Comparison of signalling mechanisms underlying UTP-evoked vasoconstriction of rat pulmonary and tail arteries

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Abstract: Asrin Tengah, Nawazish-i-Husain Syed, Siti Tajidah Abdul Talip, Siti Nur Basirah Bujang and Charles Kennedy. Comparison of signalling mechanisms underlying UTP-evoked vasoconstriction of pulmonary and systemic-like arteries

The endogenous nucleotide, UTP, acts at smooth muscle P2Y receptors to constrict rat pulmonary and tail arteries, but the underlying signalling pathways are poorly understood. The aim was to characterise the contribution of Ca^{2+} release and influx, rho kinase and protein kinase C to these contractions. Isometric tension was recorded from endothelium-denuded rat intralobar pulmonary and tail artery rings mounted on a wire myograph. Contractions were evoked by UTP and peak amplitude measured. Thapsigargin (1 µM), but not ryanodine (10 µM), significantly depressed contractions in both by 30-40% (P<0.05). Nifedipine (1 µM) significantly reduced contractions in tail artery by ~60% (P<0.01). Y27632 (10 µM), a rho kinase inhibitor and GF109203X (10 µM), a protein kinase C inhibitor, each significantly reduced pulmonary vasoconstriction by ~20%, and tail artery contractions by ~80% and ~40%, respectively (P<0.01). In pulmonary artery, Y27632, GF109203X and thapsigargin, acted in an additive manner, but nifedipine less so. Adding all four together abolished the UTP response. In tail artery, Y27632 plus thapsigargin or GF109203X or nifedipine abolished contractions. Thapsigargin, GF109203X and nifedipine, coapplied pair-wise, acted additively and applying all three together abolished UTP-evoked contractions. So, Ca^{2+} release from the sarcoplasmic reticulum and influx through $Ca_v1.2$ channels, but not ryanodine receptors, play significant roles in UTP-evoked vasoconstriction of rat pulmonary and tail arteries. Rho kinase and protein kinase C are also involved, but more so in tail artery. Thus UTP activates multiple signalling mechanisms that lead to vasoconstriction, but their relative importance differs in pulmonary compared with systemic arteries.

Key words: P2Y receptor, pulmonary artery, tail artery, Ca^{2+}, rho kinase, protein kinase C

1. Introduction

Endogenous nucleotides, such as uridine 5'-triphosphate (UTP), modulate vascular tone via P2Y receptors, a family of G protein-coupled receptors (GPCR) (Abbracchio et al., 2006) present throughout the cardiovascular system (Erlinge and Burnstock, 2008; Burnstock and Kennedy, 2011; Mitchell et al., 2012b: Kennedy et al., 2013). For example, we reported that UTP acts at P2Y receptors to contract rat isolated intralobar pulmonary (rIPA) (Chootip et al., 2002) and tail (rTA) (Evans and Kennedy, 1994; McLaren et al., 1998) arteries. How they produce these effects, however, is poorly understood.

One possible mechanism underlying contraction is a rise in cytoplasmic $[Ca^{2+}]$ and sarcoplasmic reticulum (SR) Ca^{2+} stores can be released by UTP-sensitive P2Y receptors via coupling to $G\alpha_{q/11}$, synthesis of IP_3, and activation of IP_3 receptors (IP_3R) (Abbracchio et al., 2006). Indeed, UTP evokes Ca^{2+} release in smooth muscle cells of many arteries, including rIPA (Guibert et al., 1996; Jernigan et al., 2006) and several studies have elegantly characterised sub-cellular changes in cytoplasmic $[Ca^{2+}]$, such as Ca^{2+} sparks and waves (Jaggar and Nelson, 2000; Zhao et al., 2008; Syyong et al., 2009). Ca^{2+} released by UTP in rIPA activates a depolarising Ca^{2+}-dependent, Cl^- current (Hartley and Kozlowski, 1997; Chootip et al., 2005), which we showed causes voltage-dependent $Ca_v1.2$ Ca^{2+} channels to open, enabling influx of extracellular Ca^{2+} and vasoconstriction (Mitchell et al., 2012a). In most arteries, however, the release of Ca^{2+} elicited by UTP has not been related to its vasoconstrictor actions. This is essential, as raised cytoplasmic $[Ca^{2+}]$ does not necessarily evoke contraction. For example, stimulation of the FP prostanoid receptor in rIPA caused IP_3-dependent Ca^{2+} release, but no change in vessel tone (Snetkov et al., 2006).

The roles of other, common signalling mechanisms have barely been studied and remain to be characterised fully. For example, in many tissues, Ca^{2+} released via IP_3R stimulates ryanodine receptors (RyR) in the SR to elicit Ca^{2+}-induced Ca^{2+} release (CICR) (Wray and Burdyga, 2010). Many GPCR increase the force/$[Ca^{2+}]$ ratio of smooth muscle contractile proteins (Ca^{2+}-

sensitisation) via rho kinase (RhoK) (Somlyo and Somlyo, 2003). Finally, protein kinase C (PKC), which is activated by UTP-sensitive P2Y receptors (Abbracchio et al., 2006), can contribute to vasoconstriction in several ways, including Ca^{2+}-sensitisation (Somlyo and Somlyo, 2003; Ward et al., 2004), potentiation of $Ca_v1.2$ Ca^{2+} currents (Ward et al., 2004; Navedo et al., 2005; Ren et al., 2010) and inhibition of voltage-dependent K^+ currents (Cogolludo et al., 2003).

In view of the sparse and fragmentary nature of our knowledge and understanding of how UTP induces vasoconstriction, our aim was to develop an integrated model of the signalling pathways involved. The effects mediated by GPCR can vary in different vascular beds because of differences in their physiological function, so the actions of UTP were compared in rIPA, a low pressure, low resistance artery that carries deoxygenated blood away from the heart, and the rTA, a high-pressure systemic artery that carries oxygenated blood away from the heart.

2. Materials and methods

2.1 Tissue preparation

The methods used conform to the ARRIVE guidelines and meet the ethical requirements of Strathclyde University (https://www.strath.ac.uk/science/biomedicalresearchatstrathclyde/). Segments of rIPA (Chootip et al., 2002) and rTA (Evans et al., 1994) were prepared for in vitro recording as described previously. 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, N-[2-hydroxyethyl] piperazine-N'-(2-ethanesulfonic acid] (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 a gas mixture of 21% O_2, 5% CO_2, 74% N_2. rIPA of internal diameter 300-500 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 37ºC. Segments of rTA (internal diameter 300-500 μm) were

dissected and mounted in a similar manner, but at a resting tension of 0.5-0.75 g. Tension was recorded with Grass FT03 isometric force transducers, connected to a PowerLab/4e system, using Chart 4.2 software (AD Instruments, UK).

