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RESEARCH PAPER

Evidence for the Expression of Multiple Uracil Nucleotide-Stimulated P2 Receptors Coupled to Smooth Muscle Contraction in Porcine Isolated Arteries

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Background and purpose: The uracil nucleotides UDP and UTP have been reported to activate $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors to cause vasoconstriction. We have performed a comparative analysis of these receptors in endothelium-denuded smooth muscle from porcine isolated coronary and ear arteries, using pharmacological and molecular tools.
Experimental approach: Tissue segments were used to construct non-cumulative concentration response curves for UTP and UDP, in the absence and presence of the P2 receptor antagonists PPADS or suramin. RT-PCR and immunoblot analyses were employed to define gene expression and immunoreactivity for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors.
Key results: In the coronary artery, UTP-evoked contractile responses were reduced in the presence of suramin, but not PPADS, while the smaller responses to UDP were unaffected by either antagonist. In the ear artery, contractile responses to UDP were much smaller than those to UTP; responses to UTP were inhibited by both PPADS and suramin. RT-PCR suggested predominant expression of $P2Y_2$ receptors in the coronary artery, while $P2Y_4$ and $P2Y_6$ receptor gene expression appeared equivalent in both tissues. Immunoblot analyses provided evidence for $P2Y_6$ receptors in both tissues, with equivocal evidence of $P2Y_2$ and $P2Y_4$ receptor immunoreactivities.
Conclusions and implications: We conclude that UTP-evoked contraction of porcine coronary artery smooth muscle appears to be predominantly $P2Y_2$-mediated, while the ear artery appears to express a uracil nucleotide-sensitive P2 receptor(s) which fails to fit readily into the current classification.
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Keywords: P2 receptors; UTP; UDP; coronary artery; vasoconstriction; suramin; PPADS

Abbreviations: ARL67156, 6-*N,N*-diethyl-D-$\beta\gamma$-dibromomethyleneATP; hprt, hypoxanthine phosphoribosyltransferase; PCA, pig coronary artery; PEA, pig ear artery; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; TBS, Tris-buffered saline; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate

Introduction

P2 receptors have been divided on the basis of primary sequence and coupling characteristics into the 7-transmembrane, G-protein-coupled P2Y and transmitter-gated channel P2X receptors (Burnstock and Kennedy, 1985; Ralevic and Burnstock, 1998; Alexander *et al.*, 2006). Both of these families of P2 receptors are found in the vasculature, where classically, P2X receptors are present on the smooth muscle and mediate contractile responses, while P2Y receptors may be on both endothelium and smooth muscle, mediating relaxant and contractile responses, respectively (Boarder and Hourani, 1998; Ralevic and Burnstock, 1998; Burnstock and Williams, 2000). Pharmacological subdivision of P2 receptors is limited, with the activity of the endogenous and synthetic nucleotides being the major means of identification of subtypes (Ralevic and Burnstock, 1998; Alexander *et al.*, 2006; von Kügelgen, 2006). The uracil nucleotides uridine 5′-triphosphate (UTP) and uridine 5′-diphosphate (UDP) are ligands for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, with little activity at P2X receptors or other subtypes of P2Y receptor (Ralevic and Burnstock, 1998; von Kügelgen, 2006). Analysis of recombinant $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors allows a pharmacological profile of uracil nucleotide activity to be generated (Table 1). Thus, UTP is up to two orders of magnitude more potent than UDP at $P2Y_2$ and $P2Y_4$ receptors,

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Table 1 Comparison of uracil nucleotide-activated P2 receptors in heterologous expression

	Agonist rank order (UTP/UDP ratio)	*PPADS (log concentration/inhibition)*	*Suramin (log concentration/inhibition)*
Recombinant P2Y2 receptors			
$hP2Y_2$	UTP≫DP (>100)[a]; UTP≫UDP (>100)[b]	−4.3/0%[c]	−4.3/50%[c,d]
$rP2Y_2$	UTP≫UDP (100)[e]	−5.2/50%[f]	−4/75%[e]; −3/30%[f]
$cP2Y_2$	UTP≫UDP (10)[g]	—	—
$pP2Y_2$	UTP>UDP (3)[h] UDP acts as a partial agonist	−5/50%[h]	−5/50%[h]
Recombinant P2Y4 receptors			
$hP2Y_4$	UTP=UDP (1)[i]; UTP≫UDP (10)[d,i]; UTP≫UDP (100)[k]; UTP≫UDP (>100)[l]	−4/73%[j]	−4/0%[j]; −3.3/0%[d]
$mP2Y_4$	UTP≫UDP (>100)[m]	−4.6/50%[n]	−4/0%[n]
$rP2Y_4$	UTP>UDP (2); UTP≫UDP (100)[p]	−4.3/50%[n]; −4/0%; −3/0%[f]	−4/10%[o]; −3/50%[f]
Recombinant P2Y6 receptors			
$hP2Y_6$	UDP>UTP (0.05)[q]; UDP≫UTP (0.01)[b]	−4/69%[r]	−4/27%[r]
$mP2Y_6$	UDP>UTP (0.05)[m]	—	—
$rP2Y_6$	UDP≫UTP[s]	—	−4/20%[s]

