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$P2Y_2$ receptor activation decreases blood pressure via intermediate conductance potassium channels and connexin 37

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Abstract

Aims—Nucleotides are important paracrine regulators of vascular tone. We previously demonstrated that activation of $P2Y_2$ receptors causes an acute, NO-independent decrease in blood pressure, indicating this signalling pathway requires an endothelial-derived hyperpolarization (EDH) response. To define the mechanisms by which activation of $P2Y_2$ receptors initiates EDH and vasodilation, we studied intermediate-conductance (KCa3.1, expressed in endothelial cells) and big-conductance potassium channels (KCa1.1, expressed in smooth muscle cells) as well as components of the myoendothelial gap junction, connexins 37 and 40 (Cx37, Cx40), all hypothesized to be part of the EDH response.

Methods—We compared the effects of a $P2Y_{2/4}$ receptor agonist in wild-type (WT) mice and in mice lacking KCa3.1, KCa1.1, Cx37 or Cx40 under anaesthesia, while monitoring intra-arterial blood pressure and heart rate.

Results—Acute activation of $P2Y_{2/4}$ receptors (0.01–3 mg kg^{-1} body weight i.v.) caused a biphasic blood pressure response characterized by a dose-dependent and rapid decrease in blood pressure in WT (maximal response % of baseline at 3 mg kg^{-1}: −38 ± 1%) followed by a consecutive increase in blood pressure (+44 ± 11%). The maximal responses in KCa3.1$^{-/-}$ and Cx37$^{-/-}$ were impaired (−13 ± 5, +17 ± 7 and −27 ± 1, +13 ± 3% respectively), whereas the maximal blood pressure decrease in response to acetylcholine at 3 μg kg^{-1} was not significantly different (WT: −53 ± 3%; KCa3.1$^{-/-}$: −52 ± 3; Cx37$^{-/-}$: −53 ± 3%). KCa1.1$^{-/-}$ and Cx40$^{-/-}$ showed an identical biphasic response to $P2Y_{2/4}$ receptor activation compared to WT.

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Conflict of interest

No conflicts of interest are declared.

Conclusions—The data suggest that the $P2Y_{2/4}$ receptor activation elicits blood pressure responses via distinct mechanisms involving KCa3.1 and Cx37.

Keywords

gap junction; hyperpolarization; K^+ channels; myogenic tone; P2 receptors; signalling

Vascular tone can be regulated via nucleotides like ATP and UTP that are derived from erythrocytes or platelets or released from the endothelium (Ralevic & Burnstock 2003). It is known that ATP is an agonist of $P2Y_2$ receptors and UTP is an agonist of $P2Y_{2/4}$ receptors (Abbracchio *et al.* 2006). A decrease in blood pressure most likely results from ATP inducing endothelium-dependent relaxation (von Kugelgen *et al.* 1987, von Kugelgen & Starke 1990, Ralevic & Burnstock 1991a, 2003). Evidence suggests that this vasodilatory response can be mediated by nitric oxide (NO; Ralevic & Burnstock 1991a), endothelial-derived hyperpolarization (EDH; Malmsjo *et al.* 1999, 2002, Wihlborg *et al.* 2003) and/or prostacyclin (Hammer *et al.* 2003, Wihlborg *et al.* 2003). $P2Y_2$ receptors are found on the endothelium and it was proposed that their activation stimulates the synthesis and release of NO (Ralevic & Burnstock 1991b, Buvinic *et al.* 2002, Burnstock 2009). Moreover, aortic rings from $P2Y_2$ receptor knockout mice ($P2Y_2^{-/-}$) exhibit impaired vasorelaxation in response to ATP, which suggests that NO release is subsequent to $P2Y_2$ receptor activation (Guns *et al.* 2005, 2006). Of note, mechanical destruction of the endothelium abolishes the ATP induced vasodilatory effect and produces a direct vasoconstrictory response on vascular smooth muscle cells (Kennedy & Burnstock 1985, Kennedy *et al.* 1985, Ralevic & Burnstock 1996b). Activation of $P2Y_4$ receptors is associated with vasoconstriction (Dietrich *et al.* 1996, McMillan *et al.* 1999, Rubino *et al.* 1999). In contrast to ATP causing vasodilation, UTP was found to vasoconstrict mouse aortic rings (Boarder & Hourani 1998, Kauffenstein *et al.* 2010) and rabbit inner ear arteries (von Kugelgen *et al.* 1987). However, depending on route of administration, species, localization within the vascular tree or vessel type, UTP can cause vasoconstriction, vasodilation or both (Ralevic & Burnstock 1996a,b, Janigro *et al.* 1997, Horiuchi *et al.* 2001, Guns *et al.* 2005, Rayment *et al.* 2007, Inscho 2009).

In recent studies, we demonstrated that $P2Y_2$ receptors play a physiological role in blood pressure regulation and, as a consequence, $P2Y_2^{-/-}$ mice were found to have salt-resistant hypertension (Rieg *et al.* 2007a, Pochynyuk *et al.* 2010). Direct intra-arterial blood pressure measurements indicated that the blood pressure responses to a $P2Y_{2/4}$ agonist result in vasodilation via a NO-independent mechanism that possibly involves EDH release subsequently to $P2Y_2$ receptor activation. This was concluded because endothelial NO synthase knockout mice ($eNOS^{-/-}$) showed an identical blood pressure effect in response to $P2Y_{2/4}$ receptor activation compared to WT mice. In contrast to WT mice, $P2Y_2^{-/-}$ mice responded to $P2Y_{2/4}$ receptor activation with an increase in blood pressure, possibly a direct effect of $P2Y_4$ receptor activation on vascular smooth muscle cells, which is independent of $P2Y_2$ receptors (Rieg *et al.* 2011).

The vasodilation mediated by EDH requires activation of calcium-activated potassium channels, including KCa2.3 (small-conductance) and KCa3.1 (intermediate-conductance),

which are expressed in most endothelial cells (Kohler & Ruth 2010). In contrast, KCa1.1 (big-conductance) is expressed in vascular smooth muscle cells (Feletou 2009, Kohler & Ruth 2010). The activation of calcium-activated potassium channels is speculated to produce hyperpolarization of the endothelium, which is then transmitted (possibly via connexins, see below) to underlying vascular smooth muscle cells causing vasodilation via EDH. Functional studies employing blockers of calcium-activated potassium channels have demonstrated the role of these channels in EDH and subsequent vascular smooth muscle relaxation (Adeagbo & Triggle 1993, Holzmann *et al.* 1994, Waldron & Garland 1994, Zygmunt & Hogestatt 1996, Eichler *et al.* 2003).

Vascular smooth muscle cells and endothelial cells are functionally linked, and the point of contact between the two cells, the myoendothelial gap junction (MEGJ), plays a key role in the regulation of vascular function (Figueroa & Duling 2009). Initially, it was assumed that a diffusible endothelial factor was the mechanism resulting in hyperpolarization; however, this view was later questioned by experiments demonstrating the involvement of the MEGJ (Griffith *et al.* 2002, Dora *et al.* 2003, Chaytor *et al.* 2005, Mather *et al.* 2005, Sokoya *et al.* 2006). It was concluded from these studies that EDH is transferred from the endothelium to the smooth muscle by direct charge transfer through the MEGJ (de Wit & Wolfle 2007, Feletou & Vanhoutte 2009, Grgic *et al.* 2009, Edwards *et al.* 2010, de Wit & Griffith 2010, Garland *et al.* 2011). Gap junction proteins found in the vasculature include as follows: Cx37, Cx40, Cx43 and Cx45 (Figueroa *et al.* 2004, Lohman *et al.* 2012); however, the MEGJ is specifically comprised of Cx37 and Cx40 which are speculated to conduct the EDH response as an electrical signal between endothelial and vascular smooth muscle cells (Chaytor *et al.* 2005, Isakson & Duling 2005, Haddock *et al.* 2006).

To further define the underlying mechanism(s) involved in the $P2Y_2$ receptor-initiated blood pressure responses, possibly via EDH, we studied blood pressure and heart rate in WT, Cx37, Cx40, KCa1.1 and KCa3.1 knockout (−/−) mice in response to systemic application of a $P2Y_{2/4}$ receptor agonist. We report that $P2Y_{2/4}$ receptor activation generates a similar biphasic blood pressure response in all mice except for $KCa3.1^{-/-}$ and $Cx37^{-/-}$ mice, which show impaired vascular reactivity. This implies that both KCa3.1 and Cx37 are required for full vascular reactivity in response to $P2Y_{2/4}$ receptor activation.

