0026-895X/04/6605-1265–1274$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 66:1265–1274, 2004

Vol. 66, No. 5
2642/1178086
Printed in U.S.A.

Cloning, Up-Regulation, and Mitogenic Role of Porcine $P2Y_2$ Receptor in Coronary Artery Smooth Muscle Cells

Jianzhong Shen, Cheikh I. Seye, Meifang Wang, Gary A. Weisman, Peter A. Wilden, and Michael Sturek

Departments of Medical Pharmacology and Physiology (J.S., M.W., P.A.W., M.S.), Biochemistry (C.I.S., G.A.W.), and Internal Medicine (M.S.), and the Center for Diabetes and Cardiovascular Health (J.S., M.W., P.A.W., M.S.), University of Missouri-Columbia, School of Medicine, Columbia, Missouri

Received May 14, 2004; accepted July 26, 2004

ABSTRACT

Previous work has shown up-regulation 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 the 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 extracellular signal-regulated kinase phosphorylation. Stimulation of subcultured CSMC with UTP, ITP, or ATP induced a concentration-dependent increase in cellular DNA content, protein synthesis, cell number, and proliferating cell nuclear antigen expression, indicating a mitogenic role for $P2Y_2$ receptors. This was supported by the finding that the treatment of CSMC with antisense oligonucleotides to the cloned cDNA sequence significantly inhibited UTP- and ATP-induced DNA and protein synthesis. In addition, reverse transcription-polymerase chain reaction analysis showed that $P2Y_2$ receptor mRNA was dramatically increased in cells of organ-cultured arteries compared with freshly harvested arteries, whereas the $P2Y_6$ receptor mRNA level was unchanged, and the $P2Y_4$ receptor mRNA was undetectable. This $P2Y_2$ subtype-specific up-regulation 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 up-regulated in CSMC of in vitro organ cultures or in vivo stented coronary arteries to mediate the mitogenic effects of nucleotides.

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 nitric oxide, endothelium-derived hyperpolarizing factor, 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 postangioplasty 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

This work was supported by National Institute of Health grants RR13223 and HL62552 to Michael Sturek, AG18357 and RR15565 to Gary A. Weisman and Cheikh I. Seye, an American Diabetes Association grant to Peter A. Wilden, and a University of Missouri-Columbia Life Science Fellowship to Jianzhong Shen. A presentation of part of this work was supported by a travel award from the American Society for Pharmacology and Experimental Therapeutics to the 2004 Experimental Biology meeting in Washington, D.C. (April 17–21).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.002642.

ABBREVIATIONS: VSMC, vascular smooth muscle cells; CSMC, coronary artery smooth muscle cells; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; LPA, lysophosphatidic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nucleus antigen; CCh, carbachol hydrochloride; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); G3PDH, glyceraldehyde-3-phosphate dehydrogenase; OGN, oligonucleotide(s); RB-2, reactive blue 2; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid.

receptors, of which eight different subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) have been cloned and pharmacologically characterized (Abbracchio et al., 2003; Dubyak, 2003). Interestingly, it turns out that, among the Gq-coupled P2Y receptors ($P2Y_{1, 2, 4, 6, 11}$), only $P2Y_2$ was up-regulated in vascular injury models, including balloon-injured rat aorta (Seye et al., 1997) and collared rabbit carotid arteries (Seye et al., 2002). Studies on subcultured 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 subcultured porcine coronary artery smooth muscle cells (CSMC) has also been reported (Wilden et al., 1998). However, because of the 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 (Malmsjo et al., 2000a,b, 2003; Hill et al., 2001). 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 antimitogen 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 up-regulation 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; Hill and Sturek, 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 up-regulated under organ culture conditions (Hill et al., 2001; Hill and Sturek, 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 sarcoplasmic reticulum Ca^{2+} pump (Hill and Sturek, 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 up-regulated in porcine CSMC of diseased coronary arteries is important, because 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 the activation of this receptor is responsible for the 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 mitogen-activated protein 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; Hill and Sturek, 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 the inhibition of mitogenesis with the $P2Y_2$ receptor antisense 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; Hill and Sturek, 2002). Dispersed cells were recovered and subcultured at 37°C with 5% CO_2 in DMEM/high-glucose medium containing 10% fetal bovine serum (FBS). Smooth muscle cell lineage was confirmed by α-smooth muscle actin immunocytochemistry. Stock cell cultures were maintained in a subconfluent 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, Valencia, CA). For the synthesis of first-strand cDNA, 1 μg of total RNA was reverse transcribed using a cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA). The cDNA was then amplified by PCR using 2.5 U of *Taq*DNA polymerase (Promega, Madison, WI). 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′ and 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 RACE cDNA amplification kit (BD Biosciences 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′-CGTCACCCGCACCCTCTACTACTC-CTT-3′) and a reverse adaptor primer provided in the kit. The PCR product was diluted 1/1000 in diethyl pyrocarbonate-treated water and used as a template for a second round of PCR. The second round of PCR was performed as seminested 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′-GAGCATCCTGA-CAGCGAGAGCAG-3′ and reverse, 5′-CTACAGCCGGATGTCTTCGC-CACG-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, Carlsbad, CA). The ligation product was used to transform One Shot TOP10F′-competent *Escherichia 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 bovine growth hormone primers. DNA sequencing was performed by the DNA Core Facility 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-p$P2Y_2$) 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 (geneticin) (Calbiochem, San Diego, CA) for ~2 to 3 weeks. The efficiency of transfection was determined by monitoring UTP-induced Ca^{2+} mobilization in transfected cells.

