The Journal of Physiology

ATP-mediated vasodilatation occurs via activation of inwardly rectifying potassium channels in humans

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Key points

- ATP is a substance in the blood vessels that can cause vasodilatation and increase blood flow and oxygen delivery in humans.
- The exact signalling pathways that ATP stimulates to cause vasodilatation are not well known.
- We show that a large portion of ATP-mediated vasodilatation occurs through the activation of inwardly rectifying potassium channels (K_{IR}).
- Our results lend insight into the vasodilator mechanisms of ATP, a substance that is important for vascular control.
- Further, our results may stimulate additional investigations in humans regarding the activation of K_{IR} channels and subsequent vascular hyperpolarization during other physiologically relevant conditions.

Abstract Circulating ATP possesses unique vasomotor properties in humans and has been hypothesized to play a role in vascular control under a variety of physiological conditions. However, the primary downstream signalling mechanisms underlying ATP-mediated vasodilatation remain unclear. The purpose of the present experiment was to determine whether ATP-mediated vasodilatation is independent of nitric oxide (NO) and prostaglandin (PG) synthesis and occurs primarily via the activation of Na^+/K^+-ATPase and inwardly rectifying potassium (K_{IR}) channels in humans. In all protocols, young healthy adults were studied and forearm vascular conductance (FVC) was calculated from forearm blood flow (measured via venous occlusion plethysmography) and intra-arterial blood pressure to quantify local vasodilatation. Vasodilator responses (%ΔFVC) during intra-arterial ATP infusions were unchanged following combined inhibition of NO and PGs ($n = 8$; $P > 0.05$) whereas the responses to KCl were greater ($P < 0.05$). Combined infusion of ouabain (to inhibit Na^+/K^+-ATPase) and barium chloride ($BaCl_2$; to inhibit K_{IR} channels) abolished KCl-mediated vasodilatation ($n = 6$; $\%\Delta FVC = 134 \pm 13$ *vs.* $4 \pm 5\%$; $P < 0.05$), demonstrating effective blockade of direct vascular hyperpolarization. The vasodilator responses to three different doses of ATP were inhibited on average $56 \pm 5\%$ ($n = 16$) following combined ouabain plus $BaCl_2$ infusion. In follow-up studies, $BaCl_2$ alone inhibited the vasodilator responses to ATP on average $51 \pm 3\%$ ($n = 6$), which was not different than that observed for combined ouabain plus $BaCl_2$ administration. Our novel results indicate that the primary mechanism of ATP-mediated vasodilatation is vascular hyperpolarization via activation of K_{IR} channels. These observations translate *in vitro* findings to humans *in vivo* and may help explain the unique vasomotor properties of intravascular ATP in the human circulation.

DOI: 10.1113/jphysiol.2012.234245

(Received 10 April 2012; accepted after revision 5 July 2012; first published online 9 July 2012)
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Abbreviations ACh, acetylcholine; COX, cyclooxygenase; FAV, forearm volume; FBF, forearm blood flow; FVC, forearm vascular conductance; HR, heart rate; IK_{Ca}, intermediate-conductance calcium-activated potassium channel; K_{ATP}, ATP-sensitive potassium channel; K_{Ca}, calcium-activated potassium channel; K_{IR}, inwardly rectifying potassium channel; MAP, mean arterial pressure; NOS, nitric oxide synthase; P, purinergic; PG, prostaglandin; SK_{Ca}, small-conductance calcium-activated potassium channel; VOP, venous occlusion plethysmography.

Introduction

Accumulating evidence indicates that circulating adenosine triphosphate (ATP) plays an important role in the regulation of local vascular tone in cerebral (Horiuchi *et al.* 2003), coronary (Farias *et al.* 2005) and skeletal muscle circulations (Gonzalez-Alonso *et al.* 2002). Specifically, data indicate that ATP is involved in the vascular response to physiological stimuli that require the matching of blood flow and oxygen supply to the metabolic demands of the tissue (Gonzalez-Alonso *et al.* 2002). In humans, intravascular ATP can cause profound vasodilatation and moreover may be important for vascular control in that it has the unique ability to limit sympathetically mediated vasoconstriction, similar to what occurs in contracting skeletal muscle (Kirby *et al.* 2008). Thus, given the role of ATP in vasomotor control, there has been substantial interest in understanding the primary downstream signalling mechanisms underlying ATP-mediated vasodilatation (Rongen *et al.* 1994; van Ginneken *et al.* 2004; Crecelius *et al.* 2011*a*).

In vitro studies have demonstrated that intravascular ATP binds to purinergic 2 (P_2) receptors on the endothelium, resulting in an increase in intracellular endothelial cell [Ca^{2+}] which then can stimulate multiple vasoactive pathways that ultimately cause relaxation of the vascular smooth muscle (Duza & Sarelius, 2003; Ellsworth *et al.* 2009). In this context, elevations in endothelial cell [Ca^{2+}] can increase the synthesis of nitric oxide (NO) and arachidonic acid metabolites such as vasodilating prostaglandins (PGs) and, consistent with this, some *in vitro* preparations have shown that these substances may contribute to the vasodilator action of ATP (Wihlborg *et al.* 2003; Ellsworth *et al.* 2009). In contrast, the majority of studies in humans indicate that NO and PGs are not the primary mediators and at best have only a modest role in the local dilatory response to intravascular ATP (Rongen *et al.* 1994; van Ginneken *et al.* 2004; Mortensen *et al.* 2009; Crecelius *et al.* 2011*a*). Thus, it appears that ATP-mediated vasodilatation involves mechanisms beyond these traditional endothelial cell signalling pathways in humans.

