European Journal of Pharmacology 702 (2013) 242–249

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Extracellular ATP signaling in equine digital blood vessels

Hector Zerpa [a,*], Carol Crawford [b], Gillian E. Knight [c], Alice F. Fordham [d], Silvia E. Janska [d], Claire M. Peppiatt-Wildman [b], Jonathan Elliott [d], Geoffrey Burnstock [c], Scott S. Wildman [b,**]

[a] Biomedical Department, Faculty of Veterinary Sciences, Central University of Venezuela, Maracay, Bolivarian Republic of Venezuela
[b] Medway School of Pharmacy, The Universities of Kent and Greenwich, Kent, UK
[c] Autonomic Neuroscience Centre, University College Medical School, London, UK
[d] Department of Veterinary Basic Sciences, Royal Veterinary College, London, UK

ARTICLE INFO

Article history:
Received 20 August 2012
Received in revised form
20 December 2012
Accepted 9 January 2013
Available online 29 January 2013

Keywords:
Adenosine 5′-triphosphate
Neurotransmission
Noradrenaline
P2 receptor
Purinoceptor
Purinergic
Sympathetic nerve
Equine digital vasculature
Laminitis

ABSTRACT

The functional distribution of ATP-activated P2 receptors is well characterized for many blood vessels, but not in the equine digital vasculature, which is a superficial vascular bed that displays thermoregulatory functions and has been implicated in ischemia-reperfusion injuries of the hoof. Isolated equine digital arteries (EDA) and veins (EDV) were submitted to isometric tension studies, whereby electric field stimulation (EFS) and concentration–response curves to exogenously applied agonists were constructed under low tone conditions. Additionally, immunofluorescent localization of P2X and P2Y receptor subtypes was performed. EFS-induced constriction was abolished by tetrodotoxin (1 μM, $n=4$). Endothelium denudation did not modify the EFS-induced constriction ($n=3$). The EFS-induced constriction in EDA was inhibited by phentolamine (67.7 ± 1.8%, $n=6$; 10 μM), and by the non-selective P2 receptor antagonist suramin (46.2 ± 1.3%, $n=6$; 10 μM). EFS-induced constriction in EDV was reduced by suramin (48.2 ± 2.4%, $n=6$; 10 μM), the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (58.3 ± 4.5%, $n=6$; 10 μM), and phentolamine (23.2 ± 2.5%, $n=6$; 10 μM). Exogenous methoxamine and ATP mimicked EFS-induced constriction in EDA and EDV. Immunostaining for P2X1, P2X2 and P2X3, and, for P2X1 and P2X7 receptor subunits were observed in EDA and EDV smooth muscle and adventitia, respectively. ATP and noradrenaline are co-transmitters in sympathetic nerves supplying the equine digital vasculature, noradrenaline being the dominant agonist in EDA, and ATP in EDV. In conclusion, P2X receptors mediate vasoconstriction in EDA and EDV, although different P2X subunits are involved in these vessels. The physiological significance of this finding in relation to thermoregulatory functions and equine laminitis is discussed.

1. Introduction

Adenosine 5′-triphosphate (ATP) contributes to the maintenance of vascular tone. It is released from perivascular nerves as a cotransmitter with noradrenaline and from endothelial cells in response to changes in blood flow (shear stress) and hypoxia (Ralevic, 2009). The P2X receptor family consists of seven ion channel subunits (P2X1–7) that can combine to form either trimeric homomers (P2X1–7) or heteromers (P2X1/2, 2/3, 2/6, 1/4, 1/5, 4/6, 4/7), all of which are ligand-gated ion channels. There are eight G protein-coupled P2Y receptor subtypes ($P2Y_{1,\ 2,\ 4,\ 6,\ 11-14}$), which may also form heteromultimeric complexes (Burnstock, 2007).

The P2X1 receptor subtype is the principal P2 receptor expressed on vascular smooth muscle (Bo and Burnstock, 1993; Hansen et al., 1999; Wallace et al., 2006). P2X2 receptor subunits are also expressed on smooth muscle cells of rat mesenteric, renal and pulmonary arteries (Hansen et al., 1999). P2X4 receptor subunits are expressed in rat aorta, vena cava, coronary, pulmonary, renal and femoral arteries (Soto et al., 1996; Nori et al., 1998). It is generally accepted that P2X receptors play a role in the maintenance of vascular tone, and depending on their location and expression, can mediate both vasoconstriction (e.g. P2X1), and/or vasodilation (e.g. P2X2 and/or P2X4) (Burnstock, 2010).

P2Y receptors are involved in mediating vasodilatation (Wallace et al., 2006). $P2Y_{1,\ 2,\ 4,\ 6}$ receptors have been shown to mediate endothelium-dependent vasodilatation (Knight et al., 2003). Interestingly, ATP-mediated vasodilatation (in the rat mesenteric bed) has been shown to be mediated via endothelial nitric oxide or endothelium-derived hyperpolarizing factor (Stanford et al., 2001). That the application of uridine 5′-triphosphate (UTP) elicits smooth muscle constriction suggests that $P2Y_{2,\ 4}$ and/or $P2Y_6$ are also players mediating vasoconstriction in some vessels (Erlinge and Burnstock, 2008; Gitterman and Evans, 2001).

* Corresponding author. Tel.: +58 424 451 5805.
** Corresponding author. Tel.: +44 1634 202 953.
E-mail addresses: hectorzerpa@gmail.com, hector.zerpa@lycos.com (H. Zerpa), s.s.wildman@kent.ac.uk (S.S. Wildman).

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.01.018

The contribution of ATP, when released from perivascular nerves as a co-transmitter with noradrenaline, varies greatly between vascular bed, vessel type and species (Knight et al., 2003; Wallace et al., 2006; Erlinge and Burnstock, 2008). For example, the rabbit mesenteric artery is predominantly purinergic, whereas the rabbit pulmonary artery is mainly noradrenergic (Knight et al., 2003). A change in the ratio of ATP to **noradrenaline** release from sympathetic nerves has also been linked to animal models of hypertension and in the postsynaptic vascular response to temperature changes (Pelleg and Burnstock, 1990; Kluess et al., 2005; Ralevic, 2009).

