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RESEARCH PAPER

A Ca^{2+}-dependent chloride current and Ca^{2+} influx via $Ca_v1.2$ ion channels play major roles in P2Y receptor-mediated pulmonary vasoconstriction

Callum Mitchell, Nawazish-i-Husain Syed*, Alison M Gurney† and Charles Kennedy

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK

Correspondence
Dr Charles Kennedy, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK.
E-mail: c.kennedy@strath.ac.uk

*Present address: University College of Pharmacy, University of the Punjab, Lahore, Pakistan.
E-mail: snihusain@yahoo.com.
†Present address: Faculty of Life Sciences, University of Manchester, Floor 2, Core Technology Facility, 46 Grafton Street, Manchester M13 9NT, UK.
E-mail: alison.gurney@manchester.ac.uk.

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BACKGROUND AND PURPOSE

ATP, UTP and UDP act at smooth muscle P2X and P2Y receptors to constrict rat intrapulmonary arteries, but the underlying signalling pathways are poorly understood. Here, we determined the roles of the Ca^{2+}-dependent chloride ion current ($I_{Cl,Ca}$), $Ca_v1.2$ ion channels and Ca^{2+} influx.

EXPERIMENTAL APPROACH

Isometric tension was recorded from endothelium-denuded rat intrapulmonary artery rings (i.d. 200–500 μm) mounted on a wire myograph.

KEY RESULTS

The $I_{Cl,Ca}$ blockers, niflumic acid and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and the $Ca_v1.2$ channel blocker, nifedipine, reduced peak amplitude of contractions evoked by UTP and UDP by ~45–50% and in a non-additive manner. Ca^{2+}-free buffer inhibited responses by ~70%. Niflumic acid and nifedipine similarly depressed contractions to ATP, but Ca^{2+}-free buffer almost abolished the response. After peaking, contractions to UTP and UDP decayed slowly by 50–70% to a sustained plateau, which was rapidly inhibited by niflumic acid and nifedipine. Contractions to ATP, however, reversed rapidly and fully. Tannic acid contracted tissues *per se* and potentiated nucleotide-evoked contractions.

CONCLUSIONS AND IMPLICATIONS

$I_{Cl,Ca}$ and Ca^{2+} influx via $Ca_v1.2$ ion channels contribute substantially and equally to contractions of rat intrapulmonary arteries evoked by UTP and UDP, via P2Y receptors. ATP also activates these mechanisms via P2Y receptors, but the greater dependence on extracellular Ca^{2+} most likely reflects additional influx through the P2X1 receptor pore. The lack of a sustained response to ATP is probably due to it acting at P2 receptor subtypes that desensitize rapidly. Thus multiple signalling mechanisms contribute to pulmonary artery vasoconstriction mediated by P2 receptors.

Abbreviations

DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; $I_{Cl,Ca}$, Ca^{2+}-dependent chloride ion current; IPA, intrapulmonary artery

Introduction

The cardiovascular actions of the endogenous nucleotides ATP, UTP and UDP are mediated by P2X and P2Y receptors (Burnstock and Kennedy, 1985; 1986; 2011; Erlinge and Burnstock, 2008), both of which are expressed in human pulmonary arteries (Liu *et al.*, 1989b). P2X receptors are ligand-gated cation channels (Khakh *et al.*, 2001) and P2X1 is the predominant subtype expressed in pulmonary vascular smooth muscle (Nori *et al.*, 1998; Hansen *et al.*, 1999; Lewis and Evans, 2001; Syed *et al.*, 2010), where it mediates vasoconstriction (Liu *et al.*, 1989a; Hasséssian and Burnstock, 1995; Rubino and Burnstock, 1996; Rubino *et al.*, 1999; Chootip *et al.*, 2002; Syed *et al.*, 2010). In contrast, P2Y receptors are G protein-coupled (Abbracchio *et al.*, 2006; Alexander *et al.*, 2011), and in rat pulmonary vessels, P2Y agonists elicit vasodilatation via endothelial receptors and vasoconstriction via smooth muscle receptors (McCormack *et al.*, 1989; Liu *et al.*, 1989a; Hasséssian and Burnstock, 1995; Rubino and Burnstock, 1996; Hartley *et al.*, 1998; Rubino *et al.*, 1999; Chootip *et al.*, 2002; Jernigan *et al.*, 2006).

In healthy individuals, the delivery of de-oxygenated blood to the alveoli is maximized by a complex balance of factors that maintains pulmonary arteries in a dilated, low-resistance state (Barnes and Liu, 1995), and nucleotides appear to contribute to this state. For example, mM ATP is present in red blood cells (Erlinge and Burnstock, 2008) and is released during passage through the lungs, where it acts via endothelial P2Y receptors to induce a nitric oxide-dependent decrease in pulmonary vascular resistance (Sprague *et al.*, 1996; 2003). Vasoconstriction mediated by the smooth muscle P2X and P2Y receptors is likely to become more prominent when endothelium-dependent relaxation is impaired, such as in hypoxia- or monocrotaline-induced pulmonary hypertension (Adnot *et al.*, 1991; Mam *et al.*, 2010) and chronic obstructive pulmonary disease (Dinh-Xuan *et al.*, 1991). Indeed, extracellular ATP is elevated in the latter disease (Lommatzsch *et al.*, 2010), which would increase the contribution of P2 receptors to artery regulation. The smooth muscle P2X and P2Y receptors also appear to play a role in hypoxic pulmonary vasoconstriction as the P2 receptor antagonist suramin inhibited this response in rabbit perfused lungs (Baek *et al.*, 2008). Given these potential pathophysiological roles of nucleotides, it is important to understand the mechanisms that couple P2 receptors to the constrictor response.

