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# Central Role of $P2Y_6$ UDP Receptor in Arteriolar Myogenic Tone

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## Abstract

**Objective—**Myogenic tone (MT) of resistance arteries ensures autoregulation of blood flow in organs and relies on the intrinsic property of smooth muscle to contract in response to stretch. Nucleotides released by mechanical strain on cells are responsible for pleiotropic vascular effects, including vasoconstriction. Here, we evaluated the contribution of extracellular nucleotides to MT.

**Approach and Results—**We measured MT and the associated pathway in mouse mesenteric resistance arteries using arteriography for small arteries and molecular biology. Of the P2 receptors in mouse mesenteric resistance arteries, mRNA expression of $P2X_1$ and $P2Y_6$ was dominant. $P2Y_6$ fully sustained UDP/UTP-induced contraction (abrogated in *P2ry6*$^{-/-}$ arteries). Preventing nucleotide hydrolysis with the ectonucleotidase inhibitor ARL67156 enhanced pressure-induced MT by 20%, whereas $P2Y_6$ receptor blockade blunted MT in mouse mesenteric resistance arteries and human subcutaneous arteries. Despite normal hemodynamic parameters, *P2ry6*$^{-/-}$ mice were protected against MT elevation in myocardial infarction–induced heart failure. Although both $P2Y_6$ and $P2Y_2$ receptors contributed to calcium mobilization, $P2Y_6$ activation was mandatory for RhoA–GTP binding, myosin light chain, P42–P44, and c-Jun N-terminal kinase phosphorylation in arterial smooth muscle cells. In accordance with the opening of a nucleotide conduit in pressurized arteries, MT was altered by hemichannel pharmacological inhibitors and impaired in *Cx43*$^{+/-}$ and *P2rx7*$^{-/-}$ mesenteric resistance arteries.

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**Disclosures**

None.

**Conclusions—**Signaling through P2 nucleotide receptors contributes to MT. This mechanism encompasses the release of nucleotides coupled to specific autocrine/paracrine activation of the uracil nucleotide $P2Y_6$ receptor and may contribute to impaired tissue perfusion in cardiovascular diseases.

**Keywords**

myocytes; smooth muscle; myogenic tone; myosin light chains; nucleotides; purinoceptor P2Y6; rhoA GTP-binding protein

Myogenic tone (MT) underlies the ability of small arteries to contract in response to increased internal pressure.[1] This mechanism ensures constant organ perfusion, reducing flow as pressure increases, and increasing flow when pressure drops to prevent tissue damage and edema from elevated pressure in capillaries. Abnormal MT has been reported in vascular dysfunctions related to vasospasm,[2] chronic heart failure (CHF),[3] cardiomyopathies,[4] and hypertension.[5] Resetting of MT has been proposed as a valuable strategy to protect sensitive vascular territories.[6]

MT is an intrinsic property of smooth muscle cells (SMCs), occurring independently of neurohumoral or endothelial input. Pressure sensing in SMCs implicates an integrated mechanotransduction, allowing conversion of wall stress into cell contraction.[6] Pressure induces conformational changes in extracellular matrix protein–binding adhesion sites in the cell membrane, mainly integrins.[7] The mechanical stimulus is transmitted to the submembrane space through several mechanosensitive structures. The opening of stretch-operated channels results in cationic (predominantly $Na^+$) current, sustaining membrane depolarization.[6] The resulting secondary calcium entry through voltage-gated $Ca^{2+}$ channels is essential for electromechanical coupling of MT. The molecular identity of cation channels leading to stretch-induced depolarization is unknown, but transient receptor potential channels may have a large role.[8]

Recent data suggest that G-protein–coupled receptors (GPCRs) contribute to MT,[9] at the fore of which is angiotensin II (AngII) type 1 receptor.[10] This receptor, proposed to be a mechanosensor activated in an agonist-independent manner,[11] directly couples to transient receptor potential through the $G_q$-protein pathway. This scheme sequentially associates GPCRs and transient receptor potential channels, mediating the effects of pharmacological inhibitors of both entities on MT.[10] However, several points remain to be clarified: the redundancy of the process with other GPCRs, the means of receptor activation, and the intracellular signaling pathways implicated.

In the vasculature, extracellular nucleotides participate in local control of blood flow through activation of P2 receptors. Two types of P2 receptors, ionotropic $P2X_{1–7}$ and G-protein–coupled P2Y,[1,2,4,6,11–14] bind both purine and pyrimidine.[12] Activation of endothelial P2 receptors induces local vasorelaxation, whereas direct activation of vascular SMC receptors promotes vaso-constriction via $P2X_1$ or pyrimidine-sensitive P2Y receptors.[12] Besides the well-known granular ATP secretion by platelets and nerve terminals, nonvesicular release of nucleotides occurs in virtually all cells. Such release occurs on agonist, chemical, or mechanical stimulation, appearing to involve a variety of anionic pore-forming membrane

proteins, such as pannexins, connexins, $P2X_7$ receptor, or ATP-binding cassette transporters.[13]

Here, we assessed the contribution of extracellular nucleotides and specific P2 receptors to the development of pressure-induced MT.

## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### Expression of P2 Receptors in Mesenteric Resistance Arteries

We evaluated the expression pattern of P2 nucleotide receptors in mesenteric resistance arteries (MRAs) by quantitative reverse transcription polymerase chain reaction. $P2Y_6$ was the most expressed P2Y receptor, followed by $P2Y_1$ and $P2Y_2$ and to a lesser extent $P2Y_{12,13}$ and $P2Y_{14}$ (no detectable $P2Y_4$). Among P2X receptors, $P2X_1$ was most expressed in MRAs, which is in agreement with previous work,[14] followed by $P2X_7$ and $P2X_4$ and to a lesser extent $P2X_5$; $P2X_6$, $P2X_2$, and $P2X_3$ were barely detected (Figure 1A).

### Impaired Contraction in P2ry6$^{-/-}$ MRAs

The contractile response to the uracil nucleotide UDP, its nonhydrolyzable analog UDPβS, and UTP were abrogated in *P2ry6*$^{-/-}$ MRAs (Figure 1B through 1D). Concentration–response curves to phenylephrine, endothelin-1, AngII, and the stable thromboxane A2/PGH2 receptor agonist U46619 were unaffected by the absence of $P2Y_6$ receptor (Figure 1E and 1H). Moreover, endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) relaxations were comparable in both mice strains (Figure 1I and 1J). As previously described in MRAs, ATP induced transient contraction through activation of the $P2X_1$ ligand-gated channel.[14] This response was prevented by pharmacological blockade (NF449) or desensitization (α,β-MeATP) of $P2X_1$ receptor. $P2X_1$ function was normal in *P2ry6*$^{-/-}$ arteries as shown by comparable contractile responses to α,β-MeATP (Figure I in the online-only Data Supplement).

### Interference With Purinergic Signaling Impairs MT: Role of $P2Y_6$ Receptors

Stepwise increases in pressure (10–75 mm Hg) induced MT in MRAs. This response was reproducible over time through 4 successive step increases (Figure II in the online-only Data Supplement). We compared the amplitude of successive myogenic responses after a 20-minute incubation period with inhibitors that interfered with purinergic signaling versus the appropriate vehicle. The ectonucleotidase inhibitor ARL67156 potentiated MT by 32% compared with control, whereas $P2Y_6$ receptor blockers reactive blue-2 or MRS2578 blunted ≈50% of the response (Figure 2A and 2B). Pharmacological blockade of $P2Y_1$ (MRS2179), $P2Y_2$ (suramin), or $P2X_1$ (NF449) receptors did not alter MT (Figure 2B). MT was strongly impaired in *P2ry6*$^{-/-}$ MRAs compared with those of *P2ry6*$^{+/+}$ (Figure 2C through 2E). In human subcutaneous arteries, MT was significantly reduced by $P2Y_6$ blockade with reactive blue-2 (Figure 2F).

