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Raised tone reveals ATP as a sympathetic neurotransmitter in the porcine mesenteric arterial bed

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Abstract

The relative importance of ATP as a functional sympathetic neurotransmitter in blood vessels has been shown to be increased when the level of pre-existing vascular tone or pressure is increased, in studies carried out in rat mesenteric arteries. The aim of the present study was to determine whether tone influences the involvement of ATP as a sympathetic cotransmitter with noradrenaline in another species. We used the porcine perfused mesenteric arterial bed, and porcine mesenteric large, medium and small arteries mounted for isometric tension recording, because purinergic cotransmission can vary depending on the size of the blood vessel. In the perfused mesenteric bed at basal tone, sympathetic neurogenic vasocontractile responses were abolished by prazosin, an α_1-adrenoceptor antagonist, but there was no significant effect of α,β-methylene ATP, a P2X receptor desensitizing agent. Submaximal precontraction of the mesenteric arterial bed with U46619, a thromboxane A_2 mimetic, augmented the sympathetic neurogenic vasocontractile responses; under these conditions, both α,β-methylene ATP and prazosin attenuated the neurogenic responses. In the mesenteric large, medium and small arteries, prazosin attenuated the sympathetic neurogenic contractile responses under conditions of both basal and U46619-raised tone. α,β-methylene ATP was effective in all of these arteries only under conditions of U46619-induced tone, causing a similar inhibition in all arteries, but had no significant effect on sympathetic neurogenic contractions at basal tone. These data show that ATP is a cotransmitter with noradrenaline in porcine mesenteric arteries; the purinergic component was revealed under conditions of partial precontraction, which is more relevant to physiological conditions.

Keywords: ATP, cotransmission, noradrenaline, P2X receptor, porcine mesenteric arteries, sympathetic nerves

Introduction

ATP is a cotransmitter with noradrenaline in perivascular sympathetic nerves, acting at smooth muscle P2X receptors, predominantly P2X1, to mediate vasoconstriction (see Burnstock and Kennedy, 1986; Burnstock, 1990; Ralevic and Burnstock, 1988, Burnstock and Ralevic, 2014). It is known that the relative importance of ATP as a functional sympathetic neurotransmitter in blood vessels is variable depending on the species, the vascular bed and type of blood vessel, and the frequency of stimulation (Ralevic and Burnstock, 1988; Burnstock and Ralevic, 2014). The level of pre-existing vascular tone (the degree of constriction the vessel experiences relative to its maximally dilated state under basal tone conditions) is also important (Pakdeechote et al., 2007; Rummery et al., 2007). In rat mesenteric arteries, raising intraluminal pressure to physiological levels (from 30 mmHg to 90 mmHg) was associated with depolarization of the resting membrane potential and an increase in the amplitude of the P2X receptor-mediated excitatory junction potential (EJP), the combination of which increased the effectiveness of ATP such that it became the predominant sympathetic neurotransmitter (Rummery et al., 2007). Moreover, in the rat isolated perfused mesenteric arterial bed, a purinergic component of sympathetic neurotransmission was absent at low tone and was revealed only at raised tone (Pakdeechote et al., 2007). These data are in accord with a study showing only a modest role for ATP as a sympathetic neurotransmitter in the rat mesenteric arterial bed under basal tone conditions (Donoso et al., 1997). Collectively, these findings suggest that the relative importance of ATP as a cotransmitter in sympathetic nerves is underestimated in blood vessels studied at relatively low tone/pressure, at least in rat mesenteric arteries. It is important to determine whether these findings extend to other species, prompting the present study in porcine mesenteric arteries.

