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Cloning, Upregulation, and Mitogenic Role of Porcine P2Y$_2$ Receptor in Coronary Artery Smooth Muscle Cells

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Non-standard abbreviations: VSMC, vascular smooth muscle cells; CSMC, coronary artery smooth muscle cells; pP2Y$_2$, porcine P2Y$_2$ receptor; 1321N1 cells, human 1321N1 astrocytoma cells; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; LPA, lysophosphatidic acid; CCh, carbachol hydrochloride; PCNA, proliferating cell nucleus antigen; OGN, oligonucleotides; 2-MeS-ATP, 2-methylthio-ATP

ABSTRACT

Previous work has shown upregulation of a UTP-sensitive P2Y receptor in porcine coronary smooth muscle cells (CSMC) of organ-cultured arteries. However, the molecular identity and functional role of this putative receptor remained undefined. Here, we report the cloning of the cDNA for this receptor that encodes an open reading frame for a protein of 373 amino acids with highest homology to the human $P2Y_2$ receptor (84%). Heterologous expression of this receptor in 1321N1 cells revealed a novel pharmacology in that UTP and ITP were full agonists and UTP was more potent and efficacious than ATP for increasing intracellular $[Ca^{2+}]$ and ERK phosphorylation. Stimulation of sub-cultured CSMC with UTP, ITP or ATP induced a concentration-dependent increase in cellular DNA content, protein synthesis, cell number, and PCNA expression, indicating a mitogenic role for $P2Y_2$ receptors. This was supported by the finding that treatment of CSMC with anti-sense oligonucleotides to the cloned cDNA sequence significantly inhibited UTP- and ATP-induced DNA and protein synthesis. In addition, RT-PCR analysis showed that $P2Y_2$ receptor mRNA was dramatically increased in cells of organ-cultured arteries as compared with freshly harvested arteries, whereas the $P2Y_6$ receptor mRNA level was unchanged and $P2Y_4$ receptor mRNA was undetectable. This $P2Y_2$ subtype-specific upregulation was confirmed in cells of coronary arteries stented *in vivo*. In conclusion, we have cloned the porcine $P2Y_2$ receptor with novel pharmacology and demonstrated that this receptor is upregulated in CSMC of *in vitro* organ cultures or *in vivo* stented coronary arteries to mediate the mitogenic effects of nucleotides.

Introduction

The acute effects of extracellular nucleotides in the regulation of vascular tone have been well documented. Specifically, ATP, ADP, UTP and UDP can induce endothelium-dependent vasorelaxation via P2Y receptor-mediated NO, EDHF, or prostacyclin production (Wihlborg et al., 2003). Direct vasoconstriction in some blood vessels can also be triggered by these naturally occurring nucleotides via activation of P2X and/or P2Y receptors expressed in vascular smooth muscle cells (VSMC) (Matsumoto et al., 1997; Malmsjo et al., 2000a,b, 2003). In addition to these acute effects, recent studies have shown that extracellular nucleotides, in particular ATP and UTP, also have potent long-term effects on VSMC, i.e. proliferation (Erlinge, 1998; Burnstock, 2002) and migration (Pillois et al., 2002), both of which are important in the development of vascular diseases such as atherosclerosis and post-angioplasty restenosis.

It is generally thought that P2X receptors, being ligand-gated ion channels, are not involved in the chronic effects of ATP and UTP, suggesting a role for G protein-coupled P2Y receptors of which eight different subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) have been cloned and pharmacologically characterized (Dubyak, 2003; Abbracchio et al., 2003). Interestingly, it turns out that among the Gq-coupled P2Y receptors ($P2Y_{1, 2, 4, 6, 11}$), only $P2Y_2$ was upregulated in vascular injury models, including balloon-injured rat aorta (Seye et al., 1997) and collared rabbit carotid arteries (Seye et al., 2002). Studies on sub-cultured rat aortic VSMC have shown that ATP and UTP, agonists of $P2Y_2$ receptors, triggered cell proliferation (Erlinge et al., 1993, 1995; Malam-Souley et al., 1993, 1996). A similar mitogenic effect of ATP on sub-cultured porcine coronary artery smooth muscle cells (CSMC) has also been reported (Wilden et al., 1998). However, due to lack of specific antagonists for most of the P2Y receptors, including $P2Y_2$, the exact P2Y receptor subtype(s) responsible for the mitogenic effects of ATP and UTP, specifically in CSMC, has not been defined. Other complications are that P2Y receptor subtype expression in VSMC is highly heterogeneous and varies with species, type of vascular bed, and developmental state (Hill et al., 2001; Malmsjo et al., 2000a,b; 2003). Therefore, it is

critical to choose the appropriate animal model to address the pathological significance of the individual P2Y receptor in the development of relevant diseases. This view is supported by the unexpected finding that UTP, an agonist of the P2Y$_2$ receptor, is a potent anti-mitogen in cultured human VSMC derived from internal mammary artery and saphenous vein (White et al, 2000). Thus, it remains to be determined whether P2Y$_2$ receptor upregulation and contribution can be generalized to other vascular disease models, specifically coronary artery disease.

Previous work in our lab has shown that CSMC dispersed from freshly harvested porcine coronary arteries do not express a functional Gq-coupled uridine nucleotide P2Y receptor, as evidenced by the lack of intracellular Ca^{2+} mobilization in response to UTP (Hill et al., 2001, 2002). However, UTP triggered robust Ca^{2+} mobilization in dispersed CSMC from organ-cultured coronary arteries, indicating that a UTP-sensitive P2Y receptor(s) was upregulated under organ culture conditions (Hill et al., 2001, 2002). This notion was further supported by the pharmacological inhibition of UTP-induced Ca^{2+} mobilization by P2Y receptor antagonists, and inhibitors of G proteins and the SR Ca^{2+} pump (Hill et al., 2002). Therefore, we attempted to determine the molecular identity and the (patho)-physiological role(s) of this UTP-sensitive P2Y receptor. The identification of the mitogenic P2Y receptor that is upregulated in porcine CSMC of diseased coronary arteries is important, since these animals are excellent models for human cardiovascular disease (Johnson et al., 1999).

Thus, the aims of this study were: 1) to clone and characterize this porcine UTP-sensitive P2Y receptor and 2) to determine whether activation of this receptor is responsible for nucleotide-induced proliferation of CSMC. Data presented herein show that the cloned porcine receptor has extensive homology with the human P2Y$_2$ receptor but a different agonist potency profile in that the porcine P2Y$_2$ receptor is more sensitive to UTP than ATP with respect to nucleotide-induced increases in the intracellular [Ca^{2+}] and MAP kinase (ERK) phosphorylation. These relative agonist potencies are similar to the UTP-sensitive receptor previously characterized in organ-cultured porcine coronary arteries (Hill et al., 2001, 2002) and in

monolayer cultures of porcine CSMC (Seiler et al., 1999). Finally, a mitogenic role for the

cloned P2Y$_2$ receptor in porcine CSMC was confirmed by inhibition of mitogenesis with P2Y$_2$

receptor anti-sense oligonucleotide, suggesting that these receptors may play a role in the

development of coronary artery disease.

