed in the revised version of the manuscript.

1- The authors' answer as to why a Thromboxane analog was administered conjointly with the contractile UDP-Glucose (Methods page 22 of 62) is not fully satisfactory since UDP glucose alone is a vasoconstrictor of the vessel studied. Additional experiments should be performed only with the UDP-glucose as a contractile agonist.

Further experiments were conducted to study the contraction to UDP-glucose at basal tone (by its own). As seen in Figure 1, UDP-glucose induced a contraction at basal tone, which was significantly inhibited in the presence of PPTN, a selective high affinity antagonist of P2Y₁₄ receptor. These data are clearly very similar to the data obtained at raised tone (although at raised tone we can see any potential vasorelaxant responses and the contractile responses are greater). We have included a statement to this effect in the revised manuscript (Page 13), and the figure has been added to the Supplementary information (Figure 4).

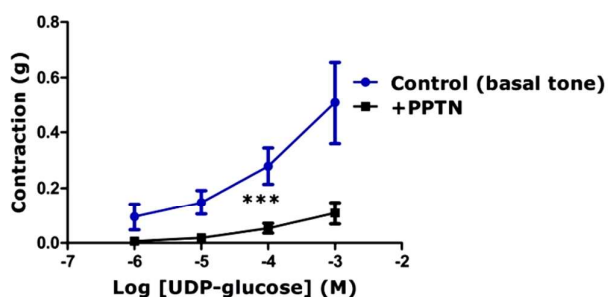


Figure 1. Concentration-dependent contraction evoked by UDP-glucose, in porcine pancreatic arteries in the presence and absence of PPTN (1 μ M) (***) $P < 0.001$, two-way ANOVA, $F = 24.37$; $n = 12$).

2- The second major concern is linked to lack of experimental evidences for the specificity characteristics of the P2Y₁₄ receptor antibody used. Both reviewers expressed valid concerns on that subject and it is suggested to the authors to provide additional data on that specific issue.

We have carried out a number of experiments to investigate the specificity characteristics of the P2Y₁₄ receptor antibody used in the western blotting and immunostaining experiments. However, the outcomes were not very satisfactory and we were unable to obtain a cell or tissue which could act as a negative control in our study. We used HL60 (Human promyelocytic leukemia cells), SHSY5Y (human neuroblastoma cell line), SK-N-MC (Human Brain Neuroepithelioma Cells) and mouse thoracic aorta; all of these cells/tissues were reported not to express the mRNA of the P2Y₁₄ receptor or to express it only at a low level. However, all of these cells showed bands, when the existence of P2Y₁₄ receptor (at protein level) was investigated using immunoblotting experiments. Therefore, the western blotting experiments and the immunostaining experiments have been removed from the manuscript and from the supplementary information.

3-Finally, as pointed out by one of the reviewers, '...all molecules need to be indicated by their correct chemical names...' on their first appearance in the text and subsequently replaced by the appropriate acronyms throughout the manuscript (i.e. Compound 2).

We have revised the nomenclature of the receptors and drugs to conform to BJP's Guide to Receptors and Channels. All correct chemical names have now been added in the text on their first appearance. Compound 2 has now been replaced by its synonym (PPTN) (Barrett MO, Sesma JI, Ball CB, Jayasekara PS, Jacobson KA, Lazarowski ER, *et al.* (2013). Human Neutrophils. *Molecular pharmacology*; <http://www.ncbi.nlm.nih.gov/pubmed/23592514>.

Reviewers' Comments to Author:

Reviewer: 2

Comments to the Author

BJP – Novel vasocontractile role of the P2Y₁₄ receptor: characterisation of its signalling in porcine isolated pancreatic arteries.

A great effort to respond the critiques and to ameliorate the paper was done. Few more comments are appended below:

1. Although the authors have tempered their data interpretation about the Abs this is not sufficient. The authors should carry out experiments with positive control proteins from both human and porcine expressing tissues, or recombinant proteins etc. together with negative controls. If the authors cannot provide this information they cannot present these data in the paper, this includes also the supplementary data. Either show the controls to convince about the specificity of the Abs, (the ones presented in Fig 11 are not sufficient unless you would already know the level of expression in those tissues), or delete all those data.

We have carried out a number of experiments to investigate the specificity characteristics of the P2Y₁₄ receptor antibody used in the western blotting and immunostaining experiments. However, the outcomes were not very satisfactory and we were unable to obtain a cell or tissue which could act as a negative control in our study. We used HL60 (Human promyelocytic leukemia cells), SHSY5Y (human neuroblastoma cell line), SK-N-MC (Human Brain Neuroepithelioma Cells) and mouse thoracic aorta; all of these cells/tissues were reported not to express the mRNA of the P2Y₁₄ receptor or to express it only at a low level. However, all of these cells showed bands, when the existence of P2Y₁₄ receptor (at protein level) was investigated using immunoblotting experiments. Therefore, the western blotting experiments and the immunostaining experiments have been removed from the manuscript and from the supplementary information.