The anatomical arrangement of the equine digital vasculature bed is complex and is involved in thermoregulatory function (Zerpa et al., 2010). Furthermore, it has been proposed that increased post-capillary resistance, due to venoconstriction, could be one of the pathophysiological mechanisms involved in the development of laminitis in horses (Moore et al., 2004). The aim of this study was to describe the extracellular ATP signaling in equine digital vessels.

2. Materials and Methods

2.1. Animals and tissues

Equine digital arteries (EDA) and veins (EDV) were collected in a local abattoir. The hind limbs of healthy mixed breed adult horses of either gender were removed within 10 min of death. The digital artery was cannulated and 120 ml of ice-cold modified Krebs Henseleit (Krebs solution) was infused through the catheter. The skin was then reflected from above the coronet band to reveal the digital coronet venous plexus and the plantar digital artery. The digital coronet venous plexus and the distal segment of the plantar digital artery were dissected and removed, placed in ice-cold Krebs solution and transported to the laboratory. The blood vessels were carefully cleared of connective tissue and cut into rings of 3–4 mm in length.

2.2. Isometric tension recording from equine digital artery and vein

Unless otherwise stated, all the experiments were conducted using endothelium-intact blood vessels. Where stated, the intimal surface of the blood vessel segments was gently rubbed using a wooden cocktail stick to remove the endothelial layer (Elliott et al., 1994). Rings of EDA and EDV were suspended between two parallel stainless steel wires contained within a jacketed organ bath maintained at 30 °C, and bathed in modified Krebs solution which was gassed with 95% O_2 and 5% CO_2. One of the wires was fixed and the other connected to an isometric force transducer (model FT03; Grass Instruments Co., USA). The output of the force transducer was fed via an amplifier to a data acquisition system (Power Lab, ADI Instruments, UK). EDA and EDV were stretched to an initial tension of 3 g and 2 g, respectively, which are optimal for force generation in these preparations as has been determined in previous experiments where the optimal length/tension relationship was established using a depolarizing high potassium solution. Afterwards, an equilibration period of 1 h was allowed prior to electrical field stimulation (EFS) or the administration of pharmacological agents. The Krebs solution was changed every 15–30 min during the equilibration period.

2.3. Frequency–response curves from equine digital arteries and veins.

Control frequency–response curves to EFS were constructed for both EDA and EDV (28 V, 0.3 ms, 1–32 Hz, 1 s stimulation) under low tone conditions. Parallel experiments using separate EDA and EDV rings from the same animal were used to evaluate the effect of the following molecules: tetrodotoxin (TTX, 1 μM) to isolate any part of the response that was due to direct stimulation of the smooth muscle, the α-adrenoceptor antagonist phentolamine (10 μM), to isolate the purinergic component, and the P2 receptor antagonists suramin (10 μM) or pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; 10 μM) to isolate the noradrenergic component of the nerve-mediated (28 V, 0.3 ms, 1–32 Hz, 1 s stimulation) responses. The effect of both antagonists (α-adrenoceptor and P2 receptor antagonists) was also evaluated.

2.4. Concentration response relationship of exogenously applied agonists to equine digital artery and vein.

The viability of each vessel segment was tested initially by exchanging the Krebs solution for one in which the sodium chloride had been replaced with potassium chloride to produce a depolarizing Krebs solution (DKS; 118 mM KCl). Once the peak tension was obtained, the tissues were washed with Krebs solution and the active tension returned to baseline. Vessels that produced less than half of their resting tension were considered non-viable and discarded. The peak response to DKS in each vessel was also used to normalize the contractile response of the vessels to the agonist.

Separate experiments were carried out to evaluate the action of each exogenously applied agonist. Cumulative concentration–response curves ($n=3$–6) were constructed to methoxamine (30 nM–300 μM), ATP (100 nM–3 mM), the slowly hydrolysable ATP analog, α,β-methylene ATP (α,β-meATP: 100 nM–300 μM), the P2X1-secific agonist L-β, γ-meATP (3 μM–100 μM), UTP (10 μM–300 μM) and ADP (30 μM–300 μM).

2.5. Immunohistochemistry

Rings (2–3 mm) of EDA and EDV were fixed with 4% paraformaldehyde overnight at room temperature and then processed for wax embedding by an automated procedure (Shandon Scientific Ltd, UK). The wax blocks were mounted and 6 μm thick sections were cut using a Microm HM360 microtome (MICROM International, UK) and then placed on microscopic slides and left to dry overnight. Sections of vessels on slides were de-waxed before being used for immunohistochemical experiments. Tissues were permeabilized with 1% triton and non-specific binding was blocked with 10% donkey serum in a solution of 0.1 M PBS for 1 h. Slides were washed in 0.1 M PBS 3×5 min at room temperature. Polyclonal antibodies for P2X1–7 (obtained from Roche Bioscience, Palo Alto, CA, USA; for epitope sequences see Wildman et al., 2008) or $P2Y_{1,\ 2,\ 4,\ 6,\ 11–13}$ (purchased from Alomone Laboratories, Jerusalem, Israel) were added to the sections at a dilution of 1:100 in a solution of 0.1 M PBS containing 1% donkey serum and left to incubate overnight. As a control to account for any non-specific immunoreactions, one slide in each experiment did not have the primary antibody added to the section. Controls were incubated overnight in PBS containing 1% donkey serum only. Donkey anti-rabbit FITC secondary antibody was diluted to 1:500 in a solution of 0.1 M PBS containing 1% donkey serum. Except in the case of P2X5 and $P2Y_1$, that had donkey anti-goat FITC secondary antibody added at a dilution of 1:500. The solution was added to the section and incubated for 2 h at 2–5 °C. After another set of 3×5 min washes with PBS, the slides were mounted using 4′,6 diamidino-2-phenylindole (DAPI)-containing mountant and cover slipped. The slides were then viewed under a microscope (Leica SP5 microscope; Leica Microsystems, UK), and a series of representative images were obtained from each slide. Images of immuno-stained EDA and EDV were independently and blindly assessed by at least three individuals. A scoring system

was applied as follows: +++ (strong signal), ++ (moderate signal), + (weak signal), and – (no signal).