In addition to stimulating the synthesis of NO and PGs, increases in intracellular endothelial cell [Ca^{2+}] can activate small- and intermediate-conductance Ca^{2+}-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively) resulting in endothelial cell hyperpolarization which then can be electrically communicated via gap junctions to other adjacent endothelial cells as well as the underlying smooth muscle cells (Edwards *et al.* 1998; Segal, 2005). Further, K^+ efflux from these channels increases K^+ concentrations in the myoendothelial space which stimulates both Na^+/K^+-ATPase and inwardly rectifying potassium (K_{IR}) channels evoking smooth muscle cell hyperpolarization (Edwards *et al.* 1998). In addition to the activation of K_{IR} channels via increases in [K^+], there is evidence that hyperpolarization of the cellular membrane can activate K_{IR} channels and thus facilitate amplification of a hyperpolarizing stimulus which spreads through the vascular wall and translates to a robust conducted or spreading vasodilator response (Smith *et al.* 2008). Interestingly, studies *in vitro* have demonstrated that application of ATP stimulates hyperpolarization of both endothelial and vascular smooth muscle cells (Malmsjo *et al.* 1999; Sheng & Braun, 2007), evokes local and conducted vasodilatation that is substantially reduced via inhibition of SK_{Ca} and IK_{Ca} channels (Winter & Dora, 2007), and that vasodilatation to direct P_2 receptor stimulation is blunted by inhibition of Na^+/K^+-ATPase (via ouabain) (Ralevic, 2001) and K_{IR} channels (via barium chloride, $BaCl_2$) (Smith *et al.* 2008).

Recently, Dawes and colleagues were able to successfully and safely administer ouabain and $BaCl_2$ via brachial artery catheter which nearly abolished potassium chloride (KCl)-mediated vasodilatation in the human forearm (Dawes *et al.* 2002). KCl mimics many of the vascular effects of ATP *in vitro* in that it causes endothelial and vascular smooth muscle cell hyperpolarization and conducted vasodilatation (Edwards *et al.* 1998; Horiuchi *et al.* 2002). Importantly, these responses are inhibited via ouabain and $BaCl_2$ (Edwards *et al.* 1998; Horiuchi *et al.* 2002; Smith *et al.* 2008). Here, we first determined the efficacy of ouabain and $BaCl_2$ to inhibit direct hyperpolarization via KCl, and then determined whether ATP-mediated vasodilatation occurs as a result of vascular hyperpolarization *in vivo*. Accordingly, in the present investigation we directly tested the hypothesis that ATP-mediated vasodilatation is largely independent of NO

and PG synthesis and occurs via Na^+/K^+-ATPase and K_{IR} channel activation in humans.

Methods

Subjects

With Institutional Review Board approval and after obtaining written informed consent, a total of 33 young healthy adults (Protocol 1: 5 men, 3 women; age, 22 ± 1 years; weight, 71.8 ± 3.6 kg; height, 174 ± 3 cm; body mass index, 23.7 ± 0.9 kg m^{-2}; forearm volume (FAV), 966 ± 64 ml; Protocol 2: 19 men, 6 women; age, 23 ± 1 years; weight, 73.7 ± 1.7 kg; height, 175 ± 1 cm; body mass index, 24.0 ± 0.5 kg m^{-2}; FAV, 960 ± 42 ml; Protocol 3: 4 men, 2 women; age, 23 ± 2 years; weight, 73.3 ± 2.8 kg; height, 172 ± 2 cm; body mass index, 24.9 ± 1.1 kg m^{-2}; FAV, 792 ± 16 ml; means ± SEM) participated in the present study. Six subjects participated in multiple protocols and all subject groups were similar in their characteristics except that those participants in Protocol 1 had a slightly larger FAV than those in Protocol 3 ($P < 0.05$).

All subjects were sedentary to moderately active, non-smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg), and not taking any medications. Studies were performed after a 4 h fast and 24 h abstention from caffeine and exercise. The subjects were in the supine position with the experimental arm abducted to 90 deg and slightly elevated above heart level upon a tilt-adjustable table. Female subjects were studied during the early follicular phase of their menstrual cycle or placebo phase of oral contraceptive use to minimize any potential cardiovascular effects of sex-specific hormones. All studies were performed according to the *Declaration of Helsinki*.

Arterial and venous catheterization

A 20 gauge, 7.6 cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anaesthesia (2% lidocaine) for local administration of study drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml h^{-1} with heparinized saline. The two side ports were used for drug infusions (Kirby *et al.* 2008; Crecelius *et al.* 2010). In addition, an 18 gauge, 3.8 cm catheter was inserted into an antecubital vein of the non-experimental arm for venous blood samples (Crecelius *et al.* 2011*b*) to be used for systemic electrolyte monitoring via clinical blood gas analyser (Siemens Rapid Point 400 Series Automatic Blood Gas System, Los Angeles, CA, USA). Saline was continuously infused through this catheter at a rate of approximately 3 ml min^{-1} for the duration of the study to keep it patent.

Forearm blood flow and vascular conductance

Forearm blood flow (FBF) was measured via venous occlusion plethysmography (VOP) using mercury-in-salistic strain gauges and techniques as previously described (Greenfield *et al.* 1963; Crecelius *et al.* 2011*a*) and was expressed as milliliters per deciliter of tissue per minute (ml dl^{-1} min^{-1}). As an index of forearm vasodilatation and to account for individual differences in baseline vascular tone, forearm vascular conductance (FVC) was calculated as (FBF/MAP) × 100 expressed as ml dl^{-1} min^{-1} (100 mmHg^{-1}). In an effort to minimize the contribution of cutaneous blood flow to FBF measurements, a fan was directed at the experimental arm throughout the experimental protocol.

Vasoactive drug infusion

All drug infusions were through the brachial artery catheter to create a local effect in the forearm. Vasodilator infusions were either ATP (P_2 receptor agonist; Sigma A7699, St Louis, MO, USA), potassium chloride (KCl; direct hyperpolarizing stimulus; Hospira, Lake Forest, IL, USA) or acetylcholine (ACh; muscarinic receptor agonist; Novartis, East Hanover, IL, USA) in the doses provided in the respective protocol (see below). Our experimental question primarily focused on ATP-mediated vasodilatation and KCl and ACh were used to test efficacy and specificity of the inhibitors employed.

To assess the contribution of NO and PGs to ATP and KCl-mediated vasodilatation, N^G-monomethyl-L-arginine (L-NMMA; nitric oxide synthase (NOS) inhibitor; Clinalfa/Bachem, Weil am Rhein, Germany) was administered to inhibit the production of NO and ketorolac (non-selective cyclo-oxygenase (COX) inhibitor; Hospira) was administered to inhibit the synthesis of PGs. Loading doses of L-NMMA and ketorolac were 25 mg (5 mg min^{-1} for 5 min) and 6 mg (600 μg min^{-1} for 10 min), respectively, and maintenance doses of 1.25 mg min^{-1} (L-NMMA) and 150 μg min^{-1} (ketorolac) were infused for the duration of the protocol to ensure continuous blockade (Crecelius *et al.* 2010, 2011*b*).