[a](Erb *et al*., 1993); [b](Nicholas *et al*., 1996); [c](Charlton *et al*., 1996b); [d](Charlton *et al*., 1996a); [e](Chen *et al*., 1996); [f](Wildman *et al*., 2003); [g](Zambon *et al*., 2000); [h](Shen *et al*., 2004); [i](Communi *et al*., 1995); [j](Communi *et al*., 1996a); [k](Nguyen *et al*., 1995); [l](Nicholas *et al*., 1996); [m](Lazarowski *et al*., 2001); [n](Suarez-Huerta *et al*., 2001); [o](Bogdanov *et al*., 1998); [p](Webb *et al*., 1998); [q](Communi *et al*., 1996b); [r](Robaye *et al*., 1997); [s](Chang *et al*., 1995).
Data are literature reports of agonist potency orders expressed as a ratio of UTP/UDP, together with indication of the extent of inhibition of responses by PPADS and suramin at the indicated concentrations.

while the reverse is true at $P2Y_6$ receptors. This difference should, therefore, allow positive identification of $P2Y_6$ receptors.

On the other hand, differentiation of $P2Y_2$ and $P2Y_4$ receptors is less straightforward. The use of antagonists to discriminate these receptors appears to be complicated by species variation in sensitivity to one of the most widely-applied antagonists, pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS, Table 1). Potentially of more use, though is another widely employed antagonist, suramin, which is effective at concentrations up to 100 μM as an antagonist of $P2Y_2$ receptors, without significant effect at $P2Y_4$ receptors (Table 1).

UDP is reported to be equipotent with UTP at constricting a range of blood vessels from different species, including the rabbit basilar artery (von Kügelgen and Starke, 1990), rat isolated pulmonary vasculature (Rubino and Burnstock, 1996) and intrapulmonary artery (Rubino *et al*., 1999; Chootip *et al*., 2002), rat mesenteric arteries (Malmsjö *et al*., 2000a), rat cerebral arteries (Horiuchi *et al*., 2001), mouse mesenteric arteries (Vial and Evans, 2002), mouse renal bed (Vonend *et al*., 2005), dog cerebral artery (Shirasawa *et al*., 1983), dog epicardial artery (Matsumoto *et al*., 1997) and human coronary arteries (Malmsjö *et al*., 2000b). In other tissues, such as the guinea-pig mesenteric vein (Mutafova-Yambolieva *et al*., 2000), UDP is 30-fold less potent than UTP. In the latter case, the interpretation would be that the guinea-pig mesenteric vein expresses $P2Y_2$ and/or $P2Y_4$ receptors, while the former list of tissues showing equipotent responses to uridine di- and triphosphates is much less straightforward to categorize in terms of P2Y receptor expression.

In the present report, we have examined porcine isolated coronary and ear artery preparations, assessing contractile responses to uracil nucleotides and P2Y receptor expression. A preliminary account of some of these data has previously been presented to the British Pharmacological Society (Rayment *et al*., 2004).

Materials and methods

Tissue preparation

Porcine material was prepared for analysis essentially as described previously (Blaylock and Wilson, 1991; Ting *et al*., 2000). Briefly, ears and hearts from Modern Hybrid White pigs (either sex, less than 6-month-old and weighing approximately 50 kg) were removed and rapidly transported at 4°C. Gross dissections of the arteries were conducted upon arrival in the laboratory before overnight storage at 4°C in gassed (95% O_2, 5% CO_2) Krebs'-Henseleit solution containing 2% Ficoll (type 70000). After allowing time for equilibration to room temperature, a fine dissection was conducted to prepare tissue rings of 2–4 mm in length. Rings were denuded of endothelium by gentle rolling of the segment on a tissue paper pad after insertion of the tip of a probe into the lumen.

Contractile responses in porcine isolated vessels

Arterial rings were transferred to a tissue bath (10 ml) containing warm (37°C), oxygenated Krebs'-Henseleit solution, mounted onto wires and connected to isometric force transducers (Grass FT03). Rings were put under tension (10 and 2 g for coronary and ear arteries, respectively) and allowed to equilibrate for 30 min. Coronary artery segments were allowed to equilibrate for a further 30 min before assessing viability with two challenges of 60 mM KCl (allowing 30 min for tissue to recover between each challenge). Ear artery segments were re-tensioned to 2 g and given a further 30 min to equilibrate before KCl challenge, as for the coronary arteries. Non-cumulative concentration–response

curves were constructed, in four to eight separate preparations of both tissues, in the presence of increasing concentrations of agonist. At appropriate times, agonists were washed out using warm oxygenated Krebs'-Henseleit solution and tissue was allowed to recover for 30 min before the addition of further agonist to ensure minimal tissue desensitization. Where the effects of antagonists were examined, parallel experiments were conducted where tissue was exposed to antagonist 30 min before agonist addition.