Materials and methods

This study is conform with the guidelines for Acta Physiologica (Persson 2013). $Cx37^{-/-}$ (Fang *et al.* 2011), $Cx40^{-/-}$ (Fang *et al.* 2012) and $KCa1.1^{-/-}$ (Sausbier *et al.* 2004, Rieg *et al.* 2007b) mice were generated and maintained as described previously. Breeder pairs of $KCa3.1^{-/-}$ mice (Begenisich *et al.* 2004) were kindly provided by Dr. Melvin, National Institutes of Health (Bethesda, MD, USA). Ear tissue DNA was used for genotyping by polymerase chain reaction using gene-specific primers (Fenton *et al.* 2014). All mice have been reproduced by heterozygous crossing and are on a hybrid SV129/C57BL6 background. Endothelial NO synthase knockout mice ($eNOS^{-/-}$) were from The Jackson Laboratory (Bar Harbor, ME, USA). Experiments were performed in male adult mice. Mice were housed in the same animal room with a 12 : 12-h light–dark cycle and free access to food (7001, Harlan Teklad, Indianapolis, IN, USA) and tap water.

The compound INS45973 (Inspire Pharmaceuticals, Raleigh, NC, USA), P1-(inosine 5′-)P_4-(uridine 5′-)tetraphosphate tetrasodium salt or $Ip_4U \times 4\ Na^+$, was previously described (Mizumori *et al.* 2009, Rieg *et al.* 2011, Trabanelli *et al.* 2012) and dissolved in 0.85% NaCl solution. The EC_{50} values for INS45973 are as follows: $P2Y_2$: approx. 280 nmol L^{-1}; $P2Y_4$: approx. 280 nmol L^{-1}; $P2Y_6$: >10 μmol L^{-1}; other P2X/P2Y receptors are not activated (Min *et al.* 2003, Mizumori *et al.* 2009). INS45973 hydrolysis by ectonucleotide pyrophosphatase/phosphodiesterase possibly results in a ≤1 min *in vivo* half-life (Vollmayer *et al.* 2003). As INS45973 does not contain adenine, there is no metabolism to adenosine and consequently adenosine receptors are not activated (Shaver *et al.* 2005).

Blood pressure and heart rate experiments

Mice were anesthetized with thiobatubarbital and ketamine as previously described (Rieg *et al.* 2004, 2005). Body temperature was maintained at 37.5 °C by a servo-controlled heating plate as part of the operating table. Mice underwent cannulation of the trachea and were exposed to 100% oxygen throughout the experiment. Cannulation of the jugular vein allowed for i.v. bolus applications and maintenance infusion of 2.25% bovine serum albumin in 0.85% NaCl at a rate of 0.4 mL $h^{-1} \times 30^{-1}$ g bw. Blood pressure was recorded via a catheter placed in the femoral artery. Urine drainage was allowed via a bladder catheter. Following surgery, mice underwent a 60-min stabilization period prior to starting experiments. Vehicle (0.5 μL g^{-1} bw of 0.85% NaCl) or INS45973 (0.01, 0.03, 0.1, 0.3, 1 or 3 mg kg^{-1} bw in escalating doses) were administered via the jugular vein catheter at a rate of 2.4 mL $h^{-1} \times 30^{-1}$ g bw over 25 s and acute blood pressure responses were measured. The interval between applications was 10 min. For certain experiments, acetylcholine (Sigma-Aldrich, St. Louis, MO, USA) was applied following the above-described protocol (0.01, 0.03, 0.1, 0.3, 1, or 3 μg kg^{-1} bw in escalating doses).

Calculations and statistical analysis

Per cent change in mean arterial pressure (MAP) or heart rate was calculated by the following equation:

$$= \frac{\text{maximal MAP response} - \text{MAP immediately before application}}{\text{MAP immediately before application}}$$

Half maximal effective dose (ED_{50}) and mean ± SEM were calculated and analysed using SIGMAPLOT® v11.0 (San Jose, CA, USA) software. Repeated-measures two-way ANOVA was used for comparison of several experimental curves with a control group followed by Dunnett's test. ED_{50} values, blood pressure and heart rate measurements were compared by one-way ANOVA followed by Bonferroni test (all data analysed via GRAPHPAD PRISM® v6.05, La Jolla, CA, USA). Significance was considered at $P < 0.05$.

Results

We have previously shown (Rieg *et al.* 2011) that acute application of INS45973 in WT mice decreased blood pressure rapidly and dose dependently (within 15 s of starting infusion, early phase, Fig. 1 and Table 1). We now provide a more comprehensive dose–

response assessment, which indicates an ED_{50} of 0.4 ± 0.1 mg kg^{-1} for the blood pressure decrease. During bolus application in WT mice, blood pressure began to rise producing a biphasic response with a marked increase in blood pressure (late phase) above baseline (maximum response +44 ± 11 mmHg) immediately after the initial decrease with an ED_{50} of 0.5 ± 0.2 mg kg^{-1}. For comparison, we show $P2Y_2^{-/-}$ mice where INS45973 induced a rapid and dose-dependent (within 15 s of starting infusion, early phase) increase in blood pressure, without showing a distinct biphasic response. In hypertensive $eNOS^{-/-}$ mice (Table 1), bolus application of INS45973 rapidly and dose-dependently decreased blood pressure comparable to WT mice; however, the maximum increase in blood pressure was reduced while the ED_{50} was not significantly different compared to WT mice (Fig. 2 and Table 1). INS45973 did not affect heart rate in WT, $P2Y_2^{-/-}$ and $eNOS^{-/-}$ mice in the early and late phase of the blood pressure responses (Tables 2 and 3). As the $P2Y_{2/4}$ receptor agonist caused biphasic blood pressure effects within seconds of application without affecting heart rate, this likely indicates that there is a direct effect on peripheral resistance. Along those lines, because the blood pressure responses were not different in $eNOS^{-/-}$ compared to WT mice, we concluded that EDH is possibly required for the decrease in blood pressure.

To test for a possible role of EDH for $P2Y_{2/4}$ receptor-initiated blood pressure responses, we studied $KCa3.1^{-/-}$ mice because KCa3.1 channels are speculated to initiate EDH. Baseline blood pressure in $KCa3.1^{-/-}$ mice was comparable to WT mice (Table 1). Bolus application of INS45973 to $KCa3.1^{-/-}$ mice showed a severely impaired dose-dependent decrease in blood pressure in the early phase as well as an impaired increase in blood pressure in the late phase compared to WT mice (Fig. 3 and Table 1). The ED_{50} values of the early and late phase were comparable to WT mice (Table 1). To exclude that the impaired $P2Y_{2/4}$ receptor-initiated blood pressure responses of $KCa3.1^{-/-}$ mice are not caused by a general vascular dysfunction, we tested in a different set of WT and $KCa3.1^{-/-}$ mice (n = 4 each) blood pressure responses to acetylcholine, a well-described vasodilator (Fig. 3). In WT mice, bolus application of acetylcholine induced a rapid and dose-dependent (within 15 s of starting infusion) decrease in blood pressure (ED_{50} of 0.4 ± 0.1 μg kg^{-1}) without showing a biphasic response (increase in blood pressure, late phase). Similarly, bolus application of acetylcholine in $KCa3.1^{-/-}$ mice dose-dependently and rapidly decreased blood pressure. Only at doses of 0.3 and 1 μg kg^{-1} bw was an attenuated acetylcholine-induced decrease in blood pressure observed compared to WT mice; however, the response at the highest dose of 3 μg kg^{-1} bw was not significantly different from WT mice. There was a significant right-shift of the dose–response curve compared to WT mice (ED_{50} of 1.0 ± 0.2 μg kg^{-1}, $P < 0.05$ vs. WT). Acetylcholine did not affect heart rate in WT and $KCa3.1^{-/-}$ mice (not shown). KCa1.1 is expressed in vascular smooth muscle cells and is important for vasodilation and regulation of vessel diameter, contributing to the hypertension found in $KCa1.1^{-/-}$ mice (Sausbier *et al.* 2005, Rieg *et al.* 2007b), a finding confirmed in the current study (Table 1). However, $KCa1.1^{-/-}$ mice showed a decrease in blood pressure in the early phase as well as an increase in blood pressure in the late phase that was comparable to WT mice (Fig. 4 and Table 1). Both the early and late phase ED_{50} values were comparable to WT mice (Table 1). INS45973 did not affect heart rate in WT, $KCa3.1^{-/-}$ and $KCa1.1^{-/-}$ mice in the early and late phase of the blood pressure responses (Tables 2 and 3). These data indicate that KCa3.1

is required for the blood pressure effects following $P2Y_{2/4}$ receptor activation while KCa1.1 in not part of the signalling pathway activated via $P2Y_{2/4}$ receptors.