We reported previously that UTP and UDP each constrict rat intrapulmonary arteries (IPA) via two P2Y subtypes, the $P2Y_6$ and either $P2Y_2$ or $P2Y_4$ receptors, while ATP acts at P2X1 receptors and a P2Y receptor, most probably the $P2Y_2$ subtype (Chootip *et al.*, 2002; 2005; Kennedy *et al.*, 2010). The signalling pathways through which these receptors produce their effects are, however, poorly characterized. P2Y receptor stimulation evokes release of intracellular Ca^{2+} stores in pulmonary artery smooth muscle cells (Bakhramov *et al.*, 1996; Drummond and Tuft, 1999; Guibert *et al.*, 1996; Jernigan *et al.*, 2006; Baek *et al.*, 2008), but the subsequent, downstream events that lead to vasoconstriction are unknown. An inward Ca^{2+}-dependent, chloride ion current ($I_{Cl,Ca}$) that is also activated by P2Y agonists (Bakhramov *et al.*, 1996; Guibert *et al.*, 1997; Hartley *et al.*, 1998; Chootip *et al.*, 2005) might be predicted to contribute, but this remains to be confirmed, particularly as $I_{Cl,Ca}$ is also activated by endothelin in rat IPA, but plays no role in endothelin's contractile activity (Kato *et al.*, 1999). Activation of P2X1 receptors results in the opening of the ligand-gated cation channel and depolarization (Khakh *et al.*, 2001), but again, how this leads to pulmonary vasoconstriction is unknown. The depolarization may promote opening of $Ca_v1.2$ ion channels, while the P2X1 pore is Ca^{2+} permeable (Egan and Khakh, 2004) and so Ca^{2+} could also potentially enter the cell directly through the pore to evoke contraction. Once more, however, the contributions of these pathways to vasoconstriction remain to be determined.

Clearly, multiple potential signalling mechanisms exist by which nucleotides may evoke pulmonary vasoconstriction, but the involvement and relative contributions of each are unknown. The aim of the present study was, therefore, to determine the extent to which the development and maintenance of nucleotide-induced constriction of the rat isolated IPA depend upon the influx of extracellular Ca^{2+} via $Ca_v1.2$ ion channels and the P2X1 pore, and the role of $I_{Cl,Ca}$ in promoting $Ca_v1.2$ activity. We present evidence that $I_{Cl,Ca}$ and Ca^{2+} influx via $Ca_v1.2$ ion channels contribute substantially and equally to both the peak and plateau of contractions evoked by UTP and UDP acting at P2Y receptors. ATP appears to act not only via P2Y receptors and these mechanisms, but also via P2X1 receptors.

Methods

Male Sprague–Dawley rats (200–250 g) were killed by cervical dislocation and exsanguination. The heart and lungs were removed *en bloc* and placed in a solution composed of (mM); NaCl 122, KCl 5, HEPES 10, KH_2PO_4 0.5, NaH_2PO_4 0.5, $MgCl_2$ 1, glucose 11, $CaCl_2$ 1.8, titrated to pH 7.3 with NaOH and bubbled with air (21% O_2, 5% CO_2, 74% N_2). IPAs of internal diameter 200–500 μm were dissected, cleaned of connective tissue and their endothelium removed gently by passing a needle and thread through the lumen. They were then cut into 5-mm rings, mounted horizontally on a pair of intraluminal wires in 1 mL organ baths and equilibrated under a resting tension of 0.5 g for 60 min at 37°C. Tension was recorded with Grass FT03 isometric force transducers, connected to a PowerLab/4e system, using Chart 4.2 software (ADInstruments, Oxford, UK).

Experimental protocols

Drugs were added directly to the tissue bath and washed out by replacement with drug-free solution. Removal of the endothelium was confirmed by loss of the relaxation to ACh (10 μM) following precontraction with UDP, UTP or ATP. The nucleotide concentration–contraction curves in rat IPA do not reach a maximum (Chootip *et al.*, 2002; 2005); therefore, they were applied at the equi-effective concentration of 300 μM to generate vasoconstriction and investigate the underlying signalling mechanisms. When studying peak contraction amplitude, each nucleotide was added for 5 min at 30 min intervals, as preliminary experiments showed that this protocol elicited highly reproducible contractions when agonist application was repeated six times.

To determine the effects of niflumic acid, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and nifedipine, control responses to an agonist were obtained. Arteries were then incubated with niflumic acid or DIDS for 10 min, nifedipine for 15 min or nifedipine plus niflumic acid or DIDS for 15 min before the agonist was re-administered. The effects of tannic acid were measured in the same way after 15 min incubation and also against contractions evoked by KCl and phenylephrine. When investigating the role of extracellular Ca^{2+}, control agonist responses were obtained in normal buffer, which was then replaced with Ca^{2+}-free solution for 10 min, before the agonists were re-administered. When studying the plateau phase of the nucleotide-evoked contractions, UDP or UTP were added for 45 min and niflumic acid or nifedipine was applied 20 min after the agonist and their effects measured a further 10 min later. Similarly, tannic acid was added 20 min after UDP or UTP and the peak amplitude of the evoked contraction measured.

Drugs and solutions

Drugs were prepared as 10 or 100 mM stock solutions and diluted in the HEPES-based buffer before applying to the tissues. ATP (Na_2 salt), UTP (Na_3 salt), UDP (Na salt), phenylephrine hydrochloride and ACh chloride (Sigma, Dorset, UK) were dissolved in distilled water. Nifedipine, niflumic acid (Sigma) and DIDS (Na_2 salt) (Invitrogen, Paisley, UK) were dissolved in dimethyl sulfoxide and tannic acid (Sigma) in ethanol. Isotonic 40 mM K^+ solution was prepared by replacing NaCl in the HEPES-buffered solution with an equimolar amount of KCl to maintain osmolarity. Ca^{2+}-free buffer was prepared by replacing the $CaCl_2$ with 3.12 mM $MgCl_2$ and 1 mM EGTA to give equimolar free [Mg^{2+}]. Free and bound [Mg^{2+}] were calculated using Maxchel (Chris Patten, Stanford University, Stanford, CA, USA).