RT-PCR and sequence analysis of porcine $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors

Total cellular RNA was isolated from endothelium-denuded vascular tissue following the Rneasy Mini protocol for heart, muscle and skin tissue (Qiagen, Crawley, UK). After elution from the column, RNA was incubated with 25 Kunitz units DNase (Qiagen) for 30 min at 37°C to remove any contaminating DNA. The RNA was further purified using the RNeasy Mini cleanup protocol (Qiagen), before conversion to cDNA using Expand RT enzyme. For each sample, control reactions were conducted to ensure that the RNA isolated was free from genomic DNA contamination. Only samples that showed no evidence of genomic DNA contamination in the reverse transcription control were used in subsequent analysis. Polymerase chain reaction (PCR) was performed using primers specific for hypoxanthine phosphoribosyltransferase (*hprt*), *p2ry2*, *p2ry4* and *p2ry6* using an Eppendorf gradient thermocycler. All primers were designed in-house and synthesized by Eurogentec (Southampton, UK). Primers for *hprt* were designed based on available sequences (Table 2). As sequence data were not available for the porcine $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptor genes, primers were initially based on sequence homology between published sequences from man, cow, rat and mouse to allow sequencing and subsequent identification of pig specific primers (Table 2). cDNA was amplified using Hotstar Taq DNA polymerase (Qiagen) using variable reaction conditions (*hprt* 1.5 mM $MgCl_2$, annealing temperature 57°C; *p2ry2* 1.5 mM $MgCl_2$, annealing temperature 58°C; *p2ry4* 2 mM $MgCl_2$, annealing temperature 57°C; *p2ry6* 1.5 mM $MgCl_2$, annealing temperature 55°C, Q solution). PCR was initiated using the following profile: one cycle of 95°C for 15 min, 57.5±5°C for 2 min, 72°C for 2 min; variable cycles of 94°C for 1 min, 57.5±5°C for 1 min and 72°C for 2 min; 1 cycle of 94°C for 1 min, 57.5±5°C for 1 min and 72°C for 7 min. Reverse transcriptase-polymerase chain reaction (RT-PCR) reaction products were fractionated through 1.5% agarose gels stained with Sybr green I, and visualized on the Chemi-doc system (Bio-Rad, Hemel, Hempstead, UK). 28 cycles of PCR were used to visualize *hprt* expression which was taken to indicate equivalent loading of tissue cDNA amounts.

Immunoblotting

Protein samples were prepared from tissue segments (from which the endothelium had been removed) by homogenization in lysis buffer (20 mM Tris, 1 mM EGTA, 320 mM sucrose, 0.1% Triton X-100, 1 mM sodium fluoride, 10 mM glycerol-2-phosphate, pH 7.6) containing protease inhibitors (Roche, Lewes, UK). Samples were diluted 1:1 in solubilization buffer (Bio-Rad) before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% acrylamide gels in 1× electrode buffer. Equal amounts of protein (5 μg) were loaded for all samples and gels electrophoresed for 50 min at 200 V. Proteins were transferred to nitrocellulose membranes at 4°C for 1 h at 100 V. Blots were blocked for 1 h in Tris-buffered saline (TBS)-Tween (0.1%) containing 5% milk before transfer and exposure to primary antibodies (diluted 1:1000 in blocking solution) and incubated overnight at 4°C. Specificity of the antibody was assessed using blocking peptides according to the manufacturer's instructions for these antibodies (incubating equal volumes of undiluted antibody and blocking peptide for 1 h before incubating with blots). Blots were washed three times in TBS-Tween for 5 min followed by three 15 min washes in TBS-Tween to remove excess primary antibody. After incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000 in blocking solution), the washing stage was repeated before visualization by chemiluminescent detection using enhanced chemiluminescence (ECL) reagent.

Data analysis

Unless specified otherwise, data reported are means±s.e.m. of results from four to eight separate preparations. Effects of antagonists on UTP-evoked responses were assessed for statistical significance by 2-way ANOVA with Bonferroni post-test, while effects on UDP responses were analysed by 1-way ANOVA with Dunnett's *post hoc* test (Prism, GraphPad, CA, USA). Evaluation of sequence data homology to known sequences was conducted using the ClustalW program.

Materials

Nucleotides were from Sigma Chemical Company (Poole, UK), while the antagonists, PPADS and suramin, were obtained from Tocris-Cookson (Bristol, UK). Expand RT enzyme was obtained from Roche (Lewes, UK). Antibodies directed against sequence-specific peptides from $P2Y_2$, $P2Y_4$

Table 2 Primers for *hprt*, *p2y2*, *p2y4* and *p2y6*, and predicted product length based on known sequence. Inter-primer sequences have been submitted to GenBank

Gene	*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*	*Length (bp)*	*GenBank accession number*
hprt	CTTTGCTGACCTGCTGGATT	TGCATTGTTTTGCCAGTGTC	210	AF143818
p2y2r	AACGCCTCCACCACGTACA	GCAGGTGAGGAACAGGATG	183	AY691681
p2y4r	ACCACTATGTGCACTTCAGCTC	GCAGTCAGCTTCCAACAGC	236	AY691682
p2y6r	CACCCACTACATGCCCTATG	AAAGGCAGGAAGCTGATGG	207	AY691683

and $P2Y_6$ receptors were from Alomone Labs/Caltag-Medsystems (Buckingham, UK), while HRP-conjugated goat anti-rabbit antibody was from DAKO/Cytomation (Ely, UK). Nitrocellulose membranes and ECL reagents were obtained from Bio-Rad (Hemel Hempstead, UK) and Amersham (Little Chalfont, UK), respectively.

