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$P2X_1$ Receptor-Deficient Mice Establish the Native P2X Receptor and a $P2Y_6$-Like Receptor in Arteries

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ABSTRACT

The contribution of P2 receptors to vasoconstriction of mouse mesenteric arteries was determined using wild-type (WT) and $P2X_1$ receptor-deficient (KO) animals. α,β-methylene ATP (α,β-meATP) and ATP evoked transient inward currents and constrictions of WT mesenteric arteries. In contrast, α,β-meATP (100 μM) and ATP (100 μM) failed to evoke responses in KO arteries from a range of vascular beds. Nerve stimulation (100 pulses at 10 Hz) evoked constrictions of mesenteric arteries. For WT arteries, the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′-5′-disulfonate (PPADS) (30 μM) reduced the amplitude of response by ~50%; the residual constriction was abolished by prazosin (0.1 μM). In KO mice, vasoconstriction induced by nerve stimulation was reduced in amplitude by ~50%, unaffected by PPADS, but was abolished by prazosin. ADP (1 mM) (a $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptor agonist) was ineffective. Because ATP had no effect on mesenteric artery tone from KO mice, this rules out the contribution of $P2Y_2$ receptors. The $P2Y_4$ receptor agonist ITP also failed to contract mesenteric arteries. However, UTP and UDP evoked sustained contractions of mesenteric arteries with similar potency (EC_{50} ~ 10 μM). Complementary studies using reverse-transcriptase polymerase chain reaction showed that mesenteric arteries express $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors. These results demonstrate that homomeric $P2X_1$ receptors underlie the artery smooth muscle P2X receptor phenotype and contribute ~50% to sympathetic neurogenic vasoconstriction and indicate the presence of a UTP- and UDP-sensitive $P2Y_6$-like receptor, but not vasoconstrictor $P2Y_2$ or $P2Y_4$ receptors, on mouse mesenteric arteries.

Purine and pyrimidine nucleotides are released from a variety of sources and act through P2 receptors (ligand-gated P2X receptor cation channels and G protein-coupled P2Y receptors) to regulate arterial tone. ATP is costored and coreleased with noradrenaline from sympathetic nerves, mediates vasoconstriction through artery P2X receptors (Burnstock, 1997), and can account for up to 65 to 100% of the neurogenic response in resistance arteries (Ramme et al., 1987; Gitterman and Evans, 2001). ATP is also released from endothelial (Dubyak, 2002) and blood cells or because of local tissue damage (Burnstock, 1997) and can produce vasoconstriction through the stimulation of P2X and P2Y receptors (Ralevic and Burnstock, 1998). Platelets release vasoactive diadenosine polyphosphates that act through P2X receptors to mediate vasoconstriction (Schluter et al., 1994; Ralevic et al., 1995). The pyrimidines UTP and UDP are released from endothelial cells and platelets and can mediate sustained vasoconstriction through the activation of pyrimidine-sensitive P2Y receptors (Ralevic and Burnstock, 1998). Thus, nucleotides may provide local and systemic control of blood flow.

Seven P2X receptors subunits ($P2X_{1-7}$) have been identified, and the subunits form a variety of homo- and heterotrimeric channels with a range of phenotypes (North and Surprenant, 2000). The $P2X_1$ receptor subunit is expressed at high levels in artery smooth muscle (Vulchanova et al., 1996), and the properties of the native artery P2X channels (α,β-meATP–sensitive transient responses that are antagonized by suramin) correspond to those of homomeric $P2X_1$ receptors (Lewis and Evans, 2000). However, other P2X receptor subunits have also been detected in arteries, e.g., $P2X_2$, $P2X_4$, and $P2X_5$ (Nori et al., 1998; Phillips et al., 1998), and there is pharmacological evidence for the presence of novel diadenosine polyphosphate-sensitive (van der Geit et al., 1999) and suramin-insensitive P2X receptors (Gitterman and Evans, 2000). This raises the distinct possibility that arteries may express heteromeric P2X receptors with properties dominated by the $P2X_1$ receptor subunit. In addition, at rest, the blood pressure of $P2X_1$ receptor-deficient mice was normal or slightly elevated (Mulryan et al., 2000), suggesting that $P2X_1$ receptors may not be essential for the expression of artery P2X receptors. Because of the lack of effective subtype-selective P2X receptor antagonists that can be used in organ-bath

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ABBREVIATIONS: α,β-meATP, α,β-methylene ATP; AP_5A, P^1,P^5-di(adenosine-5′)pentaphosphate; iso-PPADS, iso-pyridoxalphosphate-6-azophenyl-2′-5′-disulfonate; WT, wild type; +/+, wild type; KO, knock-out ($P2X_1$ receptor-deficient); −/−, $P2X_1$ receptor-deficient; bp, base pair; RT-PCR, reverse transcriptase-polymerase chain reaction.

studies, it has been difficult to examine directly the role of the $P2X_1$ receptor in physiological responses or to determine whether other P2X receptor subunits contribute to native artery P2X receptors; therefore, we studied arteries from $P2X_1$ receptor-deficient mice (Mulryan et al., 2000).

