Purinergic 2X<sub>1</sub> Receptors Mediate Endothelial Dependent Vasodilation to ATP


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ABSTRACT

ATP is an important endogenous mediator in the cardiovascular system. It induces endothelium dependent vasodilation, but the precise receptor pathway activated in this response is currently under debate. We have used traditional bioassay techniques to show that ATP-induced vasodilation in mesenteric vessels is endothelium-dependent. Furthermore, ATP-induced vasodilation was inhibited by both suramin and 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), consistent with a P2X1-, P2X2-, or P2X<sub>3</sub>-mediated event and was not potentiated by ivermectin, indicating that these responses were not P2X<sub>1</sub> receptor-mediated. ATP did not induce vasodilation in vessels from P2X<sub>1</sub>−/− mice, confirming an absolute requirement for this receptor. Finally, in pure cell populations of mouse mesenteric artery endothelial cells, we show that P2X<sub>1</sub> mRNA is specifically expressed. However, in line with observations in the brain, the P2X<sub>1</sub> present in endothelial cells does not seem to be recognized by conventional antibodies. Together, these results show that ATP-induced vasodilation is mediated by P2X<sub>1</sub> receptor activation on mesenteric arterial endothelial cells. These observations establish a critical role for P2X<sub>1</sub> receptors in the ATP vasodilator pathway.

Purinergic control of vascular tone is complex, because ATP released in response to vascular insult has dual effects. ATP acts on smooth muscle cells to cause contraction and on endothelial cells to cause vasodilation. ATP and other purines act on purinergic receptors (P2), which are either membrane G-coupled proteins (P2Y) or ion channels (P2X). ATP induces vasoconstriction via activation of P2X<sub>1</sub> (Vial and Evans, 2002) receptors, whereas vasodilator responses induced by ATP are commonly thought to be mediated by P2Y receptors (Carter et al., 1988; Ralevic and Evans, 2002). ATP did not induce vasodilation in vessels from P2X<sub>1</sub>−/− mice, confirming an absolute requirement for this receptor. For example, in the mesenteric circulation, P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>6</sub> receptors are more active in the larger vessels (Carter et al., 1988; Gitterman and Evans, 2000). Furthermore, in addition to P2Y receptors, evidence now suggests that functional P2X<sub>1</sub> receptors are present on human endothelial cells (Yamamoto et al., 2000) and their activation by sheer stress or ATP mediates vasodilation in the mouse (Yamamoto et al., 2006).

Likewise, we have recently published pharmacological evidence suggesting that ATP induces vasodilation via the activation of P2X receptors on mesenteric arteries (Stanford et al., 2001; Harrington and Mitchell, 2004). However, opinion is divided over whether P2X<sub>1</sub> receptors are actually located on the endothelium and smooth muscle (Hansen et al., 1999) or solely on smooth muscle (Vial and Evans, 2002) of blood vessels. This apparent anomaly may be explained by the lack of availability of anti-P2X<sub>1</sub> antibodies, which recognize all forms of P2X<sub>1</sub> (L. S. Harrington and J. A. Mitchell, unpublished observations; Ashour et al., 2006). In the current study, we have used mesenteric vessels from wild-type (P2X<sub>1</sub>−/−) and P2X<sub>1</sub>−/− mice (Mulryan et al., 2006) as a control.
al., 2000; Vial and Evans, 2002) to determine the role of this receptor in the dilator effects of ATP and selective P2X pharmacological tools. The presence of P2X, mRNA in primary isolates of mesenteric endothelial cells was confirmed using molecular techniques.

### Materials and Methods

**Male Black 6 C57 (P2X,+/−, wild-type) or P2X, receptor-deficient (P2X,−/−) mice** (Mulryan et al., 2000) 18 to 24 weeks old were euthanized by lethal exposure to CO₂. The mice were maintained and killed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

The entire mesenteric bed was removed using ligatures, and placed into physiological salt solution (PSS): 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.027 mM EDTA, and 5.5 mM glucose. The mesentery was pinned flat on a dissecting dish containing PSS to allow first-order arteries to be cleaned of fat and connective tissue; these arteries were stored in fresh PSS solution at room temperature until use.

**Isometric Myograph Recordings.** Using tungsten wire, 2-mm segments of artery were mounted in a four channel Mulvany-Halpern myograph (model 610M; Danish Myo Technology, Aarhus, Denmark). The vessels were equilibrated to 37°C, and the solution was bubbled with 95% O₂ and 5% CO₂ for 30 min. The tension of the vessel was normalized, and changes in arterial tone were recorded via a PowerLab/800 recording unit (ADI Instruments Pty Ltd., Sydney, Australia) and analyzed using Chart 4.0 acquisition system (ADI Instruments). In this study, the first-order mesenteric arteries had a mean normalized internal diameter of 198.5 μm.