Elastic properties of MRAs—passive diameter, media thickness, cross-sectional compliance, distensibility, and passive wall tension—were not modified in *P2ry6*$^{-/-}$ mice (Figure III in the online-only Data Supplement). MT was decreased in *P2ry6*$^{-/-}$ arteries compared with *P2ry6*$^{+/+}$vessels with comparable diameters (Figure IIIf in the online-only Data Supplement), confirming an intrinsic defect in contractility. $P2Y_6$ receptor contribution to MT may vary along the vasculature. We measured $P2Y_6$ receptor expression level, pharmacological contraction, and MT in tail arteries: in *P2ry6*$^{-/-}$, when compared with *P2ry6*$^{+/+}$, lower receptor expression correlated with the absence of uracil nucleotide–induced contraction (data not shown) and comparable MT (Figure IV in the online-only Data Supplement).

## P2ry6 Deletion Protects Against Pathological Increase in MT

To evaluate the potential impact of $P2Y_6$ receptor on blood pressure in vivo, we monitored cardiovascular parameters in conscious, freely moving mice. Three days of continuous recording showed no significant changes in blood pressure of *P2ry6*$^{-/-}$ animals, but heart rate slightly increased (Figure V in the online-only Data Supplement). In contrast, blood pressure in anesthetized (isoflurane) animals significantly increased. When subjected to AngII treatment (1 mg/kg per day for 3 weeks), *P2ry6*$^{-/-}$ mice displayed reduced hypertension compared with wild-type littermates (Figure VI in the online-only Data Supplement). Resistance to AngII-dependent hypertension was recently reported,[15] consistent with a $P2Y_6$ receptor effect on blood pressure regulation in pathological conditions.

CHF is known to induce increased peripheral vascular resistance, partly through enhanced MT.[3] In *P2ry6*$^{+/+}$animals, CHF (induced by coronary artery ligation; Figure VII in the online-only Data Supplement) was associated with a significant increase in MT ( range, 50–100 mm Hg), an increase not observed in *P2ry6*$^{-/-}$ animals (Figure 3A). Concentration–response curves to phenylephrine were comparable in both genotypes and not modified by CHF (Figure 3B).

## $P2Y_6$ Receptor-Induced Signaling Pathways

Besides intracellular calcium mobilization, several signaling pathways participate in MT, including RhoA/Rho kinase, P38 and P42–P44 (extracellular signal–regulated kinases 1 and 2) mitogen-activated protein kinase, and PI3-kinase-$\gamma$.[16–18] We evaluated the ability of $P2Y_6$ receptor to trigger these pathways. UDP, UTP, and ATP induced intracellular calcium increase in wild-type arterial SMCs in a concentration-dependent manner. In *P2ry6*$^{-/-}$ cells, calcium mobilization was abrogated in response to UDP but only slightly diminished in response to UTP and normal in response to ATP, suggesting that $P2Y_2$ receptors (UTP/ATP) are functional and can mobilize calcium in the absence of $P2Y_6$ (Figure 4A). As the expression pattern of receptors in arterial SMCs may vary in culture, we compared nucleotide-induced calcium mobilization in freshly dissociated and early passage (P2) SMCs isolated from MRAs, which gave similar results (Figure VIII in the online-only Data Supplement).

The active form (GTP bound) of the small G-protein RhoA was quantified by pull-down after nucleotide stimulation. UDP, UTP, or UDPβS increased GTP binding to Rho in aortic rings. This response was abrogated in *P2ry6*$^{-/-}$ arteries, whereas response to the thromboxane-A2 analog U46619 was preserved (Figure 4B).

In *P2ry6*$^{+/+}$ SMCs, $P2Y_6$ stimulation (UDP) induced concentration-dependent phosphorylation of myosin light chain associated with a trend toward increased myosin light chain phosphatase (ser-696 and ser-853) and mitogen-activated protein kinase extracellular signal–regulated kinase (P42–P44), P38, and c-Jun N-terminal kinase. These phosphorylations were abrogated in *P2ry6*$^{-/-}$ cells (Figure 4C; Figure X in the online-only Data Supplement).

### Hemichannel-Dependent Nucleotide Release

Endogenous extracellular nucleotide release can occur through the opening of membrane pore-forming proteins belonging mainly to the Panx and Cx families. We evaluated the expression and potential contribution of these proteins to MT. Quantitative reverse transcription polymerase chain reaction performed on RNA isolated from mouse MRAs revealed the presence of Cx, commonly described in the vasculature (*Cx37*>*Cx45*≈*Cx40*>*Cx43*) and Panx1, but not in Panx2 and Panx3 (Figure 5A). Panx1 expression was further shown by immunolabeling (Figure XIII in the online-only Data Supplement). Pharmacological blockers of connexin channels (18α-glycyrrhetinic acid, flufenamic acid, and carbenoxolone) reduced MT significantly, whereas the more selective Panx1 blockers (probenecid, mefloquine, or $^{10}$Panx-blocking peptide) had no effect (Figure 5B), consistent with normal MT in *Panx1*$^{-/-}$ mice despite reduced phenylephrine-induced contraction (Figure 5C and 5D), as previously described.[19] Although Cx37 deletion did not diminish MT (Figure 5E), the response was significantly reduced in *Cx43*$^{+/-}$ and *P2rx7*$^{-/-}$ animals (Figure 5F and 5G). MT was inhibited by KN62, a $P2X_7$ antagonist (Figure 5B). However, *P2rx7*$^{-/-}$ arteries did not display obvious dysfunction, as shown by contraction and relaxation induced by phenylephrine and ACh, respectively (Figure XI in the online-only Data Supplement). ATP, BzATP (benzoylbenzoyl ATP; P2 receptor agonist with good affinity for $P2X_7$), and α,β-MeATP ($P2X_1$ receptor agonist) induced comparable contraction in *P2rx7*$^{+/+}$ and *P2rx7*$^{-/-}$ mice arteries, suggesting the sole involvement of $P2X_1$ in arterial contraction but no involvement of $P2X_7$ receptors (Figure XI in the online-only Data Supplement).

## Discussion

In addition to the well-described neurogenic release of ATP with noradrenaline that contributes to sympathetic tone, uracil nucleotides are vasoconstrictor mediators in many vascular territories through activation of $P2Y_{2,4,6}$ receptors. Despite many in vitro investigations of purinergic signaling in the cardiovascular system, the pathophysiological implications remain misunderstood.[12] In this study, we show that $P2Y_6$ UDP receptor, the most expressed P2Y receptor in mouse resistance arteries, is required for maintaining proper arterial tone. This receptor fully supports in vitro arterial contraction in response to UDP and UTP and substantially contributes to MT through an autocrine/paracrine activation loop.

Our results indicate that MT was also sensitive to pharmacological inhibition of $P2Y_6$ by reactive blue-2 in human subcutaneous arteries, suggesting that some human small arteries may develop MT, at least in part, through the mechanism described here.

This observation adds to the emerging concept that GPCRs participate in MT.[9] A comprehensive scheme of $P2Y_6$ receptor activation and its contribution to MT is represented in Figure 6.