As gap junctions play a critical role in EDH, we studied the blood pressure response to INS45973 in animals lacking either Cx37 or Cx40, the two most abundant connexins found in the MEGJ. Baseline blood pressure in $Cx37^{-/-}$ mice was, as previously described (Figueroa & Duling 2008), comparable to WT mice (Table 1). Bolus application of INS45973 to $Cx37^{-/-}$ mice showed an impaired dose-dependent decrease in blood pressure in the early phase as well as an impaired increase in blood pressure in the late phase (Fig. 5 and Table 1). The early and late phase ED_{50} values were not significantly different in $Cx37^{-/-}$ vs. WT mice (Table 1). In a different set of $Cx37^{-/-}$ mice ($n = 4$), blood pressure responses to acetylcholine were tested (Fig. 5). Bolus application of acetylcholine induced a rapid and dose-dependent (within 15 s of starting infusion) decrease in blood pressure (ED_{50} of 0.6 ± 0.2 μg kg^{-1}, not significant vs. WT) without showing a biphasic response (increase in blood pressure in the late phase). Acetylcholine did not affect heart rate in $Cx37^{-/-}$ mice (not shown). Baseline blood pressure in $Cx40^{-/-}$ mice was significantly higher compared to WT mice (Table 1), a finding possibly related to the activated renin-angiotensin system in these mice (Krattinger *et al.* 2007). Bolus application of INS45973 to $Cx40^{-/-}$ mice showed a decrease in blood pressure in the early phase as well as an increase in blood pressure in the late phase that was comparable to WT mice (Fig. 6 and Table 1). Both, the early and late phase ED_{50} values, were comparable to WT mice (Table 1). INS45973 did not affect heart rate in WT, $Cx37^{-/-}$ and $Cx40^{-/-}$ mice in the early and late phase of the blood pressure responses (Tables 2 and 3). These data indicate that Cx37, but not Cx40, takes part in the regulation of vascular tone in response to $P2Y_{2/4}$ receptor activation.

Discussion

In this study, we utilized a $P2Y_{2/4}$ receptor agonist and UTP analog, INS45973, to better understand the role of $P2Y_2$ and $P2Y_4$ receptors for acute systemic blood pressure responses. These are, to our knowledge, the first *in vivo* studies providing direct evidence about the involved proteins mediating vascular effects, possibly via EDH, by $P2Y_{2/4}$ receptor activation. Our data indicate that KCa3.1 and Cx37 take part in $P2Y_{2/4}$ receptor-initiated blood pressure responses. As the $P2Y_{2/4}$ receptor agonist caused biphasic blood pressure effects within seconds of application without affecting heart rate, this likely indicates that there is a direct effect on peripheral resistance. In Figure 7, we depict a model summarizing the blood pressure effects of the studied proteins in this work in response to $P2Y_{2/4}$ receptor activation.

In the current study, we confirmed our previous findings (Rieg *et al.* 2007a) and expanded on the acute effects of $P2Y_{2/4}$ receptor activation on blood pressure. In mice tested in this study, activation of $P2Y_{2/4}$ receptors was able to decrease blood pressure. The findings of the current study are conform with other studies where acute i.v. application of ATP and UTP in anesthetized mice induced a dose-dependent decrease in blood pressure, while other receptors like $P2Y_1$, $P2Y_4$ and $P2X_1$ were excluded by the use of pharmacological compounds (Shah & Kadowitz 2002). In aortas of $P2Y_2^{-/-}$ mice, ATP-evoked relaxation was impaired (Guns *et al.* 2006) and, vice versa, ATP- and UTP-induced vasorelaxation

were not different in aortas of WT and $P2Y_4^{-/-}$ mice (Guns *et al.* 2005). The hypothesis that EDH may mediate such blood pressure responses comes from studies in $eNOS^{-/-}$ mice which show that the blood pressure decrease in response to $P2Y_{2/4}$ receptor activation was unaffected compared to WT mice (Rieg *et al.* 2011), as well as from studies in the human forearm vasculature where eNOS blockade had no effect on UTP-mediated changes in forearm blood flow or vascular resistance (Hrafnkelsdottir *et al.* 2001, Crecelius *et al.* 2011). Along those lines, it was proposed that EDH is a major mediator for nucleotide-induced vasodilation in the peripheral vascular bed (Malmsjo *et al.* 2002) and human vascular endothelial cells (Raqeeb *et al.* 2011).

Which signalling pathways mediate the $P2Y_2$ receptor-initiated blood pressure decrease? In EDH-related signalling, hyperpolarization via KCa3.1 and KCa2.3 are key steps and are also required to initiate gap junction-dependent vasodilations (Kohler & Ruth 2010). KCa3.1 and KCa2.3 have been shown to be crucial in agonist-induced hyperpolarization and vasodilation, according to experiments employing pharmacologic, electrophysiological and genetic approaches (Si *et al.* 2006, Brahler *et al.* 2009). Our experiments in $KCa3.1^{-/-}$ mice provide new evidence for such a contribution: the blood pressure decrease as well as blood pressure increase was significantly impaired in response to selective $P2Y_{2/4}$ receptor activation. The severely impaired vasodilation indicates that KCa3.1 has a prominent role for electrically mediating this response. Of note, in conscious dogs activation of KCa3.1 via SKA-31, an activator of KCa3.1 (EC_{50} approx. 0.26 μmol L^{-1}) and KCa2 channels (EC_{50} approx. 2.9 μmol L^{-1}), rapidly decreased blood pressure by an immediate electrical vasodilator mechanism (Damkjaer *et al.* 2012) and application of SKA-31 potentiated EDH-mediated vasodilations in carotid arteries of WT mice but not $KCa3.1^{-/-}$ mice (Sankaranarayanan *et al.* 2009). Our *in vivo* data are the first linking $P2Y_{2/4}$ receptors to KCa3.1-mediated hyperpolarization. Our data did not show an increased blood pressure in $KCa3.1^{-/-}$ mice that was described before (Brahler *et al.* 2009). Of note, the increased blood pressure in $KCa3.1^{-/-}$ mice was restricted to the dark phase (activity phase) of the mice, which could explain why the blood pressure in our $KCa3.1^{-/-}$ mice was not significantly different from WT mice because our experiments were performed during the light phase (quiet phase). Our results confirm other data studying acetylcholine responses in $KCa3.1^{-/-}$ mice. While at intermediate doses the acetylcholine-induced decrease in blood pressure was impaired, the response at the highest dose was not significantly different from WT mice (this study). Consistent with this finding, at lower doses acetylcholine-induced vasodilation of $KCa3.1^{-/-}$ mice cremaster microcirculation and carotid arteries was impaired, while higher doses induced equivalent vasodilation compared to WT mice (Brahler *et al.* 2009, Wolfle *et al.* 2009). Along those lines, arguing against the possibility that the abrogated $P2Y_{2/4}$ agonist-induced decrease in blood pressure is due to an incapability of $KCa3.1^{-/-}$ mice to vasodilate are the findings that sodium nitroprusside- (direct NO donor) and adenosine-induced vasodilation was not impaired in $KCa3.1^{-/-}$ compared to WT mice (Si *et al.* 2006, Wolfle *et al.* 2009). In contrast to KCa3.1 localized to endothelial cells, KCa1.1 is localized to vascular smooth muscle cells and was shown to be important for the regulation of myogenic tone (Sausbier *et al.* 2005, Rieg *et al.* 2007b, Kohler & Ruth 2010). Even though $KCa1.1^{-/-}$ mice show a complete lack of membrane hyperpolarizing spontaneous potassium outward currents (Sausbier *et al.* 2005) our data indicate that the acute blood pressure

responses via $P2Y_{2/4}$ receptor activation do not require functional KCa1.1 because neither vasodilation or vasoconstriction were different from WT mice. Along those lines, adenosine-induced relaxation of myogenic tone of tibial arteries in KCa1.1$^{-/-}$ mice was similar to WT mice (Sausbier *et al.* 2005).

One of the major aims of the current study was to determine whether Cx37 and/or Cx40 are required for $P2Y_{2/4}$ receptor-initiated blood pressure responses. Our results show that $P2Y_{2/4}$ receptor activation requires fully functional Cx37 because Cx37$^{-/-}$ mice show impaired vasodilation and vasoconstriction, suggesting that Cx37 possibly contributes to connecting the EDH signal between endothelial and vascular smooth muscle cells. In contrast, the acetylcholine-induced decrease in blood pressure was not different between Cx37$^{-/-}$ and WT mice. Consistent with our data, the acetylcholine-induced local or conducted vasodilator response was unaffected in Cx37$^{-/-}$ mice (Figueroa & Duling 2008). The blood pressure responses in Cx40$^{-/-}$ mice were comparable to WT mice, indicating that $P2Y_{2/4}$ receptor-initiated EDH-type vasodilatations may be completely independent of Cx40 expression in the MEGJ. Consistent with this finding, arteriolar dilatations induced by SKA-31 were not attenuated in endothelial-specific Cx40$^{-/-}$ mice, indicating that the KCa3.1-mediated EDH signal is able to induce vasodilatation even in the absence of endothelial Cx40 (Radtke *et al.* 2013). Notably, lack of Cx40 in endothelial cells was associated with a decrease in Cx37 expression (Simon & McWhorter 2003, Jobs *et al.* 2012). The reason why only Cx37$^{-/-}$ mice and not Cx40$^{-/-}$ mice show impaired blood pressure responses after $P2Y_{2/4}$ receptor activation remains to be determined.