[^{3}H]Thymidine Incorporation. The incorporation of [^{3}H]thymidine into DNA was carried out as described previously (Wilden et al., 1998). Cells were cultured in 12-well culture plates as described above. 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 (PerkinElmer Life and Analytical Sciences, Boston, MA) 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 the 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 under *[^{3}H]Thymidine Incorporation*, except 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 2000 cells/well in cell culture medium containing 10% FBS, and then serum-starved for 48 h. Agonists were added and present for 4 days. By the end of cell stimulation, 20 μl of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 100 μl of fresh DMEM was added for 4 h at 37°C according to the instructions in the MTT Cell Growth Assay Kit (Chemicon International, Temecula, CA). MTT reduced to formazan by intracellular dehydrogenases was determined at 490 nm with an enzyme-linked immunosorbent assay 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-polyacrylamide gel electrophoresis 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 Technology Inc., Beverly, MA) or anti-PCNA (1:1000; Cell Signaling Technology Inc.) overnight in 20 mM Tris, pH 7.4, 150 mM NaCl, 3% bovine serum albumin, 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, Rockford, IL). As a loading control, membranes were stripped of original antibodies and reprobed with primary anti-actin antibody (1:1000; Cytoskeleton, Denver, CO).

Organ Culture of Coronary Arteries and Dispersion of Smooth Muscle Cells. Pig hearts were isolated and prepared as described previously (Hill et al., 2000, 2001; Hill and Sturek, 2002). Right coronary arteries (distal end) were either 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 (Invitrogen) without serum, which was changed every 2 days. CSMC were enzymatically isolated as described previously (Hill et al., 2000, 2001; Hill and Sturek, 2002) and identified morphologically (Hill et al., 2000).

Porcine Model of Coronary Artery Stenting. Protocols conformed to the Animal Care and Use Guidelines of the University of Missouri-Columbia. A baseline coronary angiogram for the pigs was performed, and the circumflex artery was selected for the implantation of an appropriately sized stent (3.0 mm in diameter and 13 mm long) centered on a 3.0 × 15-mm balloon (Guidant Corporation, Indianapolis, IN). To avoid overinflation 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 anesthetized and sacrificed. The 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^{2+} calcium imaging system (Intracellular Imaging, Inc., Cincinnati, OH). Cells were grown on glass coverslips for 2 days, serum-starved for another 24 h, and then used at about 40% confluence. After incubation with 2.5 μM Fura-2/acetoxymethyl ester at 37°C for 30 min, the cells were superfused in a constant-flow superfusion chamber mounted on an inverted epifluorescence microscope (model TMD; Nikon, Melville, NY) using physiological salt solution containing 138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4, in the presence or absence of the indicated concentrations of nucleotides, LPA, or carbachol hydrochloride (CCh). Fura-2 was excited at 340 and 380 nm, and emitted fluorescence (510 nm) was collected using a monochrome charge-coupled device camera (Cohu Electronics, San Diego, CA). 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; Hill and Sturek, 2002).

Semiquantitative RT-PCR. Total RNA was isolated from cultured or dispersed cells, and RT-PCR protocols were performed as described in *PCR Cloning and RACE*. Sets of primers used for detecting and semiquantifying mRNA for individual P2Y receptor subtypes were porcine $P2Y_2$ receptor (GenBank accession no. AY620400): forward, 5′-CGTCACCCGCACCCTCTACTACTCCTT-3′ and reverse, 5′-CTACAGCCGGATGTCTTCGCC-ACG-3′, yielding a 340-bp product; porcine $P2Y_6$ receptor (GenBank accession no. AY620399): forward, 5′-CTGCATCAGCTTCCAGCGCTACCT-3′ and reverse, 5′-GTCCAAGACGCTGTTGGCACTGGC-3′, yielding a 538-bp product; porcine $P2Y_4$ receptor (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 of 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 the Quantity One computer program.

Antisense Oligonucleotide Experiments. Phosphorothioate-modified oligonucleotides (OGN) designed according to the cDNA sequence of the porcine $P2Y_2$ receptor cloned in this study were antisense 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 antisense OGN was added to the culture medium 24 h before and after serum starvation. Cellular

uptake of OGN was verified using fluorescein isothiocyanate-conjugated OGN under the same experimental conditions. Nucleotide-induced Ca^{2+} mobilization was assayed to verify inhibition of porcine $P2Y_2$ receptor expression by antisense OGN.

Materials. Fura 2-AM was purchased from Molecular Probes (Eugene, OR). Cell culture media were obtained from Invitrogen. Unless indicated, all other reagents and compounds were purchased from Sigma-Aldrich (St. Louis, MO). PCR primers and sense/antisense OGN were synthesized and purified by Integrated DNA Technologies (Coralville, IA). All 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.