As a $G_q$-coupled receptor, $P2Y_6$ activates phospholipase-Cβ and increases intracellular calcium through inositol triphosphate-sensitive stores[20] and protein kinase C activity through diacyl glycerol formation. We showed that calcium increase was abrogated in *P2ry6*$^{-/-}$ arterial SMCs (freshly dissociated and cultured cells) stimulated by UDP but only marginally affected after UTP/ATP stimulation. These results suggest efficient coupling of $P2Y_2$ (UTP/ATP) receptor to calcium mobilization, whereas $P2Y_6$ modestly contributes to this response. Unlike *P2ry6*$^{-/-}$ animals, *P2ry2*$^{-/-}$ mice did not display altered MT (Figure IX in the online-only Data Supplement) highlighting a nonredundant role of $P2Y_6$ receptor in MT where intracellular calcium increase is minimally involved.[21]

$P2Y_6$ receptor activation induced phosphorylation of mitogen-activated protein kinase, including P38, P42–44, and c-Jun N-terminal kinase. P38 is involved in MT[17] and contributes to blood pressure rise after CHF, a condition characterized by elevated peripheral resistance with a major myogenic component. [22] In contrast, P42/44 extracellular signal–regulated kinase 1/2 is activated by cell stretch with no causal relationship with MT.[23] c-Jun N-terminal kinase is a stress-activated protein kinase sensitive to UV irradiation, heat, and osmotic shock.[24] $P2Y_6$ receptor has also been implicated in the cellular response to these stresses[25,26]; thus, c-Jun N-terminal kinase activation by $P2Y_6$ receptor may represent a cellular stress response, such as SMC response to stretch.

$G\alpha_{12/13}$ proteins activate the small G-protein RhoA and constitute, with Rho-kinase, a calcium-sensitizing pathway, promoting myosin light chain phosphorylation through inhibition of phosphatase. GTP binding to RhoA in response to UDP/UTP was suppressed in *P2ry6*$^{-/-}$ mice. We have already shown that the RhoA–Rho kinase pathway contributes to MT.[27] Thus, activation of the $G_{12/13}$ Rho-kinase pathway may explain a large part of the exclusive contribution of $P2Y_6$ to MT.

Although the real trigger of MT remains to be established, in physiology, maintenance of the response (myogenic contraction) is consistent with protection of downstream capillaries pressure overload: MT lasts when pressure remains elevated. How the same mechanism participates in both triggering and sustaining the response to pressure is an old debate. Although the molecular determinants remain to be identified, membrane tensegrity seems to be central to mechanosensation. Tensegrity stabilizes cell shape by providing continuous tension that depends on cytoskeletal integration of mechanical forces through interactions with the extracellular matrix and adhesion molecules; it results in tangential forces that develop at the membrane.[28] The resulting signal transduction converges on actin–myosin interaction and cellular contraction.[1] Our hypothesis (Figure 6) places the $P2Y_6$ activation loop below mechanical perception, which should therefore be maintained as the tangential

forces are applied to the membrane. Moreover, $P2Y_6$ displays slow desensitization[29]; this, combined with the fact that the receptor activates the calcium-sensitizing pathway, suggests that a single activation after UDP release may last over time.

Mechanical strains are well known to trigger nucleotide release.[30] Here, we propose that vascular cell stretch caused by a rise in intraluminal pressure induces the release of nucleotides that stimulate $P2Y_6$ and promote SMC contraction. This hypothesis may diverge from recent data showing that AngII type 1 receptor can be activated by stretch in an agonist-independent manner.[10] Key observations argue in favor of autocrine/paracrine nucleotide release rather than direct activation by stretch. First, diminishing extracellular nucleotide hydrolysis with an ectonucleoside triphosphate diphosphohydrolase-1 (or CD39) inhibitor, the dominant arterial ectonucleotidase, enhanced MT, in agreement with our previous work showing that MT is exacerbated in *Entpd1*$^{-/-}$ mouse arteries.[31] Second, pharmacological interference with nucleotide-releasing molecules reduced MT similarly to $P2Y_6$ receptor blockade or deletion, in accordance with the effect of a pharmacological connexin channel blockade on MT.[32,33]

Candidate protein-releasing nucleotides should be permeable to anions and mechanosensitive. Such properties are held by connexins and pannexins, suggesting that they open in response to mechanical forces.[34] Unlike connexins, pannexins do not form cell-to-cell channels but only single-membrane channels.[35] Notably, Panx1 is involved in ATP release in response to adrenergic receptor stimulation.[19] Nevertheless, MT was not diminished in *Panx1*$^{-/-}$ arteries and not affected by Panx1 inhibitors, ruling out its contribution to MT.

Similarly, Cx37 can function as a hemichannel.[36] A reverse relationship exists between Cx37 expression level and arterial diameter[37]; we found that Cx37 displayed the largest expression level in MRAs. However, MT was not reduced in *Cx37*$^{-/-}$ arteries—it was potentiated—ruling out its contribution in mechanosensitive-nucleotide release. However, MT was reduced in *Cx43*$^{+/-}$ mice arteries. Notably, the MRAs of *Cx43*$^{-/-}$ mice could not be studied because of the lethality of these mice at birth.[38] The opening of Cx43 hemichannels may involve other partners, among them $P2X_7$ receptors.[39] MT was similarly reduced in MRAs from *P2rx7*$^{-/-}$ mice, and a pharmacological blockade of $P2X_7$ strongly reduced it. $P2X_7$ receptors can form large pores,[40] and heterologous expression allows cells to release ATP in response to hypotonic swelling.[41] The receptor has a long C-terminal cytoplasmic tail that binds to integrins or cytoskeletal elements (α-actin, α-actinin, supervillin)[42] putatively implicated in mechanosensation. These data suggest that the $P2X_7$ opening is sensitive to membrane stretch. $P2Y_6$ and $P2X_7$ receptors are characterized by slow desensitization[29,40]; thus, their contribution to MT may occur in a nontransient manner. Their sensitivity to mechanical deformation and their ability to release nucleotides suggest that $P2X_7$ and Cx43 are involved in MT through cellular nucleotide release. A direct molecular association of the 2 proteins was described in macrophages[39] and could potentially occur in arterial SMCs. Moreover, Cario-Toumaniantz et al[43] reported $P2X_7$-dependent vascular contraction in human saphenous veins: $P2X_7$ activation formed membrane pores permeable to large molecules, promoting myocyte contraction followed by cell lysis when stimulation was maintained. For unknown reasons, we did not observe $P2X_7$-

dependent contraction in our setting (Figure XI in the online-only Data Supplement). $P2X_7$ receptor was initially described as an apoptosis promotor; since then, it has been implicated in many complex cellular processes, including potassium efflux and NLRP3/inflammasome activation,[44] transglutaminase-2 secretion,[45] permeability to chloride,[46] and amyloid precursor protein secretion.[47] Intriguingly, these mechanisms could be dissociated from cellular depolarization. Hence, $P2X_7$ function remains enigmatic; characterization of its activation mechanism in the context of MT represents a challenging future investigation.

Thus, besides the existing therapeutic tools targeting arterial tone, drugs targeting $P2Y_6$ or associated pressure-sensitive pores could more selectively target MT. This is especially important as MT so far cannot be selectively targeted, although resetting it may represent a new therapeutic option.[6] The emergence of GPCRs, such as sphingosine-1-phosphate, prostanoids, cysteinyl leukotriene, or AngII receptors[9,48] as MT modulators, may offer such opportunity.

As demonstrated in this study, purinergic signaling, often presented as a danger signal,[49] is involved in acute arterial response to pressure. This may be important in various vascular disorders at the foreground CHF, a condition associated with increased peripheral vascular resistance and exacerbated MT.[3] We found that the absence of $P2Y_6$ receptor protects against the increase in MT in heart failure after myocardial infarction. In the long-term, chronic overstimulation of $P2Y_6$ may play a role not only in defective tissue perfusion (ie, brain)[50] but also in deleterious arterial remodeling associated with ischemic/inflammatory arteriopathies.[51] Interestingly, recent investigations reported that $P2Y_6$ is also involved in long-term arterial remodeling associated with aging and hypertension through specific synergistic interaction with AngII receptors.[15] Altogether these data indicate that $P2Y_6$ receptor might constitute a valuable target in vascular diseases associated with impaired tissue perfusion.