In vivo, UTP was found to constrict, independent of the endothelium, murine pial arterioles (Rosenblum & Nelson 1990, Rosenblum *et al.* 1990) and activation of $P2Y_{2/4}$ receptors located on vascular smooth muscle cells also causes vasoconstriction (Tölle *et al.* 2010). Data from our previous study employing the $P2Y_{2/4}$ agonist in $P2Y_2{}^{-/-}$ mice (shown for comparison in Fig. 1) indicate that activation of $P2Y_4$ receptors can induce vasoconstriction and increase blood pressure (Rieg *et al.* 2011). The blood pressure increase in $P2Y_2{}^{-/-}$ mice was observed during the timeframe WT mice respond with a blood pressure decrease in the early phase. In all mice tested in this study, except for $P2Y_2{}^{-/-}$ mice, we observed an acute blood pressure decrease that started to recover during administration of the $P2Y_{2/4}$ agonist, perhaps reflecting an opposing action of $P2Y_4$ receptors on vascular smooth muscle cells. Of note, eNOS$^{-/-}$, KCa3.1$^{-/-}$ and Cx37$^{-/-}$ mice show an impaired blood pressure increase in the late phase in response to $P2Y_{2/4}$ receptor activation compared to WT mice. The reason for this might relate to: (i) vasoconstriction consecutively following vasodilation may depend on the action and release of an endothelial-derived vasodilator which, when impaired, results in a reduced vasoconstrictor response; (ii) in eNOS$^{-/-}$ mice vascular tone is increased (Scotland *et al.* 2001), possibly reducing the maximal vasoconstrictor response via $P2Y_4$ receptor activation. Along those lines, increased EDH activity in eNOS$^{-/-}$ mice in order to normalize myogenic tone may contribute to the reduced vasoconstrictor effect.

We are aware that this study has limitations. Our data only allow us to show *in vivo* blood pressure effects of $P2Y_{2/4}$ agonist responses without defining the involved vascular beds or measuring electrical signals. Along those lines, *in vivo* EDH signalling might be more complex compared to the pharmacological response evoked via $P2Y_{2/4}$ receptor activation.

This might explain why our studies show no involvement of Cx40, as $Cx40^{-/-}$ mice are hypertensive and exhibit diminished conduction of arteriolar dilatation in response to acetylcholine and bradykinin (de Wit *et al.* 2000). Resistance vasculature in skeletal muscle, which comprises approx. 40% of total body mass in non-obese humans and animals, is essential to blood pressure regulation. However, our data cannot completely exclude that blood pressure actions of the $P2Y_{2/4}$ agonist are caused by acute effects on cardiac output or changes in nervous activity. Also, our data for $P2Y_4$-mediated vasoconstriction are indirect and future plans include testing in $P2Y_4{}^{-/-}$ mice. The development of selective and stable $P2Y_2$ and $P2Y_4$ agonists, which are not degraded into other vasoactive compounds, will help to test these ideas and additional studies, possibly involving double knockout mice and intravital microscopy, are required to better understand the role or $P2Y_{2/4}$ receptors in vascular reactivity.

In summary, our results clearly demonstrate that $P2Y_2$ receptor activation *in vivo* causes an acute blood pressure decrease, which is likely mediated by EDH and independent of endothelial NO. The EDH response after $P2Y_{2/4}$ receptor activation requires functional KCa3.1 and Cx37 to mediate its full vascular effects. Thus, $P2Y_2$ and $P2Y_4$ receptors may counteract each other in the vasculature and for blood pressure regulation. Effects of $P2Y_2$ receptors on the vasculature as well as in the kidney on renal sodium excretion make $P2Y_2$ agonists a potential target for the treatment of hypertension.

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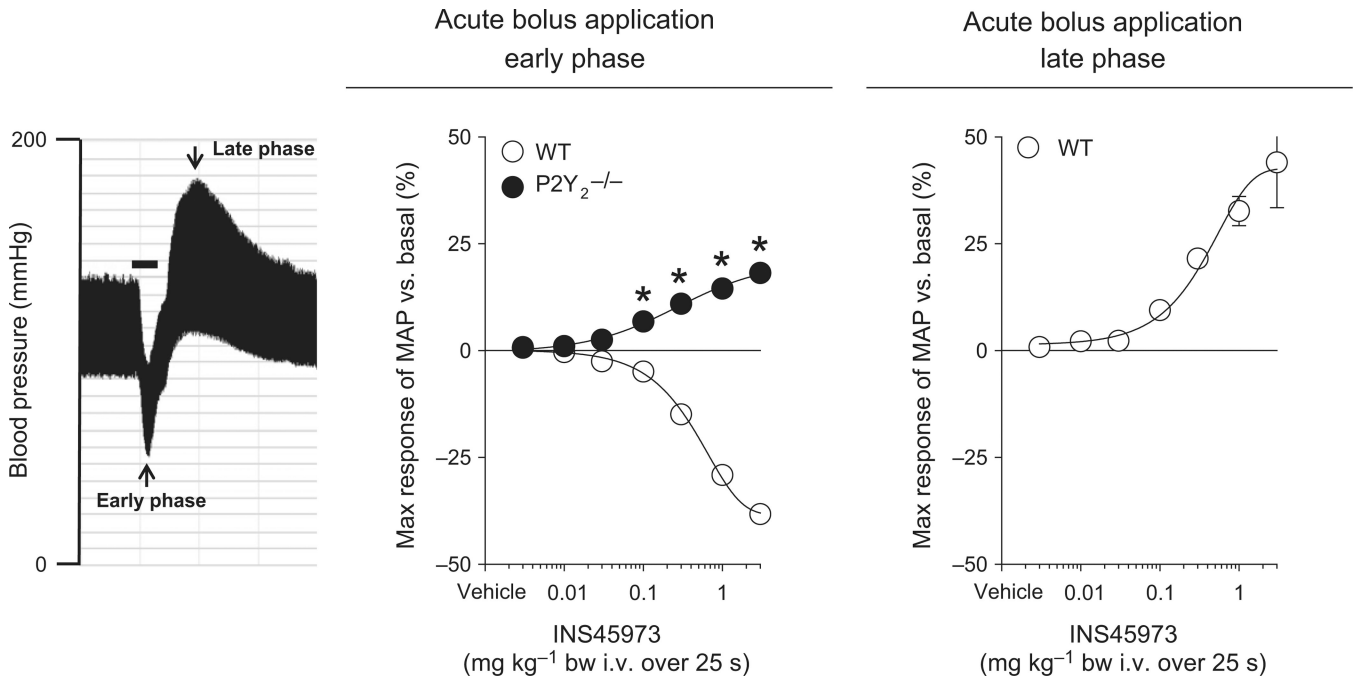

Figure 1.

Maximal responses in mean arterial blood pressure (MAP) to acute bolus application of INS45973 ($P2Y_{2/4}$ agonist) in wild-type (WT, $n = 8$) and $P2Y_2$ receptor knockout mice ($P2Y_2^{-/-}$, $n = 8$). Original recording of INS45973-induced blood pressure effects at 3 mg kg^{-1} body weight (bar = 25 s) in a WT mouse (left). In WT, application of INS45973 dose-dependently and rapidly decreased blood pressure (middle, early phase), which started to partially recover during drug application and then continued into a dose-dependent increase in blood pressure (right side, late phase). In contrast, INS45973 in $P2Y_2^{-/-}$ mice dose-dependently and rapidly increased blood pressure, which was sustained during drug application and thereafter recovered to baseline within 1–2 min, consistent with the short half-life of INS45973 [for an original blood pressure trace see Rieg *et al.* (2011)]. Some error bars are covered. *$P < 0.05$ vs. WT (two-way ANOVA with repeated measures followed by Dunnett's test).

