



Uridine adenosine tetraphosphate induces contraction and relaxation in rat aorta

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ABSTRACT

Uridine adenosine tetraphosphate (Up_4A) has been recently reported as an endothelium-derived vasoconstrictor and plasma levels of this dinucleotide are increased in juvenile hypertensive subjects. This study aimed to evaluate the vascular actions of Up_4A , typify the putative purinergic receptors that might mediate these effects and characterize the intracellular signaling pathways that may govern Up_4A responses. Up_4A induced a modest endothelium-dependent relaxation of rat aortic rings contracted with phenylephrine. From baseline, Up_4A induced concentration-dependent contractions that were significantly potentiated by endothelium removal or nitric oxide synthase inhibition. The contractile response induced by Up_4A was not tachyphylactic and was significantly reduced in the presence of P1 or P2X receptor antagonists, L-type Ca^{2+} channel blocker and Rho-kinase inhibitor. Up_4A -induced contraction apparently involves superoxide anion formation since it was significantly reduced by treatment with apocynin or tempol. This study presents the unique findings that the endogenous compound Up_4A is able to induce relaxation in addition to contraction of rat aorta. Up_4A -induced contraction is modulated by nitric oxide production, mediated by P1 and P2X receptor activation, and involves L-type Ca^{2+} channels, Rho-kinase pathway and superoxide formation.

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1. Introduction

Extracellular nucleotides contribute to the local regulation of vascular tone (Buvinic et al., 2006; Inscho and Cook, 2002; Ralevic and Burnstock, 1991). Uridine adenosine tetraphosphate (Up_4A) has been recently described as a novel non-peptidic endothelium-derived vasoconstrictive factor (Jankowski et al., 2005). This factor, isolated from human endothelial cells, increases mean arterial pressure and perfusion pressure in isolated kidneys of rats, suggesting a role for Up_4A in the regulation of vascular tone.

Purinoceptors have been classified into two subtypes: P1 and P2 receptors, based on their pharmacological properties and molecular clonings (Dalziel and Westfall, 1994; Knight and Burnstock, 2001). Adenosine and its phosphates, ATP and ADP, have been identified as the endogenous ligands for P1 and P2 receptors, respectively (Fredholm et al., 1994; Malec, 1996). Four subtypes of P1 receptors, all metabotropic, have been cloned, namely A1, A2A, A2B and A3 (Ralevic and Burnstock, 1998). The P2 receptors for nucleotides exist in two major families: metabotropic (P2Y receptors) and ionotropic (P2X receptors). There are at least 8 cloned P2Y and 7 cloned P2X receptors (Boarder and Hourani, 1998; Burnstock, 2004; Communi et al., 2000; North, 2002). Most of these receptors are capable of mediating

responses to several nucleotides, resulting in multiple receptors having overlapping ligand preferences (Ralevic and Burnstock, 1998).

In isolated perfused rat kidney, Up_4A was found to induce vasoconstriction through P2X1, P2Y2 and P2Y4 receptor activation (Jankowski et al., 2005). The vasodilatory effect of this compound has not been tested.

Because Up_4A is secreted by human endothelial cells and is present in effective concentrations in human plasma, a role for Up_4A in the regulation of vascular tone and in cardiovascular disease seems to be likely. More recently it has been reported that plasma Up_4A levels are increased in human juvenile hypertension and has a significant proliferative effect on human smooth muscle cells (Jankowski et al., 2007).

The present experiments were carried out in order to characterize the responses induced by Up_4A in rat aorta. We hypothesized that Up_4A induces contraction of rat aortic smooth muscle. In an attempt to characterize Up_4A -induced contraction, P1 and P2 receptor antagonists, inhibitors of Ca^{2+} channels and Rho-kinase were tested. The involvement of the endothelium, nitric oxide and superoxide anion was also evaluated.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (250–300 g, Harlan Laboratories, Indianapolis, IN) were maintained on a 12 h light/dark cycle with access to water and standard rat chow *ad libitum*. On the day of an experiment, rats were anesthetized with CO_2 and the thoracic aorta

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was excised. All experiments were conducted in accordance with the Medical College of Georgia's Animal Use for Research and Education Committee.

2.2. In vitro measurement of isometric force generation in rat aortic rings

After removal of fat and connective tissue, aortic rings (3 mm long) were mounted in an organ chamber (Danish MyoTechnology, Aarhus, Denmark) for isometric tension recordings (ADInstruments, Castle Hill, Australia) and bathed in physiological salt solution (PSS) of the following composition: 130.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.6 mM CaCl_2 , 14.9 mM NaHCO_3 , 0.03 mM EDTA, and 5.5 mM glucose, which was maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2 . The aorta segments were stretched at a tension of 30 mN with periodic washes every 15 min. After a 1-hour equilibration period, vessels were contracted with phenylephrine (10^{-7} M) and subsequently challenged with acetylcholine (10^{-5} M) to test tissue viability and integrity of the endothelium. Aortic rings that relaxed at least 70% of contraction induced by phenylephrine were used as retaining functional endothelium [E(+)]. Endothelium denudation [E(-)] was mechanically induced by gently rubbing the intimal surface of the aortic rings with a stainless steel wire. The absence of functional endothelium was confirmed by the complete lack of relaxation to acetylcholine.

2.3. Experimental protocols

To investigate the ability of Up_4A to induce contraction of rat aortic rings, cumulative concentration–effect curves to Up_4A (10^{-9} to 3×10^{-5} M) were constructed in endothelium-intact [E(+)] and endothelium-denuded [E(-)] aortic rings.