Data Analysis. Data are expressed as means ± S.E.M. All concentration-response curves were fitted by logistic (Hill equation), nonlinear regression analysis using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Means of two groups were compared using Student's *t* test (unpaired, two-tailed), and one-way analysis of variance was used for the 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 that 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 four 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 protein kinase C, protein kinase A, and G protein-coupled receptor kinase (Zambon et al., 2000) are conserved (annotated in Fig. 1). Interestingly, an integrin-binding Arg-Gly-Asp acid 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-p$P2Y_2$) was stably transfected into human 1321N1 astrocytoma cells devoid of any endogenous P2Y receptors (Parr et al., 1994). Figure 2A shows that 100 μM

```
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.RRLGLR S..TD..R.E
                                    •                •
          DVLGSSEDSR RTESTPAGSE NTKDIRL 377
          DS-ASSEDSR RTEITPARGE ---DIRL 373
          D...SSEDSR RTE.TPA..E ...DIRL
              ▲▲   •       •
```

Fig. 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 ●. !!!, conserved integrin-binding Arg-Gly-Asp acid motif; *, and a consensus site for *N*-linked glycosylation.

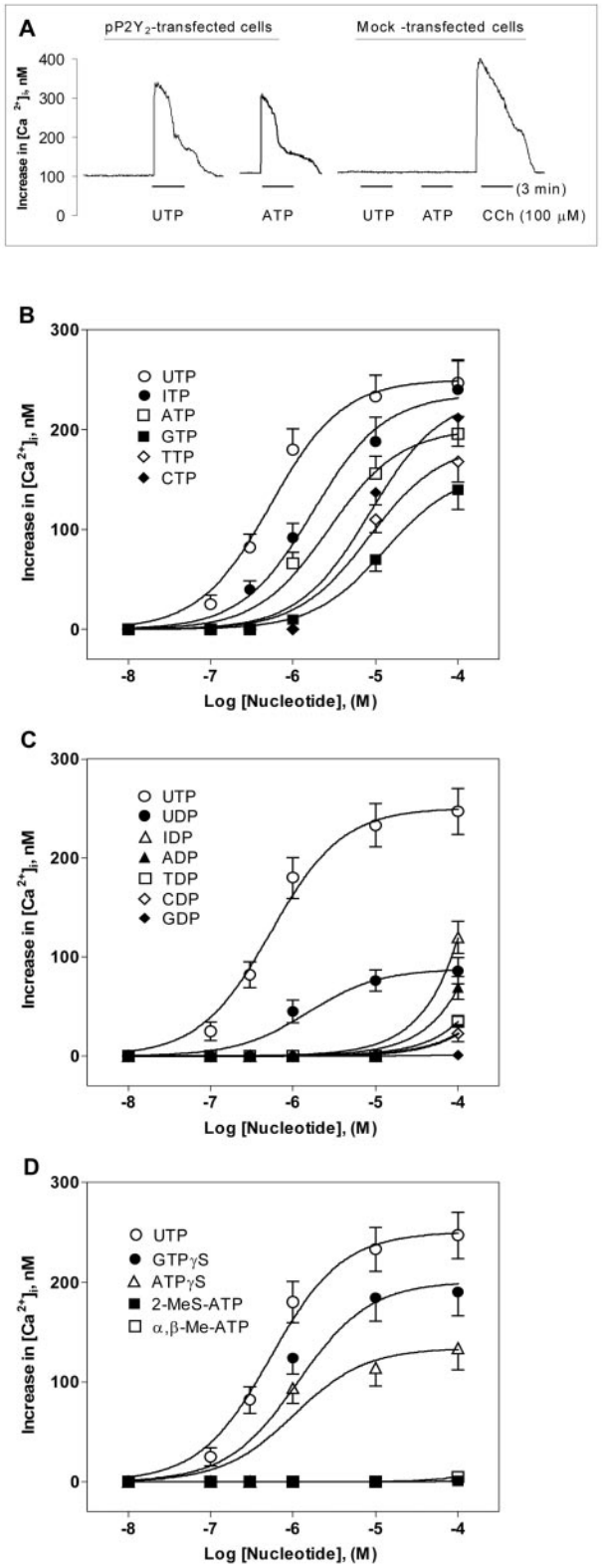

Fig. 2. Nucleotide-induced changes in $[Ca^{2+}]_i$ in human 1321N1 astrocytoma cells expressing porcine P2Y$_2$ receptors. Human 1321N1 cells, transfected with recombinant plasmid pCR$^{3.1}$-pP2Y$_2$ or mock plasmid pCR3.1, were used for Ca^{2+} mobilization assays with UTP, ATP, or CCh as agonists (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 through 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.