2.2 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 acetylcholine (10 μM) following precontraction with UTP. The UTP concentration-contraction curves in rIPA (Chootip et al., 2002) and rTA (Evans et al., 1994) do not reach a maximum. UTP, therefore, was applied at the equi-effective concentrations of 300 μM and 1 mM, respectively, for 5 min at 30 min intervals, as preliminary experiments showed that this protocol elicited highly reproducible contractions when UTP was added repeatedly.

To investigate the role of Ca^{2+} release via RyR, control responses to UTP, then caffeine (10 mM) were obtained. Ryanodine (10 μM) was added for 10 min before caffeine (10 mM) was readded for 5 min. After washing, ryanodine (10 μM) was added for another 10 min before readdition of caffeine (10 mM) for 5 min. Preliminary experiments showed that this protocol abolished contractions induced by caffeine. The tissues were again washed and ryanodine (10 μM) added for 10 min before being challenged with UTP. The effects of thapsigargin were studied in the same way. To determine the effects of (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride monohydrate (Y27632), 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide (GF109203X) and nifedipine, control responses to UTP were obtained. Arteries were then incubated with one or more of the inhibitors for 15 min before UTP was re-administered.

2.3 Drugs and solutions

UTP (Na_3 salt), caffeine, acetylcholine chloride (Sigma, UK) and ryanodine (Calbiochem, U.S.A.)

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were dissolved in distilled water. Nifedipine (Sigma, UK), thapsigargin, phorbol 12-myristate 13-acetate (PMA), Y27632 and GF109203X (Tocris, U.K.) were dissolved in DMSO. Drugs were prepared as 10 or 100 mM stock solutions and diluted in the HEPES-based buffer before applying to the tissues.

2.4 Data Analysis

Contractions are expressed as mg tension or a percentage of the control response produced by UTP, as appropriate. Data are shown as mean ± S.E.M. 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.

3. Results

3.1 Effect of ryanodine and thapsigargin

Activation of UTP-sensitive P2Y receptors induces release of intracellular SR Ca^{2+} stores (Kennedy et al., 2013). The aim of the initial experiments was, therefore, to determine the contribution of these stores to the contractions elicited by UTP using ryanodine, which, at the concentration used, causes the RyR channels to remain in a partially conducting state and thapsigargin, which, at the concentration used, inhibits Ca^{2+} uptake into the SR (Wray and Burdyga, 2010).

UTP evoked contractions of rIPA (Fig. 1A,B) and rTA (Fig. 1C,D) that reached a peak within 2-4 min. Ryanodine (10 μM) abolished contractions induced by the RyR agonist caffeine (10 mM) (not shown), but had no significant effect on the peak amplitude of the UTP-evoked responses: rIPA (Fig. 1A, 2A-1st column), rTA (Fig. 1C, 2B-1st column).

In contrast, thapsigargin (1 μM), depressed significantly the responses in both tissues by 30-40%: rIPA ($P < 0.01$, Fig. 1B, 2A, 2nd column), rTA ($P < 0.05$, Fig. 1D, 2B, 2nd column). Thus Ca^{2+} release from thapsigargin-sensitive SR stores plays a significant role in the UTP-evoked vasoconstriction in both arteries, but ryanodine-sensitive Ca^{2+} stores are not involved.

3.2 Effect of nifedipine

We reported that the $Ca_v1.2$ Ca^{2+} channel blocker, nifedipine, inhibited contractions of rIPA evoked by UTP by nearly 50% (Mitchell et al., 2012a). In the rTA nifedipine (1 μM), a concentration that maximally inhibits $Ca_v1.2$ ion channels (Clapp and Gurney, 1991), significantly reduced the peak amplitude of the UTP response by approximately 60% (P<0.01) (Fig. 2B, 3rd column), which was not significantly different from its inhibitory effects in the rIPA. Thus Ca^{2+} influx via nifedipine-sensitive Ca^{2+} channels plays a significant role in the UTP-evoked vasoconstriction in both arteries.

3.3 Effect of GF109203X and Y27632

P2Y receptors can mediate activation of RhoK and PKC (Abbracchio et al., 2006), so their involvement in UTP-evoked contractions was investigated using Y27632, an inhibitor of RhoK (Uehata et al., 1997), and the PKC inhibitor, GF109203X (Toullec et al., 1991). Initial experiments determined an effective concentration of GF109203X and the selectivity of Y27632. The PKC activator, PMA (10 μM), induced very slowly developing contractions of the rIPA of variable amplitude (range = 58 - 390 mg) that in most cases reached a peak (168 $\pm$ 20 mg, n=18) within 60 min and were maintained for at least another 30 min (Fig. 3A-E). These were partially suppressed by 3 μM GF109203X (Fig. 3A) and abolished by 10 μM GF109203X, added 15 min before (Fig. 3B) or 60 min after (Fig. 3C) PMA. In contrast, Y27632 (10 μM), a concentration that produces near maximal inhibition of RhoK (Uehata et al., 1997; Davies et al., 2000), had no effect on the PMA response under the same conditions (Fig. 3D,E), indicating a lack of effect on PKC. 10 μM Y27632 and GF109203X were, therefore, used in subsequent experiments.

Y27632 (10 μM) significantly reduced the peak amplitude of contractions evoked by UTP by about 20% in rIPA (P<0.01, Figs. 2A-3rd column, 4A) and by more than 80% in rTA (P<0.01, Figs. 2B-4th column, 4B) and the inhibition was significantly greater in the rTA (P<0.001). GF109203X (10 μM) also significantly reduced the peak amplitude of the UTP response in rIPA by over 20%

(P<0.01, Fig. 2A-4th column) and by around 40% in rTA (P<0.001, Fig. 2B-5th column). Inhibition was again significantly greater in rTA (P<0.05). Thus Y27632 and GF109203X both inhibit UTP-evoked contractions in both arteries, but more so in the rTA.

3.4 Combined inhibition of signalling components in rIPA

These data, together with our published data (Chootip et al., 2005; Mitchell et al., 2012a) show clearly that multiple signalling components contribute to UTP-evoked vasoconstriction and that their relative contributions are artery-specific. The next experiments investigated how the individual components combine, by applying two or more of the inhibitors together, first in the rIPA.

Adding Y27632 (10 μM) plus GF109203X (10 μM) significantly reduced UTP contraction peak amplitude by about 45% (P<0.01) (Fig. 5A-1st column), which was significantly greater than the effect of either agent alone (P<0.05) and virtually equivalent to the sum of their individual effects. When thapsigargin (1 μM) was added along with Y27632 (10 μM) and GF109203X (10 μM), the inhibitory effects of the three drugs also appeared to be additive, as the peak response was significantly depressed by around 85% (P<0.01) (Fig. 5A-2nd column). This was significantly more than the inhibition produced by Y27632 plus GF109203X (P<0.001).