Seven mammalian P2Y receptor subtypes have been cloned: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ (Ralevic and Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001). Because of the paucity of selective antagonists, the attribution of molecular correlates of native phenotypes is often taken from agonist potencies. For mouse isoforms, these potencies are the following: $mP2Y_1$ and $mP2Y_{12}$ are ADP-sensitive (Fabre et al., 1999; Leon et al., 1999; Foster et al., 2001), $mP2Y_2$ UTP $\geq$ ATP (Homolya et al., 1999), $mP2Y_4$ UTP $\geq$ ATP $>$ ITP, and $mP2Y_6$ is a UDP receptor (Lazarowski et al., 2001). Mouse orthologs of $P2Y_{11}$ and $P2Y_{13}$ receptors have yet to be cloned, so their exact agonist sensitivities remain to be determined; the human $hP2Y_{11}$ receptor is ATP-sensitive and pyrimidine-insensitive (Communi et al., 1997), and the canine $cP2Y_{11}$ receptor is more sensitive to ADP than to ATP (Qi et al., 2001). The $hP2Y_{12}$ and $hP2Y_{13}$ receptors are ADP-sensitive and negatively coupled to adenylate cyclase (Communi et al., 2001; Hollopeter et al., 2001), and they are unlikely to contribute to vasoconstriction. $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors have been shown to mediate vasoconstriction in a variety of species (von Kugelgen and Starke, 1990; Erlinge et al., 1998; Hartley et al., 1998). However, the expression and properties of arterial P2Y receptors in mice is unclear and remains to be determined. Our preliminary studies on mouse mesenteric arteries indicated that there are marked differences in the complement of vasoconstrictor P2Y receptors on these arteries compared with those in the rat, and this may have important considerations for the use of transgenic mouse models for circulation studies.

In this study, we compared P2 receptor-mediated vasoconstriction in mouse mesenteric arteries from normal and $P2X_1$ receptor-deficient mice to 1) determine the contribution of $P2X_1$ receptor subunits to the native P2X receptor phenotype in arterial smooth muscle and whether, in the absence of $P2X_1$ receptors, there is a residual P2X receptor and 2) characterize P2Y receptor-mediated vasoconstrictions in mouse mesenteric arteries.

Materials and Methods

Adult wild-type (WT, +/+) or $P2X_1$ receptor-deficient (KO, −/−) mice (Mulryan et al., 2000) were killed by cervical dislocation and exsanguinated. A portion of the gut with attached mesenteric arcade was removed and placed in Ringer's solution containing 120 mM NaCl, 11 mM glucose, 25 mM $NaHCO_3$, 5 mM KCl, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 2 mM $MgCl_2$, and then the solution was gassed with 95% O_2/5% CO_2. Mesenteric arteries of different diameters were dissected; large vessels were from the superior mesenteric artery, and medium-sized vessels were from the second- or third-order branches. Femoral, uterine, and tail arteries were also studied.

Immunohistochemical Studies. Mesenteric arteries were dissected as described above and processed for immunohistochemical detection of $P2X_1$ receptors (Vial and Evans, 2000). The primary antibody directed against the $P2X_1$ receptor subtype was obtained from Alomone Labs (Jerusalem, Israel).

RT-PCR Studies. Mesenteric arteries were dissected and disrupted using a sterile blade. Isolation of total RNA was processed with RNeasy Mini Kit (QIAGEN, Dorking, Surrey, UK). Total RNA was then treated with deoxyribonuclease I (amplification grade; Sigma Chemical, Poole, Dorset, UK), and cDNA was synthesized using Superscript II Rnase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA). Amplification of P2Y receptor subtypes was carried out using BIOTAQ DNA Polymerase (Bioline, London, UK) and the primer pairs shown in Table 1. Amplification of the murine β-actin was used as a control of cDNA quality. The PCR thermal profile comprised 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C. The identity of PCR products was confirmed by sequencing.

Constriction Studies. Changes in arterial diameter were measured in vitro using video-imaging microscopy as described previously (Gitterman and Evans, 2000). Data were presented as changes in internal diameter. Agonists were added to the superfusate at 30-min intervals for purine compounds and 15-min intervals for noradrenaline or KCl. Agonists were washed out after a peak/sustained response was observed. Antagonists were superfused for 15 min before being applied concomitantly with the agonist. Trains of electrical-field stimulation (100 pulses at 10 Hz, 50V, 0.25-ms pulse width) were given at 5-min intervals as described previously (Gitterman and Evans, 2001). Electrically evoked constrictions were reversibly abolished by treatment with tetrodotoxin (0.3 μM), demonstrating that they resulted from nerve stimulation.

Patch-Clamp Recording. Medium mesenteric artery smooth muscle cells were dissociated, and patch-clamp recordings were made in response to rapid U-tube application of drugs, as described previously (Lewis and Evans, 2000). Experiments were performed at

TABLE 1
Primer pairs

Receptor	Primer	Amplification Fragment
$P2Y_1$		
Forward	5′-TGGCGTGGTGTACCCTCTCAAGTC-3′	410 bp
Reverse	5′-ACCGTGCTCGCAAATTCATCGTT-3′	
$P2Y_2$		
Forward	5′-ACCAGCGTGCGGGGAACC-3′	440 bp
Reverse	5′-GCATCTCGGGCAAAGCGGACAAGT-3′	
$P2Y_4$		
Forward	5′-TGCCTCGTGCCCAACCTCTTCTTT-3′	499 bp
Reverse	5′-CAGTTGTTCGGCGCTTAGGTGTGC-3′	
$P2Y_6$		
Forward	5′-CCTGGCACTGGCGGACCTGAT-3′	452 bp
Reverse	5′-GGCGGGCCATGCGACAATAAC-3′	
β-actin (control)		
Forward	5′-ATCCATGAAACTACATTCAATTCCAT-3′	199 bp
Reverse	5′-ACCGATCCACACAGAGTACTTGCGC-3′	

a holding potential of −60 mV at room temperature. Voltage-dependent potassium currents were evoked in voltage jumps to +20 mV.

