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

Asrin Tengah<sup>1,2,4</sup>, Nawazish-i-Husain Syed<sup>1,3,4</sup>, Siti Tajidah Abdul Talip<sup>1,2,5</sup>, Siti Nur Basirah  
Bujang<sup>1,2,5</sup> and Charles Kennedy<sup>1\*</sup>

<sup>1</sup>Strathclyde Institute of Pharmacy and Biomedical Sciences,  
University of Strathclyde,  
161 Cathedral Street,  
Glasgow G4 0RE,  
United Kingdom

\* Author for correspondence: Dr. Charles Kennedy, Strathclyde Institute of Pharmacy and  
Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, United  
Kingdom.

Tel: +44 (0)141 548 2664

Fax: +44 (0)141 552 2562

E-mail: [c.kennedy@strath.ac.uk](mailto:c.kennedy@strath.ac.uk)

<sup>2</sup>Current address: PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Jalan  
Tungku Link, Gadong BE1410, Brunei Darussalam

E-mail: [asrin.tengah@ubd.edu.bn](mailto:asrin.tengah@ubd.edu.bn) (A.T.), [waney224@hotmail.com](mailto:waney224@hotmail.com) (S.T.A.T.),  
[nurbasirahghazali@hotmail.com](mailto:nurbasirahghazali@hotmail.com) (S.N.B.B)

<sup>3</sup>Current address: University College of Pharmacy, University of the Punjab, Lahore, Pakistan.

E-mail: [snihusain@yahoo.com](mailto:snihusain@yahoo.com)

<sup>4</sup>A.T. and N.S. contributed equally to this work.

<sup>5</sup>S.T.A.T. AND S.N.B.B. contributed equally to this work.

**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<sup>2+</sup> 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<sup>2+</sup> release from the sarcoplasmic reticulum and influx through Ca<sub>v</sub>1.2 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<sup>2+</sup>, 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<sup>2+</sup>-sensitisation (Somlyo and Somlyo, 2003; Ward et al., 2004), potentiation of Ca<sub>v</sub>1.2 Ca<sup>2+</sup> currents (Ward et al., 2004; Navedo et al., 2005; Ren et al., 2010) and inhibition of voltage-dependent K<sup>+</sup> 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<sub>2</sub>PO<sub>4</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 0.5, MgCl<sub>2</sub> 1, glucose 11, CaCl<sub>2</sub> 1.8, titrated to pH 7.3 with NaOH and bubbled with a gas mixture of 21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>. 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 37°C. 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  $\mu$ M) 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  $\mu$ M 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  $\mu$ M) was added for 10 min before caffeine (10 mM) was readded for 5 min. After washing, ryanodine (10  $\mu$ M) 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  $\mu$ M) 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<sub>3</sub> salt), caffeine, acetylcholine chloride (Sigma, UK) and ryanodine (Calbiochem, U.S.A.)

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  $\pm$  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  $\mu$ M) 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-1<sup>st</sup> column), rTA (Fig. 1C, 2B-1<sup>st</sup> column).

In contrast, thapsigargin (1  $\mu$ M), depressed significantly the responses in both tissues by 30-40%: rIPA ( $P < 0.01$ , Fig. 1B, 2A, 2<sup>nd</sup> column), rTA ( $P < 0.05$ , Fig. 1D, 2B, 2<sup>nd</sup> 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  $\text{Ca}_v1.2$   $\text{Ca}^{2+}$  channel blocker, nifedipine, inhibited contractions of rIPA evoked by UTP by nearly 50% (Mitchell et al., 2012a). In the rTA nifedipine (1  $\mu\text{M}$ ), a concentration that maximally inhibits  $\text{Ca}_v1.2$  ion channels (Clapp and Gurney, 1991), significantly reduced the peak amplitude of the UTP response by approximately 60% ( $P < 0.01$ ) (Fig. 2B, 3<sup>rd</sup> column), which was not significantly different from its inhibitory effects in the rIPA. Thus  $\text{Ca}^{2+}$  influx via nifedipine-sensitive  $\text{Ca}^{2+}$  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  $\mu\text{M}$ ), 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  $\mu\text{M}$  GF109203X (Fig. 3A) and abolished by 10  $\mu\text{M}$  GF109203X, added 15 min before (Fig. 3B) or 60 min after (Fig. 3C) PMA. In contrast, Y27632 (10  $\mu\text{M}$ ), 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  $\mu\text{M}$  Y27632 and GF109203X were, therefore, used in subsequent experiments.