In order to determine the role of Na^+/K^+-ATPase and K_{IR} channels, ouabain octahydrate (Sigma 03125) was infused at 2.7 nmol min^{-1} for 15 min as a loading dose and continued throughout vasodilator infusion (4 additional minutes) at the same dose to inhibit Na^+/K^+-ATPase (Dawes *et al.* 2002; Dwivedi *et al.* 2005). Barium chloride (10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ,

USA) was infused at 4 μmol min^{-1} for 3 min as a loading dose and continued throughout vasodilator infusion (4 additional minutes) at the same dose to inhibit K_{IR} channels (Dawes *et al.* 2002; Dwivedi *et al.* 2005). Importantly, Dawes and colleagues (2002) determined that this same dose of $BaCl_2$ for 6 min caused an increase in venous plasma [Ba^{2+}] to 50.00 ± 8.00 μmol l^{-1}, a concentration that is within the range (<100 μmol l^{-1}) of $BaCl_2$ to specifically inhibit K_{IR} channels (Jackson, 2005).

ATP, ouabain and $BaCl_2$ were prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS, USA) prior to use. FAV used for normalization for specific vasoactive drugs was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc., Bedford, MA, USA).

Experimental protocols

In general, 2 min of resting data were acquired prior to the start of all vasodilator infusions and 15 min of rest separated each trial, as in our experience, this is more than a sufficient length of time for baseline FBF to return to pre-vasodilator levels. Saline was used as a control infusate at matched rates to the vasodilators and inhibitors.

Protocol 1: effect of combined NOS and COX inhibition. In eight subjects, dose–response trials were performed as 2 min infusions at each progressive dose of either ATP (1.25, 2.50 and 5.00 μg (dl FAV)$^{-1}$ min^{-1}) or KCl (0.05, 0.10 and 0.20 mmol min^{-1}), thus a total of 6 min of vasodilator infusion. Dose–response was performed in control conditions and during combined L-NMMA and ketorolac administration. The order of ATP and KCl was balanced between subjects. A subgroup of these subjects ($n = 4$) also received progressive doses of acetylcholine (ACh; 4, 8 and 16 μg (dl FAV)$^{-1}$ min^{-1}) in control and blockade conditions to test the efficacy of the combined NOS + COX inhibition.

Protocol 2: effect of Na^+/K^+-ATPase and K_{IR} channel inhibition. Due to safety concerns with $BaCl_2$ administration (Dawes *et al.* 2002), subjects received either KCl or ATP infusions. Two doses of KCl ($n = 6$; 0.10 and 0.20 mmol min^{-1}) or ATP ($n = 8$; 1.25 and 5.00 μg (dl FAV)$^{-1}$ min^{-1}) were administered for 2 min each, thus a total of 4 min of vasodilator infusion. Dose–response was performed in control conditions and with combined ouabain and $BaCl_2$ administration. Additional subjects ($n = 8$) were studied at a lower range of ATP doses (0.625 and 1.25 μg (dl FAV)$^{-1}$ min^{-1}).

A small group of subjects ($n = 4$) received ACh (2 μg (dl FAV)$^{-1}$ min^{-1}) for 2 min before and after combined ouabain and $BaCl_2$ administration in order to confirm previous findings that the effects of ouabain and $BaCl_2$ are selective and thus do not impair vasodilator responses in a non-specific manner (Dawes *et al.* 2002; Dwivedi *et al.* 2005). We chose to administer ACh because, similar to ATP, it is an endothelium-dependent agonist; however, ACh is primarily dependent on the NOS and COX vasodilator pathways in humans (see Results). The dose of ACh (2 μg (dl FAV)$^{-1}$ min^{-1}) was based on an anticipated vasodilator response to match that observed with our lower doses of ATP.

Protocol 3: effect of independent K_{IR} channel inhibition. Given our findings from Protocol 2 (see Results) and the ability for K_{IR} channel activation to amplify hyperpolarization that occurs independent of Na^+/K^+-ATPase activity (Smith *et al.* 2008), we were interested in the independent role of K_{IR} channels. Therefore, in six subjects, we determined the influence of $BaCl_2$ alone on ATP-mediated vasodilatation. Two doses of ATP (0.625 and 1.25 μg (dl FAV)$^{-1}$ min^{-1}) were given for 2 min each in control (saline) conditions or with concomitant administration of $BaCl_2$.

Data acquisition and analysis

Data were collected and stored on a computer at 250 Hz and were analysed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). MAP was determined from the arterial pressure waveform and heart rate (HR) was determined via the standard three-lead ECG. FBF was determined from the derivative of the forearm plethysmogram signal, resulting in one FBF measurement every 15 s. FBF, HR and MAP represent an average of the last minute of steady-state conditions (i.e. 4 FBF measures). FVC was used as our standard index of forearm vascular tone, and we present both absolute FVC and percentage changes in FVC (%ΔFVC) in response to the vasoactive drug infusions. Given the existence of individual differences in baseline vascular tone, individual differences in forearm vascular tone during vasodilator infusion, as well the potential influence of the pharmacological inhibitors on baseline vascular tone, we were especially interested in the %ΔFVC as this tracks changes in blood vessel radius independent of the initial level of vascular tone and is therefore the most appropriate index of changes in vasomotor tone (Buckwalter & Clifford, 2001). The percentage increase in FVC due to vasodilator infusion in each trial was calculated as:

$$(\text{FVC vasodilator infusion} - \text{FVC baseline}) / (\text{FVC baseline}) \times 100.$$

The magnitude of inhibition of vasodilator responses was calculated as:

$$(\%\Delta\text{FVC inhibition} - \%\Delta\text{FVC control}) / (\%\Delta\text{FVC control}) \times 100.$$

Statistics

Data are presented as mean ± SEM. Differences within and between conditions for each vasodilator were determined via two-way (dose and condition (control, inhibition)) repeated-measures analysis of variance (ANOVA). When significance was observed, the Fisher's LSD method was used to make individual comparisons. To compare the effect of combined ouabain and $BaCl_2$ administration *vs.* $BaCl_2$ alone as well as compare subject characteristics between protocols, unpaired Student's *t* tests were used. Significance was set *a priori* at $P < 0.05$.