Nifedipine (1 μM) inhibited UTP contractions in rIPA by around 45% (Mitchell et al., 2012a) and co-adding it with Y27632 (10 μM) plus GF109203X (10 μM) significantly reduced the peak UTP response by nearly 70% (P<0.01) (Fig. 5A-3rd column). Although this was significantly greater than that produced by Y27632 plus GF109203X (P<0.05), the inhibitory effects of the three drugs were less than additive.

Finally, adding all four inhibitors together abolished the response to UTP (Figs. 5A-4th column, 5B). Thus all of the signalling components targeted by these inhibitors are involved in UTP-evoked contractions of rIPA and in a partly additive and overlapping manner.

3.5 Combined inhibition of signalling components in rTA

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The data reported above in the rTA show that Y27632 has by far the greatest inhibitory effect on UTP-evoked contractions. The mediator(s) of the Y27632-resistant component was, therefore, investigated next. The contractions elicited by UTP were abolished by coapplying Y27632 (10 μM) with nifedipine (1 μM) (Fig. 6A,B-1st column. thapsigargin (1 μM) (Fig. 6B-2nd column) or GF109203X (10 μM) (Fig. 6B-3rd column).

Finally, thapsigargin, GF109203X and nifedipine were coapplied in the absence of Y27632. Each pair significantly inhibited UTP-evoked contractions and in an approximately additive manner. Thus in the presence of the two least effective inhibitors, thapsigargin (1 μM) plus GF109203X (10 μM), nearly 30% of the peak response to UTP remained ($P<0.01$) (Fig. 6B-4th column), while coapplication of thapsigargin (10 μM) plus nifedipine (1 μM) ($P<0.01$) (Fig. 6B-5th column) or GF109203X (10 μM) plus nifedipine (1 μM) ($P<0.01$) (Fig. 6B-6th column) substantially depressed the UTP response, but did not abolish it. In each case the inhibition was significantly greater than the effect of either inhibitor alone ($P<0.05$- thapsigargin plus GF109203X; $P<0.001$- thapsigargin plus nifedipine; $P<0.001$- GF109203X plus nifedipine). Applying all three inhibitors together abolished UTP-evoked contractions (Fig. 6B-7th column).

4. Discussion

In this study, the vasoconstriction of rIPA and rTA induced by UTP was inhibited by a similar amount by thapsigargin, but unaffected by ryanodine, indicating that release of SR Ca^{2+} stores via IP$_3$R plays a role in the contractions, but RyR are not involved. Ca^{2+} influx via $Ca_v1.2$ ion channels also contributes to the response in both arteries and to a similar extent. Contractions were also suppressed by inhibitors of RhoK and PKC, but significantly more so in the rTA. Simultaneously inhibiting two or more of these signalling components depressed the contractions in a vessel-specific manner. Thus these data indicate that stimulation of P2Y receptors by UTP activates multiple signalling mechanisms that lead to vasoconstriction, but their relative contributions differ in pulmonary, compared with systemic, arteries.

4.1 Signalling mechanisms underlying UTP-evoked vasoconstriction of rIPA

In this study, pretreatment with thapsigargin, a SERCA inhibitor that depletes SR Ca^{2+} stores (Wray and Burdyga, 2010), inhibited the peak amplitude of vasoconstriction elicited by UTP by around 40%, showing for the first time that Ca^{2+} release from the SR contributes substantially to UTP-evoked contractions of rIPA. This is important, as GPCR-mediated Ca^{2+} release does not always result in vasoconstriction (Snetkov et al., 2006). Ryanodine, at a concentration that abolished caffeine-induced contractions, had no effect on the UTP-evoked contractions, indicating that Ca^{2+}-induced Ca^{2+} release via RyR was not involved in the UTP response. The role of RyR in GPCR-mediated pulmonary vasoconstriction is very variable. A similar lack of involvement was seen in α_1- and 5-HT_{2A}-receptor-mediated contractions of dog IPA (Jabr et al., 1997; Wilson et al., 2005), but depleting ryanodine-sensitive Ca^{2+} stores partially reduced noradrenaline-evoked vasoconstriction of rIPA (Zheng et al., 2005) and abolished contractions of rabbit IPA by $PGF_{2\alpha}$ (Dipp et al., 2001) and mouse IPA by 5-HT (Perez and Sanderson, 2005).

The lack of effect of ryanodine in the present study indicates that UTP elicits release of Ca^{2+} via IP_3R in rIPA, consistent with the coupling of UTP-sensitive P2Y receptors to $G\alpha_{q/11}$ (Abbracchio et al., 2006). We reported that UTP activates a Ca^{2+}-dependent Cl^- current in rIPA myocytes (Chootip et al., 2005) and that blocking this current depressed the peak of the UTP-evoked vasoconstriction by almost half (Mitchell et al., 2012a). Furthermore, the contractions evoked by UTP were depressed by nifedipine by a similar amount and concomitant blockade of Cl^- and $Ca_v1.2$ ion channels produced no further inhibition. Our current working model is, therefore, that the major function of Ca^{2+} released from the SR is to activate the Ca^{2+}-dependent Cl^- current, which depolarises rIPA smooth muscle cells, causing $Ca_v1.2$ ion channels to open and extracellular Ca^{2+} to flow into the cell and cause contraction (Fig. 7A).

These experiments show that the selective inhibitor, Y27632 (Uehata et al., 1997; Davies et al., 2000), depressed the contractions by about 20%, indicating that RhoK likely contributes to the

UTP response (Fig. 7A). This is a relatively minor effect compared with that seen against responses evoked by other GPCR in pulmonary arteries. For example, Y27632 inhibited by 70% or more, TP receptor-mediated contractions of rat (Martin et al., 2004), rabbit (Fu et al., 1998) and cow (Alapati et al., 2007) IPA and α_1-adrenoceptor-mediated vasoconstriction of dog IPA (Janssen et al., 2001), whilst contraction of rIPA induced by sphingosylphosphorylcholine (Thomas et al., 2005) and of the mouse perfused pulmonary vascular bed by sphingosine 1-phosphate (Szczpaniak et al., 2010) were both depressed by about 65%. RhoK induces vasoconstriction by Ca^{2+}-sensitisation (Somlyo and Somlyo, 2003) and consistent with this mechanism, Y27632 virtually abolished UTP contractions in permeabilised pulmonary arteries (Jernigan et al., 2004).