2.6. Analysis of data

The effect of different antagonists on frequency response curves was expressed as percentage of the maximum response obtained in the absence of the antagonist. When a maximum constriction was reached within the chosen agonist concentrations, the cumulative concentration–response curves to the agonists were fitted by computerized non-linear regression to a one process logistic equation using GraphPad Prism (Version 5.1 for Windows; GraphPad Software, USA). The equation used was

$$E = \frac{E_{max} A^{nH}}{(A^{nH} + EC_{50})}$$

where E is the observed response and A is the agonist concentration used. The best fit value for EC_{50} expressed as pD_2 ($-\log [EC_{50}]$) and the E_{max} (the maximum contractile response) and nH (Hill coefficient) obtained from each vessel segment were used to calculate the arithmetic mean and 95% confidence limits (pD_2) or the arithmetic mean ± S.E.M. (E_{max}).

Statistical analyses of the data to assess differences between two mean values were performed using two-tailed Student's t test for either paired or unpaired observations as appropriate, with $P \leq 0.05$ being considered as significant. Additionally, the effect of different antagonists on frequency response curves was analyzed by a two-way analysis of variance with Bonferroni's post hoc test.

2.7. Drugs and solutions

All reagents were of analytical grade. The modified Krebs–Henseleit solution had the following composition (mM): $CaCl_2$ 1.27, $MgS0_4$ 1.19, $NaHCO_3$ 25.0, NaCl 118, KH_2P0_4 1.19, KCl 4.57, glucose 5.55 (pH=7.34 (95% CL: 7.28–7.39). All drugs (purchased from Sigma Aldrich, Dorset, UK, unless specifically stated) were freshly prepared on the day of the experiment. Unless otherwise stated, drugs were dissolved in distilled water. Appropriate vehicle controls were conducted in each experiment.

3. Results

3.1. Frequency–response curves from equine digital arteries and veins.

EFS of autonomic nerves in rings of EDA and EDV caused frequency-dependent constriction, with peak responses obtained at 32 Hz. The EFS-induced constriction was abolished by pre-treatment with TTX (1 μM) in EDA (96.8 ± 0.5% inhibition, $n=4$, $P<0.05$, Fig. 1A), and in EDV (97.8 ± 0.9% inhibition, $n=4$, $P<0.05$, Fig. 1C). The presence of the endothelium did not modify the EFS-induced constriction in either EDA or EDV (Fig. 1B and D, respectively).

EFS, in the presence of the non-selective α-adrenoceptor antagonist phentolamine (10 μM), caused a significant reduction of the peak EFS-induced constriction in EDA (67.7 ± 1.8% inhibition, $n=6$, $P<0.05$, Fig. 2A). The incubation with the P2 receptor antagonist, PPADS (10 μM), failed to inhibit the peak EFS-induced constriction in EDA (4.3 ± 3.4%; $n=3$), but suramin (10 μM) caused a significant inhibition (46.2 ± 1.2%, $n=6$, $P<0.05$, Fig. 2A) in the EDA. When EFS-induced constriction was evaluated in the presence of a combination of phentolamine (10 μM) and suramin (10 μM), almost entire inhibition was observed (93.7 ± 5.4%, $n=3$, $P<0.05$, Fig. 2A).

In the case of EDV, phentolamine caused a partial but still significant reduction of the EFS-induced peak constriction (23.2 ± 2.5%, $n=6$, $P<0.05$, Fig. 2B). The P2 receptor antagonists, PPADS and suramin (10 μM), each attenuated the EFS-induced

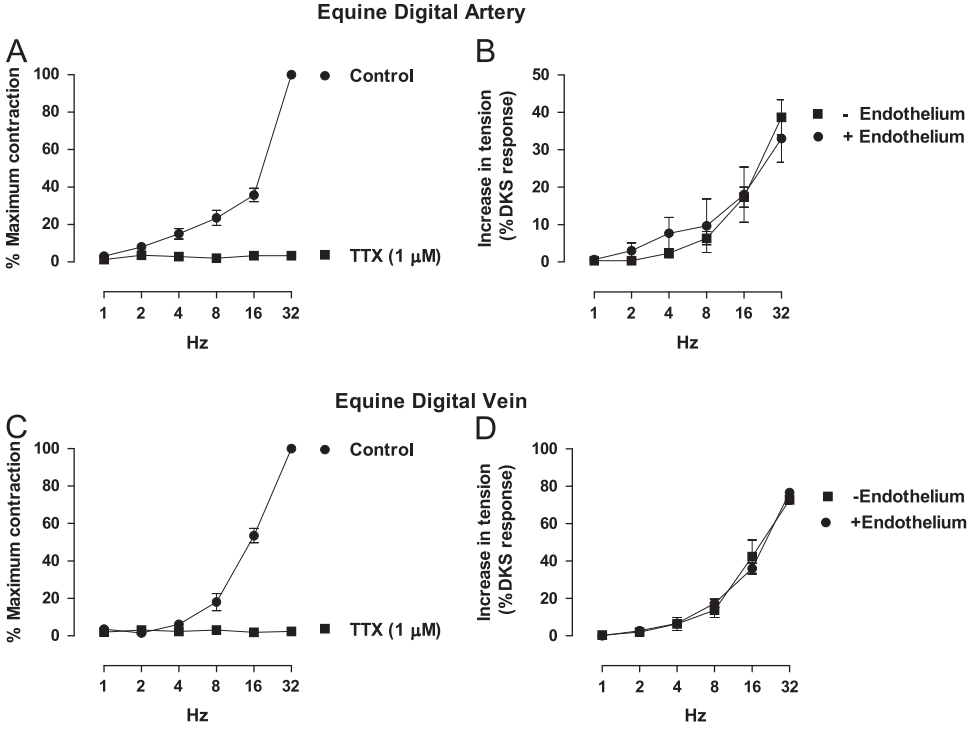

Fig. 1. Frequency–response curves showing frequency-dependent constriction to electrical field stimulation (EFS; 28 V, 0.3 ms, 1–32 Hz, 1 s stimulation) in the equine digital blood vessels. (A) The effect of tetrodotoxin (TTX) on endothelium-intact rings of equine digital artery (EDA), (B) EFS-evoked constriction in EDA either with endothelium-intact (+ endothelium) or endothelium-denuded (−endothelium), (C) the effect of TTX on endothelium-intact rings of equine digital vein (EDV), and (D) EFS-evoked constriction in EDV either with endothelium-intact or endothelium-denuded. (A,C), symbols represent mean percentage of the maximum response ± S.E.M. ($n=4$; TTX), and (B,D), represent mean percentage of constriction evoked by depolarizing Krebs solution (DKS 118 mM KCl) ± S.E.M. ($n=3$).