Results

Uracil nucleotide-evoked contraction in vascular smooth muscle

UTP evoked a poorly maintained contractile response in endothelium-denuded segments from both coronary and ear arteries (data not shown). Therefore, non-cumulative additions of UTP and UDP at concentrations between 10 μM and 3 mM were assessed for contractile responses in these tissues, with at least 20 min between additions of agonist. Peak responses to UTP and UDP in the two arterial preparations were concentration-dependent (Figure 1), although responses to UDP in the ear artery were very small compared to UTP-evoked contractions (Figure 1d). Comparison of equieffective concentrations (i.e. concentrations of UDP or UTP which evoked a standard response) indicated an approximate threefold higher potency for UTP compared to UDP in the coronary artery. The reduced responsiveness to UDP in the ear artery made comparison difficult, although UTP was at least 100-fold more potent than UDP (Figure 1).

Responses to UTP in the coronary artery (Figure 1a) were reduced in the presence of suramin (100 μM), but not PPADS (10 μM). In the coronary artery, the effect of 100 μM suramin appeared to be non-competitive, with a reduction in the response to high concentrations of UTP (e.g. 3 mM), without altering responses at lower concentrations (e.g. 100 μM UTP). In the ear artery, both suramin and PPADS inhibited responses to UTP (Figure 1c, Table 3). Responses to 1 mM UDP were unaffected in either tissue in the presence of suramin or PPADS (Figure 1, Table 3).

In preliminary experiments in which the ecto-ATPase inhibitor ARL67156 (6-*N*,*N*-diethyl-D-$\beta\gamma$-dibromomethylene-ATP) at 100 μM (Crack *et al*., 1995) was incubated for 15 min before application of agonists, 1 mM UDP elicited contractile responses were unchanged in either the coronary (79 ± 26, $n=4$) or ear artery (71 ± 26% control, $n=5$). In contrast, ARL67156 evoked a significant enhancement of the response to 300 μM UTP in both coronary (288 ± 64, $n=4$, $P<0.01$) and ear artery (173 ± 29% control, $n=6$, $P<0.05$) segments (1-way ANOVA with *post hoc* Newman-Keuls multiple comparison test).

Expression of mRNA encoding uracil nucleotide-sensitive P2 receptors

Although $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor sequences have previously been identified from a variety of species including man, mouse and rat, no sequence data were available at the time of conducting these experiments for porcine versions of these receptors. PCR primers were, therefore, initially designed on the basis of similarities between species orthologues. PCR analysis and subsequent sequencing of the derived product allowed refinement of pig-specific