Materials and Methods

Isolation and culture of porcine CSMC. Porcine hearts were obtained from domestic farm or Yucatan pigs. Coronary arteries dissected from the heart and denuded of endothelium using aseptic techniques were placed in a physiological buffer, and smooth muscle cells were isolated enzymatically (Hill et al., 2001; 2002). Dispersed cells were recovered and sub-cultured at 37°C with 5% CO_2 in DMEM/high-glucose medium contained 10% fetal bovine serum (FBS). Smooth muscle cell lineage was confirmed by α-smooth muscle actin immunocytochemistry. Stock cell cultures were maintained in a sub-confluent state and used before passage 10 (Wilden et al., 1998).

PCR cloning and RACE. The cDNA for the porcine P2Y$_2$ receptor was cloned by a PCR cloning strategy. Total RNA was extracted from cultured CSMC using the RNeasy Total RNA Mini Kit (QIAGEN). For the synthesis of first strand cDNA, 1 μg of total RNA was reversed transcribed using a cDNA synthesis kit (CLONTECH). The cDNA was then amplified by PCR using 2.5 units *Taq* DNA polymerase (PROMEGA). The initial PCR reaction was performed with a pair of degenerate primers based on the conserved regions of human and rat P2Y$_2$ receptor sequences (forward: 5'-TGC/TCGCTTCAACGAGGACTTCAAGTA-3', reverse: 5'-AG/CGCCATGTTGATGGCGTTGAGGGT-3'). After sequencing several of the obtained PCR products of the expected size, new sets of primers specific to the putative porcine P2Y$_2$ receptor were synthesized and used to obtain the full-length cDNA.

To obtain the 3'-end of the coding sequence of the porcine P2Y$_2$ receptor cDNA, we used a SMART^{TM} RACE cDNA amplification kit (CLONTECH). In brief, total RNA was reverse transcribed using an oligo(dT)$_{20}$ primer with adaptor sequence. The first round of PCR was carried out with a porcine P2Y$_2$ receptor cDNA specific forward primer (5'-CGTCACCCGCACCCTCTACTACTCCTT-3'), and a reverse adaptor primer provided in the kit. The PCR product was diluted 1/1000 in DEPC-treated water and used as a template for a second round of PCR. The second round of PCR was performed as semi-nested PCR, in

which the same reverse primer was used with a different gene specific forward primer (5'-CCTGAGCTACGGCGTGGTGTGCGTG-3') downstream of the original primer. The PCR conditions were as follows: jump start for 1 min at 94°C, denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension at 72°C for 1 min, for 35 cycles.

To obtain the 5'-end of the coding sequence of the porcine $P2Y_2$ receptor cDNA, we designed a degenerate forward primer based on the one conserved sequence located in the 5'-untranslated region (5'-UTR) of human and rat $P2Y_2$ receptor cDNAs. The degenerate forward primer (5'-GGA/GACCTGTTT/CTTCCTGTTTCC-3') and a gene specific reverse primer (5'-CGGCACAGGAAGATGTAGAGCGCC-3') were used to amplify a new PCR product. After sequencing several products of the anticipated size, partial cloning of the 5'-UTR sequence was achieved. Then, a final set of primers (forward: 5'-GAGCATCCTGACAGCGAGAGCAG-3', reverse: 5'-CTACAGCCGGATGTCTTCGCCACG-3'), based on the 5'-UTR and 3'-UTR sequence flanking the open reading frame, was used for amplification of the full-length porcine $P2Y_2$ receptor cDNA. The PCR products resolved on an agarose gel were isolated using a QIAquick Gel Extraction Kit (QIAGEN), and the purified cDNA was ligated into the plasmid vector pCR3.1 (a modified form of pcDNA3.1; INVITROGEN). The ligation product was used to transform One Shot TOP10F' competent *E. coli* cells from the TA cloning kit (INVITROGEN). The cDNA inserts from at least three different clones were sequenced in both directions using T7 and BGH primers. DNA sequencing was performed by the DNA Core of the University of Missouri-Columbia.

Heterologous expression. The purified pCR3.1 plasmids harboring the coding sequence of the porcine $P2Y_2$ receptor (pCR3.1-pP2Y$_2$) were transfected into human 1321N1 astrocytoma cells in DMEM with 10% FBS, using Effectene Transfection Reagent (QIAGEN). Stable transfectants were obtained by selection in medium containing 0.5 mg/ml G418 (CALBIOCHEM) for ~2-3 weeks. The efficiency of transfection was determined by monitoring UTP-induced Ca^{2+} mobilization in transfected cells.

[^{3}H]-Thymidine incorporation. Incorporation of [^{3}H]-thymidine into DNA was carried out as previously described (Wilden et al., 1998). Cells were cultured as above in 12-well culture plates. Cells near confluence were treated with or without nucleotides or LPA for 24 h at 37°C in DMEM/high-glucose medium containing 0.5% FBS followed by the addition of 1 µCi of [*methyl*-^{3}H]-thymidine (NEN, Wilmington, DE) for an additional 24 h at 37°C. The cells were washed three times in ice-cold PBS and solubilized in 0.1% SDS. Trichloroacetic acid was added to a final concentration of 10%, and the precipitate was collected by filtration on glass-fiber disks for determination of radioactivity by liquid scintillation counting.

Protein synthesis. [^{3}H]-Leucine incorporation was used for measuring total cellular protein synthesis. The method is similar to that described in the *"[^{3}H]-thymidine incorporation"* assay with the exception that 1 µCi of [^{3}H]-leucine instead of [^{3}H]-thymidine was added to the medium in the presence or absence of UTP, ATP, UDP or LPA.

Colorimetric determination of cell number. Cultured CSMC were suspended by trypsinization, counted, replated in 96-well plates at a density of 2,000 cells/well in cell culture medium containing 10% FBS, and then serum-starved for 48 h. Agonists were added and were present for 4 days. By the end of cell stimulation, 20 µl of tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 100 µl of fresh DMEM were added for 4 h at 37°C, according to the instructions in the *MTT Cell Growth Assay Kit* (CHEMICON). MTT reduced to formazan by intracellular dehydrogenases was determined at 490 nm with an *ELISA* plate reader, and was directly proportional to the number of living cells.

Determination of p-ERK and PCNA by Western analysis. Cells were serum-deprived for 24 h (48 h for PCNA) before stimulation with agonists at the indicated concentration for 5 min (ERK assay), or 24 h (PCNA assay). Then, cells were washed with ice-cold phosphate-buffered saline, solubilized in Laemmli sample buffer containing 200 mM dithiothreitol, and boiled. Lysates were sonicated to disrupt DNA, and proteins were separated on 10% SDS-

PAGE gels. The proteins were electrophoretically transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 20% methanol, and 0.02% SDS. The nitrocellulose was blocked with 5% nonfat milk in 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Tween 20. The membranes were probed with the primary antibody anti-p-ERK1/2 (1:2000; CELL SIGNALING) or anti-PCNA (1:1000; CELL SIGNALING) overnight in 20 mM Tris, pH 7.4, 150 mM NaCl, 3% BSA, and 0.01% Tween 20. The blots were washed in 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Tween 20, and the bound antibody was detected by horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence (PIERCE). As a loading control, membranes were stripped of original antibodies, and re-probed with primary anti-actin antibody (1:1000; CYTOSKELETON).