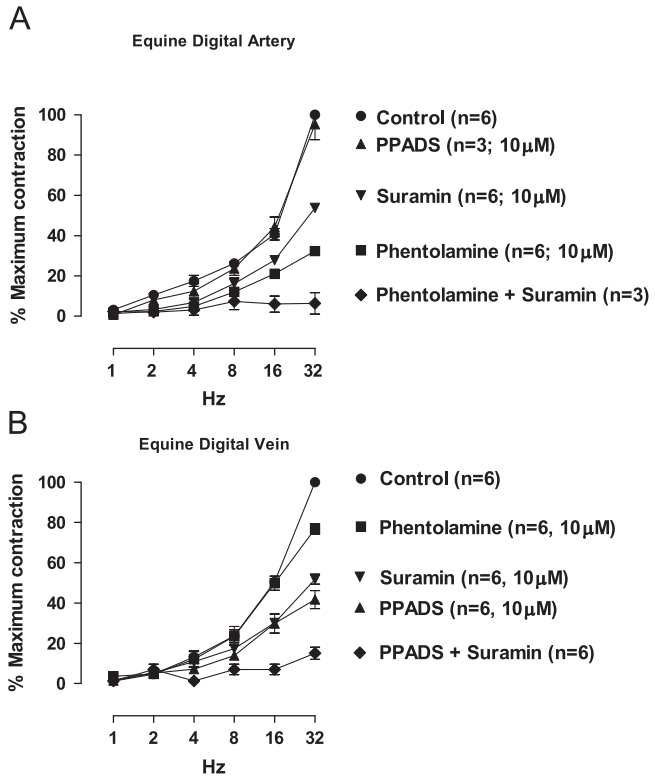

Fig. 2. Frequency–response curves showing frequency-dependent constriction to electrical field stimulation (28 V, 0.3 ms, 1–32 Hz, 1 s stimulation) in rings of equine digital artery (A) and equine digital veins (B). The effect of P2 receptor antagonists (PPADS and suramin), the α-adrenoceptor antagonist (phentolamine), or a combination of antagonists is shown. All symbols show mean percentage of maximum contraction in the absence of antagonists ± S.E.M. (n=3–6).

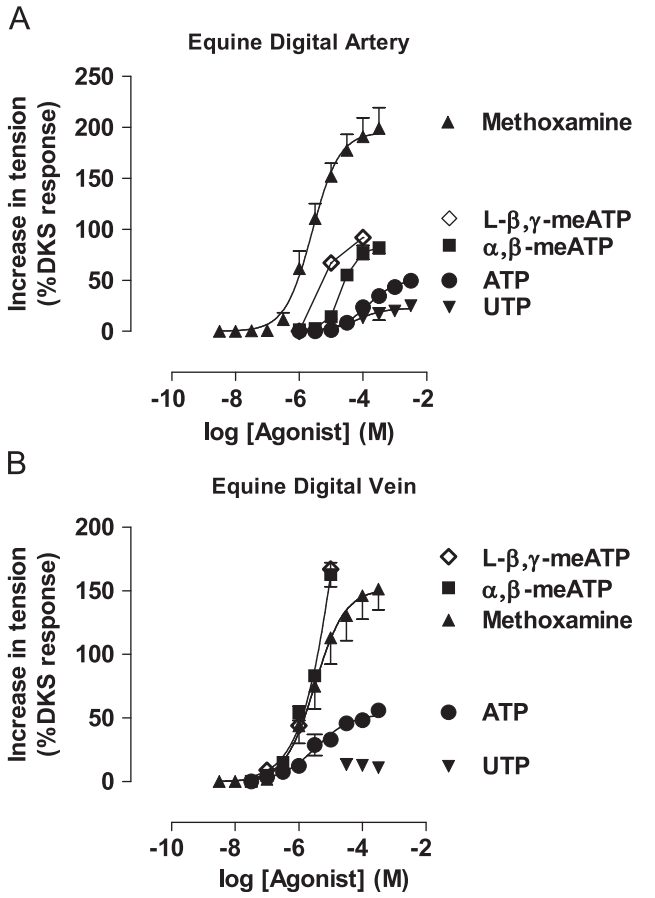

Fig. 3. Concentration–response curve for α,β-methylene ATP (α,β-meATP), L-β, γ-meATP (n=1), ATP, UTP and methoxamine on rings of isolated equine digital artery (A) and equine digital vein (B). All symbols show mean increase in tension as percentage of the maximum contraction to the depolarizing Krebs solution (DKS 118 mM KCl) ± S.E.M. (n=3–6).

peak constriction, to a greater extent: 58.3 ± 4.5% (n=6, P<0.05) and 48.2 ± 2.4% (n=6, P<0.05), respectively (Fig. 2B). The combination of P2 receptor antagonists [PPADS and suramin (10 μM)] caused a greater reduction of the EFS-induced peak constriction than the individual effect of each antagonist on its own (81.0 ± 1.0%, n=6, P<0.05, Fig. 2B).

3.2. Concentration–response relationship of exogenously applied agonists to equine digital artery and vein.

The addition of methoxamine (n=6) and the P2 receptor agonists, ATP (n=3) and α,β-meATP (n=3), caused concentration-dependent constriction in both EDA and EDV (Fig. 3 and Table 1). In contrast, UTP caused concentration dependent constriction in EDA only (n=3, Fig. 3A and Table 1), and ADP failed to elicit a constriction in both EDA and EDV (n=3; data not shown). The order of potency for P2 receptor agonists in the EDA was α,β-methylene-ATP > UTP=ATP. The order of efficacy for P2 receptor agonists with EDV was: α, β-methylene-ATP > ATP > UTP. The efficacy and potency of methoxamine were similar between EDA and EDV.