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

( $P < 0.01$ , Fig. 2A-4<sup>th</sup> column) and by around 40% in rTA ( $P < 0.001$ , Fig. 2B-5<sup>th</sup> 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  $\mu\text{M}$ ) plus GF109203X (10  $\mu\text{M}$ ) significantly reduced UTP contraction peak amplitude by about 45% ( $P < 0.01$ ) (Fig. 5A-1<sup>st</sup> 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  $\mu\text{M}$ ) was added along with Y27632 (10  $\mu\text{M}$ ) and GF109203X (10  $\mu\text{M}$ ), 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-2<sup>nd</sup> column). This was significantly more than the inhibition produced by Y27632 plus GF109203X ( $P < 0.001$ ).

Nifedipine (1  $\mu\text{M}$ ) inhibited UTP contractions in rIPA by around 45% (Mitchell et al., 2012a) and co-adding it with Y27632 (10  $\mu\text{M}$ ) plus GF109203X (10  $\mu\text{M}$ ) significantly reduced the peak UTP response by nearly 70% ( $P < 0.01$ ) (Fig. 5A-3<sup>rd</sup> 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-4<sup>th</sup> 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*



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  $\mu$ M) with nifedipine (1  $\mu$ M) (Fig. 6A,B-1<sup>st</sup> column), thapsigargin (1  $\mu$ M) (Fig. 6B-2<sup>nd</sup> column) or GF109203X (10  $\mu$ M) (Fig. 6B-3<sup>rd</sup> 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  $\mu$ M) plus GF109203X (10  $\mu$ M), nearly 30% of the peak response to UTP remained ( $P < 0.01$ ) (Fig. 6B-4<sup>th</sup> column), while coapplication of thapsigargin (10  $\mu$ M) plus nifedipine (1  $\mu$ M) ( $P < 0.01$ ) (Fig. 6B-5<sup>th</sup> column) or GF109203X (10  $\mu$ M) plus nifedipine (1  $\mu$ M) ( $P < 0.01$ ) (Fig. 6B-6<sup>th</sup> 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-7<sup>th</sup> 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  $IP_3R$  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  $\text{Ca}^{2+}$  stores (Wray and Burdyga, 2010), inhibited the peak amplitude of vasoconstriction elicited by UTP by around 40%, showing for the first time that  $\text{Ca}^{2+}$  release from the SR contributes substantially to UTP-evoked contractions of rIPA. This is important, as GPCR-mediated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  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  $\alpha_1$ - and 5-HT<sub>2A</sub>-receptor-mediated contractions of dog IPA (Jabr et al., 1997; Wilson et al., 2005), but depleting ryanodine-sensitive  $\text{Ca}^{2+}$  stores partially reduced noradrenaline-evoked vasoconstriction of rIPA (Zheng et al., 2005) and abolished contractions of rabbit IPA by PGF<sub>2 $\alpha$</sub>  (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  $\text{Ca}^{2+}$  via IP<sub>3</sub>R 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  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  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  $\text{Cl}^-$  and Ca<sub>v</sub>1.2 ion channels produced no further inhibition. Our current working model is, therefore, that the major function of  $\text{Ca}^{2+}$  released from the SR is to activate the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current, which depolarises rIPA smooth muscle cells, causing Ca<sub>v</sub>1.2 ion channels to open and extracellular  $\text{Ca}^{2+}$  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  $\alpha_1$ -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  $\text{Ca}^{2+}$ -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  $\alpha_1$ -adrenoceptor-mediated vasoconstriction of dog IPA (Damron et al., 2002). Like RhoK, PKC can induce vasoconstriction via  $\text{Ca}^{2+}$ -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  $\text{IP}_3\text{R}$  (Narayanan et al., 2012) is also unlikely to be involved, as using thapsigargin to deplete SR  $\text{Ca}^{2+}$  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  $\text{Ca}_v1.2$  ion channels (Fig. 7A), the activity of which can be potentiated by GPCR. For example, in rIPA, sphingosylphosphorylcholine increased  $\text{Ca}^{2+}$  influx via  $\text{Ca}_v1.2$  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  $\text{Ca}_v1.2$  ion currents indirectly by inhibiting voltage-dependent  $\text{K}^+$  currents in pulmonary arterial smooth muscle (Cogolludo et al., 2003), so inducing depolarisation and  $\text{Ca}_v1.2$  ion channel opening. Indeed, endothelin-1 depolarised rIPA smooth muscle cells by inhibiting voltage-dependent  $\text{K}^+$  channels (Salter and Kozlowski, 1996) via activation of PKC (Shimoda et al., 1998). In the present study, blocking  $\text{Ca}^{2+}$  influx via  $\text{Ca}_v1.2$  ion channels was less than additive with inhibition of PKC and RhoK, indicating that  $\text{Ca}_v1.2$  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  $\text{Ca}^{2+}$  from SR stores and influx of extracellular  $\text{Ca}^{2+}$  via  $\text{Ca}_v1.2$  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  $\text{Ca}^{2+}$  is important in the rTA, as nifedipine inhibited UTP vasoconstriction by about 60%, consistent with the depressant effect of  $\text{Ca}^{2+}$ -free buffer (McLaren et al., 1998).  $\text{Ca}_v1.2$  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  $\text{Ca}^{2+}$  release is not the only stimulus that opens  $\text{Ca}_v1.2$  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  $\text{K}^+$  currents in rat coronary artery (Welsh and Brayden, 2001) and depolarised rat cerebral artery smooth muscle cells by inhibiting a voltage-dependent  $\text{K}^+$  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  $\text{Ca}^{2+}$  influx. Together, this will build a comprehensive and integrated model of the signalling pathways that couple P2Y receptor activation by UTP to vasoconstriction.