Data analysis

Contractions are expressed as mg tension, a percentage of the peak amplitude or a percentage of the control response produced by a given agonist, as appropriate. Data are shown as mean ± SEM of experiments on vessels from n animals and were compared using Student's paired t-test or one-way ANOVA with Tukey's comparison of the mg tension values, as appropriate. Values of $P < 0.05$ were considered to be statistically significant.

Results

Contribution of $I_{Cl,Ca}$, $Ca_v1.2$ ion channels and extracellular Ca^{2+} to peak contraction amplitude

The channel that mediates $I_{Cl,Ca}$, TMEM16A, has only recently been cloned (Caputo *et al.*, 2008; Schroeder *et al.*, 2008; Yang *et al.*, 2008) and is functionally expressed in rat pulmonary artery smooth muscle cells (Manoury *et al.*, 2010). Little is known of P2 receptor function in mouse pulmonary artery and mice lacking a functional TMEM16A gene die within days of birth (Rock *et al.*, 2008). A pharmacological approach was, therefore, taken using the most potent and most commonly used $I_{Cl,Ca}$ blocker, niflumic acid (Hogg *et al.*, 1994b), and the structurally unrelated inhibitor, DIDS (Hogg *et al.*, 1994a) to determine the role of TMEM16A in nucleotide-evoked pulmonary vasoconstriction in rat.

Contractions evoked by UDP (300 μM) and UTP (300 μM) reached a peak within 2–3 min (Figure 1), while responses to ATP (300 μM) peaked within 1–2 min (Figure 3A). Niflumic acid (1 μM) and DIDS (100 μM) had no effect on basal tone of rat IPA or on contractions to KCl (40 mM) (103 ± 11 % of control, $n = 5$ and 96.5 ± 3.9 % of control, $n = 4$, respectively), indicating that at these concentration they do not interact directly with $Ca_v1.2$ ion channels or the myofilaments to depress smooth muscle contractility. Both, however, reduced significantly the peak responses to UDP ($P < 0.01$) (Figures 1A, 2A) and UTP ($P < 0.05$) (Figures 1B, 2B) by approximately 40–55% of their control values. Niflumic acid (1 μM) depressed responses to ATP by a similar amount ($P < 0.05$) (Figure 3B) and the degree of inhibition did not differ significantly between the nucleotides. Higher concentrations of niflumic acid (10 and 100 μM) depressed contractions to KCl (40 mM), indicating an action of niflumic acid at other sites and so were not studied further.

Nifedipine (1 μM), a concentration that maximally inhibits $Ca_v1.2$ ion channels in pulmonary arteries (Clapp and Gurney, 1991), had no effect on basal tone, but virtually abolished contractions to KCl (40 mM) (99 ± 1 % inhibition, $n = 4$) and significantly inhibited the peak response to UDP ($P < 0.001$) (Figures 1A, 2A), UTP ($P < 0.001$) (Figures 1B, 2B) and ATP ($P < 0.01$) (Figure 3B) by a similar amount as niflumic acid. There was no significant difference in the degree of inhibition of each nucleotide response. Co-administration of niflumic acid (1 μM) and nifedipine (1 μM) inhibited contractions to the same extent as either agent alone (Figures 2A,B, 3B). Likewise, adding DIDS (100 μM) and nifedipine (1 μM) together did not elicit greater inhibition of the responses to UDP (Figure 2A) or UTP (Figures 1B, 2B).

Bathing tissues in Ca^{2+}-free buffer for 10 min had no effect on basal tone, but abolished contractions to KCl (40 mM) ($n = 4$, not shown) and significantly decreased the peak amplitude of the contractions to UDP ($P < 0.001$) (Figure 2A) and UTP ($P < 0.001$) (Figure 2B) by nearly 70%. Responses to ATP were depressed by more than 90% ($P < 0.05$) (Figure 3) and now appeared to take longer, 2–3 min, to reach peak. The inhibition of the ATP response was significantly greater than that of UTP and UDP ($P < 0.05$). For each nucleotide, removing extracellular Ca^{2+} caused a significantly larger reduction in the peak response than did the ion channel blockers (UDP and UTP, $P < 0.05$, ATP, $P < 0.01$). Thus, $I_{Cl,Ca}$, $Ca_v1.2$ ion channels and Ca^{2+} influx each make a similar contribution to the peak amplitude of contractions of rat IPA evoked by UDP and UTP, whereas influx of extracellular Ca^{2+} plays a relatively greater role in the response to ATP.

Contribution of $I_{Cl,Ca}$ and $Ca_v1.2$ ion channels to contraction plateau

Once the contractions to UTP and UDP had reached a peak, they decayed slowly and significantly ($P < 0.001$), and with a similar time course over the next 10–15 min to a plateau of around 30–50% of the peak (Figure 4A,C). Thereafter, the responses were generally stable and while they tended to decrease slightly between 20, 30 and 40 min after agonist