We describe a new mechanism contributing to MT development that shares a common pathway with cellular stress (inflammation and swelling). GPCRs to date represent the target for nearly half of currently available drugs, but drugs exploit only a limited number of receptors[52]; thus, unraveling the expression and contribution of arterial GPCRs is mandatory. Specific targeting of $P2Y_6$ may be advantageous in a pathological context in which MT is impaired (heart failure, diabetes mellitus, and hypoxia).

## METHODS

### Ethical policies

The investigation for animals handling was performed in agreement with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes (authorization of the laboratory # 00577). Human subjects gave informed consent that was approved by an institutional review committee.

### Chemicals

18α-glycyrrhetinic acid, flufenamic acid, carbenoxolone, probenecid, mefloquine (QUO24-1, Bioblocks, CA, USA). Reactive Blue-2 (RB-2) was from Tebu (Le Perrayen-

Yvelines, France). Antagonists, NF449, MRS2578, MRS2179, ARL67156 and suramin were from Tocris Bioscience (R&D Systems Europe, Lille, France). Other compounds were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Stock solutions of drugs were prepared according to the manufacturers' protocol, and appropriate vehicle controls were used. The effect of pharmacological blockers was tested after an incubation period of at least 15 minutes vs control vehicle.

## Animals

Mice genetically invalidated for $P2Y_6$ (*P2ry6*$^{-/-}$), $P2Y_2$ (*P2ry2*$^{-/-}$), P2X7 (*P2rx7*$^{-/-}$), pannexin1 (*panx1*$^{-/-}$), connexin37 (*Cx37*$^{-/-}$) [1] and connexin43 (*Cx43*$^{+/-}$) were generated as previously described [2–4]. All animals were manipulated in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de l'Agriculture, France, authorization No. 6422). The protocol was approved by the Committee on the Ethics of Animal Experiments of "Pays de la Loire" (permit # CEEA. 2011.14).

## Quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Mesenteric arterial field were dissected in ice cold PSS, dried and stored at −20°C in RNAlater Stabilization Reagent (Qiagen). RNA extraction was performed using the RNeasy® micro kit (Qiagen). 100 ng of RNA extract were used to synthesize cDNA using the QuantiTect® Reverse Transcription kit (Qiagen). Quantitative real-time PCR was performed with Sybr® Select Master Mix (Applied Biosystems) using a Light cycler 480 Real-Time PCR System (Roche). Primer pairs were designed using primer 3 and those presenting a single peak of dissociation and an efficacy ranging from 1.85 to 2.1 were retained (Table 1). Gapdh, Gusb and Hprt were used as housekeeping genes for normalisation. Results were expressed as: $2^{(\text{Ct target gene} - \text{Ct mean of ref genes})}$.

## Animal model of heart failure

Heart failure (HF) was induced by experimental myocardial infarction. Preoperative analgesic buprenorphine injection (Temgesic® 0.1mg/kg subcutaneous) was performed. Animals were anaesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and Xylazine (6 mg/kg) and intubated-ventilated at 120–130 breaths per minute (tidal volume 200 μL). Myocardial infarction was performed by permanent ligation of the left anterior descending coronary artery. In sham-operated controls, the thorax and pericardium were opened, but no ligation was performed. After either procedure, the chest was closed and the mice were extubated and allow recovering on spontaneous respiration. After 6 to 8 weeks, mice developed altered left ventricular dilatation and reduced shortening fraction.

## Functional analysis of MRA

Animals were sacrificed by $CO_2$ inhalation. Mesenteric arteries were dissected in ice-cold physiological salt solution (PSS) of the following composition (mmol/L): 130.0, NaCl; 15.0, $NaHCO_3$; 3.7, KCl; 1.6, $CaCl_2$; 1.2, $MgSO_4$ and 11.0, glucose.

Pharmacological study was performed on 2-mm-long arterial segments mounted on a wire-myograph (DMT, Aarhus, DK) [5]. Cumulative concentration-dependent contraction was

tested on arteries with a disrupted endothelium (flushed with 1ml Triton X-100 0.04 %). Cumulative concentration-response curve to acetylcholine was performed on arteries contracted with phenylephrine (1 μM). Endothelium-independent relaxation was tested at the end of the protocol in response to the nitric oxide donor, sodium-nitroprusside (SNP).

For pressure myography, third order mesenteric arteries (internal diameter 140–220 μm) were cannulated between two glass pipettes and bathed in PSS (pH 7.4, $PO_2$ 160 mmHg, and $PCO_2$ 37 mmHg). Pressure was controlled by a servo-perfusion system and diameter changes and arterial wall thickness were measured continuously. MT was determined in response to stepwise increases in intra luminal pressure from 10 to 125 mmHg using a video-monitored perfusion system (LSI) [6]. At each pressure, a 5–6 min-equilibration period was allowed to achieve stable vessel diameter. The experiment was repeated with $Ca^{2+}$-free PSS containing ethylenbis-(oxyethylenenitrolo) tetra-acetic acid EGTA (2 mM), the non specific phosphodiesterases inhibitor papaverin (100 μmol/L) and sodium nitroprusside (10 μM) to ensure complete arterial relaxation, and the passive diameter was recorded for each pressure. MT at a given perfusion pressure was defined as the magnitude of the percent myogenic tone (%MT) at that pressure. The %MT was expressed by the active (AD) and passive vessel diameters (PD) such that $\%MT = [(PD - AD)/PD] \cdot 100\%$.

### Calcium mobilization assay

Early passages (P3–4) VSMC cultures prepared from enzymatically digested mesenteric arteries were used for in vitro experiments [7]. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics. Intracellular calcium mobilization was measured on Fura-2-acetoxymethyl ester (Invitrogen) loaded cells. Dual excitation at 340/380 nm with single emission at 510 nm was assessed using a Flexstation-3 Microplate Reader (Molecular Devices). Data represent area under the curve of the ratio 340/380 signal normalized to maximal signal obtained by cells permeabilization with 0.1% saponine.

Freshly dissociated cells were used to avoid any bias linked to cell culturing. Smooth muscle cells were dissociated from arteries using a HEPES-buffered isolation solution containing: (in mM) 140 NaCl, 80 sodium glutamate, 5.6 KCl, 2 MgCl2, 10 HEPES, and 10 glucose (pH 7.35 with NaOH). Briefly, mesenteric arteries were placed into isolation solution containing 1 mg/ml papain, 1 mg/ml dithioerythreitol and 1 mg/ml bovine serum albumin (BSA) for 25 minutes at 37°C. Arteries were then immediately transferred to isolation solution containing 0,7 mg/ml collagenase F and 0,3 mg/ml collagenase H (Sigma), 100 μM CaCl2 and 1 mg/ml BSA for 8 minutes at 37°C. Arteries were subsequently washed in isolation solution and dispersed using a pipette to yield single smooth muscle cells. Cells were allowed to adhere in 96 well micro plate coated with type I rat-tail collagen (Santa Cruz) in DMEM cultured medium containing 10% FBS and antibiotics for one night. Intracellular calcium mobilization was measured as described above.

### Measurement of GTP-bound RhoA

RhoA activation was assessed in agonists-stimulated aorta. Thoracic aortas were dissected from perivascular fat and adventitial tissue in icecold PSS and the endothelium was disrupted by perfusion of PSS containing Triton X-100 (0.04 %, 1mL). Aortic rings (2 mm)

were stimulated for 10 min in 37°C heated PSS containing nucleotides or the thromboxane $A_2$ analogue U46619. Tissues were snap frozen in liquid nitrogen, reduced to powder and resuspended in ice-cold homogenization buffer. GTP-bound RhoA content was determined with using G-LISA$^{TM}$ (Cytoskeleton, Denver, CO) according to the manufacturer instructions.