First described in 1965 by DD McGregor, the isolated perfused mesenteric arterial bed of the rat allows the effects of sympathetic nerve stimulation and of drugs to be studied in a setting where the responses generated are representative of those controlling vascular resistance in the whole bed. Robust responses of the whole bed to electrical stimulation of the superior mesenteric artery and its nerve plexus were described, which were abolished by the sympathetic blocker guanethidine and mimicked by exogenous noradrenaline (McGregor, 1965). This preparation is now widely used by pharmacologists. In the present study we describe for the first time a porcine isolated perfused mesenteric arterial bed preparation, modified from the method of McGregor (1965), which we have used to investigate purinergic neurotransmission. We also assessed purinergic neurotransmission in different sized porcine isolated mesenteric arteries, because the involvement of ATP in sympathetic

neurotransmission has been reported to increase as vessel diameter decreases (Gitterman and Evans, 2001). The investigations were carried out in arteries at basal and raised tone, to determine whether the effects of tone on purinergic neurotransmission in rat arteries (Pakdeechote et al., 2007; Rummery et al., 2007) are also evident in porcine arteries. There is a dearth of information on sympathetic cotransmission in the porcine vasculature and its characterisation is relevant because of the similarities of the human and porcine cardiovascular systems (Swindle et al., 2011). A preliminary report of some of these data has been published in abstract form (Shatarat et al., 2009, 2010a,b).

Methods

Perfused mesenteric arterial bed preparation

Mesenteries were obtained from male or female large white Danish pigs, aged about 10 weeks and about 80 kg in weight, after slaughter at a local abattoir. The mesenteries (which had been separated from the intestines) were immediately transported to the laboratory on ice in Krebs-Henseleit solution with the following composition (mM): NaCl 128, KCl 4.8, $NaNCO_3$ 25, $MgSO_4$ 1.1, KH_2PO_4 1.2, $CaCl_2 2H_2O$ 1.25 and glucose 12, that had previously been gassed with 95% O_2, 5% CO_2. The superior mesenteric artery was identified and one of the "first order" branches, designated "large", was dissected out together with part of the mesentery containing the vascular tree (i.e. the large branch artery and the rest of its vascular tree) (see Figure 1).

Tissue was then placed in Krebs solution containing 2% Ficoll and refrigerated overnight at 4^{o}C (preliminary experiments showed that the responses to EFS obtained in fresh tissues and in overnight incubated tissues were similar in size). The next day, fine dissection was used to expose the large mesenteric branch artery, which was cannulated using a blunted hypodermic needle (No. 21). This acted as the site of perfusion and as the positive electrode. The porcine perfused mesenteric arterial bed preparation (present study) was modified from the method described for the rat perfused mesenteric arterial bed preparation (McGregor, 1965), with the main difference being that the porcine preparation is a mesenteric vascular arcade perfused via a large branch artery arising from the superior mesenteric artery (ie. not the whole mesenteric arterial bed but a part of it), while the rat mesenteric arterial bed is the whole-bed preparation perfused via the superior mesenteric artery. The cannulated porcine preparation was then placed on a stainless steel grid (which acted as the negative electrode) in a humid chamber, in which it was perfused at a flow rate of 5 ml/min using a peristaltic pump (model 755-30, Cole-Parmer, Chicago, IL). In order to monitor changes in contractility of the mesenteric bed, a pressure transducer (model P23XL, Viggo-

Spectramed, Oxnard, CA) was used to monitor the perfusion pressure (mmHg). Changes were recorded using a powerlab (ADInstruments, Pty Ltd., Castle Hill, Australia). The tissue was equilibrated for 60 min before experimentation. A Grass SD9 stimulator was used to apply electrical field stimulation (EFS) (2-16 Hz, 1 ms, 90 V, 30 s), and the neurogenic component was identified by EFS in the presence of a sympatholytic, guanethidine (1 μM). In some experiments, α,β-methylene ATP (α,β-meATP, 1 μM) was added to desensitize P2X receptors, or prazosin (0.1 μM) an α_1-adrenoceptor antagonist was added, at least 30 min prior to EFS. U46619 was added to precontract some of the mesenteric beds (to between 10 and 30 mmHg, requiring different concentrations of U46619 between individual beds); in some of these preparations EFS was investigated both before and after U46619 addition to determine the effect of tone on the neurogenic contractile responses, and in other preparations responses to EFS were investigated only during U46619-induced raised tone, in the presence and absence of guanethidine, α,β-meATP and prazosin.