To test whether Up_4A was able to induce relaxation, E(+) and E(-) aortic rings were stimulated with phenylephrine (10^{-7} M) until the plateau of the contraction was obtained followed by a cumulative concentration–effect curve to Up_4A (10^{-9} to 10^{-5} M).

To evaluate the contribution of nitric oxide to Up_4A -induced contraction, concentration–effect curves to Up_4A were constructed in E(+) and E(-) aortic rings in the presence and absence of the nitric oxide synthase inhibitor L-NNA (10^{-4} M).

To assess whether Up_4A -induced contraction is reversible and induces desensitization or tachyphylaxis, two consecutive cumulative concentration–effect curves to Up_4A (10^{-9} to 3×10^{-5} M) were constructed in aortic rings E(-) and E(+) in the presence of L-NNA (10^{-4} M). For that, after the maximum response for the last concentration was achieved in the first curve, the rings were washed several times with PSS or PSS + L-NNA for approximately 60 min until the tension returned to baseline. Subsequently, the second curve to Up_4A was then constructed.

To determine which receptors mediate the contraction induced by Up_4A , concentration–effect curves to Up_4A were constructed in the presence of the P1 purinoceptor antagonist 8-PST (10^{-4} M); and the P2X1/P2X3 receptor antagonist NF279 (10^{-4} M).

To investigate the involvement of L-type Ca^{2+} channels in the Up_4A -induced contraction of rat aortic rings, concentration–effect curves to Up_4A in E(-) aortic rings were evaluated in the presence of the L-type Ca^{2+} channel blocker nifedipine (10^{-5} M).

To examine the contribution of RhoA/Rho-kinase pathway to Up_4A -induced contractions, concentration–effect curves to Up_4A in E(-) aortic rings were evaluated in the presence of the Rho-kinase inhibitor Y-27632 (10^{-7} M).

To study the role of superoxide anion in the Up_4A -induced contraction, E(-) aortic rings were contracted with Up_4A (10^{-6} M) and a concentration–effect curve (3–12 mM) to the superoxide anion scavenger (tempol) was performed. To confirm the involvement of superoxide anion in the Up_4A -induced contraction of rat aortic ring, a concentration–effect curve to Up_4A was performed in the presence and in the absence of the NADPH oxidase inhibitor apocynin (10^{-4} M).

All inhibitors were added to the organ chambers 30 min before the start of the concentration–effect curves to Up_4A , and were maintained in the organ chamber until the end of the experiment.

2.4. Drugs

Phenylephrine hydrochloride [(R)-(-)-1-(3-hydroxyphenyl)-2-methylaminoethanol hydrochloride], acetylcholine chloride, 8-(*p*-sulphophenyl)-theophylline (8-PST), 4'-hydroxy-3'-methoxyacetophenone (apocynin), 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (tempol), 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (nifedipine) were purchased from Sigma-Aldrich, St Louis-MO, USA. The compounds [(R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide-2HCl] (Y-27632), N^G -nitro-L-arginine (L-NNA), [8,8'-(carbonylbis(imino-4, 1-phenylene-carbonylimino-4,1-phenylene-carbonylimino)) bis(1,3,5-naphthalenetrisulfonic acid)] (NF279) were acquired from Calbiochem (San Diego, California, USA). Uridine adenosine tetraphosphate (Up_4A) was purchased from Biolog – Life Science Institute (Bremen, Germany) and dimethyl sulfoxide (DMSO) from Fisher Scientific (Fair Lawn, NJ, USA). Stock solutions were prepared using sterile HPLC grade water with the exception of apocynin and nifedipine that were prepared using DMSO.

2.5. Data analysis

Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls post hoc test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant. The points represent the mean \pm standard error of the mean (SEM) of the contraction in mN or as % of relaxation of agonist-induced force generation.

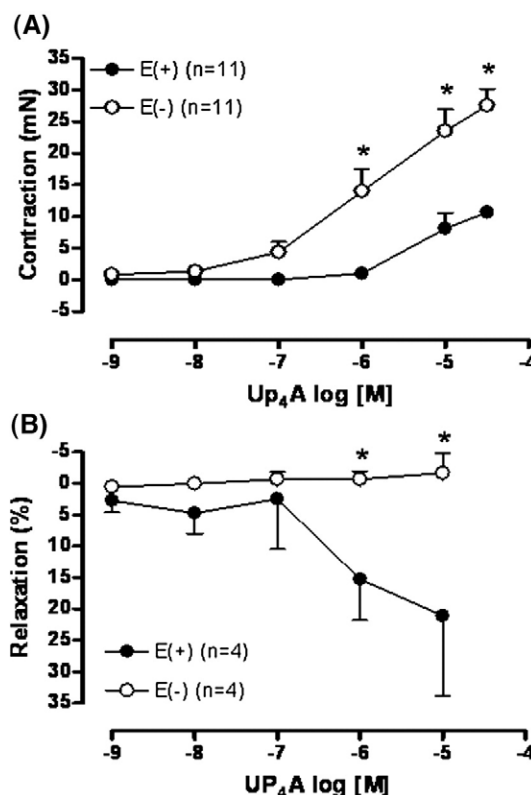


Fig. 1. Up_4A induces contraction and relaxation in rat aorta. Concentration–effect curves to Up_4A (10^{-9} to 3×10^{-5} M) were performed in endothelium-intact [E(+)] and endothelium-denuded [E(-)] rat aortic rings. In (A) the rings were treated from the baseline and in (B) the rings were contracted in response to phenylephrine 10^{-7} M. The points represent the mean \pm S.E.M. of the force displacement in mN (for contraction) or as % of relaxation of the contraction induced by phenylephrine (10^{-7} M). “n” represents the number of animals. * represents P value < 0.05 compared to endothelium-denuded rat aortic rings.