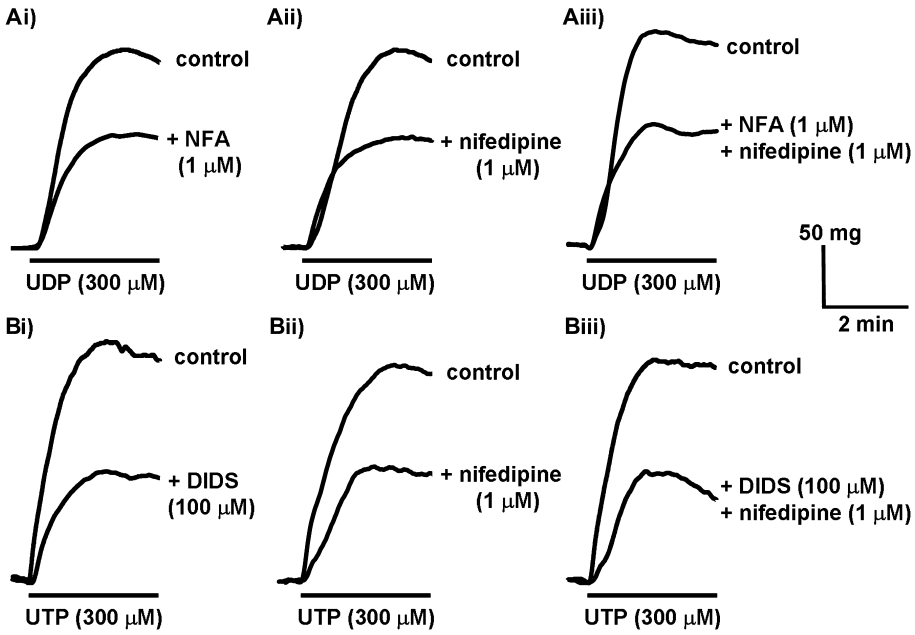

Figure 1

Inhibition of the peak amplitude of contractions evoked by UDP and UTP. (A) The superimposed traces shows typical contractions of endothelium-denuded rat isolated IPA evoked by (A) UDP (300 μM) in the absence of (upper traces) and after incubation with (Ai) niflumic acid (1 μM), (Aii) nifedipine (1 μM) and (Aiii) niflumic acid (1 μM) plus nifedipine (1 μM) for 10 min (lower traces). (B) The superimposed traces show responses evoked by UTP (300 μM) in the absence of (upper traces) and after incubation with (Bi) DIDS (100 μM), (Bii) nifedipine (1 μM) and (Biii) DIDS (100 μM) plus nifedipine (1 μM) for 10 min (lower trace). UDP and UTP were applied as indicated by the solid bar. Each pair of traces was obtained in a separate tissue.

administration, the tensions recorded at these times were not significantly different. The contractions to ATP also decayed significantly from peak, but compared with UTP and UDP, the decay was faster and the tone returned to the basal level within 40 min (Figure 4B,D).

The signalling mechanisms that underlie the initiation and development of contraction may differ from those that mediate the maintained response, therefore, the roles of $I_{Cl,Ca}$ and $Ca_v1.2$ ion channels in maintaining the UTP and UDP plateaux was determined by adding the channel inhibitors 20 min after the agonists and measuring the tension a further 10 min later. Under these conditions, niflumic acid (1 μM) rapidly and significantly reduced the plateau response to both nucleotides by about half ($P < 0.001$) (Figure 5). Nifedipine (1 μM) significantly relaxed the tissue by a similar amount (UDP, $P < 0.001$; UTP, $P < 0.01$) and the effects of co-administration of the two inhibitors were not additive (Figure 5). Thus $I_{Cl,Ca}$ and $Ca_v1.2$ ion channels contribute substantially and equally to the plateau of UTP- and UDP-evoked contractions of rat IPA.

Effects of tannic acid

Tannic acid was shown recently to inhibit $I_{Cl,Ca}$, with a maximal effect at 100 μM (Namkung *et al.*, 2010; 2011). At this concentration it induced small, rapid, transient contractions in 18/24 tissues (mean = 26 ± 5 mg, range = 10 – 84 mg) and significantly potentiated the peak amplitude of the contractions evoked by UTP, UDP and ATP in all tissues tested, by approximately 30–40% ($P < 0.05$) (Figure 6A). Contractions to phenylephrine (1 μM) and KCl (40 mM) were, in contrast, significantly depressed by about 30% ($P < 0.05$) (Figure 6A). When applied during the plateau phase of the UTP- and UDP-induced contractions (two tissues each), tannic acid (100 μM) caused a significantly larger, rapid, transient contraction (mean = 78 ± 12 mg, range = 52 – 110 mg, $n = 4$, $P < 0.001$), but no relaxation (Figure 6B).

Discussion

In this study, the vasoconstriction of the rat isolated IPA induced by UTP and UDP was inhibited by almost half by the $I_{Cl,Ca}$ blockers, niflumic acid and DIDS, suggesting that $I_{Cl,Ca}$ plays a major role in the vasoconstriction elicited via P2Y receptors. The $Ca_v1.2$ Ca^{2+} channel blocker, nifedipine, depressed the contractions by a similar amount and co-application with either $I_{Cl,Ca}$ blocker had no additional effect. This is consistent with a model whereby the depolarization induced by $I_{Cl,Ca}$ causes voltage-dependent $Ca_v1.2$ Ca^{2+} channels to open and Ca^{2+} to enter the cells. The contractions evoked by ATP were likewise inhibited by niflumic acid and nifedipine, whereas bathing tissues in Ca^{2+}-free buffer almost abolished the response. Moreover, contractions to ATP reached a peak more quickly and showed faster and greater decay following the peak, which suggests that ATP acts at both P2X and P2Y receptors. Thus, these data indicate that stimulation of P2X and P2Y receptors activates multiple signalling mechanisms to induce pulmonary artery vasoconstriction.