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-transfected 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, with the exception of UDP, which was a partial agonist (Fig. 2C), most of the nucleoside diphosphates were inactive at 10 μM. Some synthetic nucleotides, including ATPγS and GTPγS, were also active with potencies and efficacies comparable with that of ATP, whereas 2-methylthio-ATP and α,β-methyl-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 mitogen-activated protein kinase signaling pathway activated by many G protein-coupled receptors, including P2Y receptors (Burnstock, 2002; Liu et al., 2004). 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 nonspecific 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 and Sturek, 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 Antisense 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 antisense OGN selective for P2Y$_2$ receptor mRNA (see *Materials and Methods*). Results obtained indicate that pretreatment of the cultured CSMC with pP2Y$_2$ antisense, but not sense, OGN dramatically suppressed (85%) the increase in $[Ca^{2+}]_i$ induced by 100 μM UTP (Fig. 5A). In contrast, pP2Y$_2$ antisense OGN did not affect the increase in $[Ca^{2+}]_i$ induced by LPA, an agonist of another family of G protein-coupled receptors. In subcultured CSMC, pP2Y$_2$ antisense 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$_2$ antisense OGN only partially suppressed ATP-induced DNA and protein synthesis, whereas UTP-induced DNA and protein synthesis were

TABLE 1

Agonist potencies and efficacies for the porcine $P2Y_2$ receptor

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 Fig. 2.

Nucleotide	EC_{50}	Efficacy
	μM	*% 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	N.D.
ADP	29.3 ± 9.8	N.D.
TDP	31.4 ± 11.0	N.D.
CDP	87.1 ± 20.3	N.D.
GDP	N.E.	N.E.
Guanosine 5′-*O*-(3-thio)triphosphate	1.2 ± 0.4	80.2 ± 5.4
Adenosine-5′-*O*-(3-thio)triphosphate	1.0 ± 0.3	53.6 ± 6.7
2-Methylthio-ATP	N.E.	N.E.
α,β-Methyl-ATP	N.E.	N.E.

N.E., no effect at 100 μM; N.D., not determined because no maximal response was reached.

nearly completely inhibited, and responses to LPA were unaffected (Fig. 5, C and D).

Up-Regulation of Porcine $P2Y_2$ Receptor mRNA in Cells from in Vitro Organ Cultures and in Vivo Stented Coronary Arteries. Because 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; Hill and Sturek, 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 subcultured porcine CSMC or organ-cultured porcine coronary arteries (Fig. 6C).

To determine whether $P2Y_2$ receptor up-regulation 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 compared with CSMC from unstented 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).

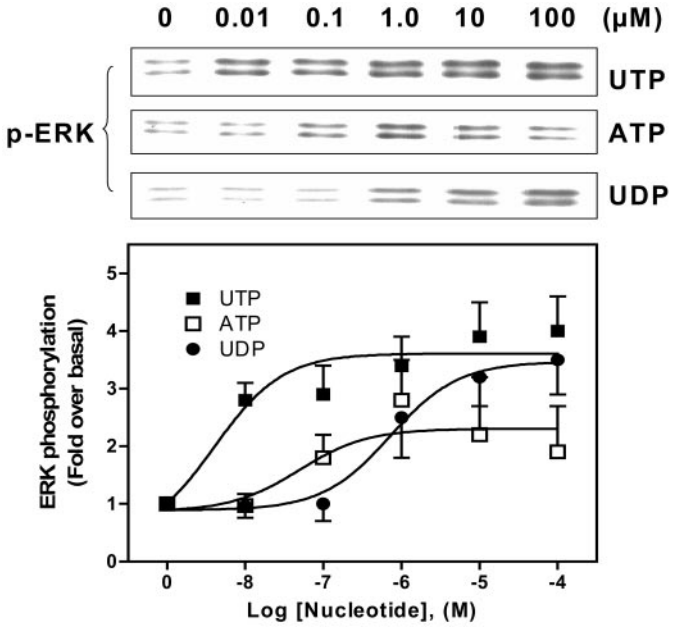

Fig. 3. Dose-response of nucleotide-stimulated tyrosine phosphorylation of ERK1/2 in 1321N1 cells expressing porcine $P2Y_2$ receptors. Confluent cultures of 1321N1 cells expressing $pP2Y_2$ receptors were serum-starved for 24 h and then stimulated for 5 min with the indicated concentrations of UTP, 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.

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 ortholog 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 the 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 up-regulation 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; Hill and Sturek, 2002), and this agonist potency relationship does not match any cloned P2Y receptors. To clone this novel receptor, we used PCR cloning and cDNA amplifi-