Data Analysis. Data are presented throughout as mean ± S.E.M., with n representing the number of observations. Concentration-response relationships are expressed as the percentage of the maximum response and were fitted by the least-squares method using Origin software (Origin LabCorp, Northampton, MA) with the following equation: response = $\alpha[A]^{n_H}/([A]^{n_H} + [EC_{50}]^{n_H})$. α is the asymptote, n_H is the Hill coefficient, and [A] is the agonist concentration. EC_{50} is the agonist concentration producing 50% of the maximum agonist response, and pEC_{50} is $-\log_{10}(EC_{50})$. Differences between means were determined by the appropriate Student's t test and were considered significant when $P < 0.05$.

Drugs. α,β-methylene ATP (α,β-meATP), ATP, cadmium chloride, collagenase, dithioerythritol, noradrenaline, hyaluronidase, papain, prazosin, suramin, UDP, UTP, P^1,P^5-di(adenosine-5′)pentaphosphate (AP_5A) (Sigma-Aldrich), and iso-pyridoxalphosphate-6-azophenyl-2′-5′-disulfonate (iso-PPADS) (Tocris Cookson Inc., Bristol, UK) were used in this study.

Results

P2X Receptor-Mediated Vasoconstriction in Medium Mesenteric Arteries. The metabolically stable ATP analog α,β-meATP evoked concentration-dependent constrictions of medium (mean internal diameter, 90.0 ± 3.5 μm; n = 55) mouse mesenteric arteries (pEC_{50} = 6.76 ± 0.09, n = 5) (Fig. 1). ATP evoked similar concentration-dependent vasoconstrictions (pEC_{50} = 4.75 ± 0.12, n = 5), albeit with a reduction in potency compared with α,β-meATP (Fig. 1). The low ATP potency probably results from the metabolic breakdown of ATP in the whole-tissue preparation (Benham and Tsien, 1987; Evans and Kennedy, 1994).

Effects of $P2X_1$ Receptor Deficiency on P2X Receptor-Mediated Vasoconstriction and Currents. $P2X_1$ receptor immunoreactivity was expressed at high levels in the smooth muscle layer of the mesenteric arterial wall of WT mice and was abolished in arteries from $P2X_1$ receptor-deficient mice or by use of the blocking peptide (Fig. 2a). α,β-meATP (10 μM) or ATP (100 μM), which evoked maximal responses in normal arteries, had no effect on the diameter of mesenteric arteries from $P2X_1$ receptor-deficient mice (Fig. 2b). Diadenosine pentaphosphate (AP_5A, 100 μM) evoked constrictions of shape and amplitude that were similar to those of α,β-meATP and ATP in WT mesenteric arteries but had no effect on artery diameter in the $P2X_1$ receptor −/− mouse (data not shown).

There may be heterogeneity in the expression of P2X receptor subtypes in different arteries. We therefore were interested to determine the contribution of the $P2X_1$ receptors to vasoconstriction in other peripheral arteries. α,β-meATP (100 μM) and ATP (1 mM) evoked transient constrictions of femoral, tail, uterine, and large mesenteric arteries; these responses were abolished in arteries taken from $P2X_1$ KO mice (n = 4–6) (Table 2).

To confirm that there was no residual P2X receptor-mediated response, we looked directly at P2X receptor-evoked currents in acutely dissociated mesenteric artery smooth muscle cells (Fig. 2c). When applied rapidly under concentration-clamp conditions, ATP (100 μM) and α,β-meATP (10 μM) evoked rapid transient inward currents (mean peak current amplitude = 688 ± 227 and 1195 ± 268 pA, respectively; n = 10 and 14). α,β-meATP (10 μM) and ATP (100 μM) had no effect on the holding current of dissociated mesenteric artery smooth muscle cells from $P2X_1$ receptor-deficient mice. There was no difference in the size of the cells between $P2X_1$ WT and KO mice (capacitance = 11.4 ± 0.8 and 12.8 ± 0.8 pF, respectively; n = 21 and 24) or in the amplitude of voltage-activated potassium currents evoked in WT and $P2X_1$ receptor-deficient mesenteric artery smooth muscle cells (581 ± 82 and 491 ± 64 pA, respectively; n = 28 and 24). These results demonstrate that the $P2X_1$ receptor is essential for the expression of functional arterial smooth muscle P2X receptors.

$P2X_1$ Receptor-Mediated Neurogenic Vasoconstriction. Sympathetic nerves corelease ATP and noradrenaline, and in the majority of peripheral arteries, nerve stimulation results in a vasoconstriction that comprises P2X and α_1-adrenoceptor–mediated components. Sympathetic nerve stimulation (100 pulses at 10 Hz) evoked arterial vasoconstriction; this consisted of an initial rapid peak that declined during the continuation of the train (63.6 ± 2.3% of the initial peak amplitude remains at the end of the 10-s train; n = 8). For WT mesenteric arteries, the P2 receptor antagonist PPADS (30 μM) reduced the amplitude of vasoconstriction by 47.9 ± 6.9% (n = 5). The residual nerve-evoked vasoconstric-