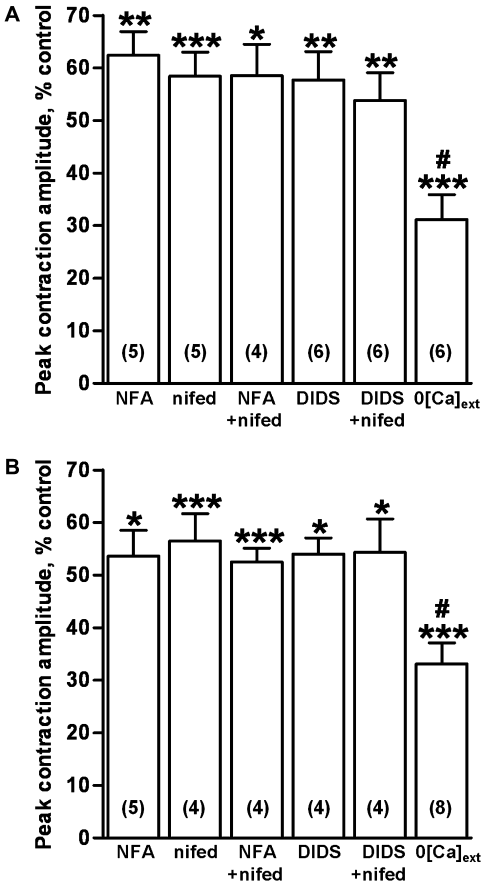

Figure 2

Inhibition of the peak amplitude of contractions evoked by UDP and UTP. The mean peak amplitude of contractions of endothelium-denuded rat isolated IPA evoked by (A) UDP (300 μM) and (B) UTP (300 μM) in the presence of niflumic acid (1 μM) (NFA), nifedipine (1 μM) (nifed), niflumic acid (1 μM) plus nifedipine (1 μM) (NFA + nifed), DIDS (100 μM), DIDS (100 μM) plus nifedipine (1 μM) (DIDS + nifed) and in Ca^{2+}-free buffer ($0[Ca]_{ext}$), expressed as a percentage of control responses, is shown. The numbers in parentheses show *n* for each. Vertical lines show SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for responses after treatment compared with control. $^{\#}P < 0.05$ for responses in Ca^{2+}-free buffer compared with the other treatments.

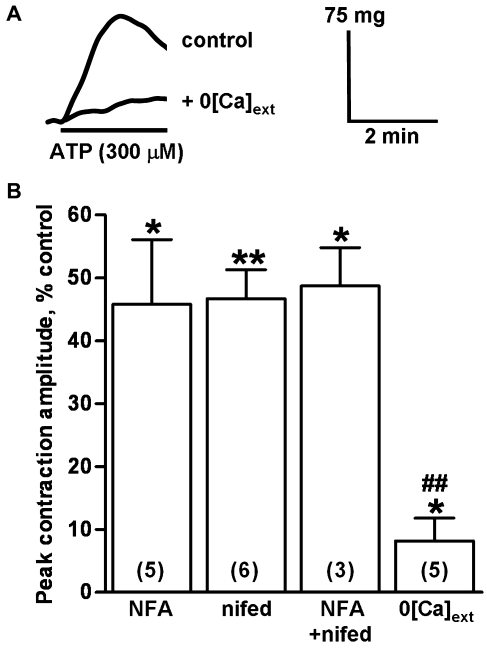

Figure 3

Inhibition of the peak amplitude of contractions evoked by ATP. (A) The superimposed traces show typical contractions of endothelium-denuded rat isolated IPA evoked by ATP (300 μM) in normal buffer (upper trace) and when bathed in Ca^{2+}-free buffer for 10 min (lower trace). ATP was applied as indicated by the solid bar. (B) The mean peak amplitude of contractions evoked by ATP (300 μM) in the presence of niflumic acid (1 μM) (NFA), nifedipine (1 μM) (nifed), niflumic acid (1 μM) plus nifedipine (1 μM) (NFA + nifed) and in Ca^{2+}-free buffer ($0[Ca]_{ext}$), expressed as a percentage of control responses, is shown. The numbers in parentheses show *n* for each. Vertical lines show SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ for responses after treatment compared with control. $^{\#\#}P < 0.01$ for the response to ATP in Ca^{2+}-free buffer compared with the other treatments.

Signalling mechanisms underlying UDP- and UTP-evoked vasoconstriction

We reported that UDP and UTP activate $I_{Cl,Ca}$ in rat IPA myocytes (Chootip *et al.*, 2005) and here we show that both the peak and plateau of the contractions to UTP and UDP were depressed by around half by the $I_{Cl,Ca}$ inhibitors niflumic acid and DIDS. This is the first demonstration that $I_{Cl,Ca}$ makes an essential contribution to the development and maintenance of P2Y receptor-mediated contractions. It is notable because activation of $I_{Cl,Ca}$ does not necessarily lead to vasoconstriction. For example, $I_{Cl,Ca}$ is activated by endothelin in, but plays no role in the concomitant contraction of rat IPA (Kato *et al.*, 1999). Thus $I_{Cl,Ca}$ is an important, but not ubiquitous, mediator of pulmonary vasoconstriction induced by GPCRs. Higher concentrations of niflumic acid (≥10 μM) can affect sites other than $I_{Cl,Ca}$, such as BK_{Ca} ion channels, non-selective cation channels, voltage-gated K^+ channels and COX (Kato *et al.*, 1999; Cruickshank *et al.*, 2003; Greenwood and Leblanc, 2007) and consistent with this, at 10 and 100 μM, it depressed contractions of rat IPA to KCl. In contrast, 1 μM niflumic acid and 100 μM DIDS had no effect on KCl-induced contractions, indicating selectivity of action at these concentrations.

The contractions evoked by UTP and UDP were also depressed by the $Ca_v1.2$ ion channel inhibitor, nifedipine and in a non-additive manner with niflumic acid and DIDS. This is consistent with a model in which $I_{Cl,Ca}$ evokes contraction by depolarizing smooth muscle cells, causing $Ca_v1.2$ ion channels to open and Ca^{2+} to flow into the cell (Large and Wang, 1996; Kitamura and Yamazaki, 2001; Leblanc *et al.*, 2005). The non-additivity of inhibition also indicates that $I_{Cl,Ca}$ was the only stimulus causing $Ca_v1.2$ ion channels to open, consistent with the inability of UTP and UDP to elicit other types of depolarizing current in rat IPA myocytes (Hartley *et al.*, 1998; Chootip *et al.*, 2005). The concentrations of $I_{Cl,Ca}$ blocker used were close to the IC_{50} values reported for niflumic acid (2.3 μM, Hogg *et al.*, 1994b) and DIDS (210 μM, Hogg *et al.*, 1994a) against $I_{Cl,Ca}$ in rabbit portal vein smooth