Organ culture of coronary arteries and dispersion of smooth muscle cells. Pig hearts were isolated and prepared, as described (Hill et al., 2000; 2001; 2002). Right coronary arteries (distal end) were either cold-stored for 4 days at 5°C or organ cultured for 4 days at 37°C in a 95% O_2, 5% CO_2 incubator. Arterial segments were denuded of endothelium, opened longitudinally to expose the lumen, and placed in a 100-mm Petri dish containing 30 ml of RPMI 1640 (LIFE TECHNOLOGIES) without serum, which was changed every 2 days. CSMC were enzymatically isolated, as described (Hill et al., 2000; 2001; 2002), and were identified morphologically (Hill et al., 2000).

Porcine model of coronary artery stenting. Protocols conformed to Animal Care and Use guidelines of the University of Missouri-Columbia. A baseline coronary angiogram for the pigs was performed and the circumflex artery (CFX) was selected for implantation of an appropriately sized stent (3.0 mm in diameter and 13 mm long) centered on a 3.0x15 mm balloon (GUIDANT). To avoid over-inflation injury, a stent-to-artery ratio of 1:1 compared with the baseline segment diameter was assessed with angiography and intravascular ultrasound. The 10 mm of artery distal to the stenting site served as a control segment. After recovery for

3 weeks, pigs were anaesthetized and sacrificed. Isolation of coronary artery and dispersion of CSMC are described above.

Intracellular [Ca^{2+}] measurements. Intracellular free Ca^{2+} concentration ([Ca^{2+}]$_i$) in transfected 1321N1 cells and cultured CSMC was measured with the InCa^{++} calcium imaging system (INTRACELLULAR IMAGING INC.). Cells were grown on glass coverslips for two days and serum-starved for another 24 h and then used at about 40% confluence. After incubation with 2.5 μM fura 2-AM at 37°C for 30 min, the cells were superfused in a constant-flow superfusion chamber mounted on an inverted epifluorescence microscope (NIKON; model TMD) using physiological salt solution (PSS) containing (mM) NaCl 138, KCl 5, CaCl$_2$ 2, MgCl$_2$ 1, HEPES 10, glucose 10, pH 7.4, in the presence or absence of indicated concentrations of nucleotides, LPA or CCh. Fura-2 was excited at 340 nm and 380 nm, and emitted fluorescence (510 nm) was collected using a monochrome CCD camera (COHU). Data were collected as a ratio of emitted light intensity at 340 and 380 nm, and then converted to [Ca^{2+}]$_i$ based on a standard curve (Hill et al., 2000; 2001; 2002).

Semi-quantitative RT-PCR. Total RNA was isolated from cultured or dispersed cells and RT-PCR protocols were performed as described above in "*PCR cloning and RACE*". Sets of primers used for detecting and semi-quantifying mRNA for individual P2Y receptor subtypes were: porcine P2Y$_2$ receptor (Shen & Sturek, GenBank accession No. AY620400), forward: 5'-CGTCACCCGCACCCTCTACTACTCCTT-3' and reverse: 5'-CTACAGCCGGATGTCTTCGCC-ACG-3', yielding a 340 bp product; porcine P2Y$_6$ receptor (Shen & Sturek, GenBank accession No. AY620399), forward: 5'-CTGCATCAGCTTCCAGCGCTACCT-3' and reverse: 5'-GTCCAAGACGCTGTTGGCACTGGC-3', yielding a 538 bp product; porcine P2Y$_4$ receptor (Shen & Sturek, GenBank accession No. AY662405), forward: 5'-GTTTGATGAGGATTT-CAAGTTCATCC-3' and reverse: 5'-CAGACAGCAAAGACAGTCAGCAC-3', yielding a 686 bp product and human G3PDH (GenBank accession No. X01677), forward: 5'-

TGAAGGTCGGAGTCAACGGATTTGGT-3' and reverse: 5'-CATGTGGGCCATGAGGT-CCACCAC-3', yielding a 983 bp product. The PCR conditions were as described in "*PCR cloning and RACE*", except for the P2Y$_4$ receptor detection for which the annealing temperature was 56°C and 100 ng porcine genomic DNA was used as a positive control. The resulting PCR products were resolved on a 2% agarose ethidium bromide gel. The amplified bands were visualized with ultraviolet light and the relative densities of individual bands were normalized to G3PDH using a computer program (QUANTITY ONE).

Anti-sense oligonucleotide experiments. Phosphorothioate-modified oligonucleotides (OGN) designed according to the cDNA sequence of the porcine P2Y$_2$ receptor cloned in this study were: anti-sense OGN (5'-TCGGGGCCTGTAGCCATCAC-3') and sense OGN (5'-GTGATGGCTACAGGCCCCGA-3'). Sequence uniqueness was checked across multiple databases using BLAST. An optimized concentration of 3 μM sense or anti-sense OGN was added to the culture medium, 24 h before serum-starvation and again after serum-starvation. Cellular uptake of OGN was verified using FITC-conjugated OGN under the same experimental conditions. Nucleotide-induced Ca^{2+} mobilization was assayed to verify inhibition of porcine P2Y$_2$ receptor expression by anti-sense OGN.

Materials. Fura 2-AM was purchased from Molecular Probes (Eugene, OR). Cell culture media were from Invitrogen. Unless indicated, all other reagents and compounds were purchased from Sigma (St. Louis, MO). PCR primers and sense/anti-sense OGN were synthesized and purified by Integrated DNA Technologies (IDT). All of the nucleoside diphosphates were prepared in stock solutions (1 mM) that contained 50 U/ml hexokinase and 110 mM glucose to eliminate the potential contamination of the corresponding nucleoside triphosphate.

Analysis of data. Data are expressed as means $\pm$ S.E.M. All concentration-response curves were fitted by logistic (Hill equation), non-linear regression analysis using GraphPad

Prism 4.0 (San Diego, CA). Means of two groups were compared using Student's t-test (unpaired, two tailed), and one-way ANOVA was used for comparison of more than two groups with $p < 0.05$ considered to be statistically significant.

Results

Cloning of the porcine P2Y$_2$ receptor. Based on the conserved regions of coding sequences for human and rat P2Y$_2$ receptors, degenerate primers were constructed and used for initial RT-PCR experiments with total RNA isolated from either cultured CSMC or coronary artery denuded of endothelium. After sequencing PCR products of anticipated size, sets of new gene-specific forward and reverse primers were designed, which enabled the isolation of full-length porcine P2Y$_2$ receptor cDNA with an open reading frame of 1119 bp (GenBank accession No. AY620400). Hydrophobicity analysis showed a deduced primary sequence for the porcine P2Y$_2$ receptor of 373 amino acids containing seven hydrophobic domains characteristic of G protein-coupled receptors (Fig. 1). Computer sequence alignment revealed that the porcine P2Y$_2$ receptor had the highest amino acid identity with human P2Y$_2$ receptors (84%), and had 4 amino acids deleted at two different positions of the C-terminus (Fig. 1). In addition, several V to A/G transitions are notable and scattered in various extracellular loops and transmembrane regions. Furthermore, a putative N-glycosylation site and most of the putative phosphorylation sites for PKC, PKA and G protein-coupled receptor kinase (Zambon et al., 2000), are conserved (annotated in Fig. 1). Interestingly, an integrin-binding RGD motif found in the first extracellular loop of the human P2Y$_2$ receptor (Erb et al., 2001) is also conserved in the porcine P2Y$_2$ receptor. However, the porcine P2Y$_2$ receptor does not contain the "PXXP" SH3 binding domain, recently identified in the C-terminus of the human P2Y$_2$ receptor (Liu et al., 2004). RT-PCR analysis showed a wide tissue distribution for porcine P2Y$_2$ receptor mRNA, including heart, kidney, intestine, skeletal muscle, and adrenal glands (data not shown).