3. Results

3.1. *Up₄A*-induced contraction of rat aortic rings is modulated by the endothelium

The ability of *Up₄A* (10^{-9} – 3×10^{-5} M) to induce contraction was tested in E(+) and E(-) rat aortic rings. *Up₄A* caused contraction of rat aortic rings in a concentration-dependent manner, and the contraction was significantly potentiated by endothelium removal (Fig. 1A). The maximum contraction observed to the highest concentration of *Up₄A* (3×10^{-5} M) corresponded to approximately 30% and 80% of the contractile response induced by phenylephrine (10^{-6} M) in rat aortic rings with and without endothelium, respectively (data not shown).

3.2. *Up₄A*-induced relaxation of rat aortic rings is endothelium dependent

Endothelium-intact and denuded rat aortic rings were contracted with phenylephrine (10^{-7} M) followed by a cumulative concentration–effect curve induced by *Up₄A* (10^{-9} – 10^{-5} M). *Up₄A* induced a modest relaxation (~25%) of the contraction to phenylephrine in endothelium-intact rat aortic rings. The absence of endothelium prevented *Up₄A*-induced relaxation (Fig. 1B).

3.3. *Up₄A*-induced contraction of rat aortic rings is modulated by NO pathway

The vasoconstriction induced by *Up₄A* (10^{-9} – 3×10^{-5} M) was tested in endothelium-intact (Fig. 2A) and endothelium-denuded (Fig. 2B) rat aortic rings in the absence and in the presence of the non-selective NO

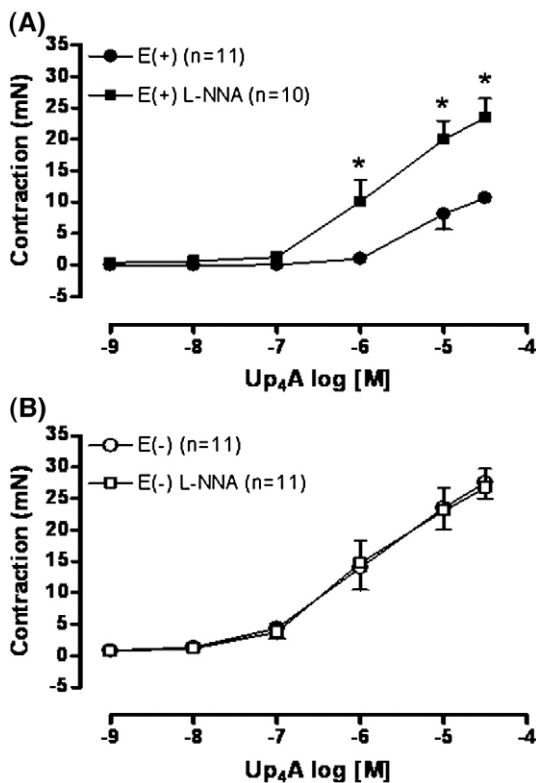


Fig. 2. *Up₄A*-induced contraction is significantly potentiated by endothelium removal and NO synthase inhibition. Concentration–effect curves to *Up₄A* (10^{-9} to 3×10^{-5} M) were performed in (A) endothelium-intact [E(+)] and (B) endothelium-denuded [E(-)] rat aortic rings in the absence and in the presence of L-NNA (10^{-4} M) for 30 min. The points represent the mean \pm S.E.M. of the force displacement in mN, “n” represents the number of animals and * represents *P* value < 0.05 compared to the response obtained in the absence of L-NNA.

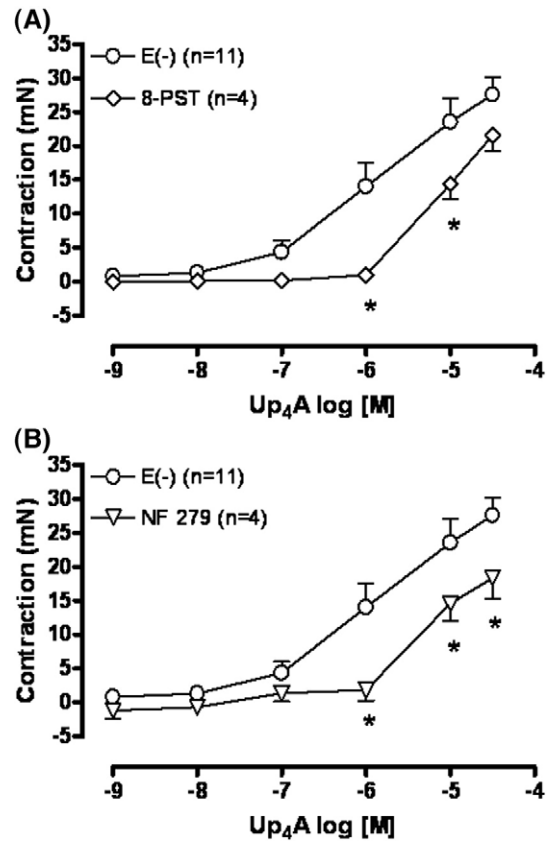


Fig. 3. *Up₄A*-induced contraction involves activation of P1 and P2X1 receptors. Concentration–effect curves to *Up₄A* (10^{-9} to 3×10^{-5} M) were performed in endothelium-denuded [E(-)] rat aortic rings in the absence and in the presence of (A) the non-selective antagonist of adenosine A1 and A2 receptors 8-PST (10^{-4} M), and (B) the non-selective P2X1 receptor antagonist NF279 (10^{-4} M). The points represent the mean \pm S.E.M. of the force displacement in mN, “n” represents the number of animals and * represents *P* value < 0.05 compared to the response obtained in the absence of the antagonist.

synthase inhibitor L-NNA (10^{-4} M) for 30 min. Incubation with L-NNA did not affect the *Up₄A*-induced contraction in E(-) rings (Fig. 2B), but significantly increased the contraction observed in E(+) rings to a similar extent observed by endothelium removal (Fig. 2A compared to Fig. 1A).