To assess the viability of the vessels, they were challenged twice with high-potassium solution: 123.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.027 mM EDTA, and 5.5 mM glucose.

A cumulative dose response to the thromboxane mimetic 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂₀ (U46619) was performed (10⁻⁹ to 3 × 10⁻⁷ M). Vessels were contracted to an approximate EC₈₀ of U46619 and the effects of accumulative additions of either ATP or ADP were determined (3 × 10⁻⁵ to 4 × 10⁻⁴ M). If no response was noted, addition of the next concentration of agonist was given after 2 to 3 min. The optimum tension that was gained using the EC₈₀ of U46619 and the effects of accumulative additions of 10⁻⁸ to 10⁻⁶ M potassium solution: 123.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.027 mM EDTA, and 5.5 mM glucose.

A cumulative dose response to ATP was added once the U46619-induced contraction had reached a plateau, and the effects were recorded for 10 min. The optimum tension that was gained using the EC₈₀ of U46619 and the effects of accumulative additions of 10⁻⁸ to 10⁻⁵ M potassium solution: 123.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.027 mM EDTA, and 5.5 mM glucose.

**Immunofluorescence Staining.** Stretched mesenteric arteries were fixed with 4% paraformaldehyde for 30 min at room temperature and stored in phosphate-buffered saline (PBS) 1% Triton X-100. To stain for P2X₇, the fixed arteries were blocked with 5% bovine serum albumin PBS with 1% Triton X-100 and then briefly incubated in 20 nM 4,6-diamidino-2-phenylindole dihydrochloride (DakoCytomation, Carpinteria, CA) in PBS for 30 min at 4°C, and then rinsed once in PBS. Arteries that relaxed in response to ATP-Mediated Dilation Is Absent in P2X₇−/− Mice and Requires an Intact Endothelium in Tissue from Wild-Type (P2X₇+/−) Mice. When added in cumulative concentrations (3 × 10⁻⁶ to 10⁻⁴ M) to precontracted mesenteric vessels from P2X₇−/− mice, ATP induced vasoconstriction followed by vasodilation (Fig. 1A). By contrast, ATP induced neither vasodilation nor vasoconstriction in vessels from P2X₇−/− mice (Fig. 1B).

The effects of ATP were not due to time-dependent loss of tone, because U46619-treated vessels maintained elevated through a 70-μm pore size cell strainer (Falcon; BD Discovery Labware, Bedford, MA).

Cells were incubated with the primary antibody, rat anti-mouse intracellular adhesion molecule II (3 μg/ml; BD PharMingen, San Diego, CA) in PBS for 30 min at 4°C, and then rinsed once in PBS. Endothelial cells were purified by positive selection using magnetic Dynabeads coated with 10 μl (4 × 10⁶) polyclonal sheep anti-rat IgG antibodies (Dynal Biotech, Bromborough, Wirral, UK), incubated for 5 min at 4°C. Intracellular adhesion molecule II positive cells were selected by placing the flask on a flat magnet and leaving for 5 min. Contaminating cells were removed by aspiration, taking care not to disturb bead-bound cells. Flasks were rinsed and placed back on the magnet for 5 min a couple of times, until only positive cells remained.

**P2X, Analysis by rtPCR.** Total RNA was extracted from brain and mesenteric endothelial cells (pooled samples from five mice) from wild-type mice and bladder from P2X₇−/− mice by means of TRizol (Invitrogen). First-strand cDNA synthesis using reverse transcriptase (Promega, Southampton, UK) were independently primed with oligo-dT. Specific primers were designed using Primer3 and Blast programs to amplify P2X₇, von Willebrand Factor (endothelial cell-specific), and smooth muscle cell heavy-chain (SMHC) myosin. The primer sequences were as follows: P2X₇, 5′-ACTGGAAGTTGTGACCGTAGCTC-3′; 5′-CAGCCTCTTGTGTCCTGTA-3′; 5′-GATGTTGTGGTCGAGGAACTG-3′; SMHC myosin, 5′-GGGACTTGAGTGAGGAGCTG; 3′-TTTGAACCTTTTCCTGGCT. The PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized with imaging software under UV light.

**Data and Statistical Analysis.** Contractile or relaxant responses were calculated as a percentage of the original U46619 induced tone, and responses were recorded once plateau was achieved. Data are given as the mean ± S.E.M. for experiments (one animal per experiment).