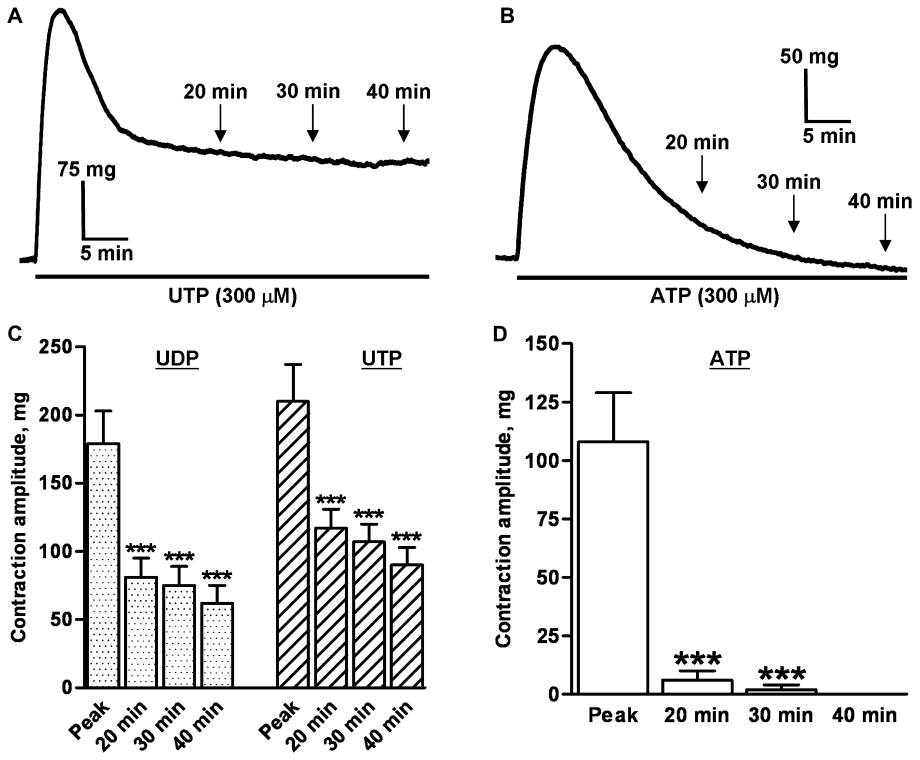

Figure 4

Time-course of contractions to UTP, UDP and ATP. The traces shows typical contractions of endothelium-denuded rat isolated IPA evoked by (A) UTP (300 μM) and (B) ATP (300 μM), applied as indicated by the solid bars. The mean amplitude of contractions evoked by (C) UDP (300 μM, $n = 5$) and UTP (300 μM, $n = 6$) and (D) ATP (300 μM, $n = 4$) at peak and 20, 30 and 40 min after agonist administration is shown. Vertical lines show SEM. ***$P < 0.001$ for contraction amplitude at 20, 30 or 40 min compared with peak.

muscle cells, so it is perhaps surprising that they depressed contractions by a similar amount to a maximally effective concentration of nifedipine. The reason for this is not clear, but it may be that relatively small changes in depolarization produce much larger changes in the opening of voltage-dependent Ca^{2+} channels. Alternatively, it may reflect the tissue variability of the potency of the $I_{Cl,Ca}$ blockers (see Large and Wang, 1996; Greenwood and Leblanc, 2007). For example, DIDS is nearly 13 times more potent against $I_{Cl,Ca}$ in rat portal vein myocytes (IC_{50} = 16.5 μM; Baron *et al.*, 1991) than in rabbit portal vein (IC_{50} = 210 μM, Hogg *et al.*, 1994a). Similarly, while 10 μM niflumic acid depressed KCl-evoked contractions in the present study, it had no effect on KCl-induced vasoconstriction of rat aorta and mesenteric vascular bed (Criddle *et al.*, 1996; 1997).

Signalling mechanisms underlying ATP-evoked vasoconstriction

The vasoconstriction evoked by ATP was also inhibited by niflumic acid in the present study, consistent with our earlier report that ATP activates $I_{Cl,Ca}$ in rat IPA myocytes (Chootip *et al.*, 2005). As seen with UTP and UDP, nifedipine inhibited ATP-induced contractions to a similar extent and in a non-additive manner. Thus, P2Y receptor-mediated activation of $I_{Cl,Ca}$ and subsequent opening of $Ca_v1.2$ Ca^{2+} channels also plays a major role in the contractile response to ATP. In contrast to UTP and UDP, however, Ca^{2+}-free buffer had a significantly greater inhibitory effect, almost abolishing the ATP response. Clearly, Ca^{2+} influx plays a greater role in the ATP vasoconstriction and appears to be via $Ca_v1.2$ ion channels, plus another route. This is most likely the P2X1 receptor, a Ca^{2+} permeable, ligand-gated cation channel (Egan and Khakh, 2004), which is the main P2X subtype expressed in rat IPA smooth muscle (Syed *et al.*, 2010) and which our pharmacological studies showed was involved in the pulmonary vasoconstrictor action of ATP (Chootip *et al.*, 2002; 2005). Consistent with an action at both P2X1 and P2Y receptors, ATP induced not only release of Ca^{2+} stores in rat pulmonary artery myocytes (responsible for activating $I_{Cl,Ca}$), but also Ca^{2+} influx (Guibert *et al.*, 1996). A similar difference between the relative roles of Ca^{2+} influx in contractions evoked by ATP and UTP was reported in the rat isolated tail artery (McLaren *et al.*, 1998) and that study also concluded that ATP acted via P2X and P2Y receptors to elicit vasoconstriction. A dual action on P2X and P2Y receptors may, therefore, be a general rule for ATP's contractile effects on vascular smooth muscle.

Signalling mechanisms underlying the decay of nucleotide-evoked vasoconstriction

The vasoconstriction elicited by UTP and UDP was biphasic, reaching a peak within 2–3 min and then decaying over 10–15 min to a plateau at about half of the peak response.