Stable expression and functional characterization of the cloned porcine P2Y$_2$ receptor. To determine whether the cloned cDNA sequence of the porcine P2Y$_2$ receptor encodes a functional receptor, a recombinant mammalian expression vector harboring the porcine P2Y$_2$ receptor cDNA (pCR3.1-pP2Y$_2$) was stably transfected into human 1321N1

astrocytoma cells devoid of any endogenous P2Y receptors (Parr et al., 1994). Fig. 2A shows

that 100 μM UTP or ATP increased $[Ca^{2+}]_i$ in 1321N1 cells expressing the porcine $P2Y_2$

receptor, whereas both nucleotides were inactive in mock-plasmid transfected cells. CCh, a

muscarinic M receptor agonist, triggered robust Ca^{2+} responses in the mock-tranfected cells.

We also tested the potential coupling of the cloned porcine $P2Y_2$ receptor to the Gs/Gi-adenylyl

cyclase-cAMP signaling pathway and found no significant change in intracellular cAMP levels

induced by UTP (data not shown), suggesting that the porcine $P2Y_2$ receptor is coupled

primarily to phospholipase C via the Gq protein.

The cloned porcine $P2Y_2$ receptor was activated by all the nucleoside triphosphates, albeit

with varying potencies (UTP > ITP > ATP > CTP ≈ TTP > GTP) and efficacies (UTP ≈ ITP ≈

CTP > ATP ≈ TTP > GTP), with ATP, TTP and GTP acting as partial agonists (Fig. 2B; Table

1). In contrast, most of the nucleoside diphosphates were inactive at 10 μM, with the exception

of UDP that was a partial agonist (Fig. 2C). Some synthetic nucleotides, including ATPγS and

GTPγS, were also active with potencies and efficacies comparable to ATP, whereas 2-MeS-

ATP and α,β-Me-ATP had no effect (Fig. 2D). We also investigated whether the cloned porcine

$P2Y_2$ receptor expressed in 1321N1 cells could activate ERK, a component of the MAP kinase

signaling pathway activated by many G protein-coupled receptors, including P2Y receptors (Liu

et al., 2004; Burnstock, 2002). In pP2Y$_2$-transfected 1321N1 cells, UTP induced robust

phosphorylation of ERK1/2 with an EC_{50} of ~10 nM, whereas ATP was a partial agonist with an

EC_{50} of ~100 nM. UDP also induced phosphorylation of ERK1/2 at concentrations ≥ 1 μM (Fig.

3). The non-specific P2Y receptor antagonists, suramin, RB-2 and PPADS at 10 μM, caused

50% inhibition of the UTP-induced increase in peak $[Ca^{2+}]_i$ in pP2Y$_2$-1321N1 cells (data not

shown), consistent with our previous results with organ-cultured CSMC (Hill et al., 2002).

Effects of UTP, ATP and ITP on proliferation of CSMC. Treatment of CSMC with UTP,

ATP or ITP caused a concentration-dependent increase in cellular DNA (Fig. 4A) and protein

synthesis (Fig. 4B), and cell number (Fig. 4C), whereas UDP (100 µM) only caused a small increase in protein synthesis (Fig. 4B).   Intriguingly, ATP was much more potent and efficacious than UTP, ITP and UDP in increasing cellular DNA synthesis (Fig. 4A) and expression of PCNA, a protein marker of cell proliferation (Fig. 4D), suggesting that another receptor may contribute to the proliferative response.

Effect of anti-sense OGN on porcine P2Y$_2$ receptor-mediated increases in [Ca^{2+}]$_i$ and proliferation in cultured CSMC. To confirm the involvement of the cloned P2Y$_2$ receptor in mediating ATP- and UTP-induced proliferation of CSMC, we employed anti-sense OGN selective for P2Y$_2$ receptor mRNA (see Methods). Results obtained indicate that pretreatment of the cultured CSMC with pP2Y$_2$ anti-sense, but not sense, OGN dramatically suppressed (85%) the increase in [Ca^{2+}]$_i$ induced by 100 µM UTP (Fig. 5A).   In contrast, pP2Y$_2$ anti-sense OGN did not affect the increase in [Ca^{2+}]$_i$ induced by LPA, an agonist of another family of G protein-coupled receptors. In sub-cultured CSMC, pP2Y$_2$ anti-sense OGN also inhibited (90%) the increase in [Ca^{2+}]$_i$ induced by 100 µM ATP (Fig. 5B).   Consistent with a role for an additional receptor, pP2Y$_2$ anti-sense OGN only partially suppressed ATP-induced DNA and protein synthesis, whereas UTP-induced DNA and protein synthesis were nearly completely inhibited, and responses to LPA were unaffected (Figs. 5C and 5D).

Upregulation of porcine P2Y$_2$ receptor mRNA in cells from *in vitro* organ-cultures and *in vivo* stented coronary arteries. Since our previous work demonstrated the up-regulation of a functional UTP-sensitive P2Y receptor in organ-cultured coronary artery smooth muscles (Hill et al., 2001, 2002), we determined whether the increased activity was associated with the increased expression of mRNA to the P2Y$_2$ receptor subtype that we have cloned. RT-PCR analysis showed that CSMC isolated from organ-cultured porcine coronary arteries expressed P2Y$_2$ receptor mRNA to a greater extent than CSMC isolated from freshly harvested or cold-stored porcine coronary arteries (Fig. 6A).   In contrast, P2Y$_6$ receptor mRNA levels

were similar in CSMC isolated from organ-cultured and freshly harvested arteries, whereas

P2Y$_6$ receptor mRNA was expressed at lower levels in cold-stored arteries (Fig. 6B). We were

unable to detect mRNA for the UTP-sensitive P2Y$_4$ receptor in either sub-cultured porcine

CSMC or organ-cultured porcine coronary arteries (Fig. 6C).

To determine whether P2Y$_2$ receptor upregulation occurs in clinically relevant arterial

injury, we assayed for P2Y$_2$ receptor mRNA expression in a porcine model of coronary artery

stent angioplasty. Interestingly, we found that P2Y$_2$ receptor mRNA levels were significantly

increased in CSMC dispersed from stented segments of coronary arteries 3 weeks after stent

angioplasty, as compared to CSMC from un-stented segments (Fig. 6D). Again, no significant

difference was observed for levels of P2Y$_6$ receptor mRNA in the stented and unstented artery

segments (data not shown), whereas P2Y$_4$ receptor mRNA was undetectable (Fig. 6C).