### Western Blot

Smooth muscle cells were obtained from the whole mesentery arterial bed. After removing adventitial peripheral fat with thin forceps MRA were enzymatiquely digested using elastase (0.125 U/ml) and collagenase (2 U/ml) (Worthington, Lakewood NJ) over night at 37°C with agitation in DMEM medium without serum. Cells between P2 and P4 were seeded in 48 well plates and starved once reached 80% of confluence for 12h. Stimulation was performed for 3 min at 37°C stopped with ice cold PBS and directly lysed and homogenized in loadding Buffer: 1% SDS, 10 mmol/L Tris, 1mmol/L Sodium orthovanadate 1 mmol/L, Sodium fluoride 10 mmol/L, β-glycerophosphate 10 mmol/L, complete protease inhibitor cocktail (Roche), 5% β-mercapto ethanol. After boiling 5 min at 90°C, proteins were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were analyzed for phosphorylated proteins using the appropriated antibodies (Table 2).

### Statistical analyses

Data are presented as mean ± SEM. Statistical analyses were performed using Graphpad PRISM (La Jolla, CA, USA). Differences between groups were assessed using two-way ANOVA followed by Fisher's LSD Multiple-Comparison Test except for single dose of inhibitors comparison where one-way ANOVA was used. P values <0.05 were considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **AngII** | angiotensin II |
| **CHF** | chronic heart failure |

| | |
|---|---|
| **GPCR** | G-protein–coupled receptor |
| **MRA** | mesenteric resistance artery |
| **MT** | myogenic tone |
| **SMC** | smooth muscle cell |

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## Highlights

- Tissue perfusion, vascular resistance, and microcirculation integrity rely on pressure-induced myogenic tone. Many aspects of mechanotransduction underlying myogenic tone remain undefined.
- Cellular nucleotides are danger signals released in cell stress conditions, including mechanical strain.
- Several P2 nucleotide receptors are expressed in vascular smooth muscle cells and coupled to vasocontraction, including $P2Y_6$ receptors.
- $P2Y_6$ is the most expressed P2Y receptor in resistance arteries and is mandatory for uracil (UTP and UDP) nucleotide–mediated constriction.
- $P2Y_6$ activation is characterized by Rho-kinase pathway activation, moderated calcium mobilization, and mitogen-activated protein kinase triggering.
- $P2Y_6$ receptors contribute to myogenic tone in physiological conditions and chronic heart failure, a condition associated with exaggerated arterial tone and vascular resistance.
- $P2Y_6$ receptor activation occurs after endogenous nucleotide release through cellular hemichannels, likely involving connexin43 and $P2X_7$ receptor but not connexin37 or pannexin.
- A clear understanding of the cellular and molecular determinants of myogenic tone is lacking. Combining a pharmacological with a gene-targeting approach, we demonstrate the contribution of extracellular nucleotides to this process. Nucleotides can be released by cellular strain and signal danger in inflammatory disease and thrombosis. We show that extracellular nucleotides participate in tonic control of resistance arteries in response to pressure increase through autocrine activation of G-protein–coupled receptor $P2Y_6$. Identification of such partners in the myogenic process may lead to new therapeutic approaches in the treatment of vascular ischemic disorders.

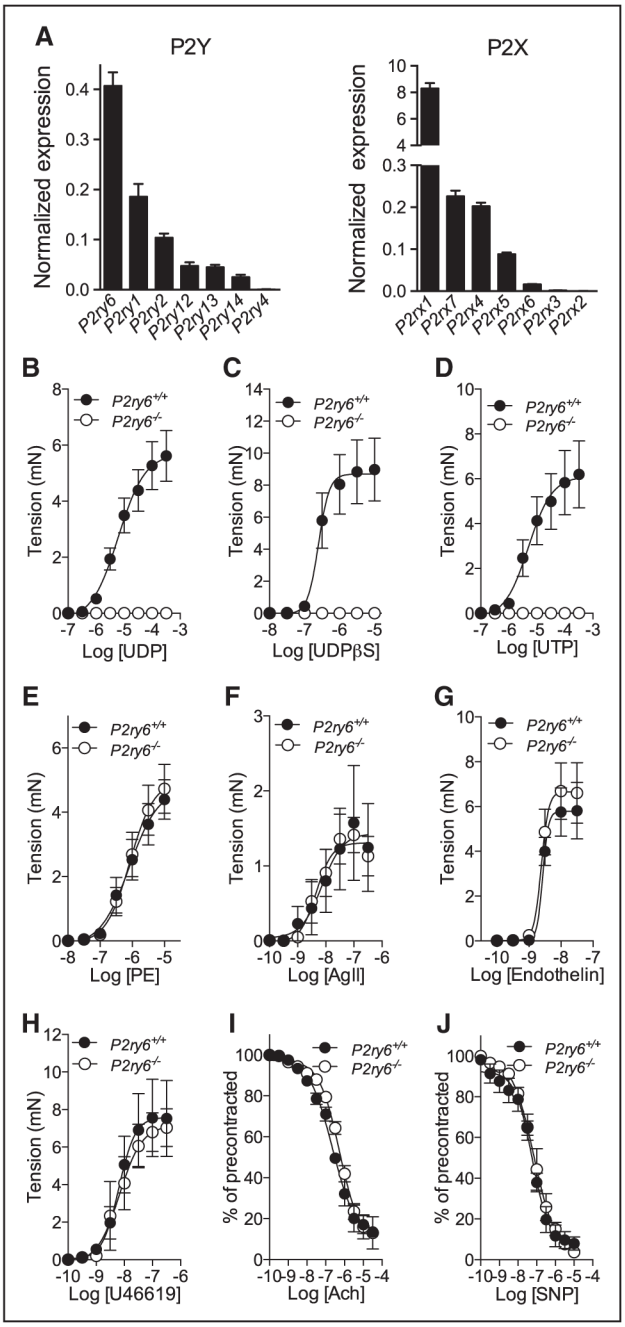


**Figure 1.**

Expression and vasoconstrictor function of $P2Y_6$ receptor in mesenteric resistance arteries. **A**, Relative expression of P2Y and P2X receptors in mesenteric resistance arteries (MRAs) determined by quantitative real-time quantitative reverse transcription polymerase chain reaction (mean±SEM of 5 independent experiments). Dose-dependent contraction developed by endothelium-denuded MRAs in response to UDP (**B**), UDPβS (**C**), UTP (**D**), phenylephrine (PE; **E**), angiotensin II (AngII; **F**), endothelin-1 (**G**), and the stable thromboxane-A2 analog (U46619; **H**) was measured by wire myography. **I** and **J**, Dose-

dependent relaxations in response to sodium nitroprusside (SNP) and acetylcholine (Ach) were measured on intact MRAs. Mean value±SEM of 5 to 8 independent experiments are shown.