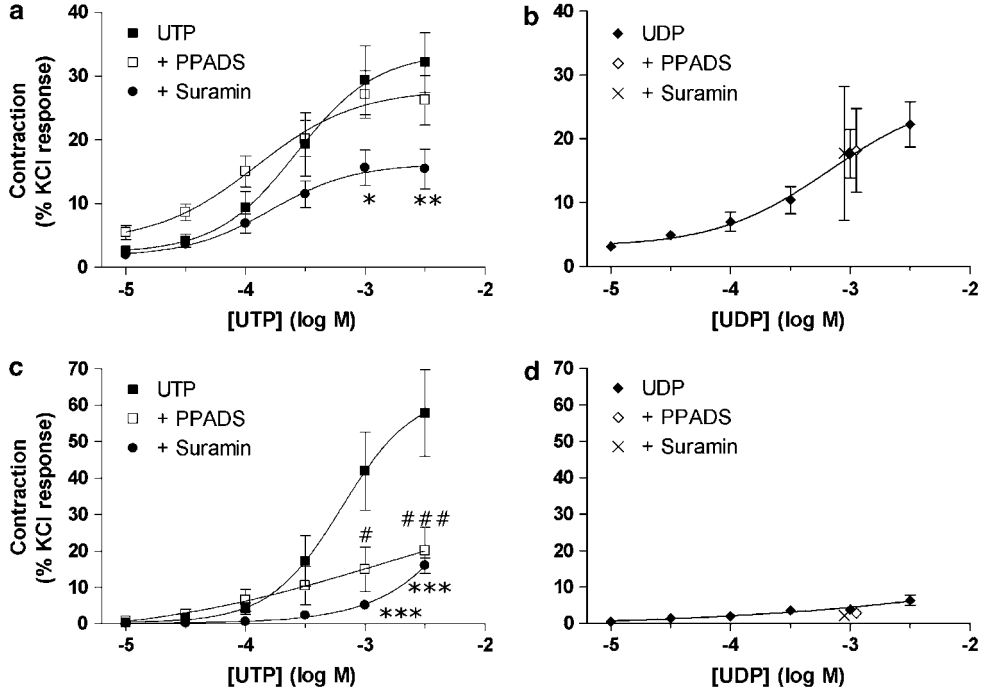

Figure 1 Contractile responses to uracil nucleotides in porcine isolated tissues. Responses to UTP (**a**, **c**) or UDP (**b**, **d**) were assessed in porcine isolated coronary (**a**, **b**) and ear (**c**, **d**) arteries in the absence and presence of PPADS (10 μM) or suramin (100 μM). Data are means$\pm$s.e.m. from 4–6 separate experiments, expressed as a percentage of the response to 60 mM KCl. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ UTP vs UTP + suramin, 2-way ANOVA; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ UTP vs UTP + PPADS, 2-way ANOVA. 1-way ANOVA of the UDP-induced responses indicated no significant difference in the presence of PPADS or suramin.

Table 3 Comparison of contractile responses in porcine isolated coronary and ear arteries to UTP and UDP in the absence and presence of PPADS and suramin

	UTP (responses at 3 mM, % KCl response)			*UDP (responses at 1 mM, % KCl response)*		
	Control	*PPADS (10 μM)*	*Suramin (100 μM)*	*Control*	*PPADS (10 μM)*	*Suramin (100 μM)*
Coronary artery	34±4	26±4	15±3**	18±4	18±10	18±7
Ear artery	58±12	20±6*	16±2**	4±1	3±1	2±1

Results were assessed for statistical significance by 1-way ANOVA with Dunnett's *post hoc* test (*$P<0.05$, **$P<0.01$).

primers which were applied in RT-PCR screening (Table 2). Sequencing of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor cDNA yielded fragments of 549, 493 and 418 bp, respectively, which have been lodged with GenBank.

The sequence obtained for the $P2Y_2$ receptor gene (AY691681) showed high levels of homology with published sequences. Human, Mongolian gerbil and dog sequences showed 89% homology to the pig sequence, while bovine, rat and mouse sequences were of a similar level of identity (84–86%). Sequence homology analysis of published $P2Y_4$ receptor gene sequences and pig sequences (AY691682), showed human sequences to have the highest homology (88%), while sequences from the cow (87%), rat, mouse and Mongolian gerbil (83%), and Chinese hamster (81%) also showed high homology. Cow and human sequences for the $P2Y_6$ receptor showed identical levels of homology to the pig sequence (AY691683, 90%), with mouse (85%), rat (84%) and chicken (69%) of lesser identity.

Agarose gel electrophoresis of the RT-PCR products for *p2y2r*, *p2y4r* and *p2y6r* using species-specific primers yielded products at the expected sizes in both pig coronary artery (PCA) and pig ear artery (PEA) (Table 2, Figure 2a–c). PCR products for all three genes were present in both coronary and ear arteries. Visual inspection suggested approximately equivalent expression of $P2Y_4$ and $P2Y_6$ receptor mRNA in the two tissues, while $P2Y_2$ receptor mRNA appeared much more abundant in the coronary artery (Figure 2a–c) than the ear artery. Cycle numbers (28–30) for visualization of $P2Y_2$ and $P2Y_6$ receptor mRNA were similar to those used for visualization of the housekeeping gene (*hprt*). Using the $P2Y_2$ receptor primers in porcine vascular tissue, it was apparent that visualization of product was optimal between 28 and 35 cycles, below which no product was observed, and above which product appeared to saturate (data not shown). A similar profile was observed for the $P2Y_6$ receptor primers. For the $P2Y_4$ receptor primers, optimization with genomic DNA allowed product identification at 35 cycles (data not shown). cDNA from vascular tissue failed to generate identifiable product at this iteration, but required an increased cycle number (40 cycles).

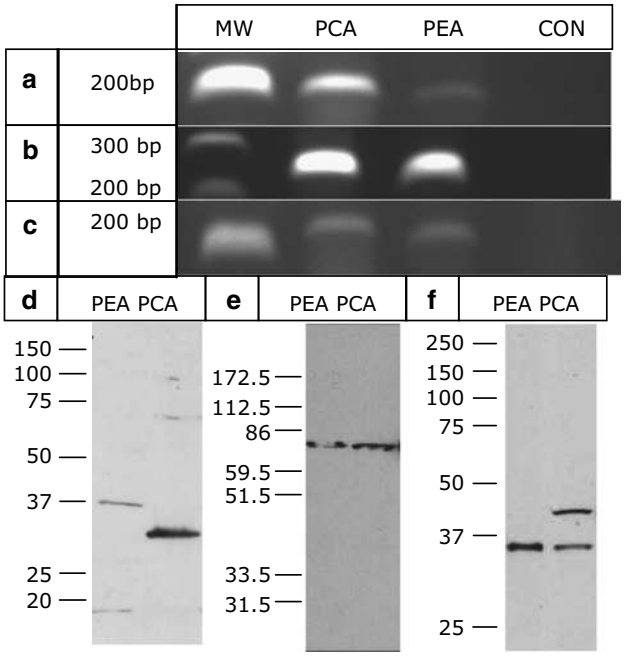

Figure 2 RT-PCR and immunoblot analysis of P2Y receptor expression in pig coronary and ear arteries. Agarose gel electrophoresis (**a–c**) showing size marker (MW) and PCR products from coronary (PCA) and ear (PEA) arteries or no template (CON) for *p2ry2* (**a**, 30 cycles), *p2ry4* (**b**, 40 cycles) and *p2ry6* (**c**, 28 cycles). Ladder markers are 200 bp (**a–c**) and 300 bp (**b**). Immunoblotting (**d–f**) was conducted using antibodies directed against $P2Y_2$ (D), $P2Y_4$ (**e**) and $P2Y_6$ (**f**) receptors.