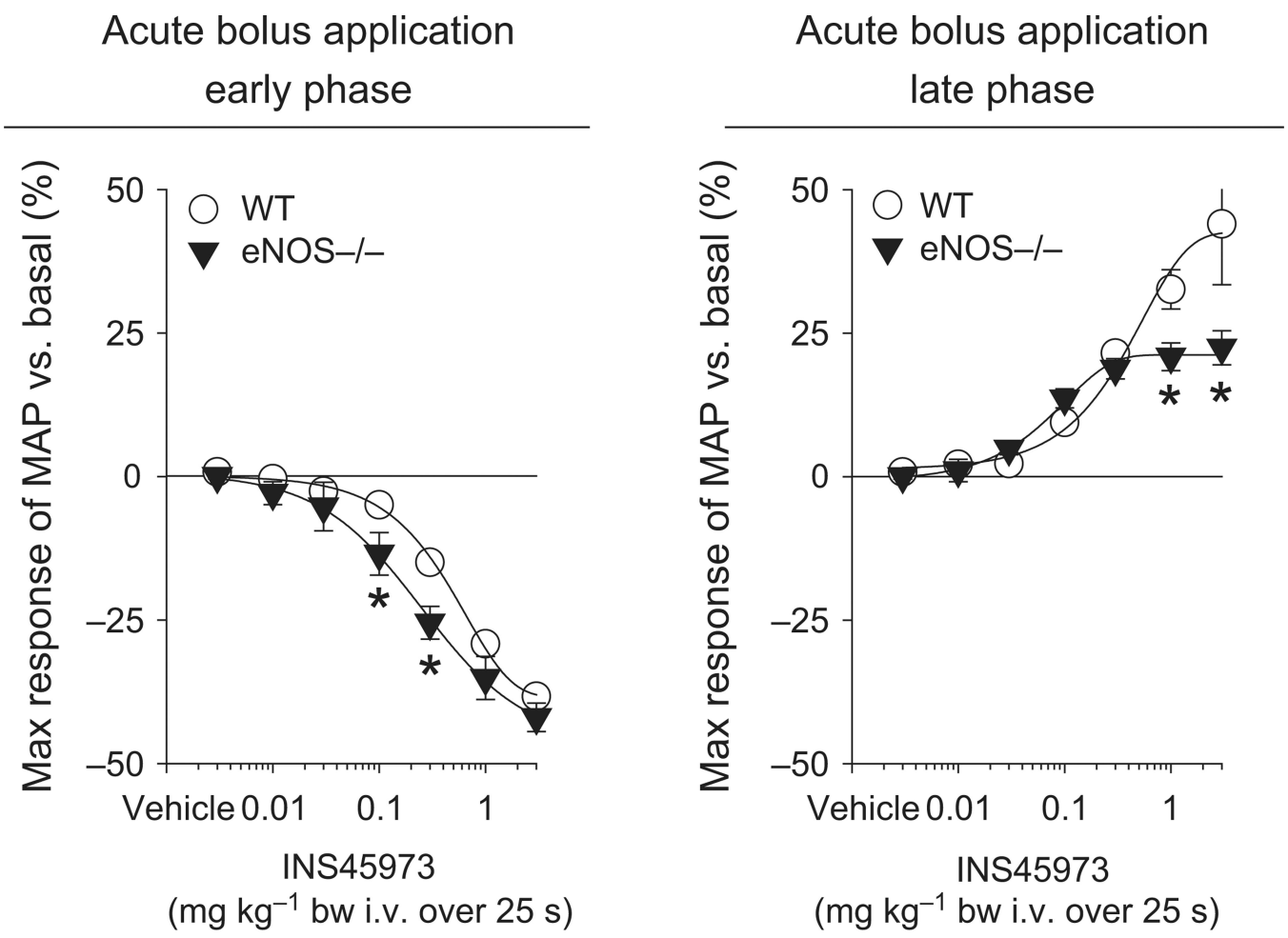

Figure 2.

Maximal responses in mean arterial blood pressure (MAP) to acute bolus application of INS45973 ($P2Y_{2/4}$ agonist) in endothelial NO synthase knockout mice ($eNOS^{-/-}$, $n = 5$). In $eNOS^{-/-}$ mice, application of INS45973 induced a dose-dependent, rapid decrease in blood pressure (left side, early phase). The dose-dependent rise in blood pressure above basal values following the initial decrease in $eNOS^{-/-}$ mice was significantly impaired compared to WT mice (right side, late phase). Some error bars are covered. *$P < 0.05$ vs. WT (two-way ANOVA with repeated measures followed by Dunnett's test).

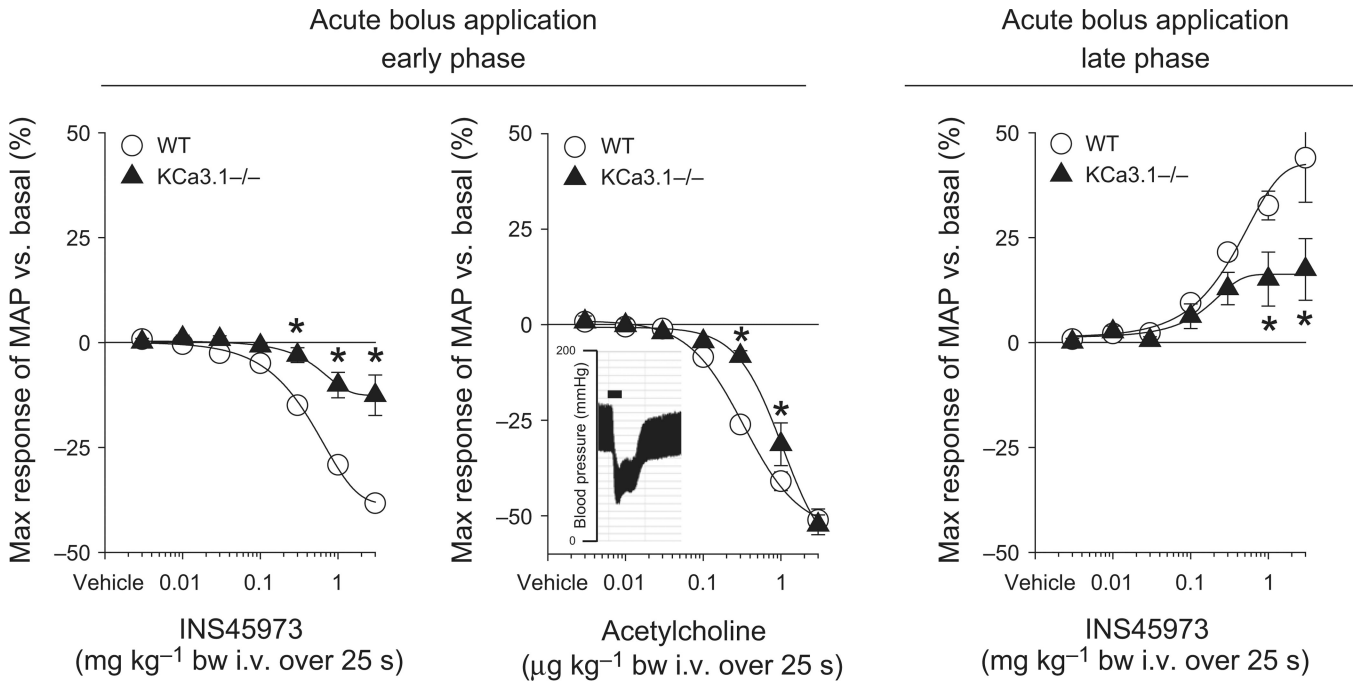

Figure 3.

Maximal responses in mean arterial blood pressure (MAP) to acute bolus application of INS45973 ($P2Y_{2/4}$ agonist) in intermediate-conductance potassium channel knockout mice ($KCa3.1^{-/-}$, $n = 5$). In $KCa3.1^{-/-}$ mice, application of INS45973 induced a dose-dependent, rapid decrease in blood pressure (left side, early phase), which was significantly impaired compared to wild-type (WT) mice. The blood pressure decrease in response to acetylcholine showed a right shift of the dose–response curve in $KCa3.1^{-/-}$ compared to WT mice; however, the maximum response was unaffected. The blood pressure decrease caused by acetylcholine is not followed by an acute blood pressure increase above basal values. *Inset:* original recording of acetylcholine-induced blood pressure effects at 3 µg kg^{-1} body weight in a WT mouse (bar = 25 s). The dose-dependent rise in blood pressure in response to INS45973 above basal values following the initial decrease in $KCa3.1^{-/-}$ mice was also significantly impaired compared to WT mice (right side, late phase). Some error bars are covered. *$P < 0.05$ vs. WT (two-way ANOVA with repeated measures followed by Dunnett's test).