Discussion

In the present study, we report the cloning of the porcine $P2Y_2$ receptor for the first time, and have demonstrated that the porcine $P2Y_2$ receptor is the first species orthologue that shows a significant difference between the agonist potencies of UTP and ATP. We also have demonstrated that ATP is more potent than UTP in stimulating proliferation of cultured CSMC, suggesting that the $P2Y_2$ receptor participates with another ATP/ADP/adenosine-related receptor(s) to regulate cell proliferation. These studies further indicated that upregulation of $P2Y_2$ receptors occurred in CSMC of organ-cultured porcine coronary arteries and in the clinically relevant model of *in vivo* coronary artery stent angioplasty.

Porcine $P2Y_2$ receptor pharmacology

A P2Y receptor subtype that is more sensitive to UTP than ATP has been reported in cultures of porcine CSMC (Seiler et al., 1999) and in CSMC from organ-cultured porcine arteries (Hill et al., 2001, 2002), and this agonist potency relationship does not match any cloned P2Y receptors. To clone this novel receptor, we used PCR cloning and cDNA amplification to isolate an open reading frame of the porcine P2Y receptor that has highest homology to the human $P2Y_2$ receptor. Pharmacological characterization of the cloned porcine $P2Y_2$ receptor expressed in 1321N1 cells devoid of endogenous P2Y receptors clearly showed that UTP was a more potent agonist than ATP at increasing $[Ca^{2+}]_i$ and phosphorylation of ERK1/2, whereas other endogenous nucleoside triphosphates were active and nucleosides except for UDP were inactive. In contrast, UTP and ATP are equipotent and efficacious agonists of $P2Y_2$ receptors in human (Parr et al., 1994), mouse (Lustig et al., 1993), rat (Chen et al., 1996), and dog (Zambon et al., 2000), receptors at which other nucleoside triphosphates are inactive (Dubyak, 2003). Differences in agonist potencies between species orthologues have been reported for other P2Y receptors. For example, the dog $P2Y_{11}$ receptor has 74% sequence identity with the human $P2Y_{11}$ receptor, but different sensitivity for ATP versus ADP (Qi et al., 2001). ATP is a full agonist at the rat $P2Y_4$ receptor, but is a competitive antagonist

of the human $P2Y_4$ receptor (Kennedy et al., 2000). Although the sensitivity of the cloned porcine $P2Y_2$ receptor to the antagonists suramin, RB-2 and PPADS was similar to the cloned rat $P2Y_4$ receptor (Kennedy et al., 2000), these antagonists are relatively non-selective. Given the fact that the primary sequence of the cloned porcine P2Y receptor has the highest identity with the human $P2Y_2$ receptor (84%), and only about 40% homology with either the rat or human $P2Y_4$ receptor, we have termed it $P2Y_2$.

The structural determinants of nucleotide selectivity (UTP *vs.* ATP) at $P2Y_2$ receptors are unknown, although mutagenesis data suggest that positively charged amino acids in the 6th and 7th transmembrane domains are required for binding the negatively charged phosphate groups of nucleotides (Erb et al., 1995). The porcine $P2Y_2$ receptor contains positively charged amino acids in these domains, and the precise positioning of them has never been shown to be important for agonist activity. It seems apparent that only a few amino acid transitions can determine the nucleotide selectivity of the $P2Y_4$ and $P2Y_{11}$ receptors (Qi et al., 2001; Herold et al., 2003), and therefore we suggest that minor differences in the primary sequence underlie variations in the agonist potency profiles between $P2Y_2$ receptor species orthologues.

We also found that there was a two-fold greater difference between the EC_{50}'s of UTP and ATP for the induction of increases in ERK phosphorylation as compared to increases in $[Ca^{2+}]_i$, although the reason for this difference is not apparent. A possible explanation is that Ca^{2+} recording was done in a constant perfusion system that minimizes nucleotide degradation by ecto-ATPases (Kennedy et al., 2000), whereas ERK phosphorylation was determined over 5 min with monolayer cell cultures, where generation of the partial porcine $P2Y_2$ receptor agonist UDP, could occur. It is also noted that ecto-ATPases can degrade ATP at different rates than UTP (Palmer et al., 1998; Kumari et al., 2003), and ADP is not an agonist of the porcine $P2Y_2$ receptor (Fig. 2C). The possibility that UDP can be converted by cell cultures into UTP (Nicholas et al., 1996), may also contribute to the relative agonist potencies.

Role of porcine $P2Y_2$ receptors in UTP- and ATP-induced proliferation of CSMC

The mitogenic effect of extracelluar nucleotides on VSMC has been known for years (Erlinge, 1998). However, a potent anti-proliferative effect of UTP on VSMC also has been reported (White et al, 2000). In either case, the P2 receptor subtype(s) responsible for these effects on proliferation of VSMC has not been determined. Earlier studies by Erlinge et al. (1993, 1995) showed that ATP or UTP increased DNA and protein synthesis in sub-cultured rat aortic VSMC. In the same cell culture model, however, Malam-Souley et al. (1993, 1996), were unable to detect increases in DNA synthesis after ATP/UTP stimulation, although ATP or UTP upregulated the expression of mRNA to several cell cycle progression-related genes. Since P2X agonists were essentially inactive, it was concluded that a P_{2U}-like receptor (now termed $P2Y_2$) was responsible for the mitogenic effects of ATP/UTP. However, the role of a $P2Y_4$ receptor cannot be excluded since the nucleotide agonist profile between rat $P2Y_2$ and $P2Y_4$ receptors is essentially indistinguishable (Wildman et al., 2003). Indeed, Harper et al. (1998) suggested that the $P2Y_4$ receptor mediated ATP/UTP-induced proliferation of rat aortic VSMC. In the present study, we found that ATP, UTP, or ITP, three agonists of the cloned porcine $P2Y_2$ receptor, increased DNA and protein synthesis and cell number in CSMC. In support of a role for the $P2Y_2$ receptor in proliferation of porcine CSMC: (1) UDP, the most potent agonist of $P2Y_6$ receptors, was inactive in stimulating cell proliferation; (2) anti-sense OGN for porcine $P2Y_2$ receptors nearly abolished UTP- or ATP-induced increases in $[Ca^{2+}]_i$, suggesting that the predominant UTP/ATP-sensitive P2Y receptor functionally expressed in sub-cultured porcine CSMC is the $P2Y_2$ subtype; (3) RT-PCR analysis showed that $P2Y_2$ receptor mRNA was highly expressed in CSMC, whereas $P2Y_6$ receptor mRNA levels were very low and $P2Y_4$ receptor mRNA was undetectable; (4) UTP or ATP, but not UDP, significantly increased expression of PCNA, a cell proliferation marker; and (5) importantly, anti-sense knockdown of $P2Y_2$ receptors inhibited UTP- or ATP-induced cellular DNA and protein synthesis. Thus, we have provided several lines of compelling data demonstrating that $P2Y_2$ receptor plays a role in CSMC proliferation.