This study also demonstrated that GF109203X, the selective PKC inhibitor (Toullec et al., 1991), depressed the peak contraction amplitude by about 20%, indicating a contribution of PKC to the UTP response. This is less than the contribution of PKC to TP receptor-mediated contraction of rIPA (~50%) (Cogolludo et al., 2003), but similar to that of α_1-adrenoceptor-mediated vasoconstriction of dog IPA (Damron et al., 2002). Like RhoK, PKC can induce vasoconstriction via Ca^{2+}-sensitisation (Somlyo and Somlyo, 2003; Ward et al., 2004), but this is unlikely to be its mode of action here, as the inhibitory actions of GF109203X and Y27632 were additive, suggesting separate sites of action. Consistent with this, GF109203X had no effect on UTP-evoked contractions in permeabilised pulmonary arteries (Jernigan et al., 2004). PKC-dependent phosphorylation of IP_3R (Narayanan et al., 2012) is also unlikely to be involved, as using thapsigargin to deplete SR Ca^{2+} stores produced an additive effect with inhibition of PKC and RhoK, suggesting that these three signalling components are arranged in parallel, rather than in series in rIPA.

The cellular target(s) of PKC in rIPA remain to be determined and possibilities include $Ca_v1.2$ ion channels (Fig. 7A), the activity of which can be potentiated by GPCR. For example, in rIPA, sphingosylphosphorylcholine increased Ca^{2+} influx via $Ca_v1.2$ ion channels in a PKC-dependent manner (Snetkov et al., 2008). These channels may also be constitutively phosphorylated by PKC,

increasing their basal activity (Navedo et al., 2005) and opening in response to depolarisation (Ren et al., 2010). PKC can also increase $Ca_v1.2$ ion currents indirectly by inhibiting voltage-dependent K^+ currents in pulmonary arterial smooth muscle (Cogolludo et al., 2003), so inducing depolarisation and $Ca_v1.2$ ion channel opening. Indeed, endothelin-1 depolarised rIPA smooth muscle cells by inhibiting voltage-dependent K^+ channels (Salter and Kozlowski, 1996) via activation of PKC (Shimoda et al., 1998). In the present study, blocking Ca^{2+} influx via $Ca_v1.2$ ion channels was less than additive with inhibition of PKC and RhoK, indicating that $Ca_v1.2$ ion channels are arranged at least in part in series with PKC and/or RhoK.

4.2 Signalling mechanisms underlying UTP-evoked vasoconstriction of rTA

The present experiments show that in rTA, as in rIPA, release of Ca^{2+} from SR stores and influx of extracellular Ca^{2+} via $Ca_v1.2$ ion channels both contribute to UTP-evoked contractions (Fig. 7B), but RyR are not involved. RyR also play no role in UTP-elicited contractions of rat basilar artery (Syyong et al., 2009). Influx of extracellular Ca^{2+} is important in the rTA, as nifedipine inhibited UTP vasoconstriction by about 60%, consistent with the depressant effect of Ca^{2+}-free buffer (McLaren et al., 1998). $Ca_v1.2$ ion channel blockers likewise depressed UTP contractions of rat cerebral artery (Luykenaar et al., 2003) and rat (Welsh and Brayden, 2001) and dog (Matsumoto et al., 1997) coronary arteries. Unlike rIPA, where the inhibitory effects of thapsigargin and nifedipine were similar, the inhibitory effect of nifedipine in rTA was twice that of thapsigargin, indicating that Ca^{2+} release is not the only stimulus that opens $Ca_v1.2$ ion channels in this artery (Fig. 7B). The identity of the other stimulus is, at present, unclear, but interestingly, UTP activated a maintained, depolarising cation current and inhibited voltage-dependent K^+ currents in rat coronary artery (Welsh and Brayden, 2001) and depolarised rat cerebral artery smooth muscle cells by inhibiting a voltage-dependent K^+ current in a RhoK-dependent, PKC-independent manner, leading to vasoconstriction (Luykenaar et al., 2003).

By far the biggest differences seen in the present study were the much greater effects of

Y27632 and GF109203X in rTA compared with rIPA. Y27632 depressed the peak response to UTP in rTA by over 80%, compared with about 20% in rIPA. To the best of our knowledge, the only other analogous study was in rat cerebral artery, where Y27632 abolished UTP-evoked contractions (Luykenaar et al., 2003). UTP, however, induced translocation of RhoA to the plasma membrane of smooth muscle cells in rat aorta (Sauzeau et al., 2000) and mesenteric artery (Nelson et al., 2008), an essential step in the activation of RhoK (Somlyo and Somlyo, 2003), so stimulation of RhoK by UTP in vascular smooth muscle is likely to be common (Fig. 7B).

GF109203X also depressed the peak amplitude of UTP-evoked contractions by twice as much in rTA than rIPA. Again, there are few reports on the role of PKC in UTP-evoked vasoconstriction in other arteries, but inhibition did not affect the response to UTP in rat cerebral artery (Luykenaar et al., 2003). Nonetheless, as UTP-sensitive P2Y receptors couple to $G\alpha_{q/11}$, stimulation of PKC by UTP in vascular smooth muscle is likely to be widespread and to play a role in rTA (Fig. 7B). As discussed above for rIPA, the cellular target(s) of PKC in the rTA remain to be determined. The component of the contraction of rTA that was resistant to Y27632 was abolished by inhibiting individually Ca^{2+} release, Ca^{2+} influx via $Ca_v1.2$ ion channels or PKC and in each case the inhibition was less than additive. In addition, blocking these three components together, in the absence of Y27632, also abolished the response to UTP. This indicates that there is substantial overlap of these signalling pathways in rTA.

5. Conclusions

In conclusion, these data have developed our knowledge and understanding of how UTP causes arteries to contract. They show that multiple signalling mechanisms are involved in UTP-evoked vasoconstriction of rIPA, a low pressure, low resistance artery, and rTA, a high pressure, systemic artery and reveal differences in their relative roles. Whilst the contributions of Ca^{2+} release from intracellular stores and Ca^{2+} influx via $Ca_v1.2$ Ca^{2+} channels are similar in the two arteries, PKC and particularly RhoK, play a much greater role in the systemic artery. How the individual signalling

components interact with each other also differs in the two systems. The limited number of reports on the signalling mechanisms underlying UTP's actions in other systemic arteries are in broad agreement with the data obtained in the rTA, but it is too early to say if they represent a general model of UTP signalling in systemic arteries. Finally, on the basis of our improved understanding of how UTP acts, further studies are now underway to investigate in greater detail how individual components are activated and how they interact with each other and with other potential mechanisms, such as voltage independent Ca^{2+} influx. Together, this will build a comprehensive and integrated model of the signalling pathways that couple P2Y receptor activation by UTP to vasoconstriction.

Conflict of Interest: none declared.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006). International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58: 281-341.

Adnot S, Raffestin B, Eddahibi S, Braquet, P, Chabrier PE (1991). Loss of endothelium-dependent relaxant activity in the pulmonary circulation of rats exposed to chronic hypoxia. J Clin Invest 87: 155-162.