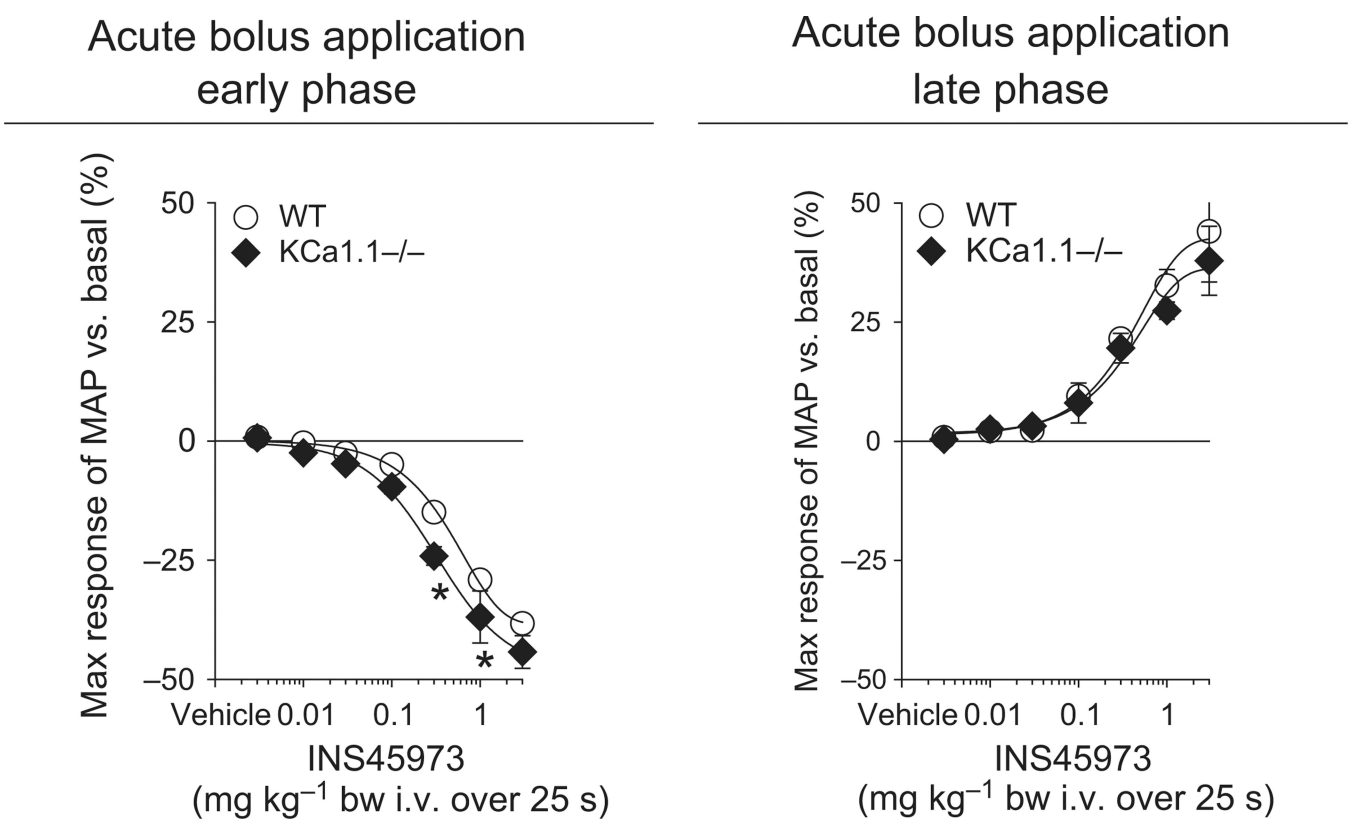

Figure 4.

Maximal responses in mean arterial blood pressure (MAP) to acute bolus application of INS45973 ($P2Y_{2/4}$ agonist) in big-conductance potassium channel knockout mice ($KCa1.1^{-/-}$, $n = 4$). In $KCa1.1^{-/-}$ mice, application of INS45973 induced a dose-dependent, rapid decrease in blood pressure (left side, early phase). The dose-dependent rise in blood pressure above basal values following the initial decrease in $KCa1.1^{-/-}$ mice was comparable to WT mice (right side, late phase). Some error bars are covered. *$P < 0.05$ vs. WT (two-way ANOVA with repeated measures followed by Dunnett's test).

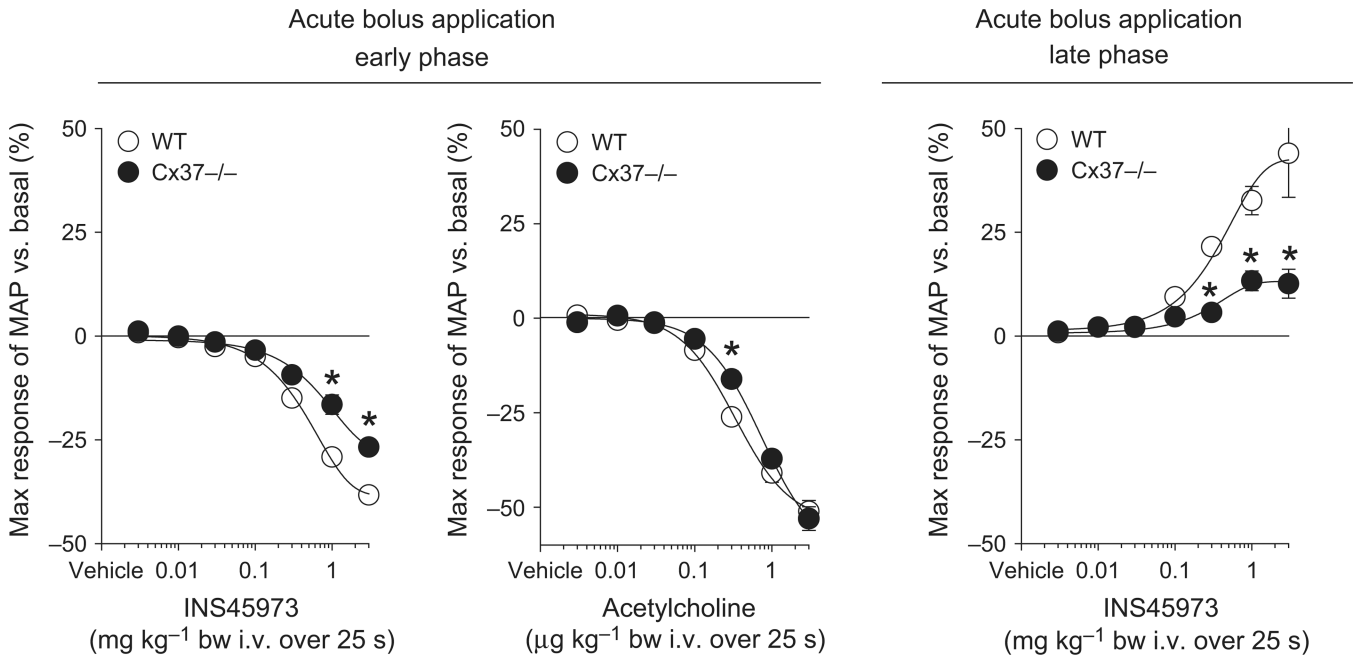

Figure 5.

Maximal responses in mean arterial blood pressure (MAP) to acute bolus application of INS45973 ($P2Y_{2/4}$ agonist) in connexin 37 knockout mice ($Cx37^{-/-}$, $n = 5$). In $Cx37^{-/-}$ mice, application of INS45973 induced a dose-dependent, rapid decrease in blood pressure (left side, early phase) which was significantly impaired compared to wild-type (WT) mice. The dose-dependent rise in blood pressure above basal values following the initial decrease in $Cx37^{-/-}$ mice was also significantly impaired compared to WT mice (right side, late phase). The dose-dependent blood pressure decrease in response to acetylcholine was comparable between $Cx37^{-/-}$ and WT mice and not followed by an acute blood pressure increase above basal values. Some error bars are covered. *$P < 0.05$ vs. WT (two-way ANOVA with repeated measures followed by Dunnett's test).