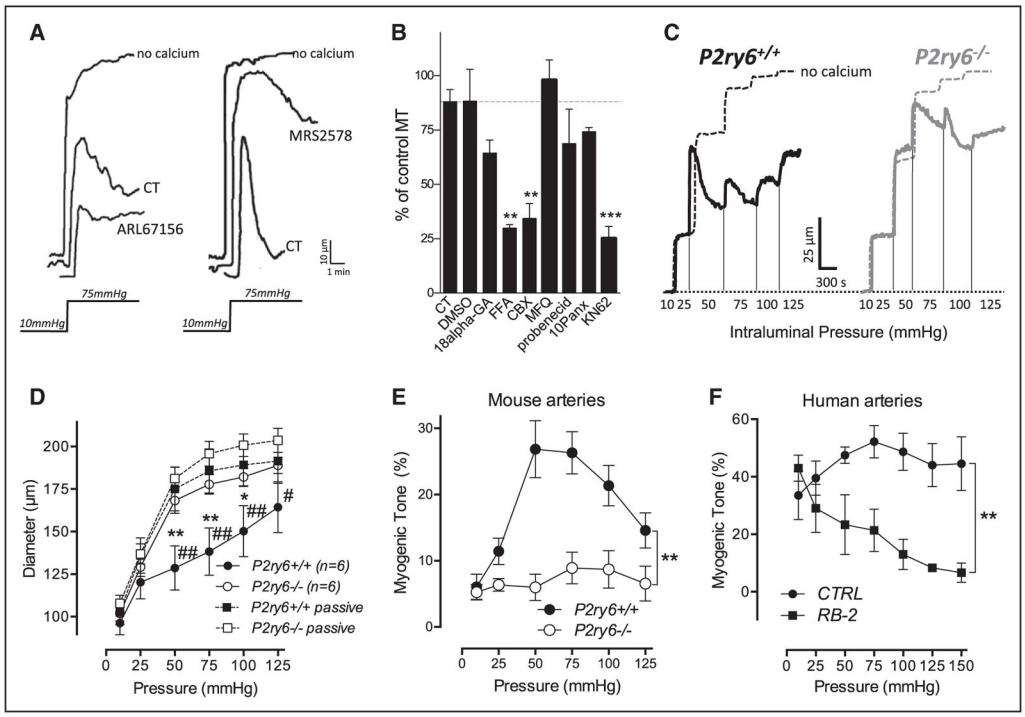


**Figure 2.**

Interference with purinergic signaling affects myogenic response—exclusive contribution of $P2Y_6$ receptor. **A**, Representative traces showing arterial diameter change after a step increase of intraluminal pressure (10–75 mm Hg). The ectonucleotidase inhibitor ARL67156 potentiates myogenic tone (MT), whereas a specific $P2Y_6$ antagonist, MRS2578, inhibits MT (CT, no inhibitors). **B**, Quantification of the effect of ARL67156 (100 µmol/L); pharmacological antagonists of $P2Y_1$ (MRS2179, 20 µmol/L), $P2Y_2$ (suramin, 100 µmol/L), $P2Y_6$ (reactive blue-2 [RB-2], 10 µmol/L; MRS2578, 10 µmol/L), and $P2X_1$ (NF449, 10 µmol/L) receptors; and DMSO (MRS2578, vehicle) on MT. Data represent mean±SEM of 3 to 5 independent experiments. $^{**}P<0.005$ and $^{*}P<0.05$ (1-way ANOVA). Representative traces (**C**) and average of pressure-diameter relationship induced by step increases in intraluminal pressure (**D**). **E**, MT calculated as a percentage of contraction normalized to passive diameter (mean±SEM of 5 independent experiments). $^{*}P<0.05$ and $^{**}P<0.005$, *P2ry6*$^{+/+}$ vs *P2ry6*$^{-/-}$; $\#P<0.05$ and $\#\#P<0.005$, *P2ry6*$^{+/+}$ active vs passive diameter. **F**, MT developed by human cutaneous arteries with or without preincubation with the $P2Y_6$ antagonist RB-2 (10 µmol/L). Data represent mean±SEM of 2 separate donors; $^{**}P<0.05$. Graphs corresponding to the measurement of raw arterial diameter in pressurized human arteries are available in Figure XIV in the online-only Data Supplement. CBX indicates carbenoxolone; CT, control; FFA, flufenamic acid; and MFQ, mefloquine.

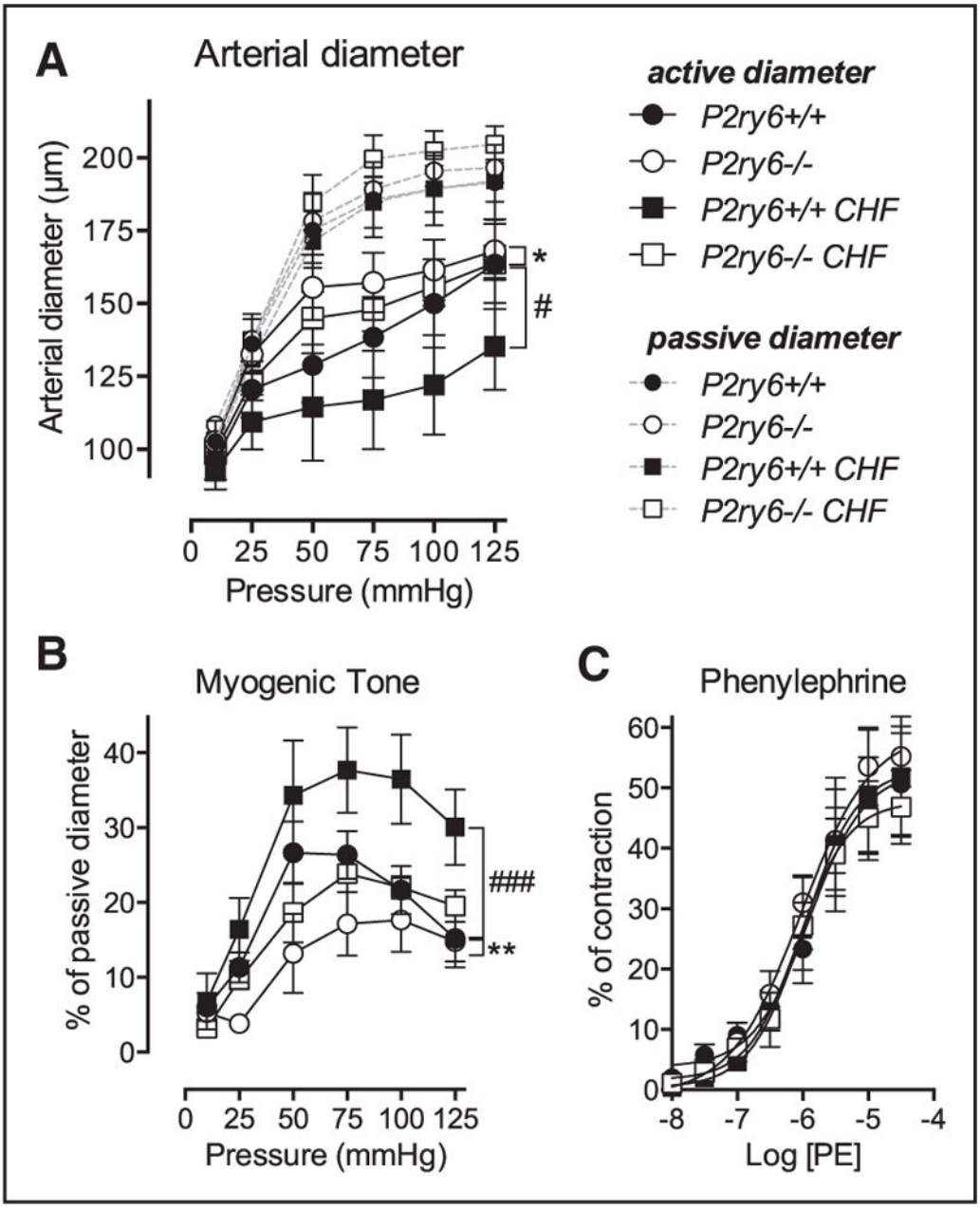


**Figure 3.**

P2ry6 deletion prevents myogenic tone (MT) increase associated with chronic heart failure. Chronic heart failure (CHF) induced by permanent coronary artery ligation significantly enhanced MT in $P2ry6^{+/+}$ but not $P2ry6^{-/-}$ in mice as shown by mesenteric resistance artery diameter changes (**A**) and calculated MT (**B**). **C**, Phenylephrine-induced contraction was equivalent in both genotypes and unaffected by CHF condition. Data represent mean±SEM of 4 to 6 experiments; *$P$<0.05 and **$P$<0.01 according to the genotype; #$P$<0.05 and ###<0.0005 according to the experimental group (CHF or control; 2-way ANOVA). PE indicates phenylephrine.