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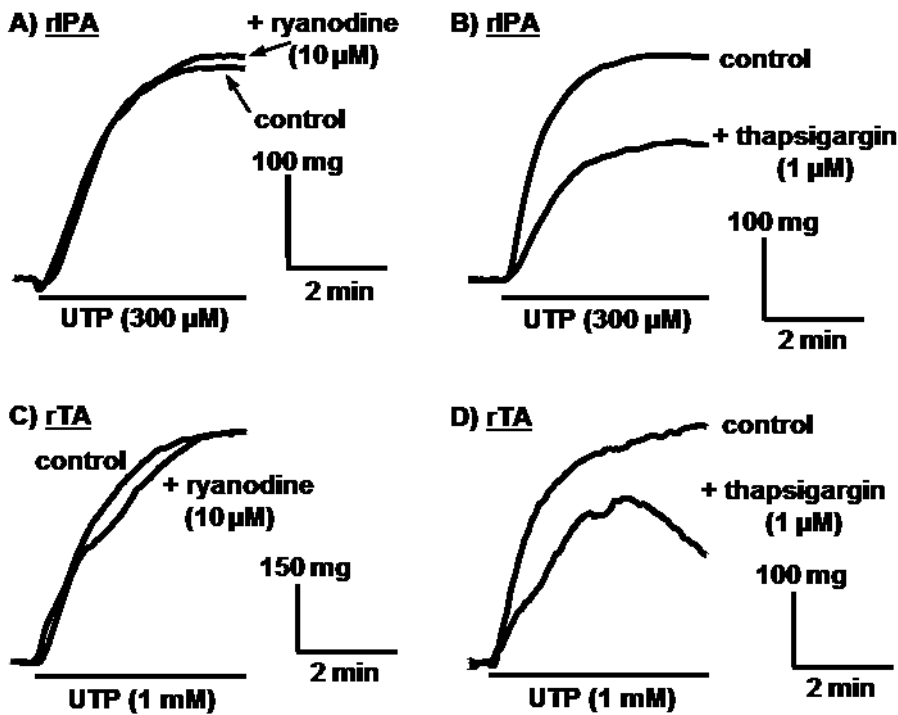
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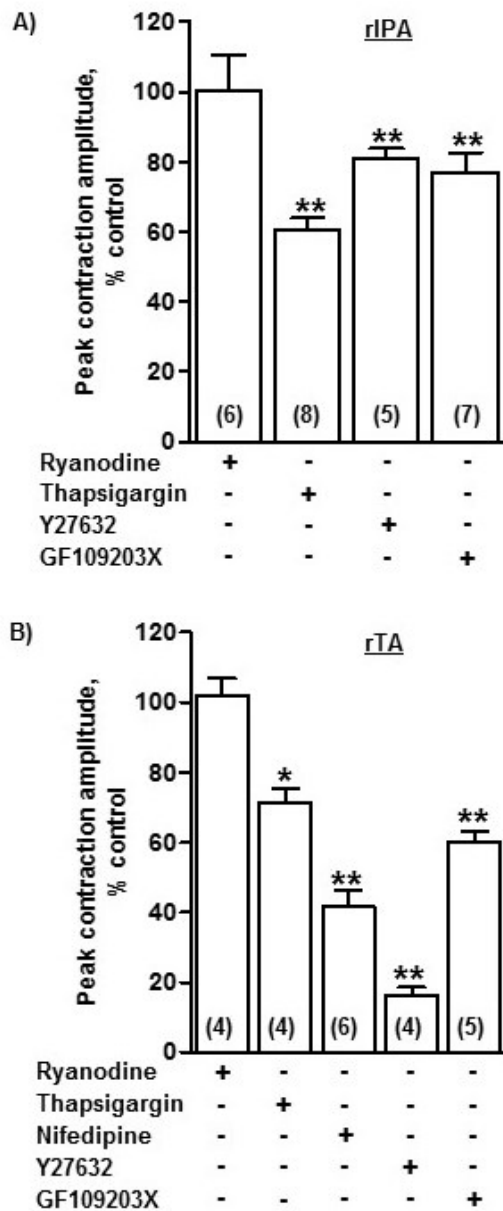