Isolated large and medium mesenteric arteries

Porcine mesenteries were obtained from an abattoir as described above. Large and medium mesenteric arteries (see Figure 1) were dissected out and placed in Krebs-Henseleit solution, gassed with 95% O_2, 5% CO_2 containing 2% Ficoll and stored overnight at 4°C. The next day, mesenteric arteries were dissected into 5 mm segments and suspended between two supports, in organ baths containing Krebs-Henseleit buffer maintained at 37°C and gassed with 95% O_2, 5% CO_2. A thin wire support was inserted through the lumen of the arterial ring while a second wire, attached to an electrode assembly, was also threaded through the lumen. The tissue was then suspended between the two supports. The electrode assembly was connected to a Grass SD9 stimulator, while the upper support was connected by a thread to a force transducer (World Precision Instruments, Sarasota, Florida, U.S.A.) linked to a Maclab data acquisition system (AD Instruments Ltd, Hastings, UK) via an amplifier. After a 15 min equilibration period, tension was applied to the large arteries (10 g) and to the medium arteries (4 g). Arteries were allowed to relax to a final resting tension of between 2-4 g for large arteries and 1-2 g for medium arteries. The tissues were contracted 2 times with KCl (60 mM) with a 30 min interval between the contractions. Tissues were then washed with Krebs-Henseleit buffer and allowed to equilibrate for 60 min. After the equilibration period, responses to EFS (2-32 Hz, 1 ms, 90 V, 30 s) were determined. The interval between the frequencies was variable (2-5 min) and was determined by the return to baseline after each stimulation, with a minimum interval of 2 min if there was no response. The neurogenic component was identified by EFS in the presence of guanethidine (1 μM). In some experiments, α,β-meATP (1 μM) was added to desensitize P2X receptors, or prazosin (0.1 μM) an α_1-adrenoceptor antagonist was added, at least 30 min prior

to EFS. In some of the arteries U46619 was added to precontract them (to 15-30% of the response to KCl, requiring different concentrations of U46619 between individual arteries); in some of these arteries EFS was investigated both before and after U46619 addition to determine the effect of tone on the neurogenic contractile responses. In other arteries responses to EFS were investigated only during U46619-induced raised tone, in the presence and absence of guanethidine, α,β-meATP and prazosin.

Isolated small mesenteric arteries

Porcine mesenteries were obtained as described above. Fine dissection was carried out to identify the small terminal branch arteries (see Figure 1) using a dissecting microscope. Small arteries (diameter 400-700 µm) were carefully dissected free of excess fat and connective tissue and separated from the veins, and then cut into segments approximately 2 mm long. Next, vessels were mounted on fine tungsten wires (40 µm diameter), and placed between the jaws of a dual channel Mulvany-Halpern wire myograph (Myo-Interface Model 410A, Danish Myo Technology, Denmark) (Mulvany and Halpern, 1977). Vessels were kept at 37°C in Krebs-Henseleit buffer, and gassed with 95% O_2, 5% CO_2. A tension of 1 g was applied and vessels were left to relax to a resting tension of 0.3-0.5 g. KCl (60 mM) was added and vessels used if they contracted by at least 0.4 g. Tension was measured and recorded on a MacLab 4e recording system (AD Instruments, UK). The perivascular nerves were activated electrically through two platinum electrodes mounted in the plastic jaws, either side of the blood vessel. EFS (2-16 Hz, 1 ms, 10 V, 30 s) was supplied by a stimulator unit (DS2, Digitimer Ltd., Welwyn Garden City, UK). In some experiments, α,β-meATP (1 µM) was added to desensitize P2X receptors, or prazosin (0.1 µM) an α_1-adrenoceptor antagonist was added, at least 30 min prior to EFS. Some of the small arteries were precontracted with U46619 (to 15-30% of the KCl-induced response, requiring different concentrations of U46619 between individual arteries); in some of these arteries EFS was investigated both before and after U46619 addition to determine the effect of tone on the neurogenic contractile responses. In other arteries responses to EFS were investigated only during U46619-induced raised tone, in the presence and absence of guanethidine, α,β-meATP and prazosin.