3.4. *Up₄A*-induced contraction is reversible and does not induce tachyphylaxis in rat aortic rings

Two consecutive curves to *Up₄A* were tested in aortic rings E(-) and E(+) treated with L-NNA. After the maximum response was obtained for the highest concentration of *Up₄A* on the first curve, the aortic rings were freed from the agonist through several wash out events. After a period of 60 min the rings returned to the baseline tension (approximately 30 mN). The second curve to *Up₄A* obtained after this re-stabilization period was similar to the first one obtained in both E(-) and E(+)/L-NNA (data not shown).

3.5. P1 and P2X receptors play an important role on *Up₄A*-induced contraction in endothelium-denuded rat aortic rings

To investigate the involvement of the P1 receptors in *Up₄A*-induced contraction, we tested the ability of the P1 receptor antagonist 8-PST with known affinity for A1 and A2 receptors (Bruns, 1981; Bruns et al., 1986; Petrack et al., 1981) to inhibit endothelium-denuded aortic ring *Up₄A*-induced contraction. Fig. 3A demonstrates that the P1 receptor antagonist 8-PST (10^{-4} M) significantly shifted *Up₄A*-induced

contraction in a parallel and competitive fashion suggesting the involvement of P1 receptors.

To study the contribution of the P2X receptors in Up₄A-induced contraction, we tested the ability of the non-selective P2X receptor antagonist NF279 with known affinity for P2X1 and P2X3 receptors (Rettinger et al., 2000) to inhibit Up₄A-induced contraction of endothelium-denuded aortic rings. NF279 (10⁻⁴ M) significantly rightward shifted the Up₄A-induced contraction confirming the involvement of P2X receptors (Fig. 3B).

3.6. Up₄A-induced contraction in endothelium-denuded rat aortic rings involves L-type Ca²⁺ channels and Rho-kinase pathway

The vasoconstriction induced by Up₄A (10⁻⁹–3 × 10⁻⁵ M) was tested in endothelium-denuded rat aortic rings in the absence and in the presence of the selective L-type Ca²⁺ channel blocker nifedipine or the Rho-kinase inhibitor Y-27632. After 30 min incubation with nifedipine (10⁻⁵ M) Up₄A-induced contraction was blunted (Fig. 4A), whereas incubation with Y-27632 (10⁻⁷ M) significantly reduced the contraction induced by Up₄A in endothelium-denuded aortic rings (Fig. 4B).

3.7. Up₄A-induced contraction in endothelium-denuded rat aortic rings involves superoxide anion formation

To investigate the involvement of superoxide anion to Up₄A-induced contraction, we tested the ability of the superoxide dismutase mimetic/superoxide anion scavenger tempol, or the NADPH oxidase inhibitor apocynin to inhibit Up₄A-induced contraction of endothelium-denuded

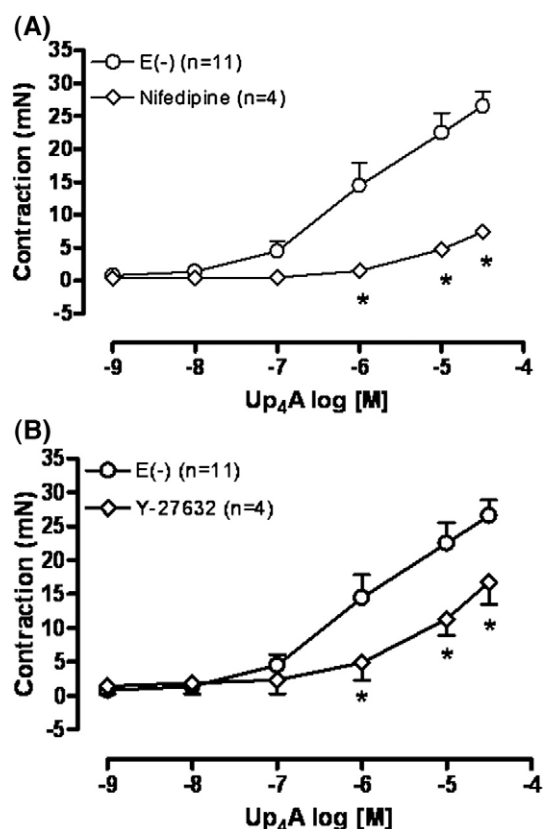


Fig. 4. Up₄A-induced contraction involves activation of L-type Ca²⁺ channels and the Rho-kinase pathway. CCEC(s) to Up₄A (10⁻⁹ to 3 × 10⁻⁵ M) were performed in endothelium-denuded [E(-)] rat aortic ring in the absence and in the presence of (A) the selective L-type Ca²⁺ channel blocker nifedipine (10⁻⁵ M) or (B) the selective Rho-kinase inhibitor Y-27632 (10⁻⁷ M). The points represent the mean ± S.E.M. of the force displacement in mN, “n” represents the number of animals and * represents P value < 0.05 compared to the response obtained in the absence of the inhibitor.