**Drugs.** All drugs were purchased from Sigma Chemical Co. (Dorset, UK). Drugs were prepared each day were prepared as a high-concentration ‘stock’ solution and were stored at −20°C until used. All drugs were dissolved in aqueous solutions, except for TNP-ATP, which was dissolved in DMSO.

### Results

**ATP-Mediated Dilation Is Absent in P2X₇−/− Mouse and Requires an Intact Endothelium in Tissue from Wild-Type (P2X₇+/−) Mice.** When added in cumulative concentrations (3 × 10⁻⁶ to 10⁻⁴ M) to precontracted mesenteric vessels from P2X₇−/− mice, ATP induced vasoconstriction followed by vasodilation (Fig. 1A). By contrast, ATP induced neither vasodilation nor vasoconstriction in vessels from P2X₇−/− mice (Fig. 1B).
force over the time course of the experiment similarly in tissues from P2X<sub>1</sub><sup>/−</sup> and P2X<sub>1</sub><sup>+/+</sup> mice (Fig. 1C).

ADP (3 × 10<sup>−6</sup> M) also induced vasodilation in mesenteric vessels from wild-type P2X<sub>1</sub><sup>+/+</sup> mice. At concentrations of 10<sup>−5</sup> M and above, ADP induced constrictor responses in mesenteric vessels from the wild-type mice (Fig. 2A). In mesenteric vessels from P2X<sub>1</sub><sup>/−</sup> mice, the vasodilator effects of ADP were enhanced and the constrictor responses abolished (Fig. 2B). Neither ATP nor ADP induced vasodilation in mesenteric vessels from which the endothelium had been removed (Fig. 3).

**P2X<sub>1</sub> Receptor Located in Purified Mesenteric Endothelial Cells.** Pure cell populations of mouse mesenteric artery endothelial cells were prepared using specific antibody-associated magnetic beads (Fig. 4A), and their purity was validated using rtPCR. Endothelial cells expressed the endothelial cell marker von Willebrand factor (Fig. 4B) but was validated using rtPCR. Endothelial cells expressed P2X<sub>1</sub> mRNA (Fig. 4D). However, validated commercial antibodies to P2X<sub>1</sub> protein purchased from Alomone (Ashour et al., 2006) were found not to recognize the form in endothelial cells (Fig. 4, E and F). Similar observations have been made of P2X<sub>1</sub> in parts of the central nervous system (Watano et al., 2004; Ashour et al., 2006).

**Lack of Functional P2X<sub>4</sub> Receptors in Mesenteric Arteries from Wild-Type Mice.** ATP, a potent agonist of P2X receptors, induced vasodilator responses in mesenteric arteries from wild-type and not P2X<sub>1</sub><sup>/−</sup> mice (Fig. 5). Suramin (3 × 10<sup>−5</sup> M), which inhibits all purinergic receptors except for P2X<sub>4</sub>, prevented ATP-induced vasodilator responses in tissues from wild-type mice (Fig. 5C). The selective P2X<sub>4</sub> inhibitor TNP-ATP (3 × 10<sup>−6</sup> M) inhibited the ATP-mediated response in vessels from wild-type mice (Fig. 5VD). Ivermectin (10<sup>−5</sup> M) potentiates P2X<sub>4</sub> receptor-mediated responses and had no effect on ATP-mediated dilation in wild-type or P2X<sub>1</sub><sup>/−</sup> mice (Fig. 5, E and F).

**Pharmacological Characterization of Responses of Mesenteric Arteries from P2X<sub>1</sub><sup>+/+</sup> and P2X<sub>1</sub><sup>/−</sup> Mice to U46619, Acetylcholine, SNP, and KCl.** U46619 was more potent at contracting mesenteric vessels from P2X<sub>1</sub><sup>/−</sup> than vessels from wild-type control mice (Fig. 6A). However, vessels from P2X<sub>1</sub><sup>/−</sup> and control mice relaxed similarly in response to the endothelium-dependent vasodilator acetylcholine (Fig. 6B) or the endothelium-independent vasodilator SNP (Fig. 6C). Contractile responses induced by high potas-
sium did not differ between vessels from wild-type or P2X\textsubscript{1}\textsuperscript{-/-} mice (Fig. 6D), and mesenteric arteries did not assume spontaneous tone at any point.

Discussion

ATP induces both constrictor and dilator responses in vessels. P2X receptors mediate the contractile responses to ATP (Vial and Evans, 2002), whereas debate surrounds the nature of the receptor(s) that mediate vasodilator responses to ATP. Here we have used genetically modified mice and selective pharmacological tools to show that P2X\textsubscript{1} receptors mediate the dilator responses to ATP in mouse mesenteric arteries. We have also used molecular techniques to show that P2X\textsubscript{1} mRNA is expressed in endothelial cells from mesenteric arteries.