Alapati VR, McKenzie C, Blair A, Kenny D, MacDonald A, Shaw AM (2007). Mechanisms of U46619- and 5-HT-induced contraction of bovine pulmonary arteries: role of chloride ions. British J Pharmacol. 151: 1224-1234.

Burnstock G, Kennedy C (2011). P2X receptors in health and disease. Adv Pharmacol 61: 333-372.

Chootip K, Gurney AM, Kennedy C (2005). Evidence for multiple P2Y receptors coupled to calcium-dependent, chloride channels in smooth muscle cells of the rat pulmonary artery. Respir Res 26: 124.

Chootip K, Ness K, Wang J, Gurney AM, Kennedy C (2002). Regional variation in P2 receptor expression in the rat pulmonary arterial circulation. British J Pharmacol 137: 637-646.

Clapp LH, Gurney AM (1991). Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. Pflugers Arch 418: 462-470.

Cogolludo A, Moreno L, Bosca L, Tamargo J, Perez-Vizcains F (2003). Thromboxane A_2-induced inhibition of voltage-gated K^+ channels and pulmonary vasoconstriction: role of protein kinase C zeta. Circ Res 93: 656-663.

Damron DS, Kanaya N, Homma Y, Kim SO, Murray PA (2002). Role of PKC, tyrosine kinases, and Rho kinase in α-adrenoreceptor-mediated PASM contraction. Am J Physiol 283: L1051-L1064.

Davies SP, Reddy H, Caivano M, Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J 351: 95-105.

Dinh-Xuan AT, Higenbottam TW, Clelland CA, Pepke-Zaba J, Cremona G, Butt AY, Large SR, Wells FC, Wallwork J (1991). Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. N Eng J Med 324: 1539-1547.

Dipp M, Nye PC, Evans AM (2001). Hypoxic release of calcium from the sarcoplasmic reticulum of pulmonary artery smooth muscle. Am J Physiol 281: L318-325.

Erlinge D, Burnstock G (2008). P2 receptors in cardiovascular regulation and disease. Purinergic Signal 4: 1-20.

Evans RJ, Kennedy C (1994). Characterization of P2-purinoceptors in the smooth muscle of the rat tail artery: A comparison between contractile and electrophysiological responses. British J Pharmacol 113: 853-860.

Fu X, Gong MC, Jia T, Somlyo AV, Somlyo AP (1998). The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPγS-, and phorbol ester-induced Ca^{2+}-sensitization of smooth muscle. FEBS Lett 440: 183-187.

Guibert C, Pacaud P, Loirand G, Marthan R, Savineau JP (1996). Effect of extracellular ATP on cytosolic Ca^{2+} concentration in rat pulmonary artery myocytes. Am J Physiol 271: 450-458.

Hartley SA, Kozlowski RZ (1997). Electrophysiological consequences of purinergic receptor stimulation in isolated rat pulmonary arterial myocytes. Circ Res 80: 170-178.

Jabr RI, Toland H, Gelband CH, Wang XX, Hume JR (1997). Prominent role of intracellular Ca^{2+} release in hypoxic vasoconstriction of canine pulmonary artery. British J Pharmacol 122: 21-30.

Jaggar JH, Nelson MT (2000). Differential regulation of Ca^{2+} sparks and Ca^{2+} waves by UTP in rat cerebral artery smooth muscle cells. Am J Physiol 279: C1528-1539.

Janssen LJ, Lu-Chao H, Netherton S (2001). Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase. Am J Physiol 280: L666-L674.

Jernigan, NL, Broughton BR, Walker BR, Resta TC (2006). Impaired NO-dependent inhibition of store- and receptor-operated calcium entry in pulmonary vascular smooth muscle after chronic hypoxia. Am J Physiol 290: L517-525.

Jernigan NL, Walker BR, Resta TC (2004). Chronic hypoxia augments protein kinase G-mediated Ca^{2+} desensitization in pulmonary vascular smooth muscle through inhibition of RhoA/Rho kinase signaling. Am J Physiol 287: L1220-1229.

Kennedy C, Chootip K, Mitchell C, Syed NH, Tengah A (2013). P2X and P2Y nucleotide receptors as targets in cardiovascular disease. Future Med Chem 5: 431-439.

Luykenaar KD, Brett SE, Wu BN, Wiehler WB, Welsh DG (2003). Pyrimidine nucleotides suppress K_{DR} currents and depolarize rat cerebral arteries by activating Rho kinase. Am J Physiol 286: H1088-1100.

Martin C, Göggel R, Ressmeyer AR, Uhlig S (2004). Pressor responses to platelet-activating factor and thromboxane are mediated by Rho-kinase. Am J Physiol 287: L250-257.

Matsumoto T, Nakane T, Chiba S (1997). UTP induces vascular responses in the isolated and perfused canine epicardial coronary artery via UTP-preferring P2Y receptors. British J Pharmacol 122: 1625-1632.

McLaren GJ, Burke KS, Buchanan KJ, Sneddon P, Kennedy C (1998). Evidence that ATP acts at two sites to evoke contraction in the rat isolated tail artery. British J Pharmacol 124: 5-12.

Mitchell C, Syed NH, Gurney AM, Kennedy C (2012a). 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. British J Pharmacol 166: 1503-1512.

Mitchell C, Syed NH, Tengah A, Gurney AM, Kennedy C (2012b). Identification of contractile $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ receptors in rat intrapulmonary artery using selective ligands. J Pharmacol Exp Therap 343: 755-762.

Narayanan D, Adebiyi A, Jaggar JH (2012). Inositol trisphosphate receptors in smooth muscle cells. Am J Physiol 302: H2190-H2210.

Navedo MF, Amberg GC, Votaw VS, Santana LF (2005). Constitutively active L-type Ca^{2+} channels. Proc Natl Acad Sci 102: 11112-11117.

Nelson CP, Willets JM, Davies NW, Challiss RA, Standen NB (2008). Visualizing the temporal effects of vasoconstrictors on PKC translocation and Ca^{2+} signaling in single resistance arterial smooth muscle cells. Am J Physiol 295: C1590-1601.

Perez JF, Sanderson MJ (2005). The contraction of smooth muscle cells of intrapulmonary arterioles is determined by the frequency of Ca^{2+} oscillations induced by 5-HT and KCl. J Gen Physiol 125: 555–567.

Ren C, Zhang J, Philipson KD, Kotlikoff MI, Blaustein M.P, Matteson DR (2010). Activation of L-type Ca^{2+} channels by protein kinase C is reduced in smooth muscle-specific Na^{+}/Ca^{2+} exchanger knockout mice. Am J Physiol 298: H1484-1491.