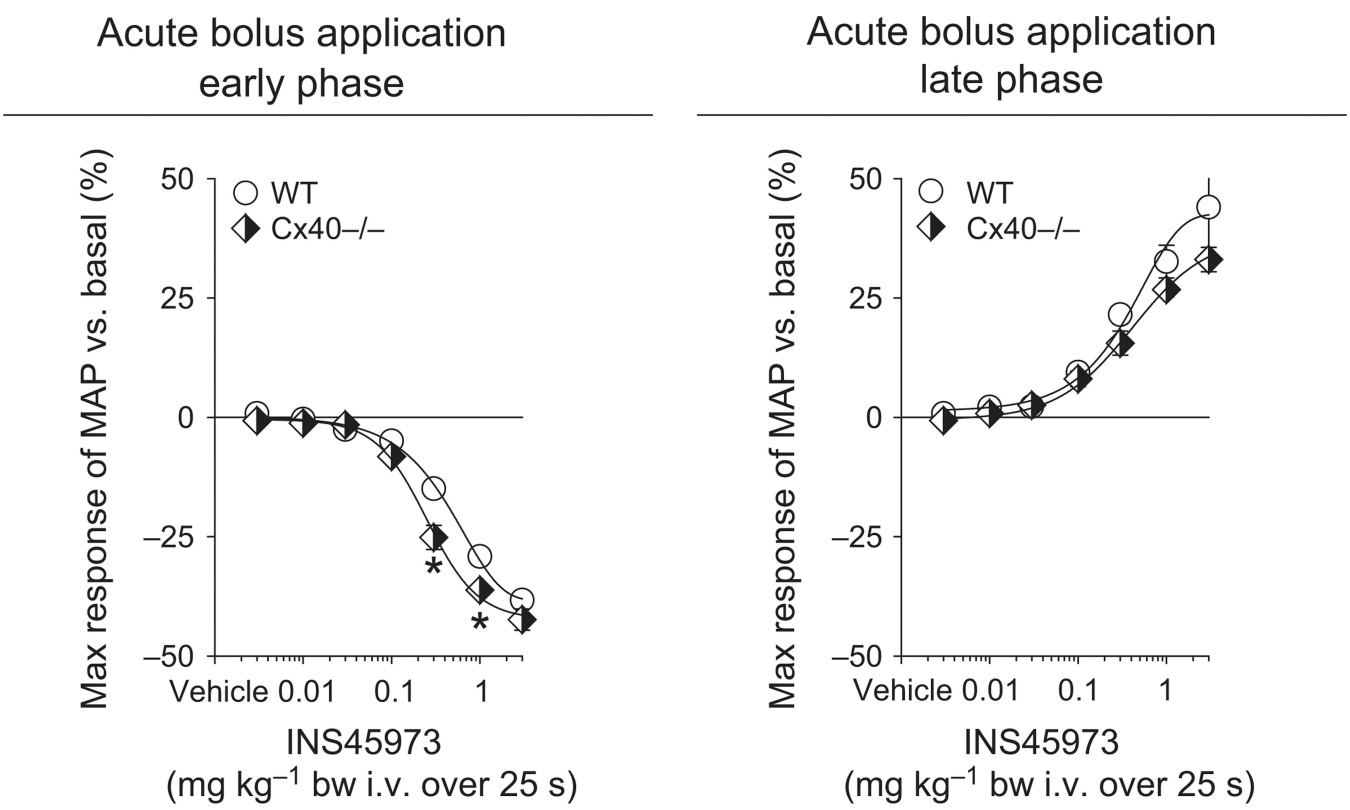

Figure 6.

Maximal responses in mean arterial blood pressure (MAP) to acute bolus application of INS45973 ($P2Y_{2/4}$ agonist) in connexin 40 knockout mice ($Cx40^{-/-}$, $n = 4$). In $Cx40^{-/-}$ mice, application of INS45973 induced a dose-dependent, rapid decrease in blood pressure (left side, early phase). The dose-dependent rise in blood pressure above basal values following the initial decrease in $Cx40^{-/-}$ mice was comparable to wild-type (WT) mice (right side, late phase). Some error bars are covered. *$P < 0.05$ vs. WT (two-way ANOVA with repeated measures followed by Dunnett's test).

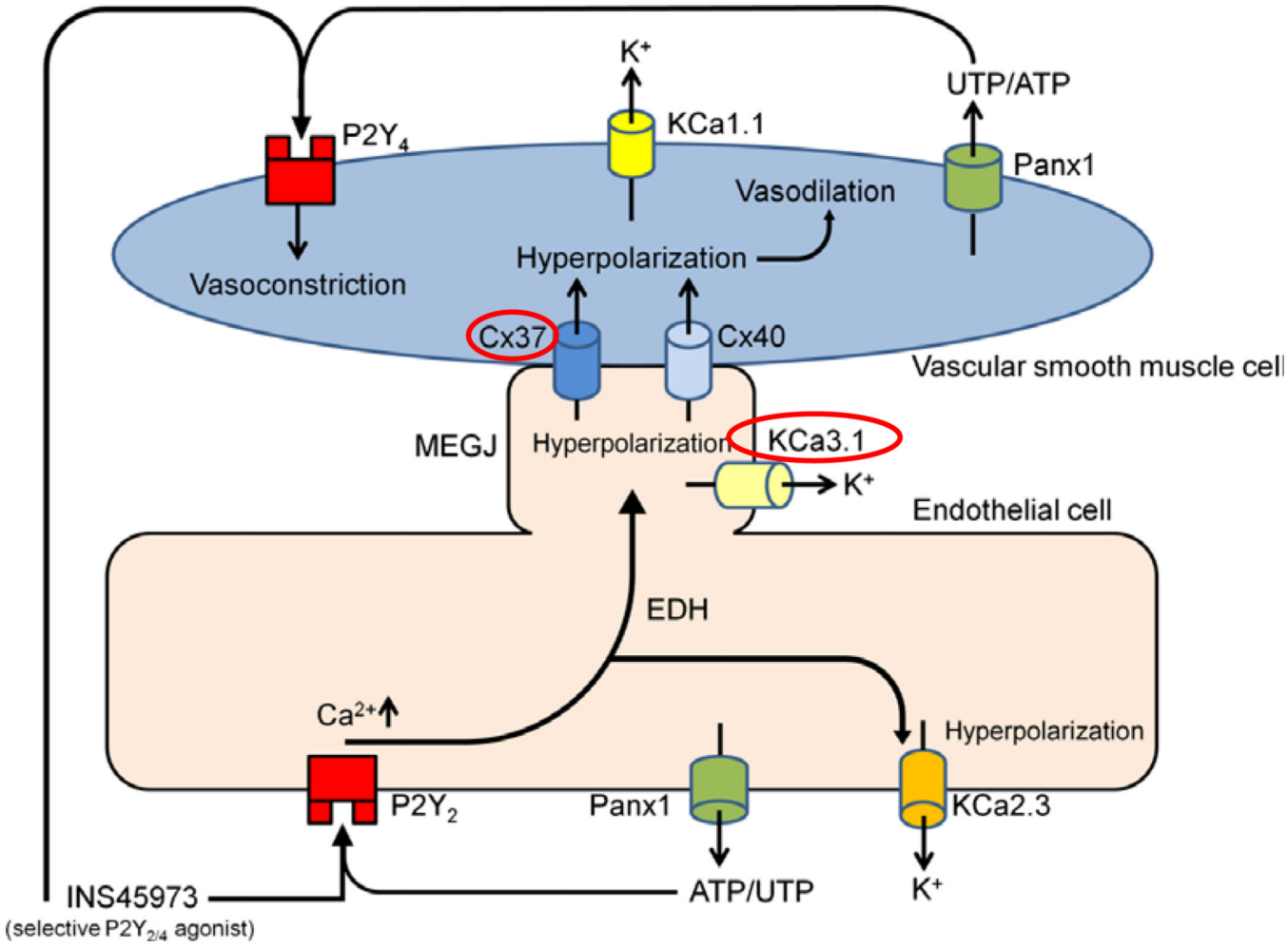

Figure 7.

A proposed model of murine blood pressure responses to $P2Y_{2/4}$ receptor activation. Our previous data implicated that the acute vasodilatory response to INS45973, a $P2Y_{2/4}$ agonist, is mediated by $P2Y_2$ receptor activation on endothelial cells. This hypothesis is supported by experiments in $P2Y_2$ receptor knockout mice which lack the observed initial blood pressure decrease and show instead an immediate increase in blood pressure (Rieg *et al.* 2011). The blood pressure increase possibly results as a consequence of $P2Y_4$ receptor activation directly on vascular smooth muscle cells resulting in vasoconstriction (Bar *et al.* 2008). The fact that endothelial NO synthase knockout mice responded with a comparable vasodilation implicated a role for endothelial derived hyperpolarization (EDH) in the acute blood pressure decrease. We speculate that $P2Y_2$ receptor activation, possibly via an increase in intracellular calcium, activates calcium-dependent intermediate-conductance potassium channels, KCa3.1, which induces EDH-type vasodilations. Lack of KCa3.1 impairs this response (red circle). The role of small-conductance potassium channels (KCa2.3) in response to $P2Y_{2/4}$ receptor activation needs to be determined. Calcium-dependent big-conductance potassium channels (KCa1.1) on vascular smooth muscle cells are not part of $P2Y_{2/4}$ receptor-initiated blood pressure responses. Gap junction proteins including connexin 37 (Cx37) and connexin 40 (Cx40) contribute to myoendothelial gap junction

(MEGJ) communication and electrically conduct EDH activity. Our data suggest that Cx37 is part of such a communication because Cx37 knockout mice show an impaired blood pressure response to $P2Y_{2/4}$ receptor activation (red circle). In contrast to the distinct response observed in $Cx37^{-/-}$ mice, Cx40 is not part of the $P2Y_{2/4}$ receptor-initiated blood pressure responses. Endothelial cells and vascular smooth muscle cells are speculated to endogenously release ATP and/or UTP via pannexin 1 (Panx1) channels (Lohman & Isakson 2014) which could consecutively activate $P2Y_{2/4}$ receptors and contribute to regulation of vascular tone.