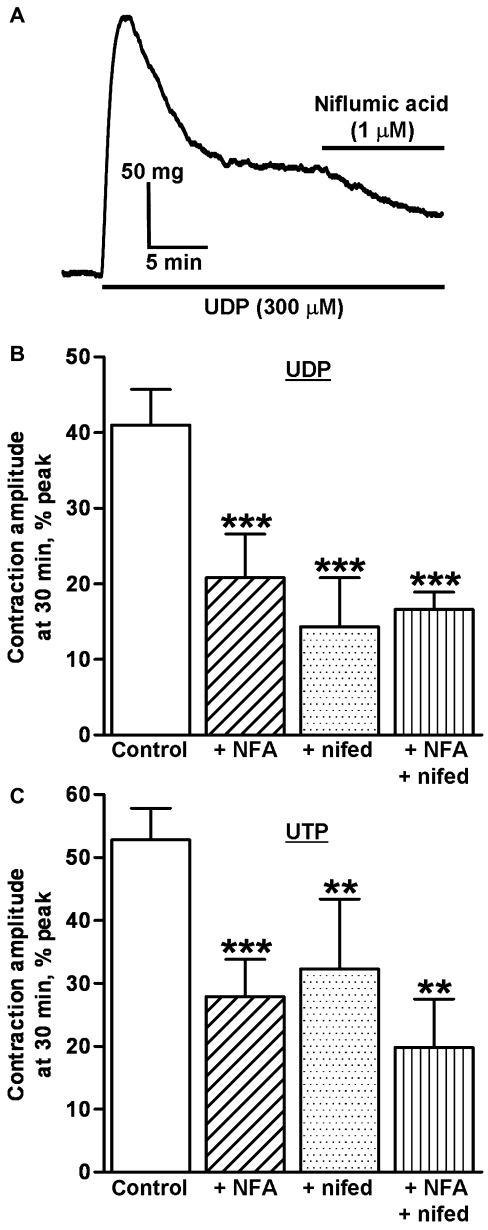

Figure 5

Inhibition of the plateau of UTP- and UDP-evoked contractions. (A) The trace show a typical contraction of endothelium-denuded rat isolated IPA evoked by UDP (300 μM) and the effect of adding niflumic acid (1 μM) for 10 min, 20 min after UDP. The drugs were applied as indicated by the solid bars. The mean amplitude of contractions evoked by (B) UDP (300 μM) and (C) UTP (300 μM) 30 min after their addition and expressed as a percentage of the peak amplitude, is shown as follows: control responses in the absence of inhibitor (UDP $n = 5$, UTP $n = 6$), responses in the presence of niflumic acid (1 μM) (NFA, UDP $n = 6$, UTP $n = 7$), in the presence of nifedipine (1 μM) (nif, UDP $n = 6$, UTP $n = 6$) and in the presence of niflumic acid (1 μM) plus nifedipine (1 μM) (NFA + nif, UDP $n = 7$, UTP $n = 5$). Vertical bars indicate SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ for response in the presence of inhibitors compared with the control response in their absence.

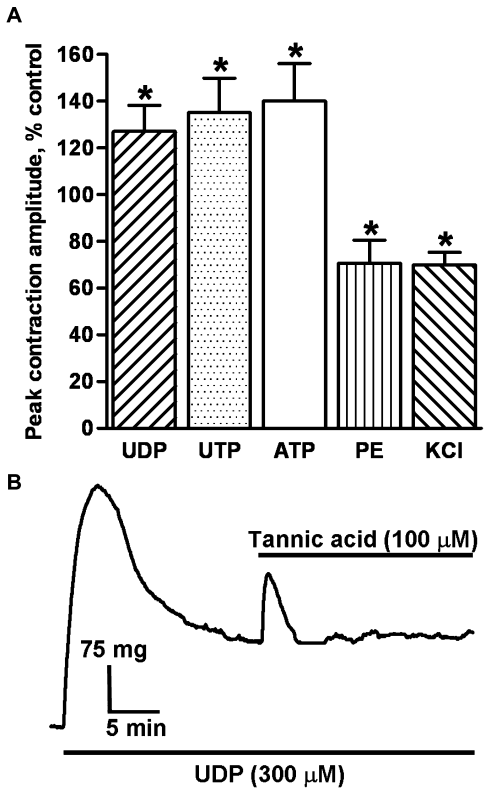

Figure 6

The effects of tannic acid. (A) The mean peak amplitude of contractions evoked by UDP ($n = 5$), UTP ($n = 4$), ATP ($n = 5$) (300 μM each), phenylephrine (PE) (1 μM) ($n = 4$) and KCl (40 mM) ($n = 6$) in the presence of tannic acid (100 μM), expressed as a percentage of the control response, is shown. Vertical lines show SEM. $^{*}P < 0.05$ for responses after treatment compared with control. (B) The trace shows a typical contraction of endothelium-denuded rat isolated IPA evoked by UDP (300 μM) and the effect of adding tannic acid (100 μM) 20 min after UDP. Drugs were applied as indicated by the solid bars.

The reason for the decay is unclear, but is unlikely to be due to the rapid decline seen in the amplitude of intracellular (Ca^{2+}) oscillations (Guibert *et al.*, 1996) and $I_{Cl,Ca}$ (Hartley *et al.*, 1998; Chootip *et al.*, 2005) evoked by UTP and UDP, as both reached a steady state within 2–3 min, which is similar to the time taken for the contractions to reach their peak. Furthermore, in the present study, niflumic acid substantially relaxed precontracted tissues, indicating that $I_{Cl,Ca}$ was active for at least 20 min after contractions peaked. Ca^{2+} oscillations and $I_{Cl,Ca}$ have yet, however, to be recorded over this longer timescale, and we cannot rule out the possibility that a slower decline in the frequency and/or amplitude of one or both of these signals accounts for the contraction decay. Similarly, we cannot exclude that the transient and sustained phases of constriction reflect temporal changes in the relative contributions of Ca^{2+} release and Ca^{2+} influx to the contractions (Lee *et al.*, 2002). Further experiments using Ca^{2+}-sensitive dyes will shed light on these possibilities.