Since the ATP analog α,β-meATP, did not reach a maximum constriction within the concentration range used in this study, a non-linear regression analysis was not performed. Similar to other, we observed rapid desensitization in experiments using ATP and α,β-meATP. However, non-linear regression analysis for ATP revealed that constriction in EDV (n=3) yielded a pD_2 value that was significantly higher than the value obtained using EDA (P<0.05, see Table 1). Preliminary experiments with the P2X1-specific agonist L-β, γ-meATP (3 μM–100 μM), caused a concentration-dependent constriction in EDA (Fig. 3A; n=1) and EDV (Fig. 3B; n=1), respectively.

3.3. Immunohistochemistry

The level of immunoreactivity assessed for both EDA and EDV is summarized in Table 2 ($n \geq 3$ animals in all cases). Staining was absent from the vessels if the primary antibody was omitted from the staining procedure; these controls were used as comparison for specific immunoreactivity.

In the EDA, P2X1 immunoreactivity was associated with the smooth muscle and adventitia (Fig. 4B). Substantial P2X2 and 3 immunoreactivity were observed in both the smooth muscle and adventitia (Fig. 4C and D). Weak reactivity of P2X6 was also observed in the smooth muscle, with moderate immunoreactivity in the adventitia (Fig. 4E). Immunoreactivity for P2X4, 5 and 7 was not observed in EDA.

Only $P2Y_1$ immunoreactivity was observed in EDA, a weak reactivity in the adventitia, and no evident reactivity in the smooth muscle (Fig. 4F). Immunoreactivity for the rest of the evaluated P2Y receptors antibodies was not observed in EDA.

P2X1 immunoreactivity was also observed in EDV; the smooth muscle showed moderate reactivity and there was weak reactivity evident on the adventitia (Fig. 5B). Moderate P2X7 reactivity was also observed in both the smooth muscle and the adventitia (Fig. 5C). Immunoreactivity in EDV for the rest of P2X and P2Y antibodies was either not observed or displayed very weak reactivity.

Table 1
Agonist activity indices for agonist-evoked vasoconstriction of equine digital blood vessels.

Agonists	*n*	pD_2 Mean (95% confidence limits)		E_{max} (%DKS 118 mM KCl Mean ± S.E.M.)	
		EDA	EDV	EDA	EDV
Methoxamine	**6**	5.6 (5.3–5.9)	5.5 (4.8–6.0)	195.3 ± 19.8	153.7 ± 13.9
α,β-methylene ATP	**3**	4.6 (4.5–4.8)	ND	82.1 ± 6.2	*162.8 ± 9.8*
ATP	**3**	3.8 (3.0–4.7)	5.6 (4.6–6.5)[a]	52.5 ± 5.3	50.8 ± 4.0
UTP	**3**	3.9 (3.0–5.0)	ND	24.2 ± 4.2	ND

Note. DKS, depolarizing Krebs solution, EDA, equine digital artery; EDV, equine digital vein; *n*, number of vessels from different animals; ND, not determinable by non-linear regression; E_{max}, maximum contraction by non-linear regression; pD_2, $-\log EC_{50}$; S.E.M., standard error of the arithmetic mean; numbers written in *italic* represents the maximum contraction reached with the highest agonist concentration.

[a] denotes a significant difference in the responses between types of blood vessels.

Table 2
Immunostaining of P2X and P2Y receptors in equine digital blood vessel samples. Key: +++, strong signal; ++, moderate signal; +, weak signal; −, no signal.

Antibody	Equine digital artery			Equine digital vein		
	Adventitia	Smooth muscle	Endothelium	Adventitia	Smooth muscle	Endothelium
P2X						
P2X1	+++	++	−	++	++	−
P2X2	++	+++	−	+	+	−
P2X3	+++	+++	−	−	−	−
P2X4	+	−	−	−	−	−
P2X5	−	−	−	−	−	−
P2X6	++	+	−	−	−	−
P2X7	+	−	−	−	++	−
P2Y						
$P2Y_1$	++	−	−	−	−	−
$P2Y_2$; $P2Y_4$; $P2Y_6$; $P2Y_{11}$; $P2Y_{12}$; $P2Y_{13}$	−	−	−	−	−	−

4. Discussion

The effect of P2 receptor antagonists on EFS-mediated constriction and the contractile response to natural and synthetic P2 receptor agonists in conjunction with the immunoreactivity for P2X receptors in the smooth muscle of EDA and EDV demonstrate the presence of P2X receptors on vascular smooth muscle mediating constriction of EDA and EDV. Furthermore, and not surprisingly given that ATP and noradrenaline are cotransmitters in sympathetic nerves, we also report dual control of both EDA and EDV constriction by adrenergic and purinergic components. Interestingly, adrenergic signaling was found to be dominant in the contractile response of EDA, and purinergic signaling was dominant in the contractile response of EDV. Our findings are similar to others, who have demonstrated a prevailing purinergic-mediated vasoconstriction of superficial veins related to thermosensitivity (Flavahan and Vanhoutte, 1986).