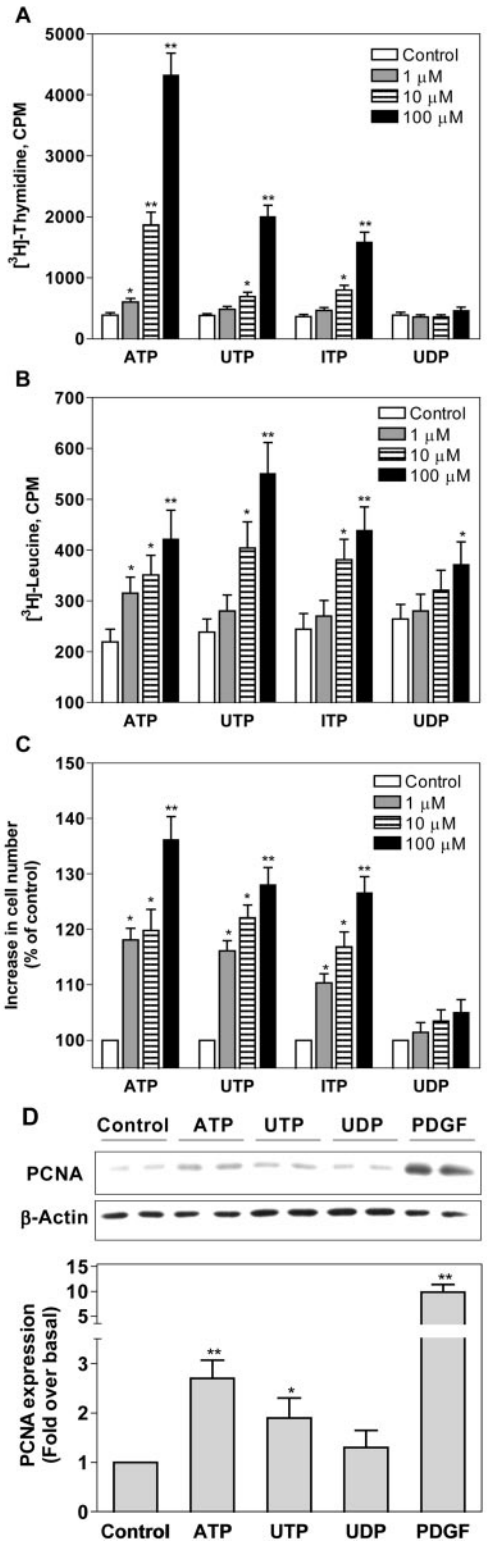

Fig. 4. Mitogenic effect of ATP, UTP, ITP, and UDP in cultured coronary artery smooth muscle cells. Cellular DNA (A) and protein synthesis (B) and cell number (C) were determined after stimulation of the CSMC with the indicated concentration of different nucleotides for 48 h (A and B) or 96 h (C). Protein 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 reprobed with anti-actin antibody (D). Data shown are the means ± S.E.M. of results from four independent experiments performed in triplicate (A, B, and C) or duplicate (D). *, $p < 0.05$ and **, $p < 0.01$ relative to the respective control.

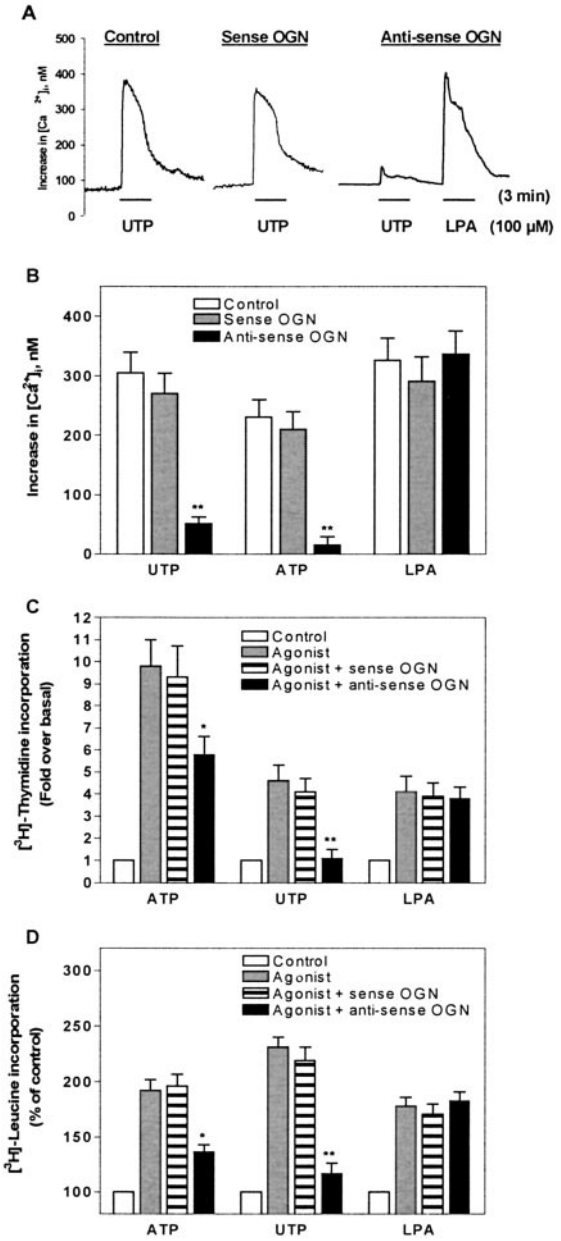

Fig. 5. Effect of pP2Y$_2$ anti-sense OGN on ATP- and UTP-induced increases in $[Ca^{2+}]_i$ and proliferation in cultured CSMC. Subcultures of CSMC were pretreated with or without sense or anti-sense OGN (3 μM) for 48 h and then incubated with 100 μM ATP, UTP, or LPA for 3 min (A and B) or 48 h (C and D), and $[Ca^{2+}]_i$ (A and B) and DNA (C) and protein synthesis (D) were determined as described under *Materials and 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 through D are the means ± S.E.M. of results from four independent experiments in triplicate (C and D). *, $p < 0.05$ and **, $p < 0.01$ relative to the response of agonist alone.