Results

Protocol 1: effect of combined NOS and COX inhibition on ATP and K^+-mediated vasodilatation

No significant changes in HR or MAP were observed across all conditions within Protocol 1 (Supplementary Tables S1 and S2). All three doses of ATP caused significant increases in FVC from rest and combined NOS–COX inhibition significantly reduced FVC at rest (Fig. 1*A*; $P < 0.05$), but not during ATP infusion ($P = 0.20$). Similarly, the vasodilator responses when quantified as a per cent change from rest were unaffected with combined L-NMMA and ketorolac infusion (Fig. 1*B*).

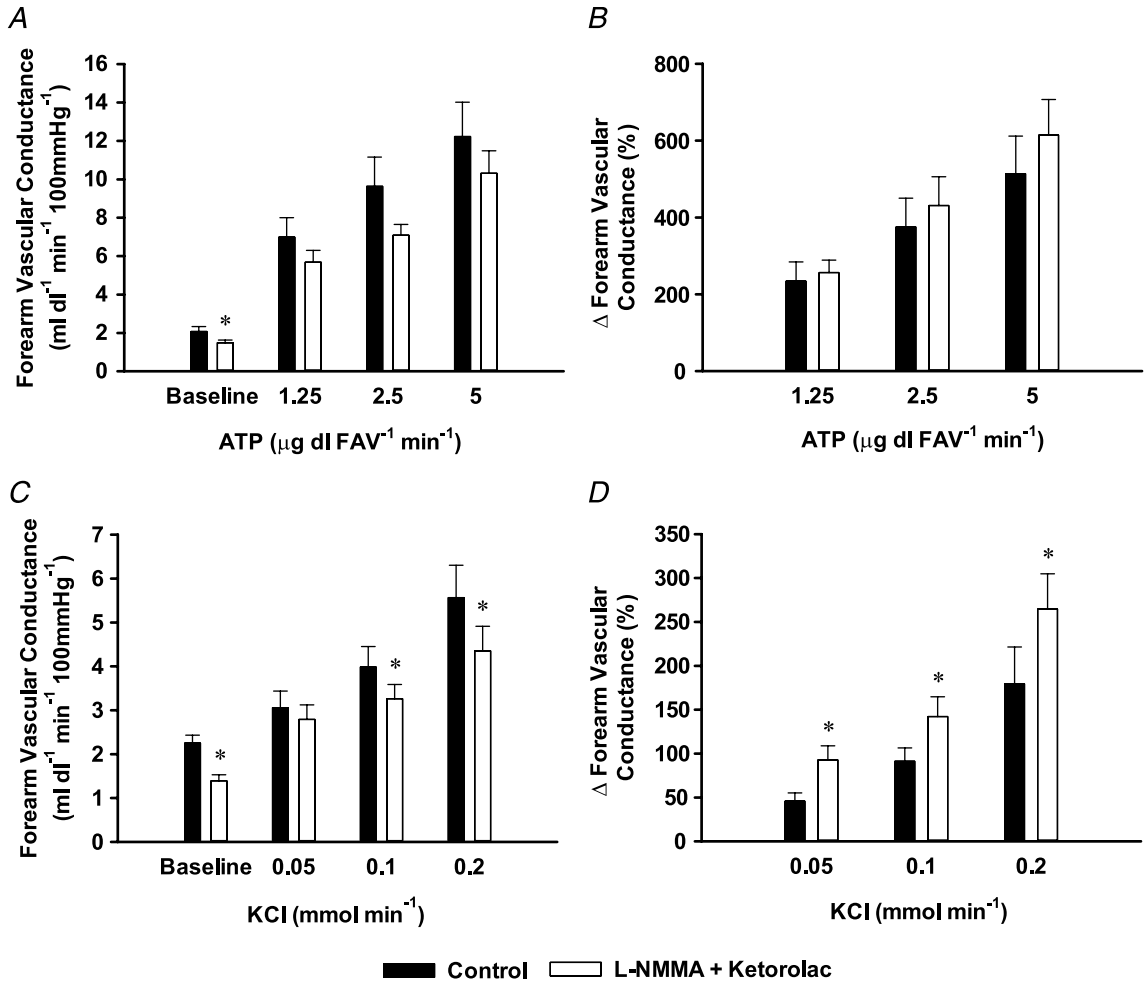

Figure 1. Protocol 1: effect of L-NMMA + ketorolac on ATP- and KCl-mediated vasodilatation
In 8 subjects, combined L-NMMA and ketorolac (open bars) to inhibit the synthesis of NO and PGs, respectively, significantly reduced absolute forearm vascular conductance (FVC) at baseline (*A*) but had no effect on absolute FVC or the per cent change in FVC (*B*) to progressive intrabrachial doses of ATP as compared with control (saline; filled bars) conditions. L-NMMA and ketorolac similarly decreased baseline absolute FVC (*C*) as well as two of three progressive intrabrachial doses of KCl. However, given the magnitude of the reduction in baseline FVC, the per cent change (*D*) was slightly augmented at all doses. *$P < 0.05$ *vs.* control.

All three doses of KCl also caused significant increases in FVC from rest and combined NOS–COX inhibition significantly reduced FVC at rest and minimally, but significantly, during the highest two doses of KCl (Fig. 1*C*; $P < 0.05$) but not the lowest dose of KCl ($P = 0.32$). The vasodilator responses when quantified as a per cent change from rest were slightly augmented with combined L-NMMA and ketorolac infusion at all doses of KCl (Fig. 1*D*).

ACh infusion resulted in significant increases in FVC from rest (2.7 ± 0.6 *vs.* 13.4 ± 3.1, 15.3 ± 3.7 and 18.3 ± 4.3 ml dl^{-1} min^{-1} (100 mmHg)$^{-1}$; $P < 0.05$) and combined NOS–COX inhibition significantly reduced FVC at rest (1.7 ± 0.3 ml dl^{-1} min^{-1} (100 mmHg)$^{-1}$; $P < 0.05$) and at all doses of ACh (4.1 ± 0.6, 3.9 ± 0.8 and 5.4 ± 1.0 ml dl^{-1} min^{-1} (100 mmHg)$^{-1}$; $P < 0.05$). On average, the vasodilator response (%ΔFVC; Supplementary Table S2B) was reduced ~60%, consistent with effective blockade of NOS (Lauer *et al.* 2001). In all conditions in this protocol, changes in FBF paralleled those observed for FVC (Supplementary Tables S1 and S2).