Our data suggest that the P2X receptor subtypes responsible for equine digital vessel constriction differ between EDA and EDV. The potency and efficacy of methoxamine were similar between EDA and EDV, however, the potency of ATP was higher in EDV. In addition, PPADS was without the effect on EFS-mediated constriction in the EDA, yet equipotent with suramin in blocking EFS-mediated constrictions in EDV. Taking into account our pharmacological and immunohistochemical data (which incidentally is in keeping with our pharmacological data despite the antibody specificity and selectivity being unknown in equine tissue), we propose that P2X1 and/or 1/2 receptors expressed on smooth muscle cells are likely to mediate vasoconstriction of EDA. However, an involvement of P2X2, 1/2, 2/3 and/or 2/6 (or the proposed heteromeric assemblies of 1/3 and/or 1/6), especially given the ability of UTP (a known weak agonist at P2X3 subunit-containing receptors) to evoke vasoconstriction, cannot be completely dismissed (King and Townsend-Nicholson, 2003). That suramin, but not PPADS, inhibited EFS-evoked constriction suggests a role for PPADS-insensitive P2 receptors, and therefore may lend weight to the involvement of an, as yet, pharmacological-uncharacterized P2X1 heteromeric assembly (e.g. 1/3 and/or 1/6). With respect to EDV, we propose that vasoconstriction is mediated via P2X1 receptors, although as with EDA we cannot rule out the involvement of other subtypes (i.e. P2X1/2 and/or P2X7 receptors). Interestingly, in the human saphenous vein a potential role has been proposed for P2X7 receptors in ATP-mediated cell lysis in smooth muscle (Cario-Toumaniantz et al., 1998). A limitation to the current study is that the non-selective antagonists used here do not fully identify the involvement of specific individual P2X subunits. Preliminary experiments with the P2X1-specific agonist L-β, γ-meATP (3 μM–100 μM), caused a constriction of both EDA and EDV (Fig. 3); whilst further experiments are needed, this does support the role for P2X1 receptors in the maintenance of vascular tone in equine digital vessels. Since agonist and antagonist profiles for P2 receptors can differ between species (Bogdanov et al., 1998; Burnstock and Knight, 2004), more comprehensive pharmacological characterization, determining activity profiles of an extended series of P2 receptor agonists and antagonists, is required to fully determine the P2X receptor subtypes responsible for vasoconstriction of EDA and EDV and in other ungulates.

In EDV, significant PPADS blockade of EFS-mediated constriction is evident at low and high frequencies (Fig. 2B). In contrast, significant suramin blockade of EFS-mediated constriction in EDV (and EDA) is only evident at higher frequencies ($\geq$ 16 Hz; Fig. 2). In EDA, the α-adrenoceptor antagonist, phentolamine, significantly blocks the EFS-mediated constriction at all frequencies, whereas in EDV phentolamine significantly blocked constriction at the higher frequencies only ($>$ 16 Hz; see Fig. 4B). The effects of suramin on both EDA and EDV on high frequency constriction are in contrast to previous studies investigating adrenergic/purinergic cotransmission

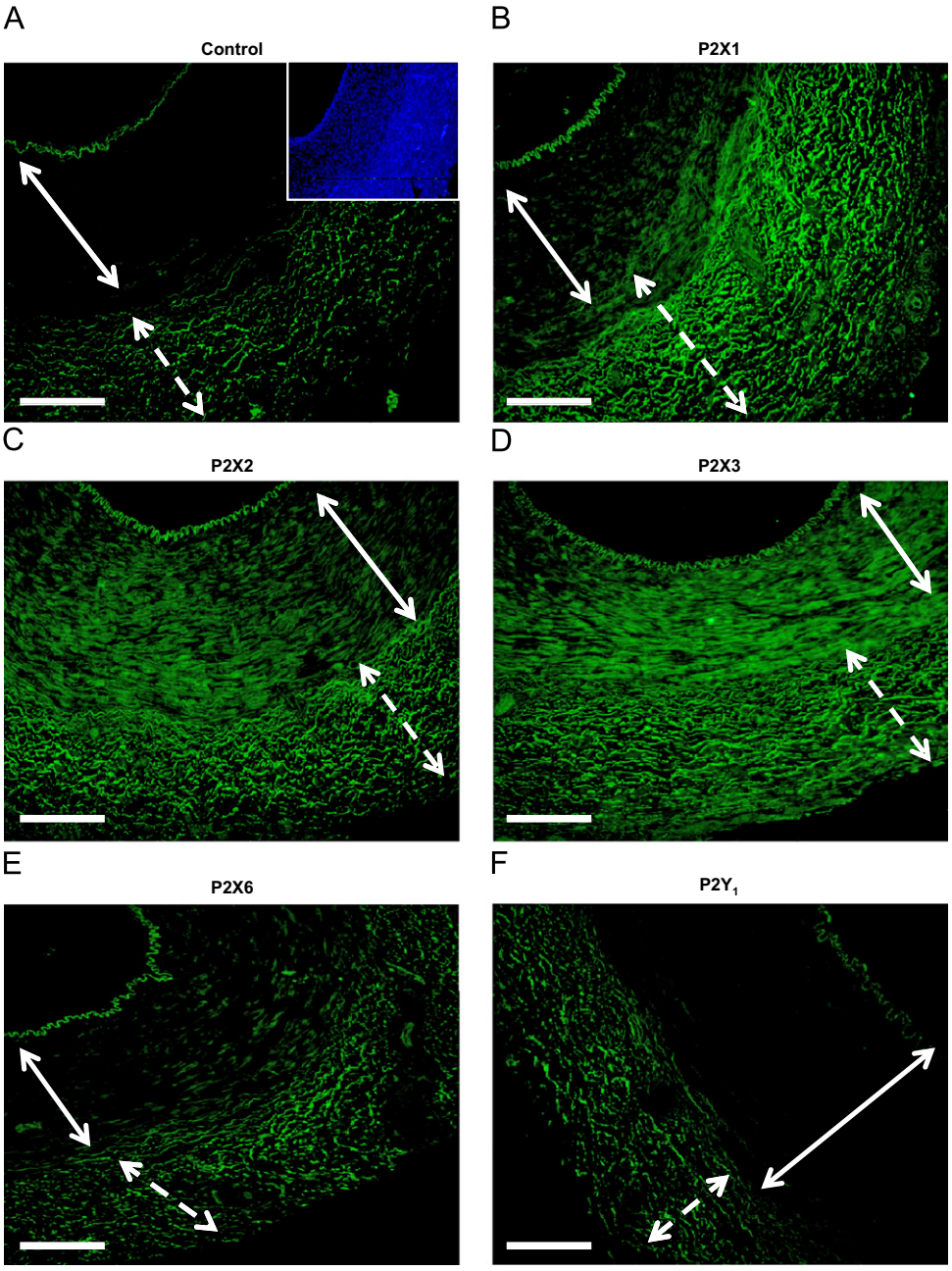

Fig. 4. P2 receptor immunostaining of the equine digital artery. (A) Photograph of negative control showing autofluorescence in adventitia (omission of the primary antibody); inset showing DAPI stain of DNA. (B) Specific immunoreactivity for P2X1 present in the smooth muscle and adventitia, (C) P2X2 immunoreactivity in the smooth muscle and adventitia, (D) specific immunoreactivity for P2X3 observed in the smooth muscle and adventitia, (E), weak reactivity of P2X6 observed in the smooth muscle and adventitia, and (F) weak reactivity of $P2Y_1$ present on the adventitia. Key: Solid line, smooth muscle layer; dashed line, adventitia layer; scale bar = 300 μm.

and arterial constriction whereby low frequency constriction was attributed to the purinergic component, and high frequency constriction to the adrenergic component (Zang et al., 2006).