Expression of P2Y receptor-like immunoreactivity

Immunoblotting using antibodies generated against peptide sequences selective for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors was undertaken using endothelium-denuded preparations from coronary and ear arteries (Figure 2d–f). Based on the human peptide sequences, immunoreactivity was anticipated at around 42.3, 41 and 36.4 kDa, respectively. In fact, the anti-$P2Y_2$ antibody yielded bands at approximately 32 and 36.5 kDa in coronary and ear artery samples (Figure 2d), respectively, which were lost after pre-incubation of the antibody with blocking peptide (data not shown). An additional band at 64 kDa was observed in PCA samples, which was also lost with blocking peptide exposure.

Immunoblotting of coronary and ear artery samples indicated a single band at 78–79 kDa using the $P2Y_4$ antibody (Figure 2e). This band was completely lost using the blocking peptide (data not shown). Densitometric analysis suggested equivalent levels of expression in the two tissues (data not shown).

The $P2Y_6$ sequence-specific antibody produced labelling of bands at 35 kDa (Figure 2f) in both arterial samples. There was an additional high molecular weight band in ear artery (42 kDa). Pre-incubation of antibody with blocking peptide greatly reduced (35 kDa) or eradicated (42 kDa) these bands (data not shown).

Discussion

In this report, we set out to define the expression of uracil nucleotide-sensitive receptors in the pig vasculature. We present evidence for expression of mRNA encoding $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in coronary and ear arteries from the pig, while immunoblotting supports the expression of $P2Y_6$ receptors in these tissues. Evidence from contractile studies suggests the presence of functional $P2Y_2$ receptors in the coronary artery, with a distinct uracil nucleotide-sensitive P2 receptor(s) in the ear artery, which fails to fit readily into the current classification.

Defining subtypes of uracil nucleotide-sensitive P2Y receptors

The interplay of P2 receptors in the regulation of vascular tone is complex. Given the widespread nature of nucleotide release, the number of P2 receptors and the influence of site of expression (endothelium vs smooth muscle), there is the possibility of multiple contractile and multiple relaxant receptors present in a particular vascular bed. This complexity is all the more challenging given the current paucity of selective ligands available to define the presence of individual subtypes. In the current study, we have used molecular and pharmacological approaches to attempt to define the presence of uracil nucleotide-sensitive P2 receptors in comparing a 'central' and 'peripheral' artery.

Although $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are often grouped as uracil nucleotide-sensitive P2Y receptors, there is differential sensitivity to endogenous agonists amongst the group (Table 1). Thus, the $P2Y_6$ receptor is often considered to be specific for UDP, although UTP fails to discriminate between $P2Y_2$ and $P2Y_4$ receptors (Table 1). As a means of defining the presence of particular receptors, agonist-evoked responses alone are far from ideal. In particular, the use of nucleotides is complicated by the potential for contamination of commercial samples and metabolic inter-conversion of extracellular nucleotides as well as the evoked release of endogenous nucleotides (Ralevic and Burnstock, 1998; von Kügelgen, 2006). A more useful approach to identify the presence of functional subtypes of uracil nucleotide-sensitive P2Y receptors is sensitivity to antagonists. However, since there are no commercially available selective antagonists for $P2Y_2$ or $P2Y_4$ receptors, we have used the widely employed antagonists suramin and PPADS, which, although generally considered as subtype non-selective, provide the potential for discriminating this pair of receptors (Table 1).

A further corollary of the use of these antagonists here is that it allows us to disregard the impact of the complications of the use of agonists mentioned above on our results. Thus, were nucleotide contamination an issue, then responses to the agonists would be much more similar in the two tissues (cf. UDP responses in the ear and coronary arteries, Figure 1b and d). Additionally, the distinct influence of suramin and PPADS as antagonists of the responses to UTP, but not UDP (Figure 1), suggests that contamination of the diphosphate with significant levels of the triphosphate is unlikely. This differential effect also militates against the possibility of metabolic inter-conversion of UTP and UDP. Furthermore, the selective effects of the ecto-ATPase inhibitor ARL67156 (Crack *et al*., 1995), which enhanced responses to UTP, but not UDP, in both ear and coronary arteries is further evidence for discounting a role for metabolic inter-conversion. Were metabolic inter-conversion of UDP to UTP an issue, then responses to UDP would also be expected to be enhanced, as well as those to UTP.

We conclude, therefore, that inter-conversion and contamination of UTP and UDP have no significant impact on contractile responses to these nucleotides in the porcine isolated coronary and ear arteries.