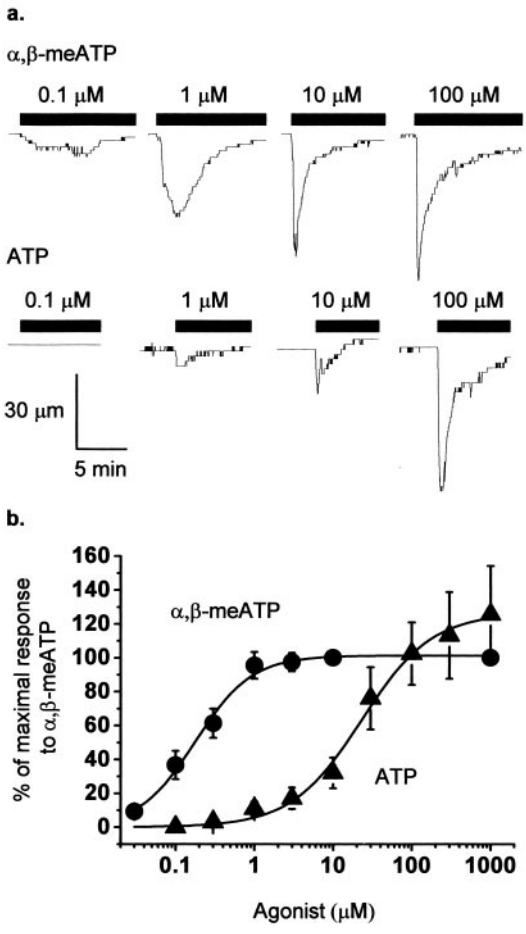

Fig. 1. Concentration dependence of α,β-meATP- and ATP-evoked vasoconstriction in mouse medium mesenteric arteries. a, α,β-meATP- and ATP-evoked transient vasoconstriction. The application period is indicated by the bar (arteries shown; resting internal diameter of arteries was 79.3 and 120 μm, respectively). b, concentration-response relationships for α,β-meATP- and ATP-evoked vasoconstrictions. The data are plotted as the mean percentage ± S.E. of the maximum response to α,β-meATP (n = 6 and 5, respectively).

tion was abolished by coapplication of PPADS and the α_1-adrenoceptor antagonist prazosin (0.1 μM). In contrast, in arteries taken from $P2X_1$ receptor-deficient mice, PPADS had no effect on the amplitude of vasoconstriction (potentiation of 7.6 ± 4.0%, $n = 3$), but the neurogenic response was abolished by prazosin (Fig. 3A). In addition, the amplitude of neurogenic vasoconstriction was significantly reduced for $P2X_1$ receptor-deficient arteries (+/+ 11.2 ± 2 μm and −/− 6.1 ± 1.6 μm, $n = 7$ and 5, respectively; $P < 0.05$). These results demonstrate that the $P2X_1$ receptor makes a substantial contribution to sympathetic nerve-evoked vasoconstriction in WT arteries.

Source of Calcium for $P2X_1$ Receptor-Mediated Vasoconstriction. $P2X_1$ receptor-mediated vasoconstrictions to applied agonists were abolished when the extracellular calcium was removed, demonstrating that calcium influx is essential for the contractile response (data not shown). Calcium could enter the cell either directly through the calcium-permeant P2X receptor and/or by the activation of voltage-dependent calcium channels as a result of P2X receptor-induced membrane depolarization. To determine the contribution of calcium influx through voltage-dependent calcium channels, we used the voltage-dependent calcium-channel blocker cadmium. Cadmium (1 mM) abolished responses to depolarization with 60 mM potassium chloride but had no effect on α,β-meATP (3 μM)-evoked $P2X_1$ receptor constrictions (101 ± 8.4% of control response, $n = 7$) (Fig. 3b). These results indicate that calcium influx directly through the $P2X_1$ receptor mediates vasoconstriction.

Does the $P2X_1$ Receptor Deficiency Result in Compensatory Changes? To investigate possible compensatory changes in artery phenotype, we compared concentration-response relationships in WT and $P2X_1$ receptor-deficient arteries with the application of KCl and noradrenaline. Potassium chloride evoked concentration-dependent vasoconstriction in all arteries tested. Fifty percent of the maximal vasoconstriction was evoked by ~28 to 34 mM KCl for all arteries (Fig. 4a). Similarly, there was no difference in the sensitivity to noradrenaline in WT compared with $P2X_1$ receptor-deficient mice (pEC_{50} = 5.27 ± 0.07 and 4.98 ± 0.13, respectively; $n = 6$ and 7) (Fig. 4B).

Characterization of P2Y Receptor-Mediated Vasoconstriction. ATP-sensitive P2Y receptor-mediated vasoconstrictions have been reported widely in many rat arteries (Ralevic and Burnstock, 1998). Therefore, it was a surprise that ATP (an agonist at recombinant $mP2Y_2$, $mP2Y_4$, and $hP2Y_{11}$ receptors) had no effect on the tone of femoral, tail, uterine, and mesenteric arteries from $P2X_1$ receptor-deficient mice. We focused on the mouse mesenteric artery to characterize the P2Y receptors present. ADP (1 mM), an agonist at $mP2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors, had no effect on the tone of medium mesenteric arteries from $P2X_1$ receptor-deficient mice ($n = 3$), and ADP (100 μM) had no effect on arteries in which the tone had been increased with noradrenaline (10 μM) ($n = 3$). Similarly, the $mP2Y_4$ receptor agonist ITP (300 μM) (Lazarowski et al., 2001) was ineffective as a contractile agonist on arteries from $P2X_1$ receptor-deficient mice ($n = 3$). In contrast, the pyrimidines UTP and UDP evoked concentration-dependent sustained vasoconstriction of normal medium mesenteric arteries with similar potency (pEC_{50} =