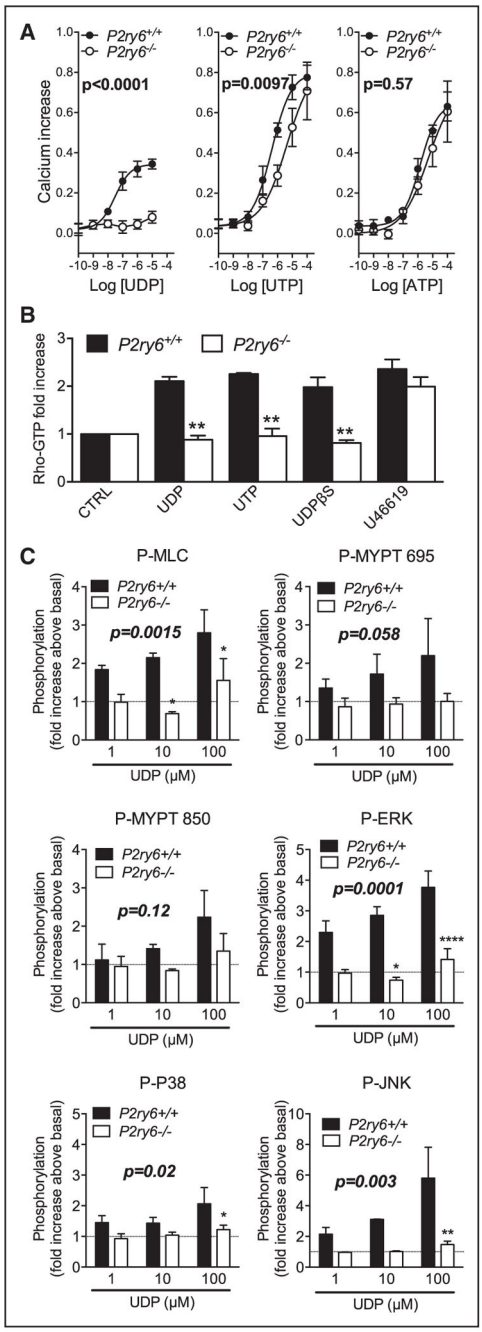


**Figure 4.**

$P2Y_6$ receptor signaling in arterial smooth muscle cells (SMCs) favors myogenic tone (MT). Dose–response curves measuring intracellular calcium were performed on FURA-2–loaded vascular SMCs isolated from *P2ry6*$^{+/+}$ *and P2ry6*$^{-/-}$ mice. **A**, Compared with wild-type cells, *P2ry6* deficiency abolished UDP-induced calcium increase and significantly reduced UTP response but did not affect ATP response in *P2ry6*$^{-/-}$ SMCs. Data represent mean ±SEM of 3 experiments performed on independent cell cultures; **P*<0.05 and ***P*<0.005 (2-way ANOVA). **B**, RhoA activation, measured through its GTP binding by pull-down, was

absent in *P2ry6*$^{-/-}$ aortic rings after stimulation with UDP (100 μmol/L), UTP (100 μmol/L), and UDPβS (10 μmol/L) but remained unaffected after U46619 (1 μmol/L) stimulation. **C**, Reduced phosphorylation assessed by Western blot of MLC, P42–44, and c-Jun N-terminal kinase (JNK) kinases in arterial SMCs isolated from *P2ry6*$^{-/-}$ mesenteric resistance arteries and stimulated with UDP. Data represent mean±SEM of 3 experiments performed on independent cell cultures; **P*<0.05 and ***P*<0.005 (2-way ANOVA). The *P* value according to the genotype is indicated. MLC indicates myosin light chain; P-ERK, phosphorylated form of extracellular signal-regulated kinase; and P-MYPT, phosphorylated form of myosin light chain phosphatase.

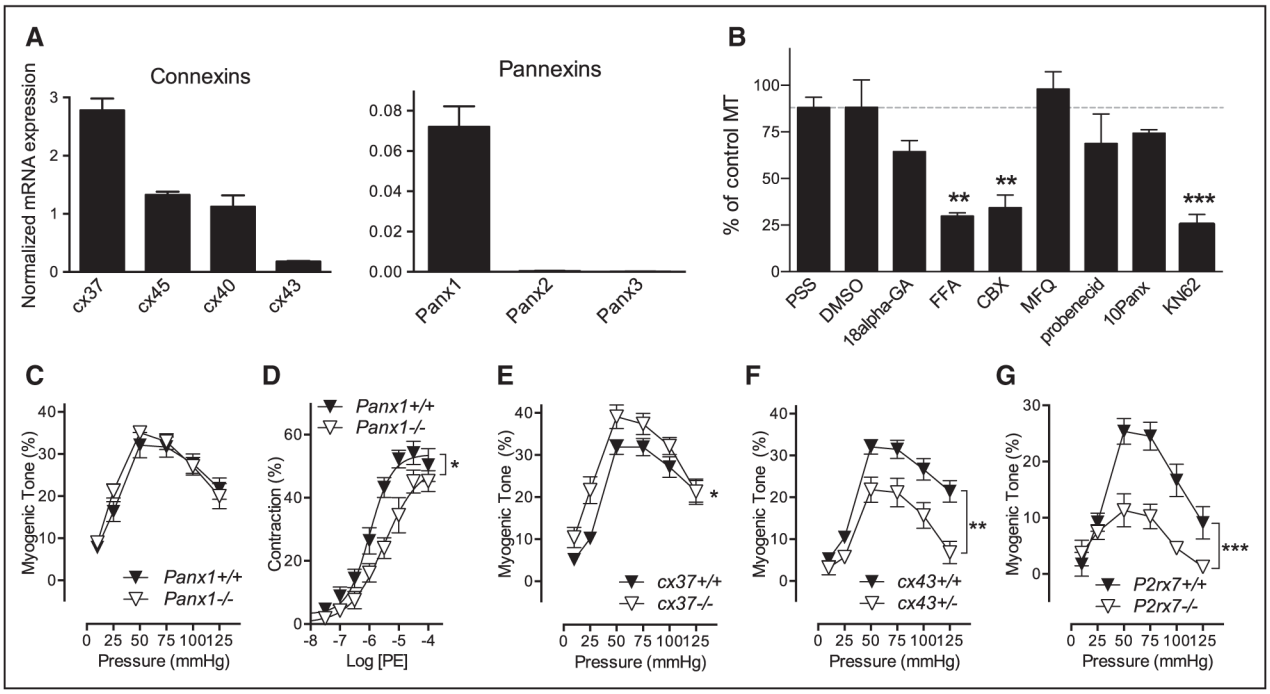


**Figure 5.**

Functional hemichannels contribute to nucleotide release in myogenic response. **A**, Determination of the relative expression of vascular connexins and pannexins by quantitative reverse transcription polymerase chain reaction in mesenteric resistance arteries (MRAs). **B**, Effect of pharmacological inhibitors on myogenic tone (MT; 75 mm Hg). Inhibition of connexins by 18α-glycyrrhetinic acid (18αGA; 100 μmol/L), flufenamic acid (FFA, 100 μmol/L), and carbenoxolone (CBX, 100 μmol/L) reduced the MT in MRAs, as did a $P2X_7$ antagonist, KN62 (0.3 μmol/L). Pharmacological inhibitors of the pannexins mefloquine (MFQ; 10 μmol/L), probenecid (2 mmol/L) and $^{10}$Panx peptide (200 μmol/L) did not affect the response. **C**, *Panx1*$^{-/-}$ mice display normal MT despite (**D**) altered contraction in response to phenylephrine. **E**, MT was slightly enhanced in *cx37*$^{-/-}$ arteries. **F**, MT is altered in *cx43*$^{-/-}$ (mean±SEM of 5 independent experiments) and (**G**) *P2rx7*$^{-/-}$ mice MRAs (mean±SEM of 5 independent experiments); **P*<0.05, ***P*<0.01, and ****P*<0.001 (2-way ANOVA). The graphs corresponding to the measurement of the raw arterial diameter in pressurized arteries is available in Figure XII in the online-only Data Supplement. PE indicates phenylephrine; and PSS, physiological salt solution.