$P2Y_2$ and $P2Y_2$-like receptors

Suramin antagonizes recombinant $P2Y_2$ receptors at concentrations up to 100 μM, without affecting $P2Y_4$ or $P2Y_6$ receptors significantly (Table 1). On this evidence, therefore, one would propose that UTP-evoked responses in coronary and ear arteries (Table 3, Figure 1a and c) are mediated via $P2Y_2$ receptors. The picture is somewhat clouded, though, by consideration of the effects of PPADS. In recombinant systems, inhibition by PPADS appears to be unpredictable, with variation between species apparent for $P2Y_2$ receptors and apparently some wide ranges in sensitivity reported for the rat $P2Y_4$ receptor (Bogdanov *et al*., 1998; Suarez-Huerta *et al*., 2001; Wildman *et al*., 2003). In the present study, PPADS antagonized UTP-evoked responses in the ear artery, without altering the concentration–response curve in the coronary artery (Figure 1a and c).

We conclude that the pig coronary artery expresses $P2Y_2$ receptors coupled to smooth muscle contraction. This suggestion is in agreement with observations of $P2Y_2$-like receptors coupled to contractile events (including calcium mobilization) in preparations from human coronary artery (Strobaek *et al*., 1996; Malmsjö *et al*., 2000b; Hill and Sturek, 2002).

The evidence from RT-PCR supports the expression of $P2Y_2$ receptors in both coronary and ear arteries of the pig, with apparently greater levels of expression in the coronary artery (Figure 2a). The immunoblotting results are more equivocal, however, since no immunoreactivity at the anticipated size (42 kDa) was observed (Figure 2d). According to the published sequence of the pig $P2Y_2$ receptor (Shen *et al*., 2004), which extends beyond our partial sequence, the human peptide sequence against which the antibody is derived is 89% identical (16/18 amino acids) to the porcine orthologue. Conceivably, this may reduce the likelihood of the antibody recognizing the porcine orthologue, however, it is noteworthy that the same antibody detected bands of 32–36, 55 and 120 kDa in human nasal epithelia samples (Kim *et al*., 2004), which were also lost after pre-incubation with blocking peptide. In rat brain and human placental cytotrophoblasts, the same antibody detected 'specific' bands at 56 and 65 kDa, respectively (Roberts *et al*., 2006). Whether any of these bands really represent the $P2Y_2$ receptor awaits further investigation.

$P2Y_4$- and $P2Y_4$-like receptors

A similar situation exists for the molecular assessment of the $P2Y_4$ receptor, in that RT-PCR provides evidence for weak expression of encoding mRNA, while immunoblotting indicates a band at approximately 79 kDa, which was lost

on pre-incubation of antibody with blocking peptide (Figure 2b and e). The antigenic peptide against which the antibody is derived is from an area of the rat $P2Y_4$ receptor which is only 71% identical (17/24 amino acids) to the human sequence. Despite this low level of identity, the antibody recognizes common bands in rat and human brain, albeit at a molecular weight of approximately 91 kDa (Roberts *et al*., 2006). Using antibodies directed against the same epitope in studies of HT29 human colon cancer cells, 'specific' bands at 33 and 80 kDa have been reported (Delbro *et al*., 2005). An antibody described as being 'raised against the N-terminus of the receptor' was employed in studies of human vascular tissue to define 'specific' bands at identical sizes of 33 and 100 kDa (Wang *et al*., 2002). In gerbil tissues, bands of molecular weight 36, 55 and 75 kDa (brain) and 42 and 56 kDa (vestibular labyrinth) were reported using the same antibody as employed in the current study (Sage and Marcus, 2002). Although the low molecular weight bands are closer to the expected molecular weight of the human $P2Y_4$ peptide sequence at 41 kDa than many of the bands described in the literature and here (78–79 kDa), it has previously been postulated that the variation in molecular weight from that expected is due to post-translational modifications (Sage and Marcus, 2002; Delbro *et al*., 2005). It remains to be established by other means whether any of the bands observed truly represents the $P2Y_4$ receptor.

$P2Y_6$- and $P2Y_6$-like receptors

Given that UDP is a relatively selective agonist for $P2Y_6$ receptors, it was employed to determine the potential for expression of these receptors coupled to contraction of these two artery preparations. The relatively minor response to UDP in the ear artery (4% of the KCl response at 1 mM) suggests an absence of $P2Y_6$ receptors or that they are not coupled to smooth muscle contraction in this tissue. An alternative explanation is that the minor response may arise simply from contamination of the UDP with a modest proportion of UTP. UDP-evoked responses in the coronary artery, however, were more substantial (ca. 50% of the UTP response) and so could be mediated either by direct action at $P2Y_6$ receptors or as a partial agonist at $P2Y_2$ receptors (Table 1). If the action were mediated through $P2Y_2$ receptors, the responses to UDP would be expected to be inhibited by the same agents which inhibit UTP action. However, neither suramin nor PPADS altered UDP responses significantly (Table 3, Figure 2b). If $P2Y_6$ receptors were the target, the literature suggests that there might be some sensitivity expected to PPADS (Table 1), although this has not been extensively investigated. In human recombinant $P2Y_6$ receptors, 100 μM PPADS evoked a substantial reversal of the response to 2 μM UDP, a concentration approximately 10-fold the EC_{50} value (Robaye *et al*., 1997). At the same concentration of agonist, 100 μM suramin evoked a less marked inhibition. Whether the lack of sensitivity to PPADS and suramin in the present study derives from the high concentrations of UDP required to evoke a measurable contractile response is difficult to determine.