Table 1

Maximal blood pressure effects at 3 mg kg^{-1} body weight and ED_{50} values of wild-type and knockout mice in response to $P2Y_{2/4}$ receptor activation

	Mean arterial blood pressure at baseline (mmHg)	Heart rate at baseline (min^{-1})	Maximal decrease in %, early phase	ED_{50} decrease early phase (mg kg^{-1})	Maximal increase in %, late phase	ED_{50} increase late phase (mg kg^{-1})
WT	93 ± 3	489 ± 11	−38 ± 1	0.4 ± 0.1	+44 ± 11	0.5 ± 0.2
$P2Y_2^{-/-}$	111 ± 3*	489 ± 19	+18 ± 1*	0.4 ± 0.1	–	–
$eNOS^{-/-}$	131 ± 2*	479 ± 17	−42 ± 2	0.3 ± 0.1	+22 ± 3*	0.2 ± 0.1
$KCa3.1^{-/-}$	91 ± 6	480 ± 28	−13 ± 5*	0.6 ± 0.2	+17 ± 7*	0.3 ± 0.1
$KCa1.1^{-/-}$	108 ± 2*	548 ± 23	−44 ± 3	0.4 ± 0.1	+38 ± 7	0.5 ± 0.2
$Cx37^{-/-}$	92 ± 4	501 ± 31	−27 ± 1*	0.6 ± 0.1	+13 ± 3*	0.3 ± 0.1
$Cx40^{-/-}$	111 ± 5*	465 ± 33	−41 ± 2	0.3 ± 0.1	+33 ± 3	0.4 ± 0.1

Values are mean ± SEM. WT, wild-type mice; $P2Y_2^{-/-}$, P2Y2 receptor knockout mice, $eNOS^{-/-}$, endothelial NO synthase knockout mice; $Cx37^{-/-}$, connexin 37 knockout mice; $Cx40^{-/-}$, connexin 40 knockout mice; $KCa1.1^{-/-}$, big-conductance potassium channel knockout mice; $KCa3.1^{-/-}$, intermediate-conductance potassium channel knockout mice. Additional analysis of previously published data (Rieg *et al.* 2011) from $eNOS^{-/-}$ and $P2Y2^{-/-}$ are included. In contrast, $P2Y_2^{-/-}$ mice show an acute blood pressure increase in the early phase and lack a biphasic response therefore not data are shown for the late phase. n = 4–8;

*$P < 0.05$ vs. WT (one-way ANOVA followed by Bonferroni test for blood pressure, heart rate and ED_{50} values, two-way ANOVA with repeated measures followed by Dunnett's test for maximal responses).

Table 2

Maximum heart rate decrease (early phase) of wild-type and knockout mice in response to $P2Y_{2/4}$ receptor activation

		INS45973 (%)					
	Vehicle (%)	**0.01 (mg kg^{-1})**	**0.03 (mg kg^{-1})**	**0.1 (mg kg^{-1})**	**0.3 (mg kg^{-1})**	**1 (mg kg^{-1})**	**3 (mg kg^{-1})**
WT	−1.1 ± 0.5	0.2 ± 0.3	−0.5 ± 0.3	−1.1 ± 0.7	2.0 ± 1.0	−0.1 ± 0.6	1.2 ± 0.8
$P2Y_2^{-/-}$	0.9 ± 0.1	0.3 ± 0.1	−1.2 ± 1.4	3.2 ± 0.8	7.7 ± 2.0	3.4 ± 0.9	6.1 ± 1.2
$eNOS^{-/-}$	0.1 ± 0.5	−0.1 ± 0.5	−0.4 ± 1.9	0.2 ± 0.5	0.6 ± 0.3	1.2 ± 0.4	1.4 ± 0.4
$KCa3.1^{-/-}$	0.8 ± 0.9	0.9 ± 0.4	−1.2 ± 0.5	0.8 ± 0.3	−0.3 ± 0.3	0.1 ± 0.6	−0.8 ± 0.5
$KCa1.1^{-/-}$	1.1 ± 0.5	0.7 ± 0.9	0.2 ± 0.1	0.6 ± 0.5	0.3 ± 0.6	0.4 ± 0.6	1.9 ± 1.2
$Cx37^{-/-}$	1.9 ± 0.8	1.1 ± 0.6	−0.3 ± 0.4	0.2 ± 0.5	0.1 ± 0.4	0.7 ± 0.4	1.2 ± 0.3
$Cx40^{-/-}$	−0.9 ± 0.6	0.4 ± 0.2	−0.2 ± 0.7	0.9 ± 0.5	1.4 ± 1.3	0.4 ± 1.4	0.1 ± 1.1

Values are mean ± SEM. WT, wild-type mice; $P2Y_2^{-/-}$, $P2Y_2$ receptor knockout mice, $eNOS^{-/-}$, endothelial NO synthase knockout mice; $Cx37^{-/-}$, connexin 37 knockout mice; $Cx40^{-/-}$, connexin 40 knockout mice; $KCa1.1^{-/-}$, big-conductance potassium channel knockout mice; $KCa3.1^{-/-}$, intermediate-conductance potassium channel knockout mice. Additional analysis of previously published data (Rieg *et al.* 2011) from $eNOS^{-/-}$ and $P2Y_2^{-/-}$ are included. n = 4–8 (two-way ANOVA with repeated measures followed by Dunnett's test).

Table 3

Maximum heart rate increase (late phase) of wild-type and knockout mice in response to $P2Y_{2/4}$ receptor activation

		INS45973 (%)					
	Vehicle (%)	**0.01 (mg kg^{-1})**	**0.03 (mg kg^{-1})**	**0.1 (mg kg^{-1})**	**0.3 (mg kg^{-1})**	**1 (mg kg^{-1})**	**3 (mg kg^{-1})**
WT	−1.1 ± 0.5	0.8 ± 0.7	0.4 ± 0.6	0.7 ± 0.9	2.5 ± 1.6	7.2 ± 2.4	7.3 ± 2.7
$P2Y_2^{-/-}$	–	–	–	–	–	–	–
$eNOS^{-/-}$	0.1 ± 0.5	0.1 ± 0.6	−0.1 ± 1.0	−1.2 ± 0.4	−0.9 ± 1.2	0.7 ± 2.0	0.1 ± 5.9
$KCa3.1^{-/-}$	0.8 ± 0.9	2.2 ± 1.0	−1.0 ± 0.3	0.6 ± 1.1	−0.5 ± 0.9	0.4 ± 2.0	−0.7 ± 1.6
$KCa1.1^{-/-}$	1.1 ± 0.5	0.7 ± 0.3	1.0 ± 0.6	1.8 ± 0.8	0.2 ± 1.5	−1.0 ± 2.4	−2.0 ± 1.8
$Cx37^{-/-}$	1.9 ± 0.8	3.8 ± 1.1	4.0 ± 2.0	0.6 ± 1.7	−2.5 ± 1.2	1.2 ± 2.1	3.3 ± 2.4
$Cx40^{-/-}$	−0.9 ± 0.6	0.6 ± 0.6	0.2 ± 0.1	3.4 ± 2.6	2.3 ± 0.9	6.1 ± 5.6	7.9 ± 4.7

Values are mean ± SEM. WT, wild-type mice; $P2Y_2^{-/-}$, $P2Y_2$ receptor knockout mice, $eNOS^{-/-}$, endothelial NO synthase knockout mice; $Cx37^{-/-}$, connexin 37 knockout mice; $Cx40^{-/-}$, connexin 40 knockout mice; $KCa1.1^{-/-}$, big-conductance potassium channel knockout mice; $KCa3.1^{-/-}$, intermediate-conductance potassium channel knockout mice. Additional analysis of previously published data (Rieg *et al.* 2011) from $eNOS^{-/-}$ and $P2Y_2^{-/-}$ are included. $P2Y_2^{-/-}$ mice show an acute blood pressure increase in the early phase and lack a biphasic response; therefore, data are not shown for the late phase. n = 4–8 (two-way ANOVA with repeated measures followed by Dunnett's test).