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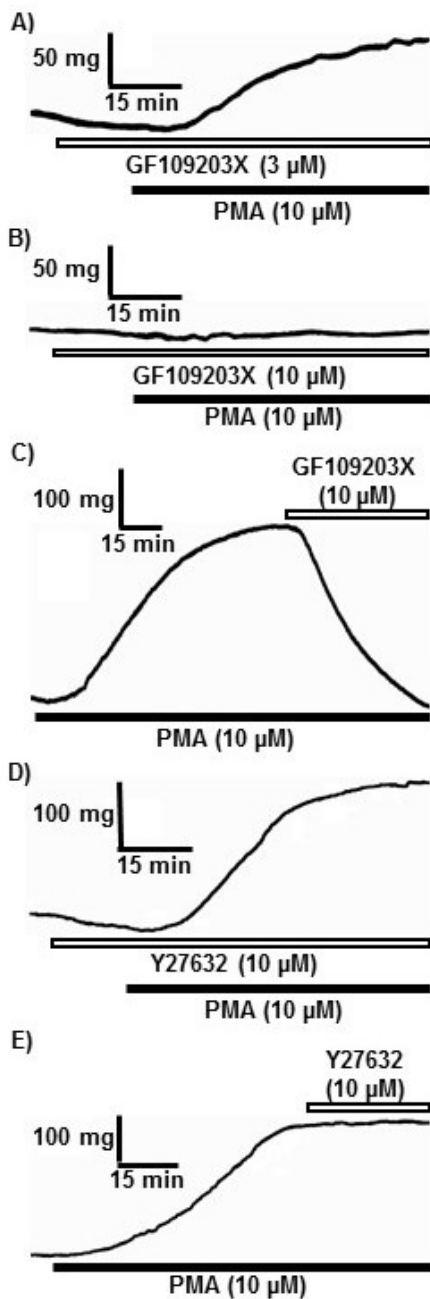
**Fig. 1. The role of SR Ca<sup>2+</sup> stores in contractions evoked by UTP.**