Protocol 2: effect of Na^+/K^+-ATPase and K_{IR} channel inhibition

No significant changes in HR or MAP were observed across all conditions within Protocol 2 (Supplementary Tables S3 and S4). Combined ouabain and $BaCl_2$ administration abolished forearm vasodilatation in response to KCl infusion ($P < 0.05$ *vs.* control; Fig. 2). At all doses of ATP, ouabain and $BaCl_2$ significantly attenuated the forearm vasodilator responses (mean effect of all doses pooled: -56 ± 4%; range: 40–70%; $P < 0.05$ *vs.* control; Fig. 3). In those subjects in which the vasodilator response to ACh was determined before and after inhibition of Na^+/K^+-ATPase and K_{IR} channels ($n = 4$), forearm vasodilatation was unchanged, demonstrating the selectivity of the blockers to KCl and ATP (Table 1). In all conditions in this protocol, changes in FBF paralleled those observed for FVC (Supplementary Tables S3 and S4).

Protocol 3: independent effect of K_{IR} channel inhibition

No significant changes in HR or MAP were observed across all conditions within Protocol 3 (Supplementary Table S5) where the impact of $BaCl_2$ alone on ATP-mediated vasodilatation was investigated. The ability of $BaCl_2$ alone to attenuate vasodilatation in response to exogenous ATP (mean effect of both doses pooled: -51 ± 3%; $P < 0.05$ *vs.* control; Fig. 4) was similar to that of combined ouabain and $BaCl_2$ ($P = 0.50$). Similar to other protocols, changes in FBF paralleled those observed for FVC (Supplementary Table S5).

Discussion

The primary novel finding from the current study is that ATP-mediated vasodilatation is largely independent of NO and PG synthesis and is significantly attenuated during combined inhibition of Na^+/K^+-ATPase and K_{IR} channels. Further, $BaCl_2$ alone reduced forearm vasodilator responses to a similar extent as combined ouabain and $BaCl_2$, thus implicating a primary role for vascular hyperpolarization via K_{IR} channel activation in ATP-mediated vasodilatation in humans.

Historically, investigations into the mechanisms of endothelium-dependent vasodilatation have focused primarily on NO and PGs, the synthesis of which can increase with elevations in intracellular endothelial cell [Ca^{2+}]. In the present study, we first aimed to determine whether KCl and ATP-mediated vasodilatation occur independently of NO and PGs in the human forearm. Our results from Protocol 1 (Fig. 1) demonstrate that combined inhibition of NO and PG synthesis does not impair the vasodilator response (%Δ) to KCl or ATP,

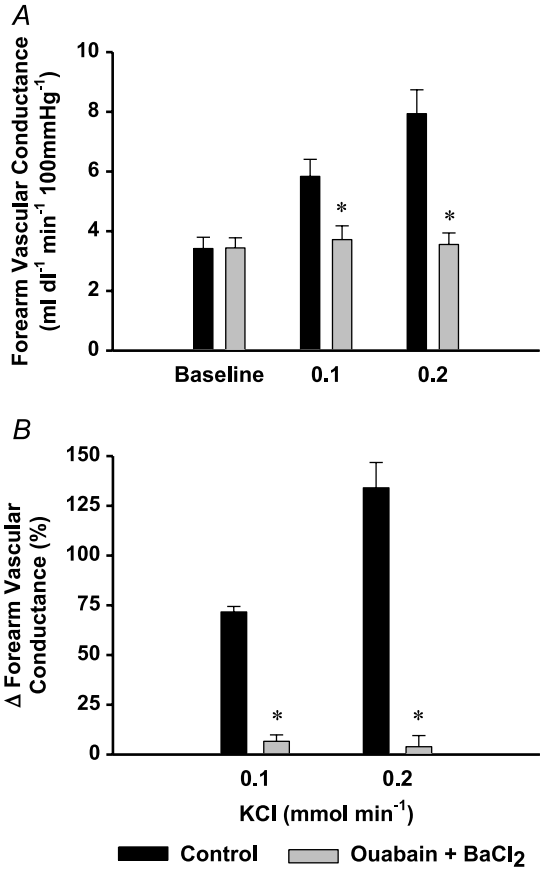

Figure 2. Protocol 2: efficacy of ouabain + $BaCl_2$ to block KCl-mediated vasodilatation
KCl-mediated vasodilatation (absolute FVC (A) and per cent change forearm vascular conductance (B)) was abolished with combined ouabain + $BaCl_2$ infusion (grey bars; to inhibit Na^+/K^+-ATPase and K_{IR} channels, respectively), indicating successful inhibition of a direct hyperpolarizing stimulus. *$P < 0.05$ *vs.* control.

Table 1. Protocol 2 subgroup: effect of ouabain + $BaCl_2$ on vasodilator response to ACh infusion

	Baseline FVC	ACh infusion FVC (2 μg dl FAV^{-1} min^{-1})	ΔFVC (%)	Magnitude of inhibition from control (%)
Control	2.3 ± 0.5	8.5 ± 1.8	274 ± 47	
Ouabain + $BaCl_2$	2.0 ± 0.2	8.0 ± 1.5	305 ± 60	+11 ± 9

$n = 4$; ACh, acetylcholine; FAV, forearm volume; FVC, forearm vascular conductance (ml dl^{-1} min^{-1} 100 mmHg^{-1}).

although there is a modest reduction in absolute FVC for KCl. To the best of our knowledge, we are the first to show this type of data for KCl in humans. The lack of a statistical effect of combined NOS and COX inhibition on ATP-mediated vasodilatation is in accordance with previous findings from our own laboratory (Crecelius *et al.* 2011*a*) and others (Rongen *et al.* 1994; van Ginneken *et al.* 2004). While we believe that %ΔFVC is the most appropriate presentation of these type of data, particularly given the reduction in baseline vascular tone (Buckwalter & Clifford, 2001) and this quantification suggests no role for NO and PGs in the vasodilator response to ATP and KCl, we acknowledge that the absolute FVC data would not completely rule out a potential role for these vasodilator pathways. Despite the slight difference in interpretation based on the method of quantification, within the present study design our data clearly indicate that the majority of the vasodilator response to intravascular ATP is beyond the traditional endothelial cell signalling pathways (e.g. NO and PGs) in humans.