It is worth noting that porcine $P2Y_2$ receptor anti-sense OGN nearly abolished UTP-induced DNA and protein synthesis, but only partially inhibited the mitogenic effect of ATP, whereas the $P2Y_2$ anti-sense OGN nearly eliminated UTP- or ATP-induced increases in $[Ca^{2+}]_i$ in cultured CSMC. This inconsistency suggests that another receptor(s) for ATP, ADP or adenosine might contribute to ATP-induced cell proliferation. The fact that ATP induced higher levels of DNA synthesis and PCNA expression than UTP, yet triggered a partial Ca^{2+} response, strongly suggests that ATP or its hydrolysates, can activate other receptors than $P2Y_2$ to mediate Ca^{2+}-independent cell proliferation.

Selective upregulation of $P2Y_2$ receptors in CSMC of organ-cultured and stented arteries

Our previous studies have shown that a functional UTP-sensitive P2Y receptor was upregulated in porcine coronary smooth muscles after several days in organ culture (Hill et al., 2001, 2002). However, the receptor subtype that was upregulated was not determined. In the present study, we found that $P2Y_2$ receptor mRNA levels were dramatically increased in CSMC from organ-cultured arteries as compared to freshly harvested arteries, whereas $P2Y_6$ receptor mRNA levels were unchanged and $P2Y_4$ receptor mRNA was undetectable. These data suggest that the $P2Y_2$ receptor is selectively upregulated in organ culture, an accepted *ex vivo* model for studying vascular biology. Our findings are consistent with a previous study showing that short-term culture of rat salivary gland epithelial cells caused upregulation of $P2Y_2$ receptors (Turner et al., 1997). The limited expression of $P2Y_2$ receptor mRNA in control CSMC is consistent with the inability of UTP or ATP to induce increases in $[Ca^{2+}]_i$ in freshly isolated CSMC from porcine coronary arteries (Hill et al., 2001, 2002). Moreover, the cloned porcine $P2Y_2$ receptor expressed in 1321N1 cells demonstrated similar relative potencies of UTP and ATP as the endogenous receptor in CSMC (Hill et al., 2002), and antagonist sensitivities (i.e., suramin, RB-2 and PPADS) were essentially the same for the endogenous and cloned receptors. Furthermore, we found that UTP desensitized ATP-induced Ca^{2+}

responses in organ-cultured CSMC (unpublished data), suggesting a common receptor target, presumably $P2Y_2$.

The high expression level of $P2Y_6$ receptor mRNA in CSMC from normal porcine coronary arteries was unexpected, since there was no detectable Ca^{2+} response to UDP stimulation in these cells. However, the data are consistent with a previous finding with human coronary artery rings, in which very high levels of $P2Y_6$ receptor mRNA were detected, yet UDP, the most potent and efficacious agonist of this receptor subtype, failed to induce contraction of the arteries (Malmsjo, et al., 2000b). These results suggest that either the $P2Y_6$ receptor is not expressed at the protein level or perhaps the receptor is expressed, but fails to couple with Gq protein.

To extend our findings to a more clinically relevant model of arterial injury (Johnson et al., 1999, for review), we compared P2Y receptor expression in CSMC of stented and unstented segments of coronary arteries. We found that $P2Y_2$ receptor mRNA levels were significantly increased in the stented CSMC, as compared to the unstented cells. This result confirms our recent findings with collared rabbit carotid arteries, in which selective upregulation of $P2Y_2$ receptors in smooth muscle cells was associated with the development of neointimal hyperplasia (Seye et al., 2002). Collectively, these studies suggest that upregulation of $P2Y_2$ receptors after vascular injury may be a general phenomenon in VSMC, which might be important in the development of vascular diseases such as atherosclerosis and angioplasty-induced restenosis, and the failure of bypass vein grafts.

In summary, we report the cloning of the porcine $P2Y_2$ receptor, which is the first species orthologue of $P2Y_2$ that exhibits different agonist potencies for UTP and ATP. In addition, we demonstrate that activation of the $P2Y_2$ receptor is fully responsible for UTP-induced proliferation of porcine CSMC, but only partially involved in the mitogenic effect of ATP, thereby highlighting an additional mechanism for ATP-induced cell proliferation. Lastly, we show that

selective upregulation of P2Y$_2$ receptors in porcine CSMC occurs in both *ex vivo* models of vessel organ cultures, and in coronary arteries after stent angioplasty *in vivo.*

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Deduced amino acid sequence of the cloned porcine P2Y$_2$ receptor and alignment with the human P2Y$_2$ receptor sequence. Putative transmembrane-spanning domains are indicated with solid lines and marked as TM I to TM VII. Sequence matches are shaded only, and conservative substitutions are shaded and marked as "**+**". Gaps (-) are introduced to best fit the alignment. A potential phosphorylation site for G protein-coupled receptor kinase is denoted with "▲▲", and potential phosphorylation sites for protein kinase A or protein kinase C are indicated by "●". A conserved RGD motif is denoted with "**!!!**", and a consensus site for N-linked glycosylation is marked with "⋆".

Figure 2. Nucleotide-induced changes in [Ca^{2+}]$_i$ in human 1321N1 astrocytoma cells expressing porcine P2Y$_2$ receptors. Human 1321N1 cells, transfected with recombinant plasmid pCR$^{3.1}$-pP2Y$_2$ or mock plasmid pCR3.1, were used for Ca^{2+} mobilization assays with UTP, ATP or carbacol (CCh) as agonists (**A**). Concentration-response relationships for nucleoside triphosphates (**B**), diphosphates (**C**), and synthetic nucleotides (**D**) for the recombinant pP2Y$_2$ receptor expressed in 1321N1 cells. Data points in panels B-D indicate the average peak increase in [Ca^{2+}]$_i$ for 8 to 16 cells on each of five coverslips and represent the means ± S.E.M..

Figure 3. Dose-response of nucleotide-stimulated tyrosine phosphorylation of extracellular signal-regulated kinase (ERK1/2) in 1321N1 cells expressing porcine P2Y$_2$ receptors. Confluent cultures of 1321N1 cells expressing pP2Y$_2$ receptors were serum-starved for 24 h and then stimulated for 5 min with the indicated concentrations of UTP, ATP or UDP. After treatment, cells were lysed and phospho-ERK1/2 was detected by Western analysis. Data shown are the means ± S.E.M. of results from three independent experiments.

Figure 4. Mitogenic effect of ATP, UTP, ITP and UDP in cultured coronary artery smooth muscle cells.   Cellular DNA (**A**) and protein synthesis (**B**), and cell number (**C**) were determined after stimulation of the CSMC with the indicated concentration of different nucleotides for 48 h (A, B) or 96 h (C). Protein expression levels of PCNA (**D**) were determined in CSMC stimulated with 100 µM nucleotide or PDGF (100 ng/mL) for 48 h followed by Western analysis. Blots were stripped and re-probed with anti-actin antibody (D). Data shown are the means ± S.E.M. of results from four independent experiments performed in triplicate (A, B, C) or duplicate (D). *, $p < 0.05$; **, $p < 0.01$ relative to the respective control.