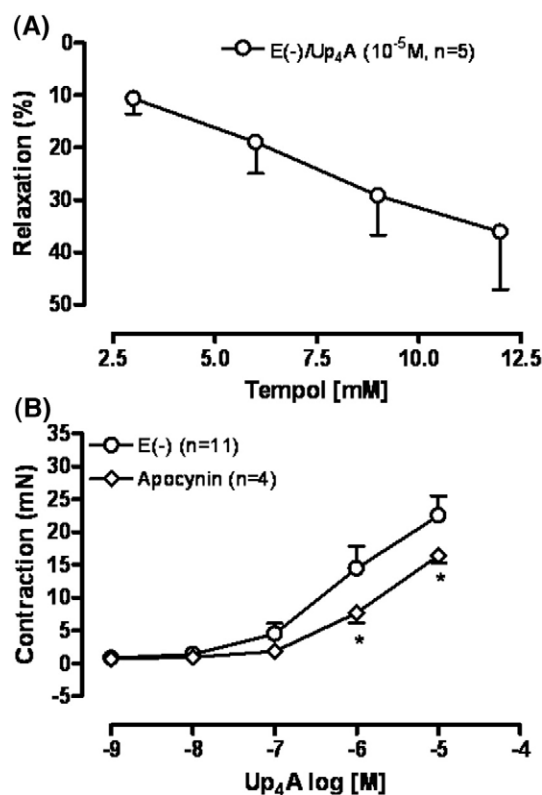


Fig. 5. Up₄A-induced contraction involves superoxide anion formation. (A) Concentration–effect curve to the superoxide anion scavenger tempol (3–12 mM) was performed in endothelium-denuded [E(-)] rat aortic ring contracted with Up₄A (10⁻⁵ M). (B) Concentration–effect curves to Up₄A (10⁻⁹ to 3 × 10⁻⁵ M) were constructed in endothelium-denuded [E(-)] rat aortic rings in the absence and in the presence of the selective NADPH oxidase inhibitor apocynin. The points represent the mean ± S.E.M. of the force displacement in mN (for contraction) or as % of relaxation of the contraction induced by Up₄A (10⁻⁵ M). “n” represents the number of animals. * represents P value < 0.05 compared to the response obtained in the absence of apocynin.

aortic rings. Fig. 5A demonstrates that contraction induced by Up₄A is inhibited (~35%) by tempol (12 mM). The concentration–effect curve induced by Up₄A is significantly shifted to the right in the presence of apocynin (10⁻⁴ M) (Fig. 5B).

4. Discussion

We present here the unique findings that the novel endothelium-derived factor Up₄A, originally described as a vasoconstrictor (Jankowski et al., 2005), also causes endothelium-dependent relaxation of rat aortic rings.

Adenine and uracil nucleotides contribute to the local regulation of vascular tone (Olsson and Pearson, 1990; Ralevic and Burnstock, 1991). Dinucleotides have also been shown to control vascular tone (Jankowski et al., 2003; Jankowski et al., 2005; Tolle et al., 2006). However, dinucleotides containing two purines as moieties are the most common naturally-occurring signaling molecules. Up₄A is the first dinucleotide containing one purine and one pyrimidine moiety found in living organisms (Jankowski et al., 2005). The potency and type of response of the dinucleotides are largely determined by the number of phosphates in the polyphosphate chain (Lewis et al., 1994; Lewis et al., 2000; Ralevic et al., 1995; Ralevic et al., 2001). In general, dinucleotides containing a phosphate chain of 2–3 compounds elicit vasorelaxation, whereas those containing a longer chain of 4 to 6 elicit vasoconstriction followed by vasodilation. Despite the characterization of Up₄A as a potent vasoconstrictor of isolated perfused rat kidney, little is known about its properties in other vascular tissues. Furthermore, the ability of Up₄A to induce relaxations has not been previously reported.

Two major families of purinoceptors, named P1 and P2 have been described. P1 and P2 receptor activation can cause both vasoconstriction and vasodilatation in the cardiovascular system. The responses induced by purines and pyrimidines varies extensively according to the tissue and specie studied. All P1 receptors are metabotropic (G-coupled protein) and specific agonists and antagonists are available for each different subtype (Burnstock, 2004). On the other hand, the lack of selective agonists and antagonists for P2 receptors has impeded a more detailed pharmacological characterization of the P2 receptors involved in the control of vascular tone.

We observed that Up₄A induced a modest endothelium-dependent relaxation of rat aortic rings contracted with phenylephrine. The current literature would support the view that endothelium-dependent relaxation of rat aorta mediated by P1 receptors may involve the A2A (Lewis et al., 1994) and A2B (Prentice and Hourani, 1996). P2-mediated endothelium-dependent rat aorta relaxation involves P2Y1- and P2Y2-like receptors (Hansmann et al., 1997). Rat vascular endothelial cells express P2Y receptors that mediate relaxation via the nitric oxide pathway. In murine aorta, in addition to P2Y1 and P2Y2, P2Y6 is also involved in the relaxing responses (Guns et al., 2005). Further studies are necessary to characterize the receptor subtype in endothelium-dependent relaxation induced by Up₄A in rat aorta.

Up₄A-induced contraction of rat aorta was significantly increased by endothelium removal or by nitric oxide synthase inhibition. Jankowski et al. (2005) also observed that the vasoconstrictor effect of Up₄A in perfused isolated kidney is increased after blockade of nitric oxide synthase (Jankowski et al., 2005). The contraction induced by certain guanine and by adenine nucleotides is modulated by the endothelium in rat aorta (Bultmann et al., 1997; Shen et al., 2000). In chorionic vessels, adenosine-induced contraction is also modulated by the endothelium (Donoso et al., 2005). Endothelium modulation of contractile responses elicited by agonists is an extensively reported phenomenon and, therefore, is not surprising to occur for Up₄A.