Alternatively, the decay of the contraction could be due to desensitization of the P2Y receptors that mediate the actions

of UTP and UDP, rather than changes in Ca^{2+} signalling *per se*. We showed that both agonists act via two P2Y subtypes to evoke contraction in this tissue, most likely $P2Y_6$, plus $P2Y_2$ or $P2Y_4$ receptors (Chootip *et al.*, 2002; 2005; Kennedy *et al.*, 2010). $P2Y_2$ (Sromek and Harden, 1998; Flores *et al.*, 2005; Lemon *et al.*, 2005) and $P2Y_4$ (Brinson and Harden, 2001) receptors both desensitize rapidly and with a time-course compatible with the decay seen in the present study, whereas the $P2Y_6$ receptor desensitizes much more slowly, reaching a steady-state over 3–6 h (Robaye *et al.*, 1997; Brinson and Harden, 2001). Thus, the activation of $P2Y_6$ and $P2Y_2$/$P2Y_4$ receptors could together lead to the peak contraction, with the subsequent decay reflecting desensitization of $P2Y_2$/$P2Y_4$ receptors, to leave $P2Y_6$ receptors dominating the plateau. This model will be investigated once potent, stable, receptor subtype-selective agonists become commercially-available.

The contraction of rat pulmonary artery evoked by ATP was transient, showing full reversal over 40 min in the continuous presence of ATP, even though ATP induces Ca^{2+} influx in addition to Ca^{2+} release in this tissue. This may be explained by ATP acting at both P2X1 and P2Y receptors (Chootip *et al.*, 2005). The P2X1 receptor desensitizes rapidly and completely during agonist exposure (Evans and Kennedy, 1994; Khakh *et al.*, 2001). The $P2Y_2$ receptor is probably the second site of action of ATP and, as discussed above, it also desensitizes rapidly. ATP is not an agonist at the slowly desensitizing $P2Y_6$ receptor (Abbracchio *et al.*, 2006), consistent with the lack of a contraction plateau. Thus the rapid desensitization of the two sites of action of ATP can explain why its contractions reverse fully during agonist exposure.

Tannic acid

As tannic acid was shown recently to be a potent inhibitor of $I_{Cl,Ca}$ carried by recombinant and native TMEM16A channels (Namkung *et al.*, 2010; 2011), we used it as an additional probe for investigating $I_{Cl,Ca}$ involvement in nucleotide-induced constriction of rat IPA. Unfortunately, it produced effects that are inconsistent with a selective inhibitory action on $I_{Cl,Ca}$, such as evoking transient contractions in 75% of unstimulated tissues and significantly potentiating, rather than inhibiting the nucleotide-evoked contractions. Moreover, although tannic acid inhibited the vasoconstriction induced by phenylephrine, which may also involve the activation of $I_{Cl,Ca}$ (Yuan, 1997), it had the same effect on the vasoconstriction evoked by KCl. Thus, at the concentration used tannic acid does not appear to be a selective inhibitor of $I_{Cl,Ca}$ in pulmonary artery.

The mechanism(s) underlying the excitatory actions of tannic acid are unclear, but it is notable that the drug inhibits a variety of ATPases, including gastric H^+,K^+-ATPase (Murakami *et al.*, 1992), mitochondrial proton F0F1-ATPase (Zheng and Ramirez, 2000) and verapamil-stimulated ATPase activity in membrane vesicles (Kitagawa *et al.*, 2007) and so appears to be a general ATPase inhibitor. Ecto-nucleoside triphosphate diphosphohydrolases (formerly known as ecto-ATPase), are a family of ecto-enzymes that inactivate ATP, UTP and UDP by dephosphorylation (Robson *et al.*, 2006). In vascular smooth muscle these enzymes decrease nucleotide potency by up to two to three orders of magnitude (Evans and Kennedy, 1994; Kauffenstein *et al.*, 2010) and inhibition of the ecto-enzymes by the specific inhibitor ARL67156 potentiated contractions of rat tail artery to UTP to a similar extent as tannic acid (McLaren *et al.*, 1998). So inhibition of nucleotide breakdown by tannic acid might explain its ability to potentiate the nucleotide-evoked contractions, thereby masking any inhibitory effect that it might have on $I_{Cl,Ca}$. Further experiments are required to address this possibility.

Summary

In conclusion, these data indicate that UTP and UDP act at P2Y receptors to elicit release of Ca^{2+} from intracellular stores, which activates $I_{Cl,Ca}$, a depolarizing current. Our working hypothesis is that the depolarization in turn causes voltage-dependent $Ca_v1.2$ Ca^{2+} channels to open, allowing Ca^{2+} to flow into the cell and induce vasoconstriction. The constrictions caused by UDP and UTP probably involve rapidly desensitizing $P2Y_2$/$P2Y_4$ receptors and slowly desensitizing $P2Y_6$ receptors, giving rise to an initial peak response that decays partially to a plateau. ATP acts at P2Y receptors to activate the same pathway, but also acts at the P2X1 receptor. Together these activated receptors elicit depolarization, $Ca_v1.2$ ion channel opening and Ca^{2+}influx, as well as Ca^{2+} entry via the P2X1 receptor pore. The complete decay of the constrictor response to ATP following its peak is consistent with ATP acting at P2X1 and $P2Y_2$ receptors, both of which desensitize rapidly. Thus purine and pyrimidine nucleotides employ different receptors and produce kinetically distinct constrictor responses in rat IPA, but in each case, both $I_{Cl,Ca}$ and Ca^{2+} influx via $Ca_v1.2$ ion channels mediate a substantial proportion of the response. It was notable that Ca^{2+}-free buffer inhibited the UDP- and UTP-evoked contractions significantly more than did blocking $I_{Cl,Ca}$ and $Ca_v1.2$ ion channels, indicating that refilling of the intracellular Ca^{2+} stores by voltage-independent Ca^{2+} channels may also play a role. This possibility and the contribution of other signalling mechanisms, such as Ca^{2+}-sensitization, in nucleotide-induced pulmonary vasoconstriction are currently being studied.

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Conflicts of interest

None.

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