We have previously shown that ATP induces an atypical vascular response (Stanford et al., 2001). At low doses, ATP induces a transient dilation mediated by the activation of P2Y receptors and the consequent corelease of NO and prostacyclin. At higher doses, we showed that the dilator response induced by ATP consisted of two discernible phases: the transient phase mentioned above followed by a sustained phase, which we showed to be mediated independently of NO and prostacyclin but consistent with the release of endothelial derived hyperpolarizing factor (Stanford et al., 2001; Harrington and Mitchell, 2004). The emergence of the second and sustained phase of endothelial dependent dilation induced by ATP coincided with the emergence of the typical P2X\textsubscript{1}-mediated vasoconstrictor response.

In the current study, we show that pure populations of endothelial cells isolated from mouse mesenteric artery express P2X\textsubscript{1} mRNA. Furthermore, we showed, using pharmacological tools and genetically modified mice, that ATP-induced vasodilator responses are mediated by P2X\textsubscript{1} in mouse mesenteric arteries. However, we found that currently used antibodies to P2X\textsubscript{1} did not seem to recognize P2X\textsubscript{1} in endothelial cells. Similar results have been published for a form of P2X\textsubscript{1} in the central nervous system (Ashour et al., 2006). It is not yet clear why certain anatomical tissues seem to express and function via P2X\textsubscript{1} without immunogenic reactivity. However, P2X\textsubscript{1} in endothelial cells and/or the central nervous tissues could be a spliced variant or present in a conforma-
tional state restricting access of antibodies to the epitope, as suggested by Ashour et al. (2006). We should also consider the theoretical possibility that, despite endothelial cells expressing P2X4 mRNA and mediating vasodilation to ATP in a P2X1-dependent manner, vascular smooth muscle mediates the initial sensing, sending a signal to the endothelium and then back again.

In addition to the data presented in the current study and in a recent report by Yamamoto et al. (2006) support the view that P2X receptors can be present on endothelial cells and mediate endothelium-dependent vasodilation. Yamamoto et al. (2006) showed that endothelial cells from the pulmonary circulation express P2X4 mRNA but not mRNA for P2X1 or other forms of P2X receptors (Yamamoto et al., 2006). P2X4 receptors can be distinguished pharmacologically by their insensitivity to suramin, an otherwise noneffective purinergic antagonist (Buell et al., 1996; Nicke et al., 2005), low sensitivity to antagonism by TNP-ATP (Virginio et al., 1998; Nicke et al., 2005), and potentiation by ivermectin (Khakh et al., 1999). We found that ATP-induced dilator response in mesenteric arteries from wild type was abolished by TNP-ATP and suramin and not potentiated by ivermectin. These new data show that P2X4 receptors are not functional on mesenteric arteries in mice used in this study. In the current study, we also show that ATP was inactive in mesenteric arteries from P2X1−/− mice.

ADP is thought to mediate endothelium-dependent vaso- dilation via activation of P2Y1 receptors (Nicholas et al., 1996; Guns et al., 2005). In rat mesenteric arteries, the vasodilator effects of ADP, but not ATP, are abolished by the P2Y1 receptor antagonist MRS2179 (Buvinic et al., 2002; Guns et al., 2006), suggesting that in this tissue, ATP does not activate P2Y1 receptors. In the current study, we show that ADP contracted and dilated vessels. The dilator effects were endothelium-dependent and independent of P2X1, consistent with the notion that they are P2Y-mediated.

The constritor effects were mediated by P2X2, indicative of contamination with ATP. The vasodilator effects of ADP were enhanced in P2X1−/−, probably because of the loss of the functional antagonism-induced P2X1-mediated constritor response. Some commercial preparations are known to be contaminated with ATP (Mahaut-Smith et al., 2000), which would seem to be the case with drugs used in this study.

Our observations are likely to have physiological relevance, perhaps at the site of inflammation or thrombosis or after ischemia reperfusion injury, where extracellular levels of ATP are elevated beyond the 10−9M range (Carty et al., 1981; Smolenski et al., 2001; Gourine et al., 2005). Our observations are not due to some unrelated phenotype distortion, because the vessels from P2X1−/− animals relax appropriately when stimulated with acetylcholine, which acts via the endothelium, or SNP, which acts directly on the smooth muscle. We also show that the vasodilator effects of ATP are mediated by the endothelium.

In conclusion, we have shown definitively that activation of P2X1 receptors, most likely located on endothelial cells, mediates ATP-induced vasodilator responses in mesenteric vessels. These observations are likely to have important biological relevance at the site of inflammation or vascular insult where extracellular ATP levels are elevated.

References

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