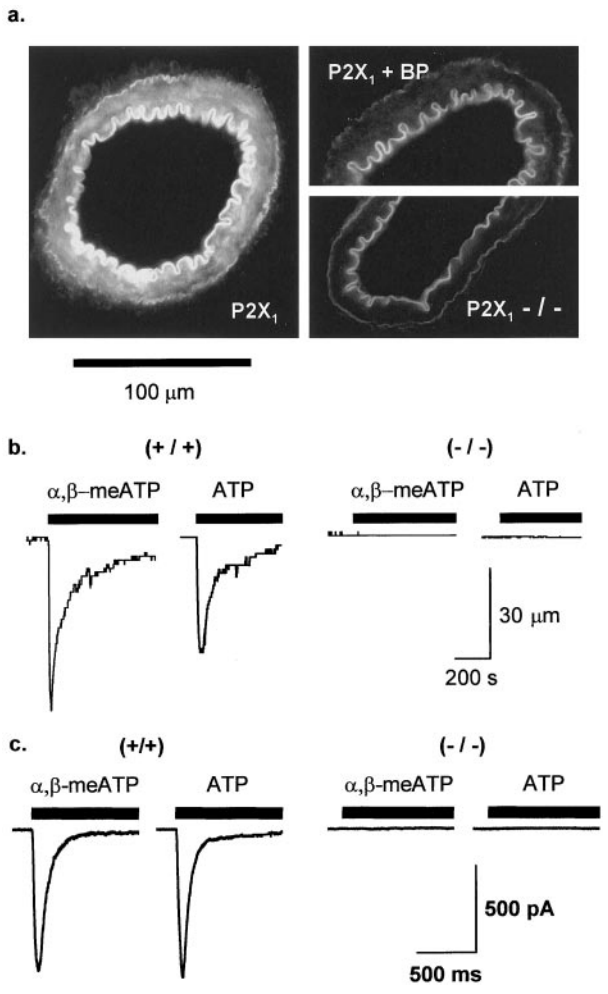

Fig. 2. $P2X_1$ receptor immunoreactivity and the effects of $P2X_1$ receptor deficiency on P2X receptor-mediated contractions and currents in mouse arterial smooth muscle. a, $P2X_1$ receptor immunoreactivity is localized to the smooth muscle layer of medium mesenteric arteries and reduced to background levels by incubation with control antigen-blocking peptide or in tissues from $P2X_1$ receptor-deficient mice (the residual fluorescence is the autofluorescence of elastic lamina). b, 10 μM α,β-meATP- and 100 μM ATP-evoked transient constrictions in WT (+/+) medium arteries were abolished in arteries from $P2X_1$ receptor-deficient (−/−) mice (arteries shown; resting internal diameter was 105 and 153 μm, respectively). c, 10 μM α,β-meATP- and 100 μM ATP-evoked transient inward currents from acutely dissociated smooth muscle cells from WT (+/+) medium arteries. There was no change in the holding current upon application of these agonists from cells from $P2X_1$ receptor-deficient (−/−) mice. Holding potential, −60 mV; drugs were applied for the period indicated by the bar.

TABLE 2
Summary of the agonist sensitivity of different peripheral mouse arteries

Artery	Agonist Sensitivity	
	ATP	α,β-meATP
WT		
Medium mesenteric	Yes	Yes
Large mesenteric	Yes	Yes
Femoral	Yes	Yes
Tail	Yes	Yes
Uterine	Yes	Yes
KO		
Medium mesenteric	No	No
Large mesenteric	No	No
Femoral	No	No
Tail	No	No
Uterine	No	No

4.74 ± 0.15; pEC_{50} = 4.97 ± 0.11; n = 5 and 4, respectively) (Fig. 5a). There was no compensatory change in the potency of UTP (pEC_{50} = 5.05 ± 0.13) or in the amplitude of responses evoked by UDP in mesenteric arteries from $P2X_1$ receptor-deficient mice. The maximum responses to UTP and UDP were 72.9 ± 14.2% and 86.6 ± 8.6%, respectively, of the maximum response to α,β-meATP. UTP and UDP responses persisted in nominally calcium-free extracellular solution (100 ± 12% of the peak response, n = 5) indicative of a G protein-coupled P2Y receptor and not a novel P2X receptor. The P2 receptor antagonist suramin (100 μM) was equally effective in antagonizing responses to an EC_{90} concentration (300 μM) of UTP or UDP (32.3 ± 9.6% and 38.0 ± 0.1% inhibition, respectively; n = 6) (Fig. 5, b and c). Similarly, the P2 receptor antagonist iso-PPADS (30 μM) had an equivalent inhibitory effect on vasoconstrictions in response to an EC_{90} concentration (300 μM) of UTP or UDP (34.0 ± 15.6% and 11.0 ± 3.2% inhibition, respectively; n = 4) (Fig. 5, b and c). These results indicate that there is a UTP- and UDP-sensitive P2Y receptor on mouse arteries.

$P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors have been described in the vasculature. In RT-PCR studies, subtype-selective primers amplified transcripts for $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors (Fig. 6). $P2Y_4$ receptor transcripts were not detected from mesenteric arteries (Fig. 6); however, the primers amplified $P2Y_4$ receptors from genomic DNA (data not shown).