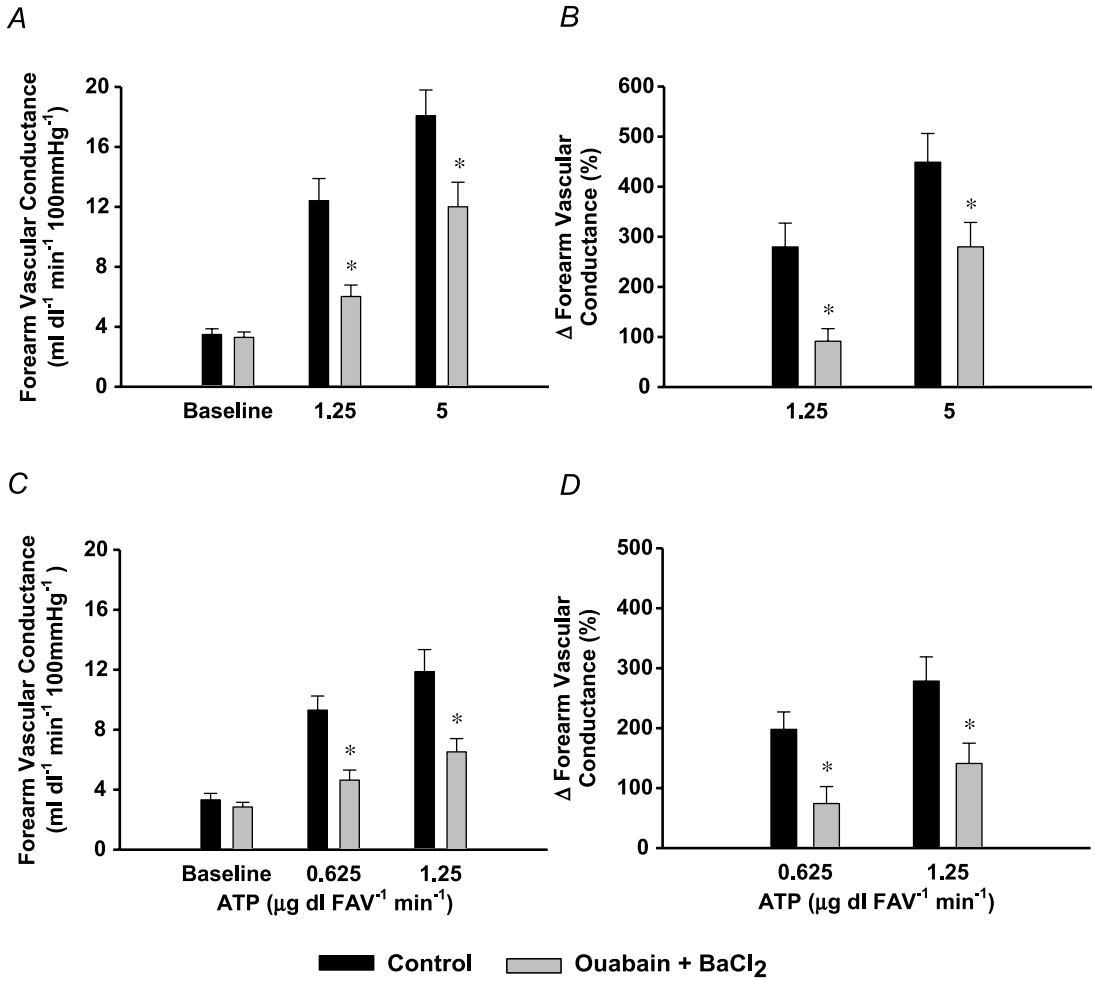

Figure 3. Protocol 2: effect of ouabain + $BaCl_2$ on ATP-mediated vasodilatation
Combined ouabain + $BaCl_2$ infusion (grey bars), significantly reduced absolute (*A*) and per cent changes (*B*) in forearm vascular conductance ($n = 8$; 1.25 μg dl FAV^{-1} min^{-1}: −69 ± 6%; 5.0 μg dl FAV^{-1} min^{-1}: −40 ± 6%). Similar findings were observed for FVC (*C*) and %ΔFVC (*D*) at two lower doses of ATP ($n = 8$; 0.625 μg dl FAV^{-1} min^{-1}: −66 ± 12%; 1.25 μg dl FAV^{-1} min^{-1}: −50 ± 8%). $P < 0.05$ *vs.* control.

Use of ouabain and $BaCl_2$ to address hyperpolarizing mechanisms of vasodilatation

On vascular smooth muscle cells, activation of the Na^+/K^+ pump leads to a hyperpolarization of the cellular membrane (net effect of efflux of 3 Na^+ ions and influx of 2 K^+ ions), as does K^+ efflux via opening of K_{IR} channels (Nelson & Quayle, 1995; Edwards *et al.* 1998). Increases in interstitial $[K^+]$ via exogenous KCl or endothelial-cell K^+ efflux from SK_{Ca} and IK_{Ca} channels has been shown to stimulate both Na^+/K^+-ATPase and K_{IR} channels (Nelson & Quayle, 1995; Edwards *et al.* 1998), whereas increased $[K^+]$ does not directly activate other potassium channels such as calcium-activated (K_{Ca}) and ATP-sensitive (K_{ATP}) potassium channels (Jackson, 2005). Further, K_{IR} channels are sensitive to changes in vascular smooth muscle cell membrane potential and are directly activated by hyperpolarization (Nelson & Quayle, 1995). Thus, inhibition of Na^+/K^+-ATPase and K_{IR} channels via ouabain and $BaCl_2$, respectively, can be used as a means to inhibit K^+-induced hyperpolarization and the spread/amplification of hyperpolarization (Smith *et al.* 2008) in experimental models where changes in membrane potential can be directly measured. Previously, Dawes and colleagues (2002) established that ouabain and $BaCl_2$ can be administered intra-arterially in the human forearm without adverse effect and can significantly inhibit KCl-mediated vasodilatation *in vivo*. Here, we have performed similar experiments in our own laboratory with the addition of another dose of KCl and demonstrate that combined ouabain and $BaCl_2$ administration abolishes KCl-mediated vasodilatation (Fig. 2). Further, we show that the effects of this pharmacological inhibition are not a general reduction in vasodilator capability as the responses to acetylcholine were unchanged during ouabain and $BaCl_2$ infusion (Table 1). These findings are consistent with previous studies which have also shown that vasodilator responses to acetylcholine, verapamil (L-type calcium channel antagonist), sodium nitroprusside (NO donor) and albuterol (β_2-adrenergic receptor agonist) are unaffected by ouabain and $BaCl_2$ (Dawes *et al.* 2002; Dwivedi *et al.* 2005).