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.



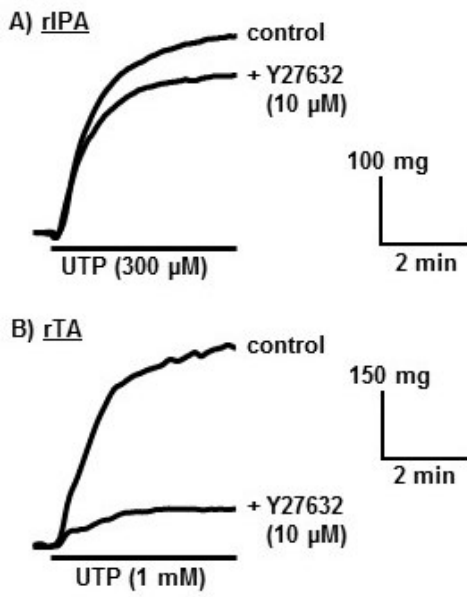
**Fig. 2. Inhibition of contractions evoked by UTP.**

The mean peak amplitude of UTP-evoked contractions of isolated, endothelium-denuded (A) rIPA in the presence of ryanodine (10  $\mu$ M) (n=6), thapsigargin (1  $\mu$ M) (n=8), Y27632 (10  $\mu$ M) (n=5) and GF109203X (10  $\mu$ M) (n=7), and (B) rTA in the presence of ryanodine (10  $\mu$ M) (n=4), thapsigargin (1  $\mu$ M) (n=4), nifedipine (1  $\mu$ M) (n=6), Y27632 (10  $\mu$ M) (n=4) and GF109203X (10  $\mu$ M) (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.



**Fig. 3. Inhibition of contractions evoked by PMA.**

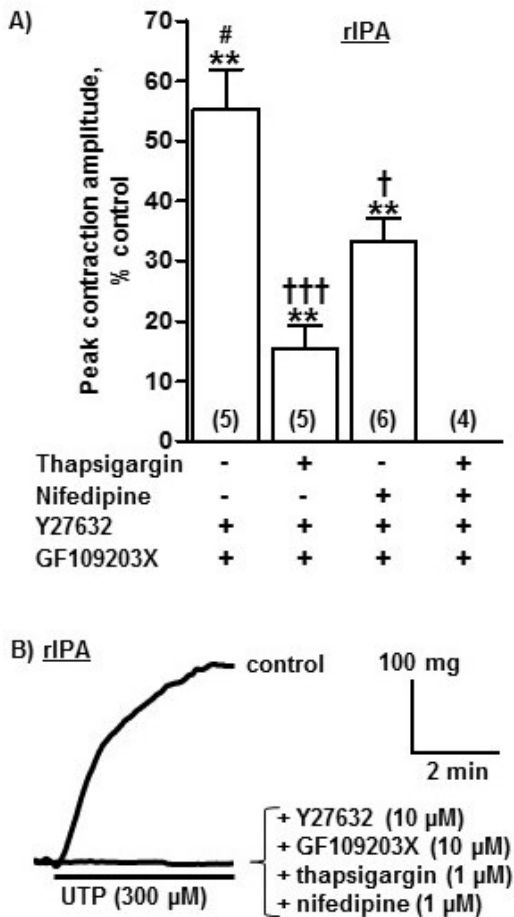
The traces show typical responses of isolated, endothelium-denuded rIPA when (A) GF109203X (3 μM) was added 15 min before PMA (10 μM); or GF109203X (10 μM) was added (B) 15 min before or (C) 60 min after PMA (10 μM); or Y27632 (10 μM) was added (D) 15 min before or (E) 60 min after PMA (10 μM). GF109203X 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  $\mu$ M) and (B) rTA evoked by UTP (1 mM) before (upper traces) and after (lower traces) incubation with Y27632 (10  $\mu$ M) for 15 min. UTP was applied as indicated by the solid bars.