The use of relatively stable (not commercially available) analogues of the uracil nucleotides has previously allowed definition of a significant contribution of $P2Y_6$ receptors to contractile responses in human coronary arteries and rat basilar artery (Malmsjö *et al*., 2000b, 2003b).

RT-PCR and immunoblot analysis suggested the expression of $P2Y_6$ mRNA and immunoreactivity in both coronary and ear artery samples (Figure 2c and f). The antigen peptide used to generate this antibody is derived from the C-terminal of the rat $P2Y_6$ receptor, which is 83% identical to the human receptor (15/18 amino acids). Given that this antibody identifies a 'specific' band at 35 kDa in porcine tissues, which is similar to the size expected of the human $P2Y_6$ peptide sequence (36.4 kDa), it appears likely that the band is indeed the $P2Y_6$ receptor (Figure 2f). Other studies which assessed anti-$P2Y_6$ antibodies in immunoblots of human tissue, however, have identified bands at 45 kDa (Wang *et al*., 2002, 2003) or (using the same antibody as in the present study) 42 kDa (Kim *et al*., 2004). Whether the higher molecular weight band we observed to be expressed selectively in the ear artery (42 kDa, Figure 2f) corresponds to this entity awaits further routes of experimentation.

Uracil nucleotide-sensitive P2 receptor expression

The RT-PCR technique has been used to detect uracil nucleotide-sensitive P2Y receptors in vessels from a number of species. For example, reports describe expression of mRNA for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in human coronary artery (Malmsjö *et al*., 2000b), $P2Y_2$ and $P2Y_6$ receptors in human omental and cerebral arteries (Malmsjö *et al*., 2003a) and $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in human placental vasculature (Buvinic *et al*., 2006).

Rat aortic smooth muscle cells were observed to express mRNA encoding $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in culture (Pillois *et al*., 2002), while, in rat pial sheets, $P2Y_2$ and $P2Y_4$ gene expression was detected (Lewis *et al*., 2000). $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were detected in the rat basilar artery (Malmsjö *et al*., 2003b), and uracil nucleotides were observed to evoke contractile responses, although the effects of antagonists were not assessed. A further report, in which $P2X_1$ receptor-deficient mice were examined, indicated the expression of mRNA encoding $P2Y_2$ and $P2Y_6$, but not $P2Y_4$, receptors in the mesenteric artery, in which $P2Y_6$ receptor-induced contraction appeared to predominate (Vial and Evans, 2002).

Thus, it appears that expression of uracil nucleotide-sensitive P2 receptors is widespread both in terms of vessels examined as well as the species.

(Patho)physiological role(s) of uracil nucleotide-stimulated P2 receptors in vascular smooth muscle

$P2Y_2$ receptors have been suggested to be present primarily in the endothelium of coronary vessels, where a vasodilatory role is served (Gödecke *et al*., 1996). It appears likely that the contractile $P2Y_2$ receptors observed in the present study using endothelium-denuded coronary arteries are more likely to play a role in response to luminally released UTP when the endothelium is damaged. A potentially significant role might be in unstable angina, in which platelets (which contain high concentrations of releasable UTP) aggregate

at the site of a ruptured atherosclerotic plaque. The vasoconstriction resulting from $P2Y_2$ receptor activation might well contribute to the associated reduction in coronary blood flow.

Measurements of extracellular levels of UTP and UDP in dense tissue such as vascular smooth muscle are technically difficult and so the physiological significance of uracil nucleotide-responsive receptors in these tissues is unclear. Circulating levels of UTP have recently been described to be elevated in myocardial infarction (Wihlborg *et al*., 2006), presumably as a result of intra-coronary thrombosis, and it is tempting to speculate on a role for the contractile phenomena described here under those circumstances.

The molecular evidence obtained in the present study suggests that $P2Y_6$ receptors are indeed expressed in both tissues, although they appear silent in terms of contractile responses. An alternative function for $P2Y_6$ receptors in these tissues could be as mediators of mitogenesis, as has been suggested in rat aortic smooth muscle cells in culture (Hou *et al*., 2002).

In summary, therefore, we present evidence for expression of $P2Y_2$ receptors in the coronary artery of the pig, associated with smooth muscle contraction. UTP also evoked concentration-dependent contractions in the pig isolated ear artery, although examination of antagonist sensitivity of these responses failed to allow identification of the receptor(s) involved. It is attractive to hypothesize that $P2Y_2$ receptors in the coronary artery may be potential targets in the treatment of unstable angina.

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Conflict of interest

The authors state no conflict of interest.

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