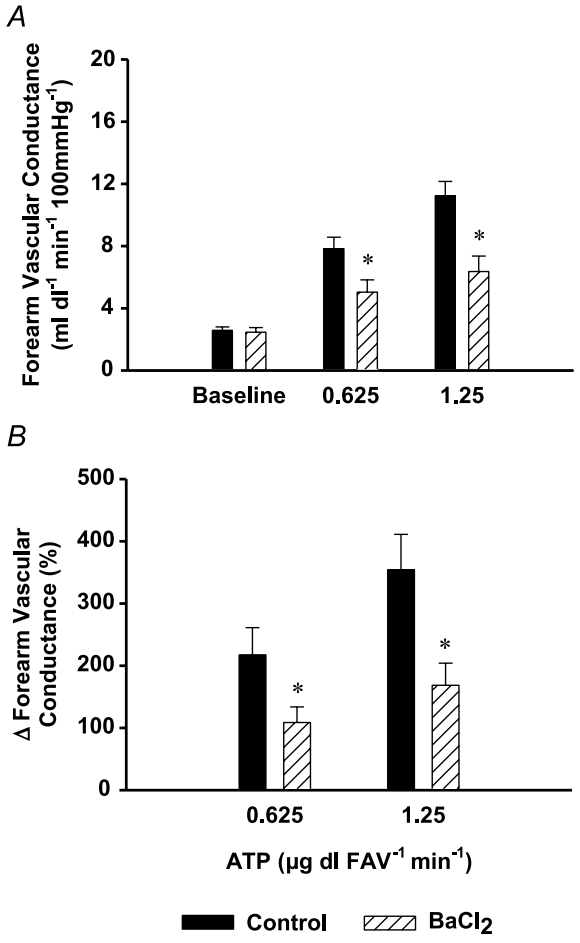

Figure 4. Protocol 3: independent effect of $BaCl_2$ on ATP-mediated vasodilatation
Inhibition of K_{IR} channels (via $BaCl_2$ infusion (striped bars)), significantly reduced absolute (*A*) and per cent changes (*B*) in forearm vascular conductance ($n = 6$; 0.625 μg dl FAV^{-1} min^{-1}: $-50 \pm 5\%$; 1.25 μg dl FAV^{-1} min^{-1}: $-54 \pm 4\%$) to a similar extent as observed with combined infusion of ouabain and $BaCl_2$. *$P < 0.05$ *vs.* control.

ATP-mediated vasodilatation: role of Na^+/K^+-ATPase and K_{IR} channels

Certain vasoactive stimuli can evoke 'conducted vasodilatation' or vasodilatation that occurs remotely from the site of agonist application (Segal, 2005; Winter & Dora, 2007). Also called 'spreading' or 'ascending' vasodilatation, this physiological mechanism is thought to provide a means for robust dilatation that arises from the microcirculation and decreases resistance to flow at the level of the upstream vasculature (Segal, 2005). It is recognized that ATP is capable of producing conducted dilatation (Duza & Sarelius, 2003; Winter & Dora, 2007; Dietrich *et al.* 2009) that can be attenuated by specific inhibition of SK_{Ca} and IK_{Ca} channels *in vitro* (Winter & Dora, 2007). Activation of these K_{Ca} channels leads to endothelial and vascular smooth muscle cell hyperpolarization that can be blocked by inhibition of Na^+/K^+-ATPase and K_{IR} channels (Edwards *et al.* 1998). Thus, we aimed to use ouabain and $BaCl_2$ which can be administered to humans in order to inhibit Na^+/K^+-ATPase and K_{IR} channels, respectively, to determine whether these hyperpolarizing pathways contribute to ATP-mediated vasodilatation *in vivo* in humans.

The data from the present study clearly indicate that the local vasodilatation observed in response to all doses of ATP administered was significantly attenuated by combined ouabain and $BaCl_2$ (Fig. 3), and as such,

represent the first data in humans to demonstrate vascular hyperpolarization via these pathways as a primary mechanism of ATP-mediated dilatation. Given one previous study in humans that demonstrated no effect of ouabain on ATP-mediated vasodilatation (van Ginneken *et al.* 2004), we then questioned whether K_{IR} channel activation alone is the predominant hyperpolarizing pathway involved in the dilatory response. Indeed, our data obtained from studies in Protocol 3 demonstrate a similar magnitude of inhibition in ATP-mediated vasodilatation with $BaCl_2$ alone (Fig. 4) as observed with combined ouabain and with $BaCl_2$. Taken together, these novel observations implicate vascular hyperpolarization via K_{IR} channel activation as a primary mechanism of vasodilatation in response to intravascular ATP.