Figure 5. Effect of pP2Y$_2$ anti-sense OGN on ATP- and UTP-induced increases in [Ca^{2+}]$_i$ and proliferation in cultured CSMC. Sub-cultures of CSMC were pretreated with or without sense or anti-sense OGN (3 µM) for 48 h and then incubated with 100 µM ATP, UTP or LPA for 3 min (A, B) or 48 h (C, D) and [Ca^{2+}]$_i$ (**A**, **B**), and DNA (**C**) and protein synthesis (**D**) were determined, as described in Methods. Data points in panel B represent the average peak increase in [Ca^{2+}]$_i$ for 8 to 16 cells on each of five coverslips. Data points in panels C-D are the means ± S.E.M. of results from four independent experiments in triplicate (C, D). *, $p < 0.05$; **, $p < 0.01$ relative to the response of agonist alone.

Figure 6. Upregulation of P2Y$_2$ receptor mRNA in organ-cultured and stented porcine coronary artery smooth muscle cells. Semi-quantitative RT-PCR for detection of P2Y$_2$ receptor mRNA expression (340bp) was performed with cultured CSMC or CSMC dispersed from freshly isolated, organ-cultured, and 5°C cold-stored porcine coronary arteries (**A**), or from *in vivo* stented and un-stented coronary artery segments (**D**), as described in Methods. P2Y$_6$ receptor mRNA expression (538bp) also was determined in cultured CSMC or CSMC dispersed from freshly isolated, organ-cultured, and 5°C cold-stored porcine coronary arteries

(**B**). The expression level of porcine $P2Y_4$ receptor mRNA (686bp) was below the detection limit in cultured CSMC, intact coronary arteries and stented artery segments. The positive control for $P2Y_4$ receptor mRNA expression was 100 ng of genomic DNA isolated from cultured porcine CSMC (**C**). RT-PCR performed without reverse transcriptase is indicated as "-RT". The piece of artery shown in panel D indicates the position of stented and unstented fragments of porcine coronary arteries. PCR products were electrophoresed on a 2% agarose ethidium bromide gel, and the relative densities of $P2Y_2$ (A) and $P2Y_6$ (B) receptor mRNAs were normalized to G3PDH (983bp). Data shown are the means ± S.E.M. of results from five (A, B, C) or four pigs (D). *, $p < 0.05$ relative to fresh arteries or unstented arteries.

TABLE 1
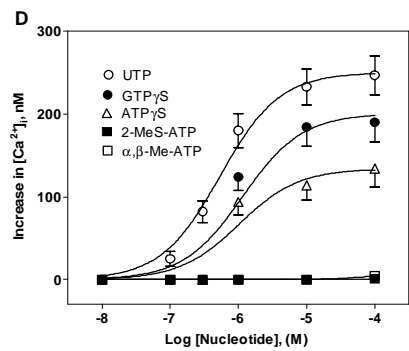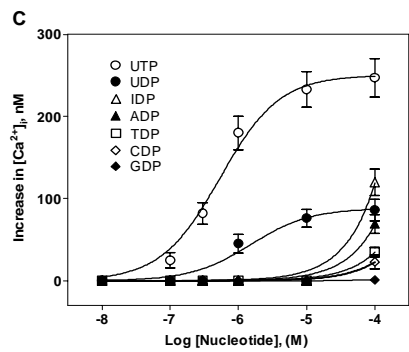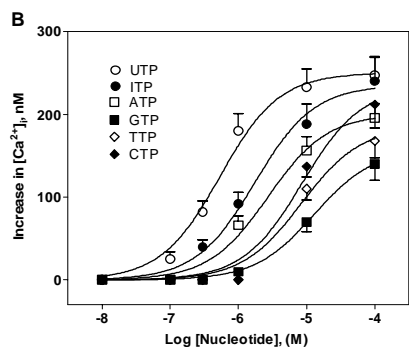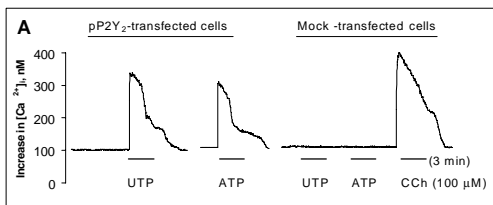
Agonist potencies and efficacies for the porcine P2Y$_2$ receptor

Nucleotide	EC$_{50}$ (µM)	Efficacy (% of UTP)
UTP	0.53 ± 0.08	100
ITP	1.7 ± 0.5	94.2 ± 6.8
ATP	2.7 ± 0.4	80.1 ± 4.9
GTP	1.3 ± 0.4	63.3 ± 5.4
TTP	8.6 ± 1.6	74.4 ± 6.0
CTP	8.4 ± 1.3	93.6 ± 5.5
UDP	1.5 ± 0.4	35.2 ± 8.7
IDP	35.7 ± 10.8	ND
ADP	29.3 ± 9.8	ND
TDP	31.4 ± 11.0	ND
CDP	87.1 ± 20.3	ND
GDP	NE	NE
GTPγS	1.2 ± 0.4	80.2 ± 5.4
ATPγS	1.0 ± 0.3	53.6 ± 6.7
2-MeS-ATP	NE	NE
α,β-Me-ATP	NE	NE

Human 1321N1 cells transfected with recombinant plasmid pCR$^{3.1}$-pP2Y$_2$ were used for Ca^{2+} mobilization assays with indicated nucleotide agonists. Data shown are the means ± S.E.M. summarized from the dose-response curves in figure 2. NE: no effect at 100 µM. ND: not determined due to no maximal response reached.