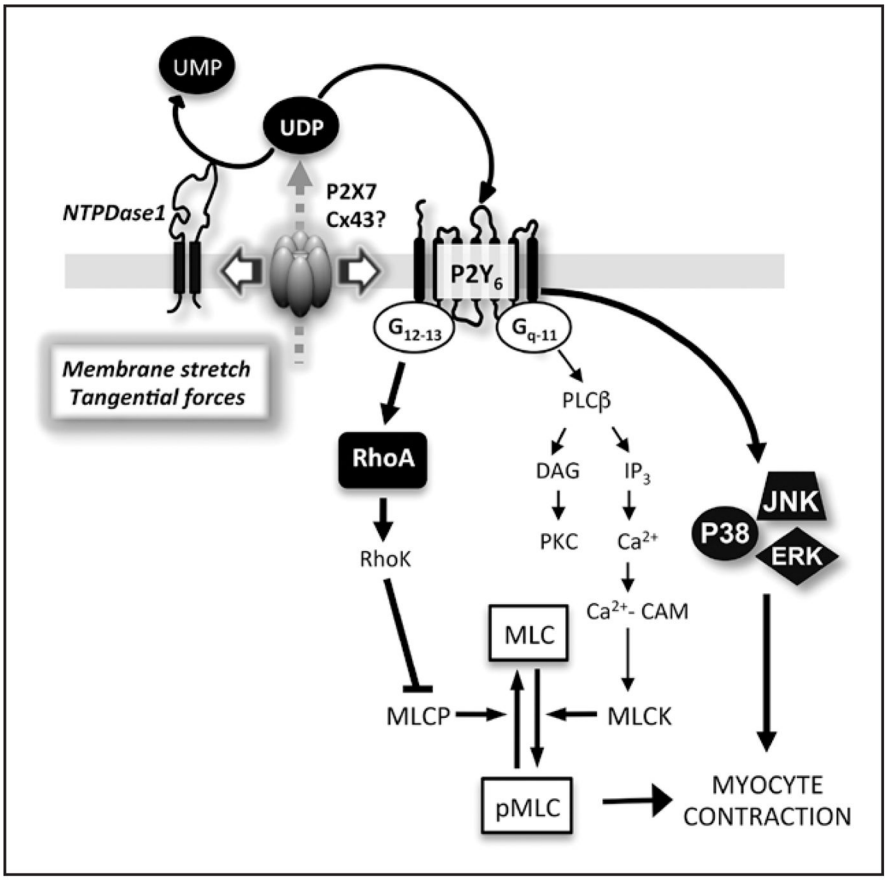


**Figure 6.**

Functional scheme integrating extracellular nucleotide release and $P2Y_6$-dependent pathway in myogenic tone. CaM indicates calmodulin; ERK, extracellular signal–regulated kinases; JNK, c-Jun N-terminal kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PKC, protein kinase C; PLCβ, phospholipase C beta; and pMLP, phosphorylated myosin light chain.

**Table 1**

| Gene | Protein | Gene ID | Amplicon size | Primers sequence | |
|---|---|---|---|---|---|
| | | | | forward | reverse |
| **Gapdh** | **GAPDH** | NM_008084.2 | 121 | ccggggctggcattgctctc | ggggtgggtggtccagggtt |
| **Gusb** | **GUSB** | NM_010368.1 | 72 | ctctggtggccttacctgat | cagttgttgtcaccttcacctc |
| **Hprt** | **HPRT** | NM_013556.2 | 125 | ggggtgggtggtccagggtt | aagacattctttccagttaaagttgag |
| **Gja4** | **Connexin37** | NM_008120 | 59 | tcctgggaaaaagcactgat | ctgtgtctgtccaggtgacg |
| **Gja1** | **Connexin43** | NM_010288.3 | 86 | tcctttgacttcagcctcca | ccatgtctgggcacctct |
| **Gja5 ter** | **Connexin40** | NM_008121.2 | 64 | acaggagttctggtgaacagg | ctagcaggcgagtcaggaag |
| **Gjc1 (Gja7)** | **Connexin45** | NM_001159382.1 | 70 | acaggagttctggtgaacagg | ctagcaggcgagtcaggaag |
| **P2rx1** | **P2X1** | NM_008771.3 | 86 | ccgaagccttgctgagaa | ggtttgcagtgccgtacat |
| **P2rx2** | **P2X2** | NM_153400.3 | 77 | agtcagcatcatcaccagga | ctgtgaaccctcatgctctct |
| **P2rx3** | **P2X3** | NM_145526 | 77 | catcattcccaccatcatca | aggatgatgtcacagagaacagtc |
| **P2rx4** | **P2X4** | NM_011026.2 | 89 | ccaacacttctcagcttggat | tggtcatgatgaagagggagt |
| **P2rx5** | **P2X5** | NM_033321.3 | 77 | cacagtcatcaacattggttcc | aggtagataagtaccaggtcacagaag |
| **P2rx6** | **P2X6** | NM_011028.2 | 64 | tgtccccagtactccttcca | caccagtgattggctgtcc |
| **P2rx7** | **P2X7** | NM_011027.2 | 76 | ctggttttcggcactgga | ccaaagtaggacagggtgga |
| **P2ry1** | **P2Y1** | NM_008772.4 | 60 | ctgtgtggaccccattcttt | tcgggacagtctccttctga |
| **P2ry2** | **P2Y2** | NM_008773.3 | 127 | tgcgctgatgaactttgttt | ggcaggaaacaggaagaaca |
| **P2ry4** | **P2Y4** | NM_020621.3 | 114 | gaagaagcagcagaacacca | caaggagtctgcactggtca |
| **P2ry6** | **P2Y6** | NM_183168.1 | 80 | tcttccatcttgcatgagaca | ggatggtgccattgtcct |
| **P2ry12** | **P2Y12** | NM_027571.3 | 94 | gagacactcatatccttcagattcag | tcgggacagtctccttctga |
| **P2ry13** | **P2Y13** | NM_028808.3 | 60 | atgtgtgagatggggaaagg | gtcccaggggagaaggtg |
| **P2ry14** | **P2Y14** | NM_001008497.2 | 59 | ctttgcctccagaggtgaga | ggctggtttggagggtct |
| **Panx1** | **pannexin-1** | NM_019482.2 | 61 | agaccaagggagaggacca | gctgctcaggtccaaatctt |
| **Panx2** | **pannexin-2** | NM_001002005.2 | 122 | gtacccctgccctacca | gatttccttctcggggagtg |
| **Panx3** | **pannexin-3** | NM_172454.2 | 63 | gaaatctctctggcctcacaa | atacatggccacagccaga |

## Table 2

| Target protein | Reference | Provider |
|---|---|---|
| Phospho-p38 (Thr180/Tyr182) | 4511 | Cell Signaling Technology |
| p38 MAPK | 9212 | Cell Signaling Technology |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | 9101 | Cell Signaling Technology |
| ERK1 | 610031 | BD biosciences |
| Phospho-Akt (Ser473) | 4060 | Cell Signaling Technology |
| Akt (pan) (C67E7) | 4691 | Cell Signaling Technology |
| Phospho-MYPT-1 (Thr850) | 04–773 | Merck Millipore |
| Phospho MYPT-1 (Thr 696) | sc-17556 | Santa Cruz Biotechnology |
| MYPT-1 | 612164 | BD biosciences |
| Phospho-SAPK/JNK (Thr183/Tyr185) | 9251 | Cell Signaling Technology |
| JNK1 | 551197 | BD biosciences |
| Phospho-Myosin Light Chain 2 (Thr18/Ser19) | 3674 | Cell Signaling Technology |
| Myosin Light Chain 2 | 3672 | Cell Signaling Technology |
| Beta-Actin | A5316 | SIGMA-ALDRICH |