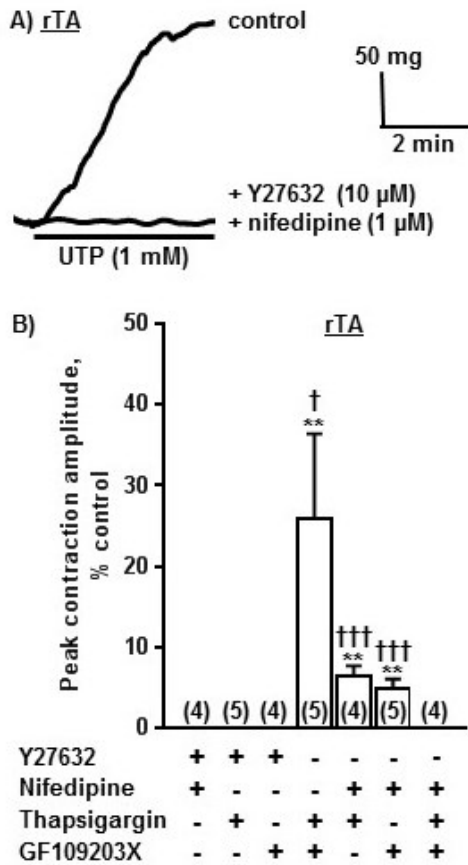




**Fig. 5. Combined application of inhibitors in rIPA.**

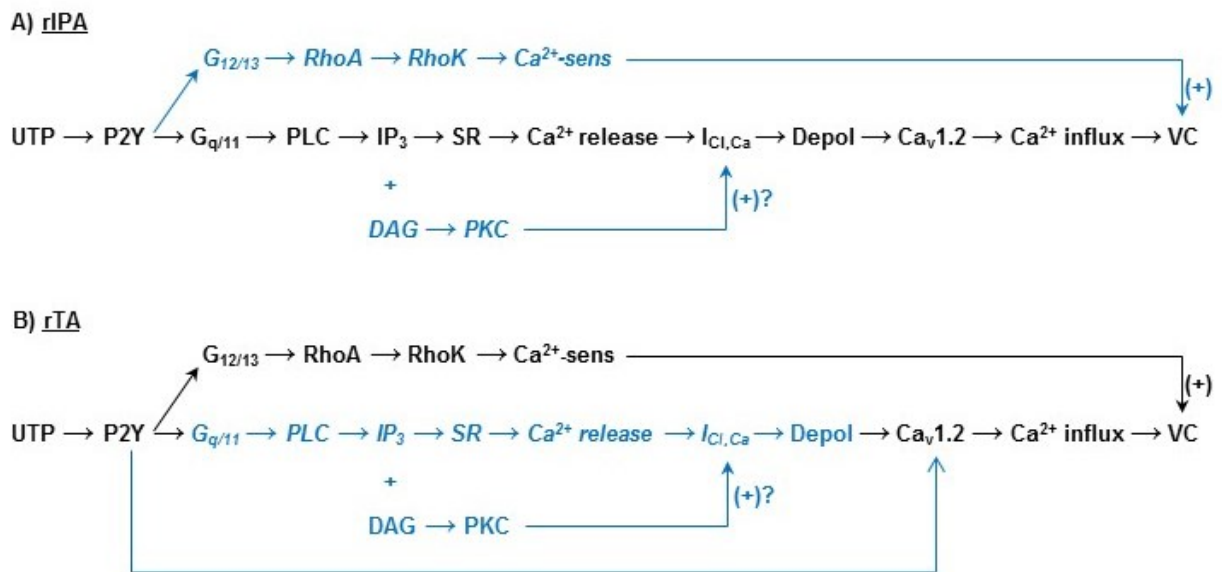
(A) The mean peak amplitude of contractions of isolated, endothelium-denuded rIPA evoked by UTP (300  $\mu$ M) in the presence of thapsigargin (1  $\mu$ M) (n=5), nifedipine (1  $\mu$ M) (n=5), Y27632 (10  $\mu$ M) (n=4) and GF109203X (10  $\mu$ M) (n=4), in combination and expressed as a percentage 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  $\mu$ M) before (upper trace) and after (lower trace) incubation with Y27632 (10  $\mu$ M), GF109203X (10  $\mu$ M), thapsigargin (1  $\mu$ M) and nifedipine (1  $\mu$ M) 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  $\mu$ M) and nifedipine (1  $\mu$ M) 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  $\mu$ M), nifedipine (1  $\mu$ M), thapsigargin (1  $\mu$ M) and GF109203X (10  $\mu$ M), 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 are 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