2. The description of Fig 1 B. is not clear and needs further details. How/When was added the “same” agonist etc.

We have now clarified the description of Figure 1B. The response to UDP-glucose (the control) was studied in the presence of UDP-glucose and UDP (both agonists at P2Y₁₄ receptors), to find out whether the response to UDP-glucose (the control) would desensitise in the presence of P2Y₁₄ receptor agonists. Both agonists (UDP-glucose or UDP) were added 10 min prior to the addition of U46619 (as stated in the method section page 8, and now in the legend) to investigate desensitisation of the contraction to UDP-glucose in the presence of P2Y₁₄ receptor agonists.

Reviewer: 1

Comments to the Author

The new data and modifications of the text have substantively improved this manuscript. Several concerns remain that should be addressed.

1. This reader remains concerned that the authors' insistence on calling the molecule compound 2 will introduce unnecessary confusion in the literature. New molecular identities in medicinal chemistry papers are by convention referred to by numbers beginning with 1 and in the paper by Robichaud et al (Bioorg and Med Chem Lett 21:4366-4368, 2011) there were 11 such derivatives (including one called compound 2) referred to by numbers. However, in the two preceding papers on P2Y₁₄ antagonists from this research group (Guay et al Biorg. Med. Chem Lett. 21:2832-2835, 2011 and Gauthier et al 21:2836-2839, 2011) there was also a “compound 2” which in fact referred to two entirely different molecules from the one that the authors in this submitted paper are calling compound 2. Why not unambiguously call it by its chemical name, which is 4-((piperidin-4-yl)-phenyl)-7-(4-trifluoromethyl)-phenyl)-2-naphthoic acid?

We have now changed the name of the P2Y₁₄ selective antagonist to PPTN according to the report of (Barrett MO, Sesma JI, Ball CB, Jayasekara PS, Jacobson KA, Lazarowski ER, *et al.* (2013). Human Neutrophils. *Molecular pharmacology*;

<http://www.ncbi.nlm.nih.gov/pubmed/23592514>, in which they used exactly the same compound/structure that we used in our study. In addition we have added the correct chemical name to the manuscript, page 6.

2. Page 19 bottom line. The information in parentheses adds confusion---Compound 2 is in fact a 4,7-disubstituted naphthoic acid derivative but this provides no information about which derivative this is---again the molecule needs to be indicated by its correct chemical name.

The correct structure is now stated in the manuscript according to the report of (Barrett MO, Sesma JI, Ball CB, Jayasekara PS, Jacobson KA, Lazarowski ER, *et al.* (2013). Human Neutrophils. *Molecular pharmacology*; <http://www.ncbi.nlm.nih.gov/pubmed/23592514>. The correct structure is 4-(4-(Piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid, and has now been added to page 6 of the manuscript.

3. The addition of new cyclic AMP data (Figure 10) greatly strengthens the mechanistic story developed here. However, basal cyclic AMP levels in the absence of added forskolin need to be added to the figure.

We have now added a column which represents the level of basal cyclic AMP in the absence of forskolin (Figure 11, Page 30) and explained it in the legend.

4. The authors' arguments that the antibody is detecting the P2Y₁₄ receptor are not convincing. That a signal is not seen in the absence of primary antibody tells nothing about whether this antibody detects natively expressed P2Y₁₄ receptor or not. The paper provides a very strong pharmacological argument for the existence of functional P2Y₁₄ receptors in porcine pancreatic artery contraction, but inclusion of the antibody data would in the opinion of this reader weaken the quality of this manuscript.

We have carried out a number of experiments to investigate the specificity characteristics of the P2Y₁₄ receptor antibody used in the western blotting and immunostaining experiments. However, the outcomes were not very satisfactory and we were unable to obtain a cell or tissue which could act as a negative control in our study. We used HL60 (Human promyelocytic leukemia cells), SHSY5Y (human neuroblastoma cell line), SK-N-MC (Human Brain Neuroepithelioma Cells) and mouse thoracic aorta; all of these cells/tissues were reported not to express the mRNA of the P2Y₁₄ receptor or to express it only at a low level. However, all of these cells showed bands, when the existence of P2Y₁₄ receptor (at protein level) was investigated using immunoblotting experiments. Therefore, the western blotting experiments and the immunostaining experiments have been removed from the manuscript and from the supplementary information.

5. The Schulte and Fredholm, 2003 citation (top of page 35) on adenosine receptors should be deleted.

Thank you. It has now been deleted.