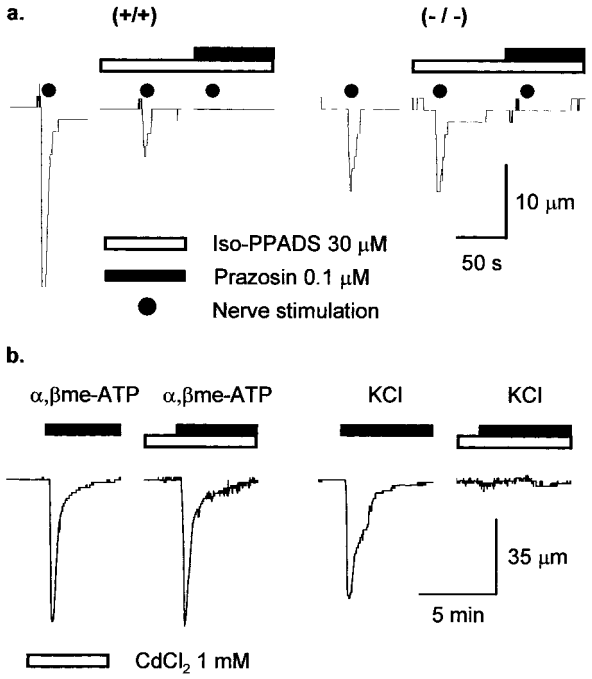

Fig. 3. The contribution of $P2X_1$ receptors to neurogenic vasoconstriction and sensitivity of P2X receptor-mediated vasoconstriction to the calcium-channel blocker cadmium. a, nerve stimulation (100 pulses, 10 Hz) evoked vasoconstriction in WT (+/+) and $P2X_1$ receptor-deficient (−/−) arteries. The P2 receptor antagonist iso-PPADS (30 μM) reduced the amplitude of vasoconstriction for +/+ but not −/− arteries (arteries shown; resting internal diameter was 87 and 102 μm, respectively). Nerve-evoked responses in both +/+ and −/− mesenteric arteries were abolished by the coapplication of iso-PPADS and the α_1-adrenoceptor antagonist prazosin (0.1 μM). b, the nonselective calcium-channel blocker cadmium (1 mM) had no effect on vasoconstrictions evoked by an EC_{90} concentration of α,β-meATP (3 μM); in contrast, similar amplitude vasoconstrictions in response to depolarization by potassium chloride (60 mM) were abolished (artery shown; resting internal diameter was 112 μm).

The RT-PCR studies indicated that mesenteric arteries express multiple P2Y receptor subtypes.

Discussion

In this study, we determined the effect of $P2X_1$ receptor deficiency on the properties of mouse mesenteric arteries and characterized the pharmacology of vasoconstrictor P2Y receptors. The lack of subtype-selective P2X receptor antagonists made it difficult to define conclusively the contribution of the $P2X_1$ receptor to the regulation of arteries. We show that the $P2X_1$ receptor underlies the native P2X receptor-mediated responses in arterial smooth muscle and contributes ~50% to sympathetic nerve-evoked vasoconstriction; in addition, a uridine nucleotide-sensitive but ATP-insensitive $P2Y_6$-like receptor mediates sustained vasoconstriction. Thus, arterial P2 receptors can provide a mechanism for both short- and long-term regulation of blood flow.

In mouse mesenteric arteries, α,β-meATP and ATP evoked transient inward currents and concentration-dependent constrictions. These properties are essentially the same as those of P2X receptor-mediated responses in the majority of arteries studied (Kennedy et al., 1986; Benham and Tsien, 1987). In the $P2X_1$ receptor-deficient mouse α,β-meATP- and ATP-evoked responses were abolished in mesenteric, femoral, uterine, and tail arteries. These results demonstrate for the first time that the $P2X_1$ receptor subunit is essential for the production of functional P2X receptors in a range of arterial

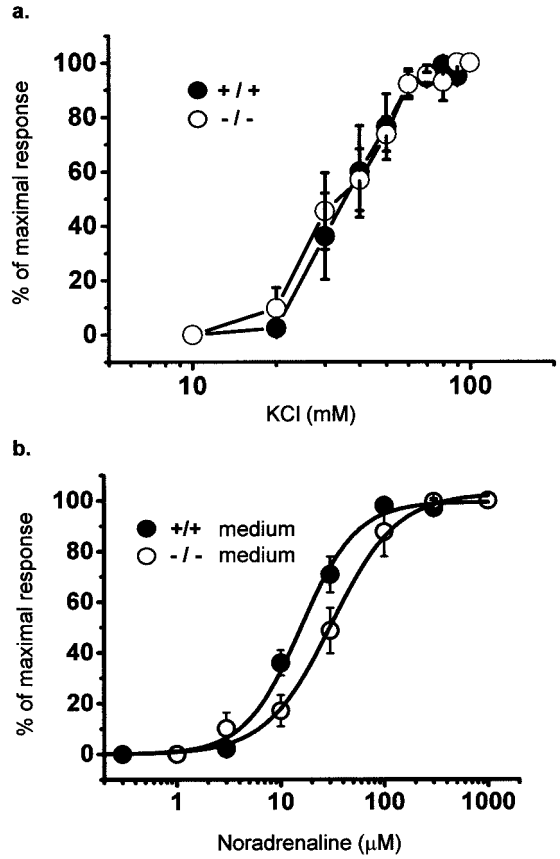

Fig. 4. Lack of compensatory changes on potassium chloride and noradrenaline evoked vasoconstrictions in $P2X_1$ receptor deficient arteries. The sensitivity to either potassium chloride (a) or noradrenaline (b) was unaffected by the $P2X_1$ receptor deficiency. Data shown are mean ± S.E. mean of the maximum response to KCl (n = 4) or noradrenaline (n = 6).