Salter KJ, Kozlowski RZ (1996). Endothelin receptor coupling to potassium and chloride channels in isolated rat pulmonary arterial myocytes. J Pharmacol Exp Ther 279: 1053-1062.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Vaillant N, Gadeau AP, Desgranges C, Scalbert E, Chardin P, Pacaud P, Loirand G (2000). $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are coupled to Rho and Rho kinase activation in vascular myocytes. Am J Physiol 278: H1751-1761.

Shimoda LA, Sylvester JT, Sham JS (1998). Inhibition of voltage-gated K^{+} current in rat intrapulmonary arterial myocytes by endothelin-1. Am J Physiol 274: L842-853.

Snetkov VA, Knock GA, Baxter L, Thomas GD, Ward JP, Aaronson PI (2006). Mechanisms of the prostaglandin $F_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ in rat intrapulmonary arteries. J Physiol 571: 147-163

Snetkov VA, Thomas GD, Teague B, Leach RM, Shaifta Y, Knock GA, Aaronson PI, Ward JP (2008). Low concentrations of sphingosylphosphorylcholine enhance pulmonary artery vasoreactivity: the role of protein kinase C delta and Ca^{2+} entry. Hypertension 51: 239-245.

Somlyo AP, Somlyo AV (2003). Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 83: 1325-1358.

Syyong HT, Yang HH, Trinh G, Cheung C, Kuo KH, van Breemen C (2009). Mechanism of asynchronous Ca^{2+} waves underlying agonist-induced contraction in the rat basilar artery. British J Pharmacol 156: 587-600.

Szczepaniak WS, Pitt BR, McVerry BJ (2010). S1P receptor-dependent Rho-kinase activation mediates vasoconstriction in the murine pulmonary circulation induced by sphingosine 1-phosphate. Am J Physiol 299: L137-145.

Thomas GD, Snetkov VA, Patel R, Leach RM, Aaronson PI, Ward JP (2005). Sphingosylphosphorylcholine-induced vasoconstriction of pulmonary artery: activation of non-store-operated Ca^{2+} entry. Cardiovasc Res 68: 56-64.

Toullec D, Pianetti P, Coste H, Bellenesque P, Grand-Perret T, Ajakane M, Baudet M, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J (1991). The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266: 15771-15781.

Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 389: 990-994.

Ward JPT, Knock GA, Snetkov VA, Aaronson PI (2004). Protein kinases in vascular smooth muscle tone-role in the pulmonary vasculature and hypoxic pulmonary vasoconstriction. Pharmacol Ther 104: 207-231.

Welsh DG, Brayden JE (2001). Mechanisms of coronary artery depolarization by uridine triphosphate. Am J Physiol 280: H2545-2553.

Wilson SM, Mason HS, Ng LC, Montague S, Johnston L, Nicholson N, Mansfield S, Hume, JR (2005). Role of basal extracellular Ca^{2+} entry during 5-HT-induced vasoconstriction of canine pulmonary arteries. British J Pharmacol 144: 252-264.

Wray S, Burdyga T (2010). Sarcoplasmic reticulum function in smooth muscle. Physiol Rev 90: 113-178.

Zhao G, Adebiyi A, Blaskova E, Xi Q, Jaggar JH (2008). Type 1 inositol 1,4,5-trisphosphate receptors mediate UTP-induced cation currents, Ca^{2+} signals, and vasoconstriction in cerebral arteries. Am J Physiol 295: C1376-1384.

Zheng YM, Wang QS, Rathore R, Zhang WH, Mazurkiewicz JE, Sorrentino V, Singer HA, Kotlikoff MI, Wang YX (2005). Type-3 ryanodine receptors mediate hypoxia-, but not neurotransmitter-induced calcium release and contraction in pulmonary artery smooth muscle cells. J Gen Physiol 125: 427-440.
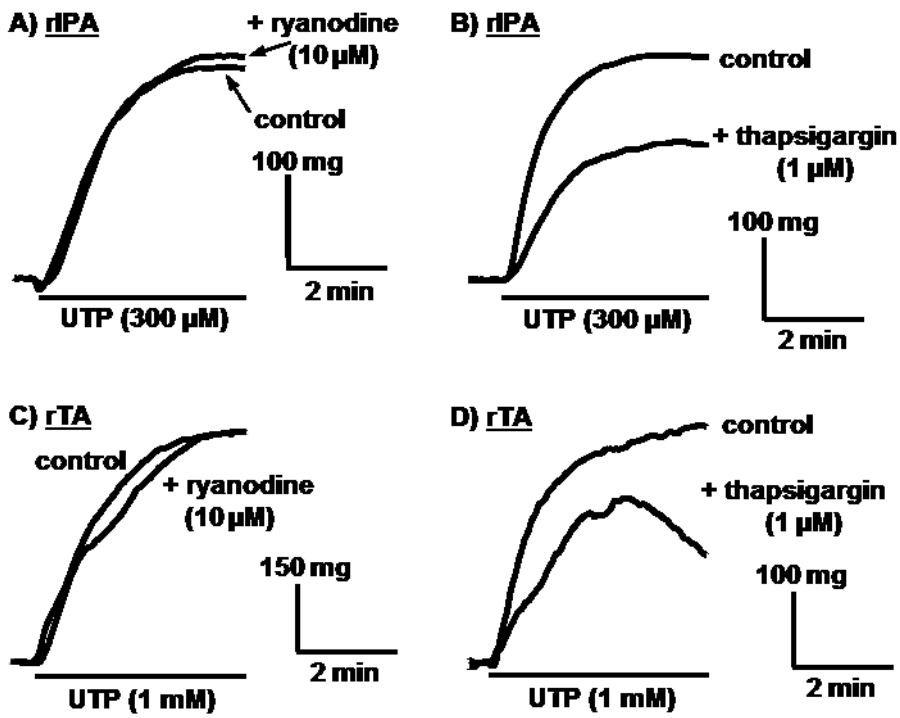
Fig. 1. The role of SR Ca^{2+} stores in contractions evoked by UTP.