cation to isolate an open reading frame of the porcine P2Y receptor that has the 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

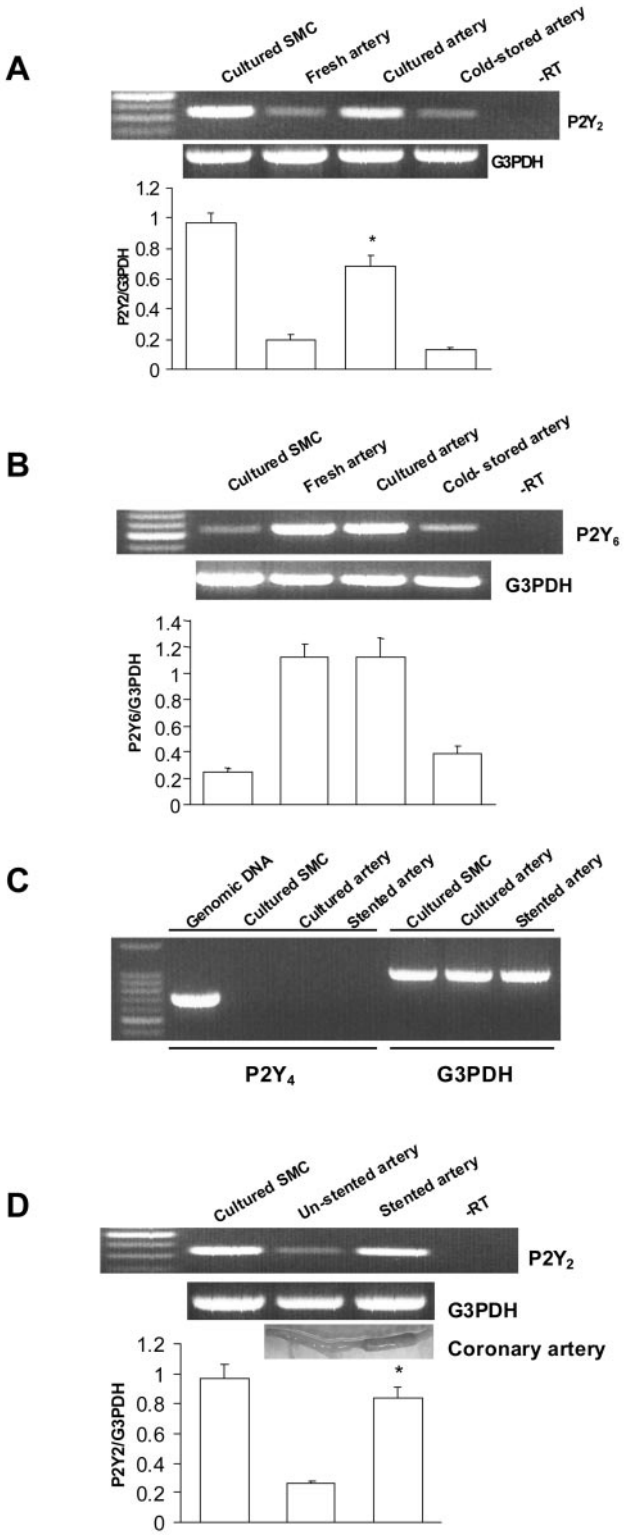

Fig. 6. Up-regulation of $P2Y_2$ receptor mRNA in organ-cultured and stented porcine coronary artery smooth muscle cells. Semiquantitative RT-PCR for detection of $P2Y_2$ receptor mRNA expression (340 bp) was performed with cultured CSMC or CSMC dispersed from freshly isolated, organ-cultured, and 5°C cold-stored porcine coronary arteries (A) or from in vivo stented and un-stented coronary artery segments (D), as described under *Materials and Methods*. $P2Y_6$ receptor mRNA expression (538 bp) was also 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 (686 bp) 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 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 (983 bp). Data shown are the means ± S.E.M. of results from five (A–C) or four pigs (D). *, $p < 0.05$ relative to fresh arteries or unstented arteries.

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 orthologs 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 nonselective. 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 versus 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 their precise positioning 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); therefore, we suggest that minor differences in the primary sequence underlie variations in the agonist potency profiles between $P2Y_2$ receptor species orthologs.