It is noteworthy that agonist-mediated constriction supports the possibility that ATP might evoke constriction in EDA and EDV not only as a neuro-cotransmitter but also when released into the extracellular compartment during inflammation or hypoxia (Burnstock et al., 2012) contributing to modify the contractile state of superficial blood vessels—which may be relevant to disease states, such as equine laminitis. An increase in post-capillary resistance due to digital venoconstriction has been proposed to occur in equine laminitis; however, the mechanisms behind the persistence of digital vasoconstriction remain to be defined (Robertson et al., 2009). While the vasodilatory effect of ATP and neurogenic peptides, such as substance-P and calcitonin gene-related peptide, have been described in the equine digital vasculature (Elliott and Soydan, 1993; Katz et al., 2003, 2011), this is the first time that the functional localization of P2X receptors linked to digital venoconstriction is reported in the equine digital vasculature. The possibility exists that the activation of P2 receptors during inflammation, as has been proposed to occur in equine laminitis (Black et al., 2006; Noschka et al., 2009), may contribute to the development of digital venoconstriction during this crippling ischemic condition of the equine digit.

The absence of P2Y receptors immunoreactivity (except for $P2Y_1$ in EDV), suggests little contribution of P2Y receptors mediating relaxation in the equine digital vasculature. Interestingly, the effect of ATP on EDV has previously been described to

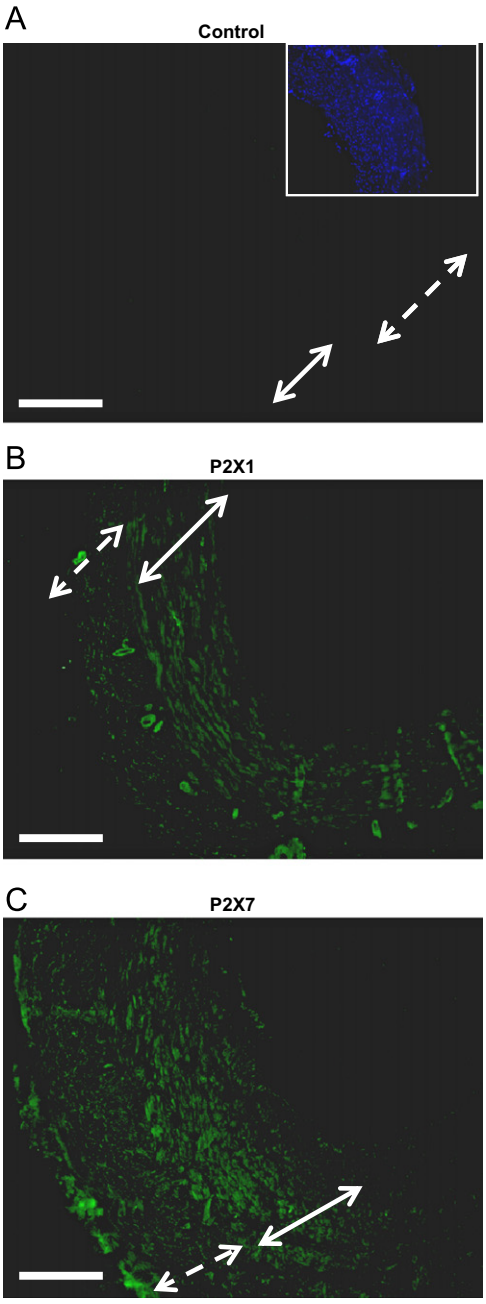

Fig. 5. P2 receptor immunostaining of the equine digital vein. (A) Photograph of negative control (omission of the primary antibody); inset showing DAPI stain of DNA, (B) specific immunoreactivity for P2X1 present in the smooth muscle and adventitia, (**C**) specific immunoreactivity for P2X7 observed in the smooth muscle and adventitia. Key: Solid line, smooth muscle layer; dashed line, adventitia layer; scale bar = 300 μm.

cause endothelium-independent relaxation in raised tone preparations, along with transient contractions at higher concentrations (Elliott and Soydan, 1993; Elliott et al., 1994). Further experimentation is necessary to establish the role of extracellular ATP signaling in raised tone equine digital preparations.

In conclusion, the presence of P2X receptors linked to constriction of the smooth muscle in the equine digital vasculature was demonstrated, showing a higher proportion of a purinergic vs. adrenergic component of the response in the EDV. Additionally, the post-synaptic activation of P2 receptors by ATP and other synthetic analogs reinforces the contractile role of these receptors, particularly in the venous side of the equine digital circulation. The pattern of P2 receptor expression in the superficial EDV not only offers some insight into the physiology of this vascular bed, which is of great interest in veterinary medicine and in particular the cause of laminitis, but also supports the potential use of these veins as a model to study the role of P2 receptors in superficial veins.

Acknowledgments

Acknowledgments go to Dr Yoel Berhane for his assistance and advice with organ bath experiments.