Fig. 1

```
Human      MAADLGPWND TINGTWDGDE LGYRCRFNED FKYVLLPVSY GVVCVPGLCL 50
Porcine    MATGPDLWNG TVNGTSDGDD WGYRCRFHED FKYVLLPLSY GVVCVLGLSL
Consensus  MA.....WN. T+NGT.DGD+ .GYRCRF.ED FKYVLLP+SY GVVCV.GL.L
                     *                              TMI

           NAVALYIFLC RLKTWNASTT YMFHLAVSDA LYAASLPLLV YYYARGDHWP 100
           NAGALYIFLC RLKTWNASTT YMFHLAVSDA LYAASLPLLV YYYARGDHWP
           NA.ALYIFLC RLKTWNASTT YMFHLAVSDA LYAASLPLLV YYYARGDHWP
                                 TMII                     !!!

           FSTVLCKLVR FLFYTNLYCS ILFLTCISVH RCLGVLRPLR SLRWGRARYA 150
           FSTALCKLVR FLFYTNLYCS ILFLTCISVH RCLGVLRPLR SLRWGHARYA
           FST.LCKLVR FLFYTNLYCS ILFLTCISVH RCLGVLRPLR SLRWG.ARYA
                          TMIII

           RRVAGAVWVL VLACQAPVLY FVTTSARGGR VTCHDTSAPE LFSRFVAYSS 200
           RRVAAAVWGL VLACQAPALY FITTTAQGGR ITCHDTSAPE LFSHFVAYSL
           RRVA+AVW.L VLACQAP.LY F+TT+A.GGR +TCHDTSAPE LFS.FVAYS.
                          TMIV

           VMLGLLFAVP FAVILVCYVL MARRLLKPAY GTSGGLPRAK RKSVRTIAVV 250
           VMLSVLFAAP FAVILVCYAL MARRLLRPAY GTAGGLPRAK RKSVRTIAVV
           VML.+LFA.P FAVILVCY.L MARRLL+PAY GT+GGLPRAK RKSVRTIAVV
                        TMV

           LAVFALCFLP FHVTRTLYYS FRSLDLSCHT LNAINMAYKV TRPLASANSC 300
           LAVFALCFLP FHVTRTLYYS FRTLDLSCHT LDAINMAYKI TRPLASANSC
           LAVFALCFLP FHVTRTLYYS FR+LDLSCHT L.AINMAYK+ TRPLASANSC
            TMVI                                        TMVII

           LDPVLYFLAG QRLVRFARDA KPPTGPSPAT PARRRLGLRR SDRTDMQRIE 350
           LDPVLYFLAG QRLVRFARDA KPPTDATPTA QACRRLGLRR SHGTDTKRTE
           LDPVLYFLAG QRLVRFARDA KPPT..+P.. .A.RRLGLRR S..TD..R.E
                                        •                    •

           DVLGSSEDSR RTESTPAGSE NTKDIRL 377
           DS-ASSEDSR RTEITPARGE ---DIRL 373
           D...SSEDSR RTE.TPA..E ...DIRL
                ▲▲  •         •
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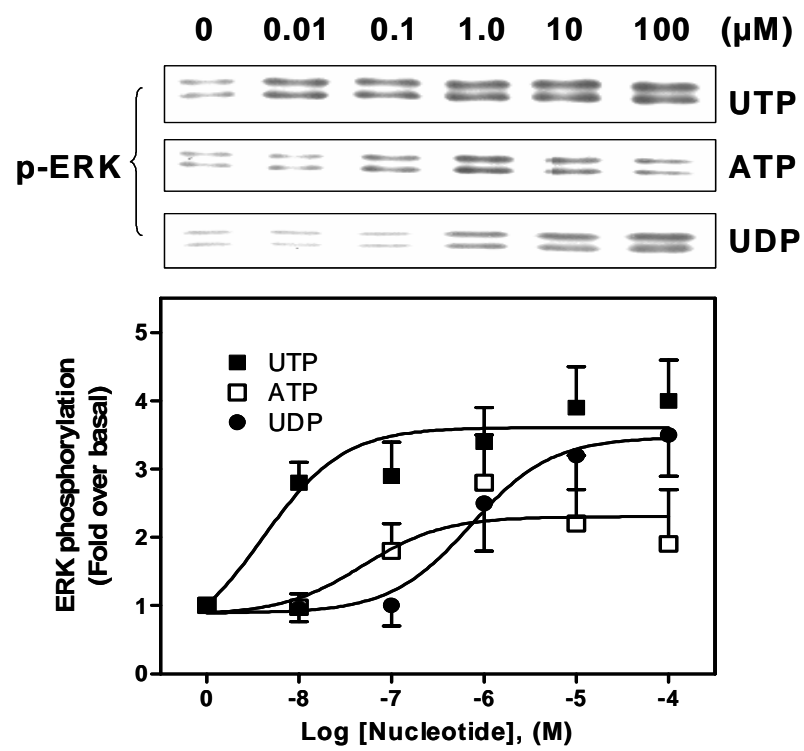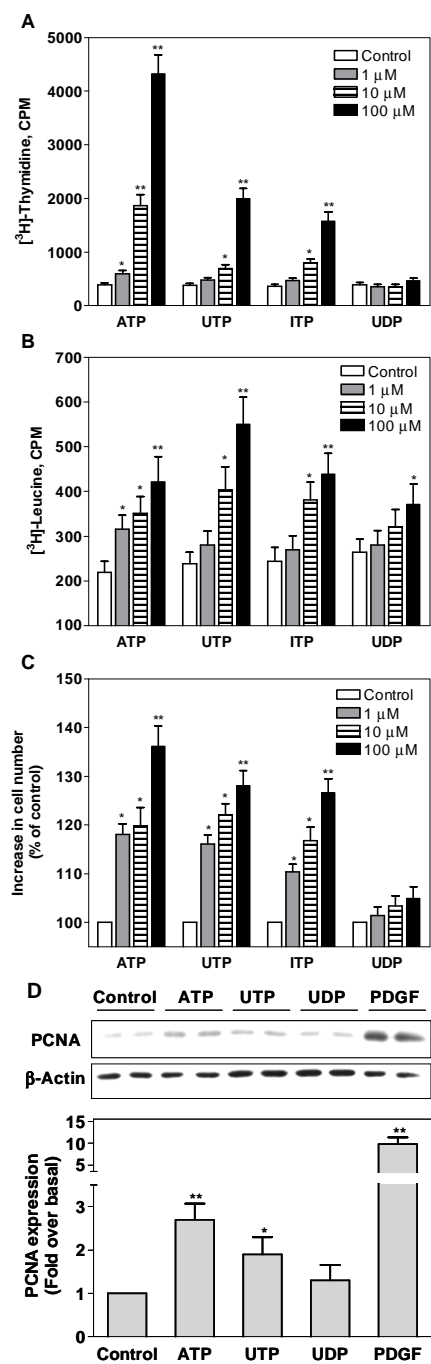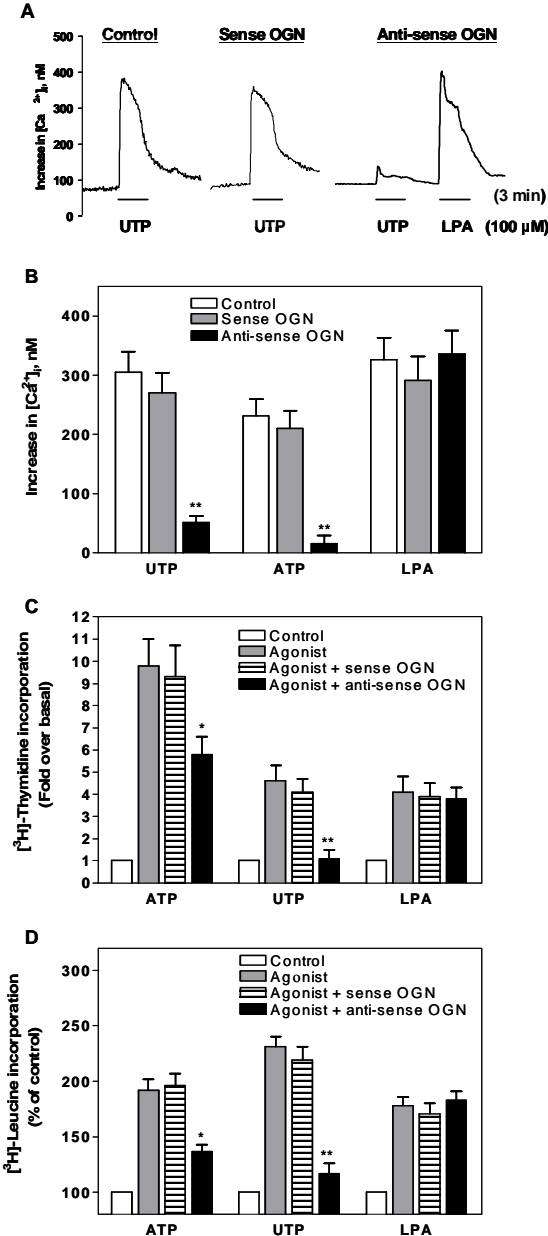
Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6