In the present study, we did not attempt to inhibit SK_{Ca} and IK_{Ca} channels, as there are no specific inhibitors of these channels approved for use in humans. Van Ginneken and colleagues (2004) recently demonstrated that the K_{Ca} channel inhibitor tetraethylammonium chloride (TEA) did not impact ATP-mediated vasodilatation in the human forearm. However, at lower concentrations, TEA may not have effectively blocked SK_{Ca} and IK_{Ca} channels but rather large-conductance K_{Ca} channels (Langton *et al.* 1991; Ledoux *et al.* 2006) which are predominantly on smooth muscle cells (Jackson, 2005), and thus may not be involved in the endothelium-dependent vasodilatation evoked via intravascular ATP. Nevertheless, our results clearly indicate that combined inhibition of Na^+/K^+-ATPase and K_{IR} channels, and K_{IR} channel inhibition alone, significantly reduced ATP-mediated vasodilatation. These data are consistent with *in vitro* data indicating that vasodilatation to luminal perfusion of ATP is substantially reduced via inhibition of SK_{Ca} and IK_{Ca} channels (Winter & Dora, 2007), and that vasodilatation to direct P_2 receptor stimulation is blunted by inhibition of Na^+/K^+-ATPase (via ouabain) (Ralevic, 2001) and K_{IR} channels (via $BaCl_2$) (Smith *et al.* 2008).

Experimental considerations

It should be acknowledged that while administration of combined ouabain and $BaCl_2$, or $BaCl_2$ alone, did block a substantial portion of ATP-mediated vasodilatation, the response was not entirely abolished. Therefore, we suggest that the remaining vasodilatation after ouabain and $BaCl_2$ infusion indicates that (1) we did not achieve complete inhibition of these pathways during ATP infusions (see below) or (2) ATP may evoke some vasodilatation independent of activation of Na^+/K^+-ATPase and K_{IR} channels. In this context, it is possible that in the present study in the forearm using VOP as our method of blood flow measurement, we may have underestimated a potential modest role for NO and PGs in the vasodilator response to exogenous ATP (Mortensen *et al.* 2009; Crecelius *et al.* 2011*a*). However, VOP is a reliable and valid technique for the measurement of limb blood flow, particularly for studies involving pharmacological responses under resting conditions (Joyner *et al.* 2001).

Further, regarding our use of VOP to measure forearm blood flow, it is important to acknowledge that this technique is reflective of total tissue blood flow and cannot be confined to the skeletal muscle vasculature which is our primary interest. Within our laboratory, we take steps to limit the amount of cutaneous blood flow (cool (18–21°C) environment with a fan directed at the experimental arm) and this is reflected in our blood flow measures that are typically on the low end of predicted total flow based on tissue mass of the forearm (2–4 ml ($100\ g^{-1}$)) (Rowell, 1993). Additionally, the hand circulation is occluded throughout all trials (see Methods), thus reducing the potential impact of additional tissue of mixed type and vascularization (e.g. cutaneous, muscle, fat, bone). It is possible that a different technique to measure limb blood flow such as dye dilution (Jorfeldt & Wahren, 1971) may have strengthened our ability to make conclusions specifically regarding skeletal muscle vasculature; however, we do not believe that this would have altered our primary conclusions regarding the vasodilator mechanisms of exogenous ATP.

The contribution of Na^+/K^+-ATPase and K_{IR} channel activation to ATP-mediated vasodilatation may appear to be somewhat dose dependent (Fig. 3) and it is possible that the highest dose of ATP was able to override the effectiveness of $BaCl_2$ during this stimulus (Armstrong & Taylor, 1980). Although we were able to abolish KCl-mediated vasodilatation with combined ouabain and $BaCl_2$, the amount of dilatation was markedly less than that for ATP, and we are unable to test the efficacy of our inhibition with KCl at higher doses due to issues regarding subject comfort and safety (Dawes *et al.* 2002). It would be of interest to increase the dose of $BaCl_2$ (Jantzi *et al.* 2006) during ATP infusions to test this directly, but again, there are issues regarding subject safety that limit the amount of $BaCl_2$ exposure to each subject (Dawes *et al.* 2002). Given that we show on average ∼50% inhibition of ATP-mediated vasodilatation with $BaCl_2$, we do not feel that this changes our primary conclusions.

Finally, based on the previous work by Dawes and colleagues (2002), we believe that our current dosing approach of $BaCl_2$ allows us to be within the range of selectivity for K_{IR} channel inhibition by $[Ba^{2+}]$ ($<100\ \mu mol\ l^{-1}$). Higher concentrations of $BaCl_2$ can act on other potassium channels, specifically K_{ATP} channels (Jackson, 2005). If we were somehow in this range, we do not believe that inhibition of K_{ATP} channels explains our results as previously there was no effect of glibenclamide (K_{ATP} antagonist) on ATP-mediated vasodilatation (van Ginneken *et al.* 2004). Further, the relative concentration

of Ba^{2+} would predictably decrease during ATP infusions (as FBF increases). Nevertheless, given that we have no direct evidence for specificity of inhibition of K_{IR} channels by $BaCl_2$, our results should be interpreted with this in mind.

Conclusions

The collective data from the present *in vivo* investigation are the first to identify vascular hyperpolarization via K_{IR} channel activation as the primary pathway underlying the vasodilator mechanisms of intravascular ATP in humans. Our novel finding of a critical role for K_{IR} channel activation in this regard align with *in vitro* studies that suggest vascular smooth muscle cell hyperpolarization via K_{IR} channel activation mediates vasomotor responses to a variety of physiological and pharmacological stimuli (Jantzi *et al.* 2006; Armstrong *et al.* 2007). Circulating ATP plays a unique and important role in the regulation of vascular control during mismatches in oxygen delivery and demand (Gonzalez-Alonso *et al.* 2002), and recent evidence suggests that endothelium-dependent ATP-mediated vasodilatation may be impaired in type II diabetics, a population at risk for cardiovascular disease (Thaning *et al.* 2010). Thus, identification of the downstream signalling pathways of ATP may prove to be of clinical interest as a means for improving blood flow and oxygen delivery in specific patient populations.

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Author contributions

A.R.C. contributed to the conception and design of the experiment, collection, analysis and interpretation of the data, and writing of this article. B.S.K. contributed to the conception and design of the experiment, collection and interpretation of the data, and critical revision of this article. G.J.L and D.G.L. contributed to the experimental design, provided invasive methodology for data collection, and critical revision of this article. F.A.D. contributed to the conception and design of the experiment, collection, analysis and interpretation of the data and writing of this article. All authors gave final approval of the article. All experiments were performed in the Human Cardiovascular Physiology Laboratory at Colorado State University.

Acknowledgements

We thank the subjects who volunteered to participate and Jennifer C. Richards and Leora J. Garcia for their assistance in conducting these studies. This research was supported by the National Institutes of Health award HL102720 (F.A.D.).