In addition to characterizing the contractile and relaxing actions of Up₄A in rat aorta, we tentatively identified the receptor subtypes and their intracellular signaling mechanisms. The contraction induced by Up₄A in endothelium-denuded rat aortic rings was significantly inhibited by the non-selective P2X1/P2X3 receptor antagonist NF 279. Vascular smooth muscle cells express multiple P2X and P2Y receptors that cause contraction. P2X1 is the primary subtype expressed on vascular smooth muscle involved in vasoconstriction. In rat aortic smooth muscle cells, the presence of P2X1, P2X2, P2X4, P2Y2 and P2Y6 has been shown [for review: (Kunapuli and Daniel, 1998)]. In addition, a study reported that purine dinucleotides containing 4 to 6 phosphates in the polyphosphate chain cause contraction mediated by P2X1 receptor present on the smooth muscle (Lewis et al., 2000). Based on these data, our results indicate that dinucleotides containing both purine and pyrimidine moieties and a long polyphosphate chain induce contraction by partially activating P2X1 receptors on the smooth muscle.

Interestingly, the non-selective P1 receptor antagonist 8-PST also inhibited Up₄A-induced contraction. Adenosine is the prototype agonist for P1 receptors. P1 receptor activation is generally associated with vasodilation (Liu et al., 2002). For many years it was accepted that adenosine evoked dilatation by stimulation of A2 receptors (particularly A2A subtype) on the vascular smooth muscle via an increase in cAMP (Olsson and Pearson, 1990). In the kidney, adenosine produces vasoconstriction and vasodilatation through activation of A1 and A2, respectively (Inscho, 2003). Exceptionally, in aorta from hypertensive rats, P1 receptor activation has been shown to mediate endothelium-dependent contraction (Fahim and Mustafa, 2001; Tawfik et al., 2005). In addition, in human chorionic vessels, A2B adenosine receptor mediates contraction (Donoso et al., 2005). There have been some reports that diadenosine tetraphosphate (Ap₄A) contractile response in rat urinary bladder is mediated by P1-purinoceptor whereas ATP responses were not affected by the P1 receptor antagonist theophylline (Khattab et al.,

2002; Khattab and Al-Hrasen, 2006). To our knowledge, this is the first report showing the involvement of P1 receptor in vascular smooth muscle mediating contraction in aorta from normotensive rats.

The calcium channel blocker nifedipine inhibited the contraction induced by Up₄A in endothelium-denuded rat aortic rings. In rat aorta, nifedipine has been reported ineffective to inhibit the contraction or increase in intracellular calcium concentration induced by P2 receptors activation (Kalthof et al., 1993; Shen et al., 2000). On the other hand, nifedipine inhibits the contraction induced by adenosine in afferent arterioles and by Ap₄A and ATP in rat urinary bladder (Hansen et al., 2007; Khattab et al., 2002). These data support the presence of a P1 receptor in the smooth muscle of rat aorta mediating contraction, dependent on extracellular calcium influx.

The Rho-kinase inhibitor Y27632 (Uehata et al., 1997) inhibited Up₄A-induced contraction of endothelium-denuded rat aortic rings. In rat aorta, nucleotides can induce contraction mediated by P2X, P2Y2 and P2Y6 receptor activation (Bultmann et al., 1997). Furthermore, P2Y1, P2Y2, P2Y4 and P2Y6 receptors have been shown to be coupled to Rho-kinase activation in rat aortic smooth muscle cells (Sauzeau et al., 2000). Based on this information, our results suggest the possible involvement of P2Y receptors in the contraction induced by Up₄A in rat aorta.

Our data demonstrate that the membrane permeable reactive oxygen species scavenger tempol, and the NADPH oxidase inhibitor apocynin significantly reduced Up₄A-induced contraction in rat aorta suggesting an important role for superoxide anion formation. To our knowledge, there have been no reports on superoxide formation after purinergic receptor activation in the vasculature. However, it has been reported that Ap₄A as well as ATP-induced contractile responses in rat urinary bladder involves superoxide formation (Khattab et al., 2002; Khattab and Al-Hrasen, 2006). Furthermore, activation of ATP receptors causes increase in Ca⁺² transients and production of superoxide anion in human eosinophil granulocytes, human prostate tumor spheroids and human tumor thyroid cell line (Dichmann et al., 2000; Pines et al., 2005; Sauer et al., 2001).

Tachyphylaxis, characterized by a rapidly decreasing response to a pharmacologically active agent after its initial administration, is a phenomenon that has been observed for many vasoconstrictor agents such as angiotensin II (Holloway et al., 2002; Linder et al., 2007; Thomas, 1999), endothelin 1 (Linder and Bendhack, 2002), serotonin (De Mey and Vanhoutte, 1981) and ATP (Shen et al., 2000). It has also been reported that ATP and diadenosine tetraphosphate (Ap₄A) exhibit significant tachyphylaxis in isolated rat urinary bladder (Khattab et al., 2002; Shen et al., 2000). The fact that Up₄A exhibits no tachyphylactic pattern indicates that the Up₄A-induced constriction of rat aortic rings cannot be explained by the presence of one of its degradation products. These data confirm the findings that ATP or UTP do not contribute to the Up₄A-induced contraction observed previously in isolated perfused rat kidney (Jankowski et al., 2005).