The superimposed traces show typical contractions of isolated, endothelium-denuded (A,B) rIPA evoked by UTP (300 μM) and (C,D) rTA evoked by UTP (1 mM) before (upper traces) and after (lower traces) incubation with ryanodine (10 μM) or thapsigargin (1 μM) for 15 min. UTP was applied as indicated by the solid bars.
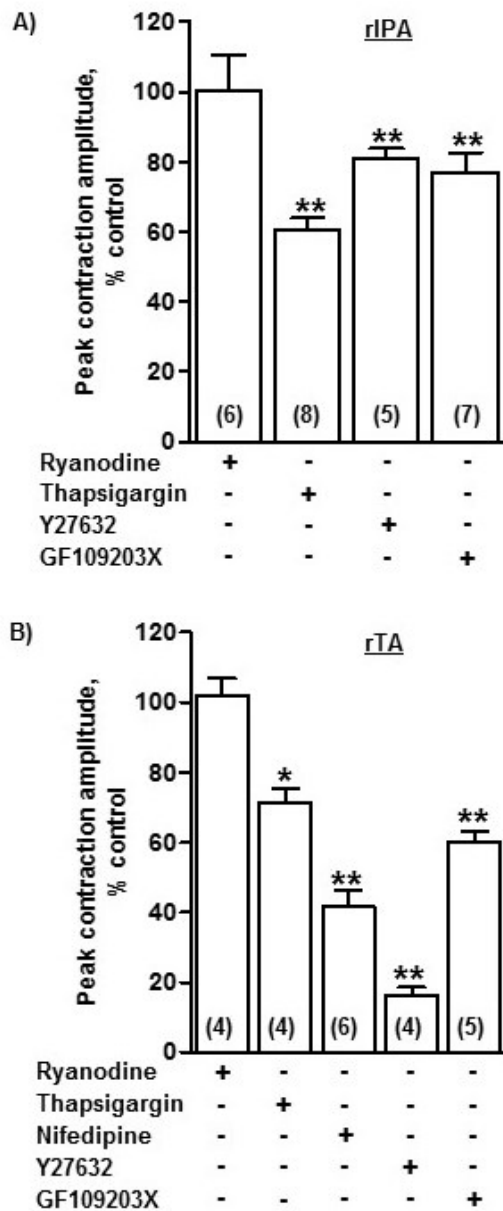
Fig. 2. Inhibition of contractions evoked by UTP.

The mean peak amplitude of UTP-evoked contractions of isolated, endothelium-denuded (A) rIPA in the presence of ryanodine (10 μM) (n=6), thapsigargin (1 μM) (n=8), Y27632 (10 μM) (n=5) and GF109203X (10 μM) (n=7), and (B) rTA in the presence of ryanodine (10 μM) (n=4), thapsigargin (1 μM) (n=4), nifedipine (1 μM) (n=6), Y27632 (10 μM) (n=4) and GF109203X (10 μM) (n=5), expressed as a percentage of control responses, is shown. The numbers in brackets show n for each. Vertical lines show S.E.M.. * $P<0.05$, ** $P<0.01$ for responses in the presence of inhibitor compared to control.

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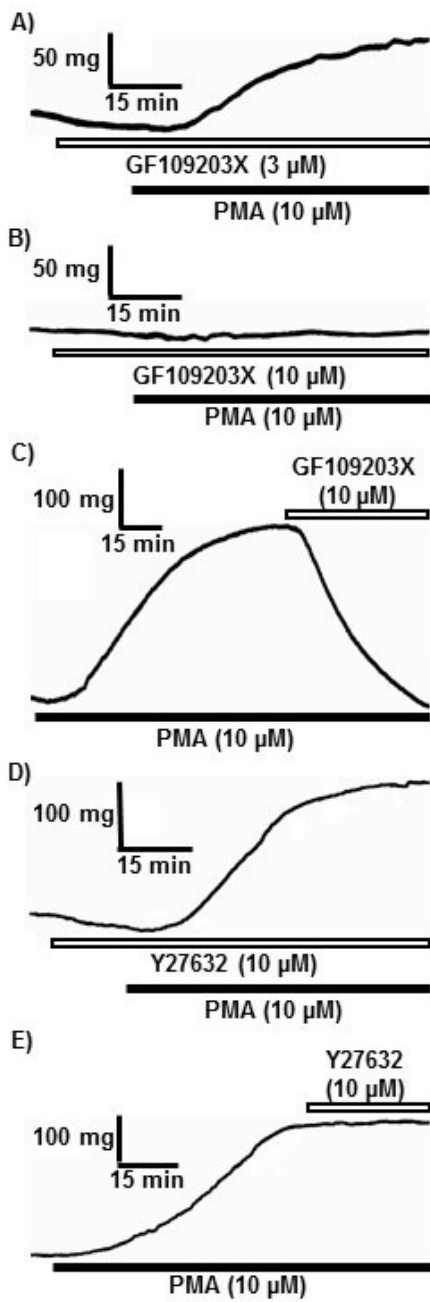
Fig. 3. Inhibition of contractions evoked by PMA.

The traces show typical responses of isolated, endothelium-denuded rIPA when (A) GF109203X (3 µM) was added 15 min before PMA (10 µM); or GF109203X (10 µM) was added (B) 15 min before or (C) 60 min after PMA (10 µM); or Y27632 (10 µM) was added (D) 15 min before or (E) 60 min after PMA (10 µM). GF109203X and Y27632 were applied as indicated by the open bars and PMA as indicated by the solid bars.
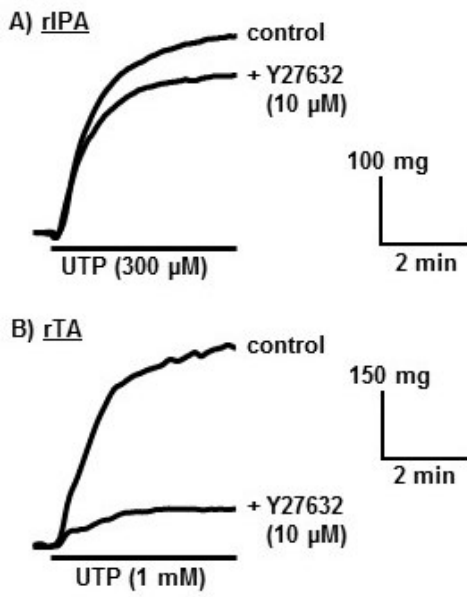
Fig. 4. Inhibition by Y27632 of contractions evoked by UTP.

The superimposed traces show typical contractions of isolated, endothelium-denuded (A) rIPA evoked by UTP (300 μM) and (B) rTA evoked by UTP (1 mM) before (upper traces) and after (lower traces) incubation with Y27632 (10 μM) for 15 min. UTP was applied as indicated by the solid bars.
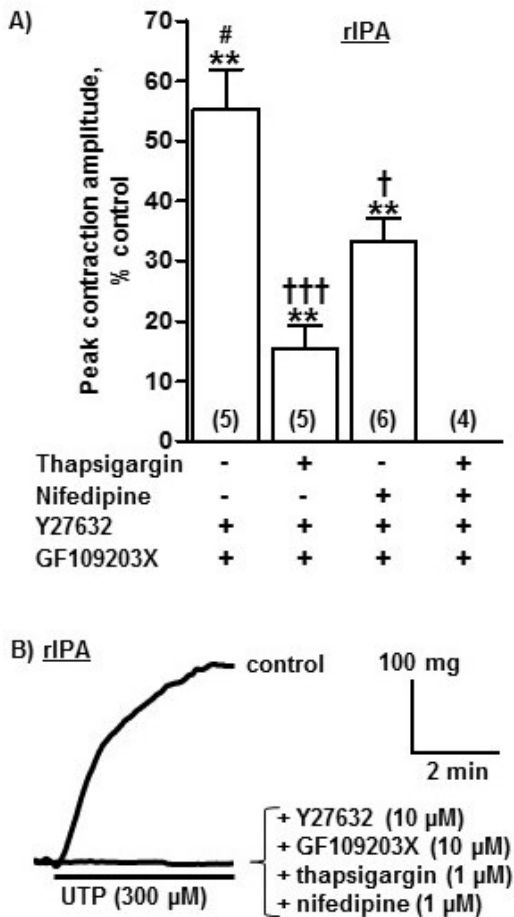
Fig. 5. Combined application of inhibitors in rIPA.