We also found that there was greater than a 2-fold difference between the EC_{50} values of UTP and ATP for the induction of increases in ERK phosphorylation compared with 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 extracellular nucleotides on VSMC has been known for years (Erlinge, 1998). However, a potent antiproliferative 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 the 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 subcultured 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 up-regulated the expression of mRNA to several cell cycle progression-related genes. Because 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, because 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. The following evidence supports 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) antisense 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 subcultured 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 importantly, 5) antisense 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 antisense OGN nearly abolished UTP-induced DNA and protein synthesis but only partially inhibited the mitogenic effect of ATP, whereas the $P2Y_2$ antisense 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 Up-Regulation 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 up-regulated in porcine coronary smooth muscles after several days in organ culture (Hill et al., 2001; Hill and Sturek, 2002). However, the receptor subtype that was up-regulated was not determined. In the present study, we found that $P2Y_2$ receptor mRNA levels were dramatically increased in CSMC from organ-cultured arteries compared with 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 up-regulated in organ culture, an accepted ex vivo model for studying vascular biology. Our findings are consistent with a previous study showing that a short-term culture of rat salivary gland epithelial cells caused up-regulation 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; Hill and Sturek, 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 and Sturek, 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, because 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 is expressed but fails to couple with Gq protein.

To extend our findings to a more clinically relevant model of arterial injury (for review, see Johnson et al., 1999), 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 compared with the unstented cells. This result confirms our recent findings with collared rabbit carotid arteries, in which selective up-regulation 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 up-regulation 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 ortholog of $P2Y_2$ that exhibits different agonist potencies for UTP and ATP. In addition, we demonstrate that the activation of the $P2Y_2$ receptor is fully responsible for the 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. Last, we show that selective up-regulation 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.

Acknowledgments

We are grateful for technical assistance and guidance from Jean M. Camden and Ann M. Schrader (Department of Biochemistry) and Drs. Robert W. Lim (Department of Pharmacology), Jianming Qiu (Department of Microbiology and Immunology), Eric Mokelke, Kaser Rex, and Zafar Nayab (Department of Cardiology and the Center for Diabetes and Cardiovascular Health).

References

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, et al. (2003) Characterization of the UDP-glucose receptor (re-named here the $P2Y_{14}$ receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24:**52–55.

Burnstock G (2002) Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* **22:**364–373.

Chen ZP, Krull N, Xu S, Levy A, and Lightman SL (1996) Molecular cloning and functional characterization of a rat pituitary G protein-coupled adenosine triphosphate (ATP) receptor. *Endocrinology* **137:**1833–1840.

Dubyak GR (2003) Knock-out mice reveal tissue-specific roles of P2Y receptor subtypes in different epithelia. *Mol Pharmacol* **63:**773–776.

Erb L, Garrad R, Wang Y, Quinn T, Turner JT, and Weisman GA (1995) Site-directed mutagenesis of P_{2U} purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. *J Biol Chem* **270:**4185–4188.

Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Perez LI, Gonzalez FA, et al. (2001) An RGD sequence in the $P2Y_2$ receptor interacts with $\alpha_v\beta_3$ integrins and is required for G_o-mediated signal transduction. *J Cell Biol* **153:**491–501.

Erlinge D (1998) Extracellular ATP: a growth factor for vascular smooth muscle cells. *Gen Pharmacol* **31:**1–8.

Erlinge D, Yoo H, Edvinsson L, Reis DJ, and Wahlestedt C (1993) Mitogenic effects of ATP on vascular smooth muscle cells vs. other growth factors and sympathetic cotransmitters. *Am J Physiol* **265:**H1089–H1097.

Erlinge D, You J, Wahlestedt C, and Edvinsson L (1995) Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol* **289:**135–149.

Harper S, Webb TE, Charlton SJ, Ng LL, and Boarder MR (1998) Evidence that $P2Y_4$ nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. *Br J Pharmacol* **124:**703–710.

Herold CL, Qi AD, Harden TK, and Nicholas RA (2003) Agonist versus antagonist action of ATP at the $P2Y_4$ receptor is determined by the second extracellular loop. *J Biol Chem* **279:**11456–11464.

Hill BJ, Katwa LC, Wamhoff BR, and Sturek M (2000) Enhanced endothelin(A) receptor-mediated calcium mobilization and contraction in organ cultured porcine coronary arteries. *J Pharmacol Exp Ther* **295:**484–491.

Hill BJ and Sturek M (2002) Pharmacological characterization of a UTP-sensitive P2Y nucleotide receptor in organ cultured coronary arteries. *Vasc Pharmacol* **39:**83–88.

Hill BJ, Wamhoff BR, and Sturek M (2001) Functional nucleotide receptor expression and sarcoplasmic reticulum morphology in dedifferentiated porcine coronary smooth muscle cells. *J Vasc Res* **38:**432–443.

Johnson GJ, Griggs TR, and Badimon L (1999) The utility of animal models in the preclinical study of interventions to prevent human coronary artery restenosis: analysis and recommendations. *Thromb Haemostasis* **81:**835–843.

Kennedy C, Qi AD, Herold CL, Harden TK, and Nicholas RA (2000) ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol Pharmacol* **57:**926–931.

Kumari R, Goh G, Ng LL, and Boarder MR (2003) ATP and UTP responses of cultured rat aortic smooth muscle cells revisited: dominance of $P2Y_2$ receptors. *Br J Pharmacol* **140:**1169–1176.

Liu J, Liao Z, Camden J, Griffin KD, Garrad RC, Santiago-Perez LI, Gonzalez FA, Seye CI, Weisman GA, and Erb L (2004) Src homology 3 binding sites in the $P2Y_2$ nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2 and growth factor receptors. *J Biol Chem* **279:**8212–8218.