A number of cardiovascular diseases involve alterations in the synthesis of vasoactive compounds. Hypertension is associated with increased vasoconstriction and decreased relaxation usually accompanied by endothelium dysfunction. It has been recently reported that the plasma of juvenile hypertensive subjects presents increased levels of Up₄A (Jankowski et al., 2007). The results of the present study indicate that the endogenous compound Up₄A may be involved in the control of vascular tone and, therefore, may have a strong impact in cardiovascular diseases. Understanding the pharmacological and physiological function and regulation of vascular tone by Up₄A may be of extreme value to understanding the pathophysiology and treatment of these diseases.

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References

- Boarder, M.R., Hourani, S.M., 1998. The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. *Trends Pharmacol. Sci.* 19, 99.
- Bruns, R.F., 1981. Adenosine antagonism by purines, pteridines and benzopteridines in human fibroblasts. *Biochem. Pharmacol.* 30, 325.
- Bruns, R.F., Lu, G.H., Pugsley, T.A., 1986. Characterization of the A2 adenosine receptor labeled by [³H]NECA in rat striatal membranes. *Mol. Pharmacol.* 29, 331.
- Bultmann, R., Hansmann, G., Tuluc, F., Starke, K., 1997. Vasoconstrictor and vasodilator effects of guanine nucleotides in the rat aorta. *Naunyn Schmiedeberg's Arch. Pharmacol.* 356, 653.
- Burnstock, G., 2004. Introduction: P2 receptors. *Curr. Top. Med. Chem.* 4, 793.
- Buvinic, S., Poblete, M.L., Donoso, M.V., Delpiano, A.M., Briones, R., Miranda, R., Huidobro-Toro, J.P., 2006. P2Y1 and P2Y2 receptor distribution varies along the human placental vascular tree: role of nucleotides in vascular tone regulation. *J. Physiol.* 573, 427.
- Communi, D., Janssens, R., Suarez-Huerta, N., Robaye, B., Boeynaems, J.M., 2000. Advances in signalling by extracellular nucleotides: the role and transduction mechanisms of P2Y receptors. *Cell. Signal.* 12, 351.
- Dalziel, H.H., Westfall, D.P., 1994. Receptors for adenine nucleotides and nucleosides: subclassification, distribution, and molecular characterization. *Pharmacol. Rev.* 46, 449.
- De Mey, C., Vanhoutte, P.M., 1981. Effect of age and spontaneous hypertension on the tachyphylaxis to 5-hydroxytryptamine and angiotensin II in the isolated rat kidney. *Hypertension* 3, 718.
- Dichmann, S., Idzko, M., Zimpfer, U., Hofmann, C., Ferrari, D., Luttmann, W., Virchow Jr., C., Di Virgilio, F., Norgauer, J., 2000. Adenosine triphosphate-induced oxygen radical production and CD11b up-regulation: Ca²⁺ mobilization and actin reorganization in human eosinophils. *Blood* 95, 973.
- Donoso, M.V., Lopez, R., Miranda, R., Briones, R., Huidobro-Toro, J.P., 2005. A2B adenosine receptor mediates human chorionic vasoconstriction and signals through arachidonic acid cascade. *Am. J. Physiol. Heart Circ. Physiol.* 288, H2439.
- Fahim, M., Mustafa, S.J., 2001. Evidence for the presence of A(1) adenosine receptors in the aorta of spontaneously hypertensive rats. *Br. J. Pharmacol.* 134, 1760.
- Fredholm, B.B., Abbraccio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., Williams, M., 1994. Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* 46, 143.
- Guns, P.J., Korda, A., Crauwels, H.M., Van Assche, T., Robaye, B., Boeynaems, J.M., Bult, H., 2005. Pharmacological characterization of nucleotide P2Y receptors on endothelial cells of the mouse aorta. *Br. J. Pharmacol.* 146, 288.
- Hansen, P.B., Friis, U.G., Uhrenholt, T.R., Briggs, J., Schnermann, J., 2007. Intracellular signalling pathways in the vasoconstrictor response of mouse afferent arterioles to adenosine. *Acta Physiol. (Oxf)* 191, 89.
- Hansmann, G., Bultmann, R., Tuluc, F., Starke, K., 1997. Characterization by antagonists of P2-receptors mediating endothelium-dependent relaxation in the rat aorta. *Naunyn Schmiedeberg's Arch. Pharmacol.* 356, 641.
- Holloway, A.C., Qian, H., Pipolo, L., Ziogas, J., Miura, S., Karnik, S., Southwell, B.R., Lew, M.J., Thomas, W.G., 2002. Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Mol. Pharmacol.* 61, 768.
- Inscho, E.W., 2003. Modulation of renal microvascular function by adenosine. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 285, R23.
- Inscho, E.W., Cook, A.K., 2002. P2 receptor-mediated afferent arteriolar vasoconstriction during calcium blockade. *Am. J. Physiol. Renal. Physiol.* 282, F245.
- Jankowski, J., Jankowski, V., Seibt, B., Henning, L., Zidek, W., Schluter, H., 2003. Identification of dinucleoside polyphosphates in adrenal glands. *Biochem. Biophys. Res. Commun.* 304, 365.
- Jankowski, V., Meyer, A.A., Schlattmann, P., Gui, Y., Zheng, X.L., Stamcou, I., Radtke, K., Tran, T.N., van der Giet, M., Tolle, M., Zidek, W., Jankowski, J., 2007. Increased uridine adenosine tetraphosphate concentrations in plasma of juvenile hypertensives. *Arterioscler. Thromb. Vasc. Biol.* 27, 1776.