smooth muscles. Previous studies have indicated the presence of additional P2X receptor subunits in rat arterial smooth muscle (Nori et al., 1998; Phillips et al., 1998). ATP (100 μM) is an effective agonist at all recombinant P2X receptors, with the possible exception of the P2X$_6$ receptor, which does not readily form functional channels in recombinant systems; when fully glycosylated, however, it can form functional channels (Torres et al., 1999; North and Surprenant, 2000; Jones et al., 2001). If the native arterial smooth muscle P2X receptor was a heteromeric receptor dominated by the properties of the P2X$_1$ receptor, one would predict that in the P2X$_1$ receptor-deficient mouse there would be a residual phenotype resulting from the expression of non-P2X$_1$ receptor subunits. The lack of residual ATP (100 μM) current or constriction in P2X$_1$ receptor-deficient mouse arteries demonstrates that the native P2X receptor phenotype in arterial smooth muscle is most likely caused by the expression of homomeric P2X$_1$ receptors.

A component of the sympathetic nerve-evoked vasoconstriction in peripheral arteries is resistant to the blockade of α-adrenoreceptors and is mediated by neurally released ATP acting through α,β-meATP–sensitive P2X receptors (Burnstock, 1997). In the present study, the purinergic component accounted for ~50% of the neurogenic response. These stimulation conditions, i.e., a long train of stimulation, have been shown to favor adrenergic transmission, and shorter bursts of stimulation correspond more closely to those recorded under physiological conditions; in resistance arteries, the purinergic component dominates under these conditions (Ramme et al., 1987, Gitterman and Evans, 2001). The characterization of the underlying P2X$_1$ receptor response to applied agonists and the abolition of P2X receptor-mediated vasoconstriction to agonist application or nerve stimulation in mesenteric arteries from P2X$_1$ receptor-deficient mice demonstrate that the P2X$_1$ receptor underlies a significant component of the neurogenic vasoconstriction. This is supported by rat in vivo studies after stimulation of the sympathetic outflow, showing an α,β-meATP–sensitive component of the vasoconstriction (Bulloch and McGrath, 1988) and suggesting that P2X receptors may be important in autoregulation in the kidney (Inscho, 2001). Thus, sympathetic nerves releasing ATP and noradrenaline can mediate vasoconstriction through the activation of P2X$_1$ and α_1-adrenoreceptors. However, at rest, the blood pressure of P2X$_1$ receptor-deficient mice was normal or slightly elevated (Mulryan et al., 2000). Similarly, in mice lacking noradrenaline, the agonist at α-adrenoreceptors and cotransmitter with ATP in sympathetic nerves have normal resting blood pressure (Cho et al., 1999). This suggests that under resting conditions, either P2X$_1$ receptor or α-adrenoreceptor–mediated responses are sufficient to maintain sympathetic regulation of blood pressure. The contribution of P2X$_1$ receptors to blood pressure under conditions of increased sympathetic tone or in disease states remains to be determined. It is interesting in coronary heart failure that P2X$_1$ receptor expression is decreased on coronary arterioles (Malmsjo et al., 1999), sug-

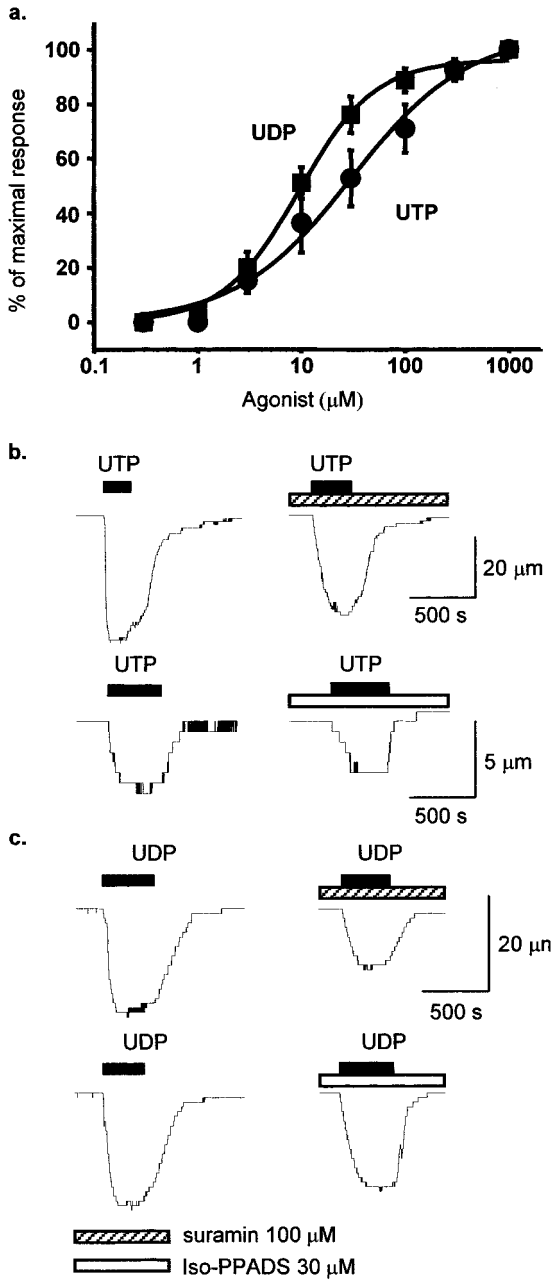

Fig. 5. Comparison of UDP- and UTP-evoked vasoconstriction of normal mouse medium mesenteric arteries. a, UDP evoked concentration-dependent vasoconstrictions of mouse medium mesenteric arteries of potency similar to those observed in response to UTP. b, the amplitude of 300 μM UTP-evoked vasoconstrictions are reduced by the P2 receptor antagonists suramin (100 μM) and iso-PPADS (30 μM) (arteries shown; resting internal diameter was 94 and 68 μm, respectively). c, suramin and iso-PPADS had a similar inhibitory effect on 300 μM UTP-evoked vasoconstriction (artery shown; resting internal diameter was 102 μm).