References

Black, S.J., Lunn, D.P., Yin, C., Hwang, M., Lenz, S.D., Belknap, J.K., 2006. Leukocyte emigration in the early stages of laminitis. Vet. Immunol. Immunopathol. 109, 161–166.
Bo, X., Burnstock, G., 1993. Heterogeneous distribution of [3H] alpha,beta-methylene ATP binding sites in blood vessels. J. Vasc. Res. 30, 87–101.
Bogdanov, Y.D., Wildman, S.S., Clements, M.P., King, B.F., Burnstock, G., 1998. Molecular cloning and characterization of rat P2Y4 nucleotide receptor. Br. J. Pharmacol. 124, 428–430.
Burnstock, G., 2007. Purine and pyrimidine receptors. Cell. Mol. Life. Sci. 64, 1471–1483.
Burnstock, G., 2010. Control of vascular tone by purines and pyrimidines. Br. J. Pharmacol. 161, 527–529.
Burnstock, G., Knight, G.E., Greig, A.V., 2012. Purinergic signaling in healthy and diseased skin. J. Invest. Dermatol. 132, 526–546.
Burnstock, G., Knight, G.E., 2004. Cellular distribution and functions of P2 receptor subtypes in different systems. Int. Rev. Cytol. 240, 31–304.
Cario-Toumaniantz, C., Loirand, G., Ladoux, A., Pacaud, P., 1998. P2X7 receptor activation-induced constriction and lysis in human saphenous vein smooth muscle. Circ. Res. 83, 196–203.
Elliott, J., Bryant, C.E., Soydan, J., 1994. The role of nitric oxide in the responses of equine digital veins to vasodilator and vasoconstrictor agents. Equine Vet. J. 26, 378–384.
Elliott, J., Soydan, J., 1993. Characterization of the responses of equine digital veins to adenosine and its analogues. Br. J. Pharmacol. 110, 146.
Erlinge, D., Burnstock, G., 2008. P2 receptors in cardiovascular regulation and disease. Purinerg. Signal 4, 1–20.
Flavahan, N.A., Vanhoutte, P.M., 1986. Sympathetic purinergic vasoconstriction and thermosensitivity in a canine cutaneous vein. J. Pharmacol. Exp. Ther. 239, 784–789.
Gitterman, D.P., Evans, R.J., 2001. Nerve evoked P2X receptor contractions of rat mesenteric arteries; dependence on vessel size and lack of role of L-type calcium channels and calcium induced calcium release. Br. J. Pharmacol. 132, 1201–1208.
Hansen, M.A., Dutton, J.L., Balcar, V.J., Barden, J.A., Bennett, M.R., 1999. P2X (purinergic) receptor distributions in rat blood vessels. J. Auton. Nerv. Syst. 75, 147–155.
Katz, L.M., Marr, C.M., Elliott, J., 2003. Characterisation of the response of equine digital arteries and veins to substance P. J. Vet. Pharmacol. Ther. 26, 361–368.
Katz, L.M., Marr, C.M., Elliott, J., 2011. Characterization of the responses of equine digital veins and arteries to calcitonin gene-related peptide. Am. J. Vet. Res. 72, 975–981.
Kluess, H.A., Buckwalter, J.B., Hamann, J.J., Clifford, P.S., 2005. Elevated temperature decreases sensitivity of P2X purinergic receptors in skeletal muscle arteries. J. Appl. Physiol. 99, 995–998.
King, B.F., Townsend-Nicholson, A., 2003. Nucleotide and nucleoside receptors. Tocris Rev. 23, 1–11.
Knight, G.E., Oliver-Redgate, R., Burnstock, G., 2003. Unusual absence of endothelium-dependent or -independent vasodilatation to purines or pyrimidines in the rat renal artery. Kidney Int. 64, 1389–1397.
Moore, R.M., Eades, S.C., Stokes, A.M., 2004. Evidence for vascular and enzymatic events in the pathophysiology of acute laminitis: which pathway is responsible for initiation of this process in horses? Equine Vet. J. 36, 204–209.
Nori, S., Fumagalli, L., Bo, X., Bogdanov, Y., Burnstock, G., 1998. Coexpression of mRNAs for P2X1, P2X2 and P2X4 receptors in rat vascular smooth muscle: an in situ hybridization and RT-PCR study. J. Vasc. Res. 35, 179–185.
Noschka, E., Moore, J.N., Peroni, J.F., Lewis, S.J., Morrow, J.D., Robertson, T.P., 2009. Thromboxane and isoprostanes as inflammatory and vasoactive mediators in black walnut heartwood extract induced equine laminitis. Vet. Immunol. Immunopathol. 129, 200–210.
Pelleg, A., Burnstock, G., 1990. Physiological importance of ATP released from nerve terminals and its degradation to adenosine in humans. Circulation 82, 2269–2272.
Ralevic, V., 2009. Purines as neurotransmitters and neuromodulators in blood vessels. Curr. Vasc. Pharmacol. 7, 3–14.
Robertson, T.P., Bailey, S.R., Peroni, J.F., 2009. Equine laminitis: a journey to the dark side of venous. Vet. Immunol. Immunopathol. 129, 164–166.

Soto, F., Garcia-Guzman, M., Gomez-Hernandez, J.M., Hollmann, M., Karschin, C., Stuhmer, W., 1996. P2X4: an ATP-activated ionotropic receptor cloned from rat brain. Proc. Natl. Acad. Sci. U.S.A. 93, 3684–3688.

Stanford, S.J., Gitlin, J.M., Mitchell, J.A., 2001. Identification of two distinct vasodilator pathways activated by ATP in the mesenteric bed of the rat. Br. J. Pharmacol. 133, 825–832.

Wallace, A., Knight, G.E., Cowen, T., Burnstock, G., 2006. Changes in purinergic signalling in developing and ageing rat tail artery: importance for temperature control. Neuropharmacology 50, 191–208.

Wildman, S.S., Marks, J., Turner, C.M., Yew-Booth, L., Peppiatt-Wildman, C.M., King, B.F., Shirley, D.G., Wang, W.-H., Unwin, R.J., 2008. Sodium-dependent regulation of renal amiloride-sensitive currents by apical P2 receptors. J. Am. Soc. Nephrol. 19, 731–742.

Zang, W.J., Zacharia, J., Lamont, C., Wier, W.G., 2006. Sympathetically evoked Ca^{2+} signaling in arterial smooth muscle. Acta Pharmacol. Sin. 27, 1515–1525.

Zerpa, H., Berhane, Y., Woodcock, H., Elliott, J., Bailey, S.R., 2010. Rho kinase activation and ROS production contributes to the cooling enhanced constriction in cutaneous equine digital veins. J. Appl. Physiol. 109, 11–18.