- Jankowski, V., Tolle, M., Vanholder, R., Schonfelder, G., van der Giet, M., Henning, L., Schluter, H., Paul, M., Zidek, W., Jankowski, J., 2005. Uridine adenosine tetraphosphate: a novel endothelium-derived vasoconstrictive factor. *Nat. Med.* 11, 223.
- Kalthof, B., Bechem, M., Flocke, K., Pott, L., Schramm, M., 1993. Kinetics of ATP-induced Ca²⁺ transients in cultured pig aortic smooth muscle cells depend on ATP concentration and stored Ca²⁺. *J. Physiol.* 466, 245.
- Khatab, M.A., A.L.-S. O., E.L.-K. H., 2002. Comparative study of the contractile activity evoked by ATP and diadenosine tetraphosphate in isolated rat urinary bladder. *Pharmacol. Res.* 45, 93.
- Khatab, M.M., Al-Hrasen, M.N., 2006. Contractile activity of ATP and diadenosine tetraphosphate on urinary bladder in the rats: role of superoxide anion and urothelium. *Autacoid Pharmacol.* 26, 149.
- Knight, G.E., Burnstock, G., 2001. Identification of P1 and P2 purinoceptors in the aorta of the lizard (*Agama sp.*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 128, 413.
- Kunapuli, S.P., Daniel, J.L., 1998. P2 receptor subtypes in the cardiovascular system. *Biochem. J.* 336 (Pt 3), 513.
- Lewis, C.D., Hourani, S.M., Long, C.J., Collis, M.G., 1994. Characterization of adenosine receptors in the rat isolated aorta. *Gen. Pharmacol.* 25, 1381.
- Lewis, C.J., Gitterman, D.P., Schluter, H., Evans, R.J., 2000. Effects of diadenosine polyphosphates (Ap(n)As) and adenosine polyphospho guanosines (Ap(n)Gs) on rat mesenteric artery P2X receptor ion channels. *Br. J. Pharmacol.* 129, 124.
- Linder, A.E., Bendhack, L.M., 2002. Endothelin-1-induced contraction is impaired in the tail artery of renal hypertensive rats. *Vascul. Pharmacol.* 39, 77.
- Linder, A.E., Thakali, K.M., Thompson, J.M., Watts, S.W., Webb, R.C., Leite, R., 2007. Methyl-beta-cyclodextrin prevents angiotensin II-induced tachyphylactic contractile responses in rat aorta. *J. Pharmacol. Exp. Ther.* 323, 78.
- Liu, S.H., Shan, L.M., Wang, H., 2002. Pharmacological characteristics of novel putative purinoceptors in vascular endothelium. *Cell Biol. Intl.* 26 (11), 963.
- Malec, D., 1996. Purinergic receptors. *Pol. J. Pharmacol.* 48, 457.
- North, R.A., 2002. Molecular physiology of P2X receptors. *Physiol. Rev.* 82, 1013.
- Olsson, R.A., Pearson, J.D., 1990. Cardiovascular purinoceptors. *Physiol. Rev.* 70, 761.
- Petrack, B., Czernik, A.J., Ansell, J., Cassidy, J., 1981. Potentiation of arginine-induced glucagon secretion by adenosine. *Life Sci.* 28, 2611.
- Pines, A., Perrone, L., Bivi, N., Romanello, M., Damante, G., Gulisano, M., Kelley, M.R., Quadrioglio, F., Tell, G., 2005. Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *Nucleic Acids Res.* 33, 4379.
- Prentice, D.J., Hourani, S.M., 1996. Activation of multiple sites by adenosine analogues in the rat isolated aorta. *Br. J. Pharmacol.* 118, 1509.
- Ralevic, V., Burnstock, G., 1991. Roles of P2-purinoceptors in the cardiovascular system. *Circulation* 84, 1.
- Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. *Pharmacol. Rev.* 50, 413.
- Ralevic, V., Hoyle, C.H., Burnstock, G., 1995. Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J. Physiol.* 483 (Pt 3), 703.
- Ralevic, V., Jankowski, J., Schluter, H., 2001. Structure–activity relationships of diadenosine polyphosphates (Ap(n)As), adenosine polyphospho guanosines (Ap(n)Gs) and guanosine polyphospho guanosines (Gp(n)Gs) at P2 receptors in the rat mesenteric arterial bed. *Br. J. Pharmacol.* 134, 1073.
- Rettinger, J., Schmalzing, G., Damer, S., Muller, G., Nickel, P., Lambrecht, G., 2000. The suramin analogue NF279 is a novel and potent antagonist selective for the P2X(1) receptor. *Neuropharmacology* 39, 2044.
- Sauer, H., Klimm, B., Hescheler, J., Wartenberg, M., 2001. Activation of p90RSK and growth stimulation of multicellular tumor spheroids are dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *Faseb J.* 15, 2539.
- Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C., Vaillant, N., Gadeau, A.P., 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. Heart Circ. Physiol.* 278, H1751.
- Shen, J.Z., Zheng, X.F., Kwan, C.Y., 2000. Evidence for P(2)-purinoceptors contribution in H(2)O(2)-induced contraction of rat aorta in the absence of endothelium. *Cardiovasc. Res.* 47, 574.
- Tawfik, H.E., Schnermann, J., Oldenburg, P.J., Mustafa, S.J., 2005. Role of A1 adenosine receptors in regulation of vascular tone. *Am. J. Physiol. Heart Circ. Physiol.* 288, H1411.
- Thomas, W.G., 1999. Regulation of angiotensin II type 1 (AT1) receptor function. *Regul. Pept.* 79, 9.
- Tolle, M., Giebing, G., Tietge, U.J., Jankowski, J., Jankowski, V., Henning, L., Horl, M.P., Weiss, W., Zidek, W., van der Giet, M., 2006. Diguanosine pentaphosphate: an endogenous activator of Rho-kinase possibly involved in blood pressure regulation. *J. Hypertens.* 24, 1991.
- 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.