Lustig KD, Shiau AK, Brake AJ, and Julius D (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci USA* **90:**5113–5117.

Malam-Souley R, Campan M, Gadeau AP, and Desgranges C (1993) Exogenous ATP induces a limited cell cycle progression of arterial smooth muscle cells. *Am J Physiol* **264:**C783–C788.

Malam-Souley R, Seye C, Gadeau AP, Loirand G, Pillois X, Campan M, Pacaud P, and Desgranges C (1996) Nucleotide receptor P_{2u} partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. *J Cell Physiol* **166:**57–65.

Malmsjo M, Adner M, Harden TK, Pendergast W, Edvinsson L, and Erlinge D (2000a) The stable pyrimidines UDPβS and UTPγS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery. *Br J Pharmacol* **131:**51–56.

Malmsjo M, Hou M, Harden TK, Pendergast W, Pantev E, Edvinsson L, and Erlinge D (2000b) Characterization of contractile P2 receptors in human coronary arteries by use of the stable pyrimidines uridine 5′-*O*-thiodiphosphate and uridine 5′-*O*-3-thiotriphosphate. *J Pharmacol Exp Ther* **293:**755–760.

Malmsjo M, Hou M, Pendergast W, Erlinge D, and Edvinsson L (2003) The stable pyrimidines UDPβS and UTPγS discriminate between contractile cerebrovascular P2 receptors. *Eur J Pharmacol* **458:**305–311.

Matsumoto T, Nakane T, and Chiba S (1997) UTP induces vascular responses in the isolated and perfused canine epicardial coronary artery via UTP-preferring P2Y receptors. *Br J Pharmacol* **122:**1625–1632.

Nicholas RA, Watt WC, Lazarowski ER, Li Q, and Harden K (1996) Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol* **50:**224–229.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA, and Harden TK (1998) Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol Pharmacol* **54:**1118–1123.

Parr CE, Sullivan DM, Paradiso AM, Lazarowski ER, Burch LH, Olsen JC, Erb L, Weisman GA, Boucher RC, and Turner JT (1994) Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc Natl Acad Sci USA* **91:**3275–3279.

Pillois X, Chaulet H, Belloc I, Dupuch F, Desgranges C, and Gadeau AP (2002) Nucleotide receptors involved in UTP-induced rat arterial smooth muscle cell migration. *Circ Res* **90:**678–681.

Qi AD, Zambon AC, Insel PA, and Nicholas RA (2001) An arginine/glutamine difference at the juxtaposition of transmembrane domain 6 and the third extracellular loop contributes to the markedly different nucleotide selectivities of human and canine $P2Y_{11}$ receptors. *Mol Pharmacol* **60:**1375–1382.

Seiler L, Matyas S, and Fleckenstein-Grun G (1999) Extracellular ATP increases $[Ca^{2+}]_i$ in primary cultured pig coronary smooth muscle cells via a P2Y purinoceptor subtype. *J Cardiovasc Pharmacol* **33:**807–813.

Seye CI, Gadeau AP, Daret D, Dupuch F, Alzieu P, Capron L, and Desgranges C (1997) Overexpression of $P2Y_2$ purinoceptor in intimal lesions of the rat aorta. *Arterioscler Thromb Vasc Biol* **17:**3602–3610.

Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, Gonzalez FA, and Weisman GA (2002) Functional $P2Y_2$ nucleotide receptors mediate uridine 5′-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* **106:**2720–2726.

Turner JT, Weisman GA, and Camden JM (1997) Upregulation of $P2Y_2$ nucleotide receptors in rat salivary gland cells during short-term culture. *Am J Physiol* **273:**C1100–C1107.

White PJ, Kumari R, Porter KE, London NJ, Ng LL, and Boarder MR (2000) Antiproliferative effect of UTP on human arterial and venous smooth muscle cells. *Am J Physiol* **279:**H2735–H2742.

Wihlborg AK, Malmsjo M, Eyjolfsson A, Gustafsson R, Jacobson K, and Erlinge D (2003) Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor. *Br J Pharmacol* **138:**1451–1458.

Wilden PA, Agazie YM, Kaufman R, and Halenda SP (1998) ATP-stimulated smooth muscle cell proliferation requires independent ERK and PI3K signaling pathways. *Am J Physiol* **275:**H1209–H1215.

Wildman SS, Unwin RJ, and King BF (2003) Extended pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions. *Br J Pharmacol* **140:**1177–1186.

Zambon AC, Hughes RJ, Meszaros JG, Wu JJ, Torres B, Brunton LL, and Insel PA (2000) $P2Y_2$ receptor of MDCK cells: cloning, expression and cell-specific signaling. *Am J Physiol* **279:**F1045–F1052.

Address correspondence to: Dr. Michael Sturek, Professor and Chair, Department of Cellular and Integrative Physiology, Indiana University, School of Medicine, 635 Barnhill Drive, MS 309, Indianapolis, IN 46202-5120. E-mail: msturek@iupui.edu