(A) The mean peak amplitude of contractions of isolated, endothelium-denuded rIPA evoked by UTP (300 μM) in the presence of thapsigargin (1 μM) (n=5), nifedipine (1 μM) (n=5), Y27632 (10 μM) (n=4) and GF109203X (10 μM) (n=4), in combination and expressed as a percentage of control responses, is shown. The numbers in brackets show n for each. Vertical lines show S.E.M. ** $P<0.01$ for responses in the presence of inhibitors compared to control. # $P<0.05$ for responses in the presence of Y27632 plus GF109203X compared to either drug alone. † $P<0.05$ for responses in the presence of nifedipine, Y27632 and GF109203X compared to Y27632 plus GF109203X. ††† $P<0.001$ for responses in the presence of thapsigargin, Y27632 and GF109203X compared to Y27632 plus GF109203X.

(B) The superimposed traces show typical contractions evoked by UTP (300 μM) before (upper trace) and after (lower trace) incubation with Y27632 (10 μM), GF109203X (10 μM), thapsigargin (1 μM) and nifedipine (1 μM) for 15 min. UTP was applied as indicated by the solid bar.
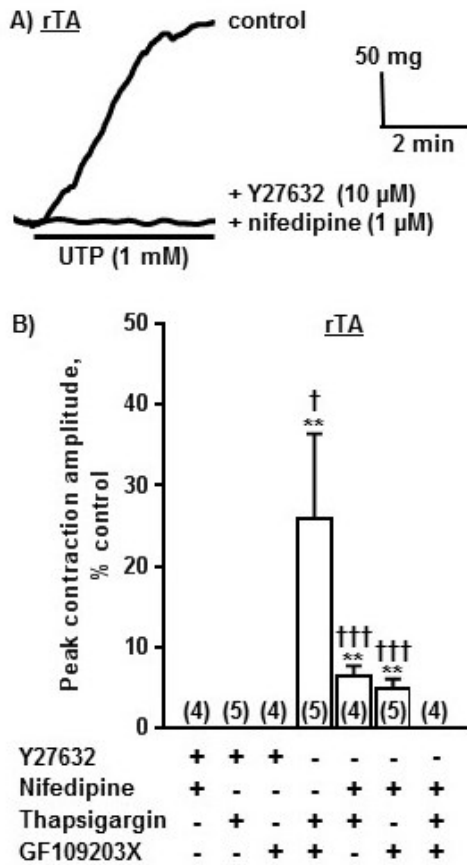
Fig. 6. Combined application of inhibitors in rTA.

(A) The superimposed traces show typical contractions of isolated, endothelium-denuded rTA evoked by UTP (1 mM) before (upper trace) and after (lower trace) incubation with Y27632 (10 μM) and nifedipine (1 μM) for 15 min. UTP was applied as indicated by the solid bar. (B) The mean peak amplitude of contractions evoked by UTP (1 mM) in the presence of Y27632 (10 μM), nifedipine (1 μM), thapsigargin (1 μM) and GF109203X (10 μM), in combination and expressed as a percentage of control responses, is shown. The numbers in brackets show n (4 or 5) for each. Vertical lines show S.E.M. ** $P<0.01$ for responses after treatment compared to control. † $P<0.05$ for responses in the presence of thapsigargin and GF109203X compared to either agent alone. ††† $P<0.001$ for responses in the presence of nifedipine plus thapsigargin or GF109203X compared to each agent alone.
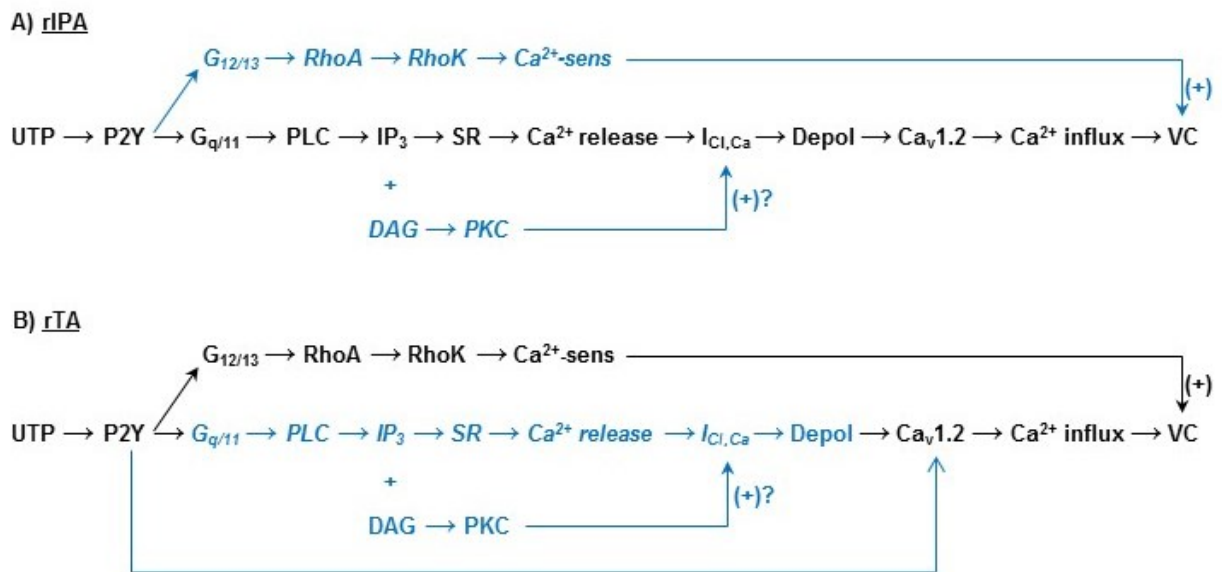
Fig. 7. P2Y signalling pathways in rIPA and rTA.

The schematics show our current understanding of the signalling pathways that mediate vasoconstriction evoked by UTP in (A) rIPA and (B) rTA. The signalling components that appear to play major roles are shown in black and less important components aer shown in blue italics. Abbreviations are the same as in the main body of text. PLC = phospholipase C, Depol = depolarisation, VC = vasoconstriction, DAG = diacyl glycerol

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