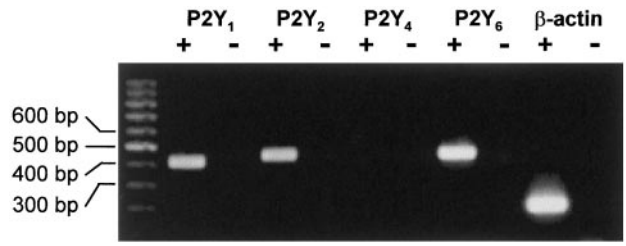

Fig. 6. Identification of P2Y receptor isoforms expressed in mouse medium mesenteric arteries by RT-PCR. RT-PCR showed that P2Y$_1$ (410 bp), P2Y$_2$ (440 bp), and P2Y$_6$ receptors (452 bp) mRNAs were expressed in the medium mesenteric arteries from mouse. However, no P2Y$_4$ receptor (499 bp) mRNA was amplified. β-Actin (199 bp) mRNA amplification was used as a positive control for RT-PCR.

gesting that the removal of this endogenous vasoconstrictor may improve blood flow to the heart. In addition, $P2X_1$ receptor immunoreactivity has been detected in human cerebral arteries (Bo et al., 1998), and $P2X_1$-like receptors mediate vasoconstriction in the cerebral microvasculature (Lewis and Evans, 2000). Because $P2X_1$ receptor-mediated arterial constrictions are resistant to α-adrenoreceptor and calcium-channel antagonists, they may provide a novel drug target for the treatment of cardiovascular disorders, including heart disease and stroke.

The analysis of native P2Y receptors in smooth muscle has been complicated previously by the presence of ATP-sensitive P2X receptors; for example, in rat arteries, ATP-sensitive P2Y receptor-mediated constriction of arteries has been described previously (Saiag et al., 1990). In the present study, ATP-mediated vasoconstrictions were abolished in a range of arteries from $P2X_1$ receptor-deficient mice. This was a surprise and indicates that there is marked species variation in P2Y receptor function. UTP and UDP were equipotent at mouse artery vasoconstrictor P2Y receptors, and the purines ADP and ATP were ineffective. RT-PCR studies indicated that $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors are expressed in mesenteric artery segments; however, whether the RNA transcript amplification corresponds to expression in vascular smooth muscle, endothelial, or blood cells remains to be determined. The lack of ADP- and ATP-evoked responses rules out the functional contribution of $P2Y_1$ (ADP-sensitive) and $P2Y_2$ (ATP-sensitive) receptor subtypes (Cressman et al., 1999; Leon et al., 1999). Three subtypes of molecularly identified P2Y receptors are sensitive to uridine nucleotides ($P2Y_2$, $P2Y_4$, and $P2Y_6$). The receptor in the mesenteric arteries cannot be a $P2Y_2$ receptor because it is insensitive to ATP. Similarly, it is unlikely to be a $P2Y_4$ receptor because this receptor is below the limit of detection by RT-PCR and because the mouse $P2Y_4$ receptor agonist ITP (Lazarowski et al., 2001) is ineffective. This leaves the $P2Y_6$ receptor as a candidate for mediating vasoconstriction.

At recombinant $mP2Y_6$ receptors, UDP is an order of magnitude more potent that UTP, although it has been suggested that the effects of UTP are actually the result of agonist breakdown to UDP, presumably by ectonucleotidases (Lazarowski et al., 2001). Nucleotidases are active in whole-tissue preparations of mesenteric arteries, and the breakdown of ATP in vasoconstriction studies reduced the apparent potency of ATP ~100-fold (ATP and α,β-meATP are equipotent at recombinant $P2X_1$ receptors and when applied under concentration-clamp conditions in patch-clamp studies to dissociated smooth muscle cells). In the present study, UTP and UDP are equipotent; this suggests that it is unlikely that the agonist actions of UTP result solely from interconversion to UDP by ectonucleotidases or from low levels of UDP contamination of commercially available UTP. Also, the high potency of the pyrimidines compared with many other arterial preparations indicates that there is limited agonist breakdown. This suggests that the receptor most probably corresponds to a $P2Y_6$-like receptor with increased potency of UTP. Recently it was shown that P2Y and adenosine receptors can dimerize, resulting in a change in their pharmacological properties (Yoshioka et al., 2001). A similar dimerization of $P2Y_6$ receptors with other P2Y receptors (e.g., $P2Y_1$ or $P2Y_2$) could provide a possible explanation of the $P2Y_6$-like response in mouse mesenteric arteries.

These studies show that arterial vasoconstriction can be rapidly and transiently regulated by ATP released after sympathetic nerve stimulation. They also have firmly established the essential role of $P2X_1$ receptor ligand-gated cation channels and have shown that these receptors may be novel molecular targets for the regulation of blood flow. Pyrimidine nucleotides are released from endothelial cells and platelets and, after damage to the arterial wall, may act through $P2Y_6$-like metabotropic receptors, giving rise to sustained vasoconstriction. This work demonstrates that there are marked species differences in P2Y receptor function in arteries. Given the increased use of transgenic mice, the characterization of the P2Y receptors in mouse arteries may have important considerations for studies on circulation.

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