Drugs

Prazosin, α,β-methylene ATP and guanethidine were obtained from Sigma (Poole, Dorset, UK). U46619 stock concentration was 10 mg/ml in methyl acetate and reconstituted in ethanol to form a 10^{-2} M solution. All other drugs were dissolved in distilled water.

Data handling and statistical analysis

Contractile responses to EFS in the perfused mesenteric arterial beds were expressed as a percentage of the response obtained at 16 Hz of the first frequency response curve. Responses to EFS in the isolated large, medium and small mesenteric arteries were expressed as g tension. Results are expressed as the mean ± S.E.M. Statistical comparisons were made by two way analysis of variance (ANOVA) with Bonferroni post-hoc test, or Student's paired or unpaired t-test. A value of $P < 0.05$ was taken to indicate statistical significance.

Results

Reproducibility experiments for the frequency response curves, carried out under basal and raised tone conditions, showed that consecutive frequency response curves to EFS were reproducible (data not shown).

Effect of prazosin and α,β-meATP on neurogenic contractile responses in porcine isolated mesenteric arterial bed under basal tone conditions

Under basal tone conditions (baseline perfusion pressure 42 ± 4 mmHg, n=14), EFS elicited frequency-dependent vasoconstrictor responses in the porcine isolated mesenteric arterial bed. Responses were expressed as a percentage of the response to 16 Hz obtained in the first FRC, because of the variability of the responses. Under basal tone conditions, prazosin (0.1 μM), an α_1-adrenoceptor antagonist, significantly attenuated the electrically-evoked vasocontractile responses (e.g. by 70 ± 8% at 16 Hz, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 2A). A small residual response remained that was not blocked by further addition of guanethidine (1 μM), a sympathetic neuron blocker, presumably due to direct smooth muscle activation (Fig. 2A). Neither prazosin nor guanethidine altered the basal perfusion pressure. α,β-meATP (1 μM), a P2X receptor desensitising agent, produced a transient contraction (23 ± 4 mmHg, n=9) and its effect on responses was measured once the perfusion pressure had returned to its original level, usually after 5-15 min. α,β-meATP had no significant effect on the vasoconstrictor responses obtained to EFS in the porcine isolated mesenteric arterial bed under basal tone conditions (e.g. the response at 16 Hz was 92 ± 20% of the control) (n=9) (Fig. 2B).

Role of α_1-adrenoceptors and P2X receptors in mediating electrically-evoked vasocontractile responses in porcine mesenteric arterial bed under raised tone conditions

The thromboxane A_2 agonist U46619 (10-50 nM) increased the perfusion pressure from 42 ± 4 mmHg to 68 ± 7 mmHg ($P < 0.05$, Student's unpaired t-test) (n=14), and significantly enhanced the electrically-evoked vasocontractile response, particularly at higher frequencies (e.g. the response was increased at 16 Hz by 236 ± 75% ($P < 0.05$, ANOVA) (n=8) (Fig. 3A, representative trace, and Fig. 3B). Under these conditions guanethidine (1 μM) almost abolished the vasoconstrictor responses evoked by EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=4) (Fig. 4A). Prazosin (0.1 μM) did not alter the raised perfusion pressure but attenuated the electrically-evoked vasocontractile responses at all frequencies reaching significance at higher frequencies (e.g. 45 ± 5% inhibition at 16 Hz, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 4B). At 16 Hz,

the inhibition produced by prazosin in the presence of U46619 was less than the 70% inhibition produced under basal tone conditions (see Fig. 2A and 4B). In experiments where prazosin and α,β-meATP were co-applied there was evidence of additivity at 8 Hz, but not at 16 Hz (data not shown).

In the presence of U46619, α,β-meATP (1 μM) produced a transient contraction (114 ± 19 mmHg, n=10). This transient contraction was much larger compared to that under basal tone conditions ($P < 0.001$, unpaired t-test, n=10). α,β-meATP pre-treatment attenuated the vasoconstrictor responses obtained after EFS in the porcine isolated mesenteric arterial bed under conditions of raised tone at all frequencies, reaching statistical significance at 16 Hz (49 ± 9% inhibition, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 4C and Fig. 4D representative trace).

Effects of prazosin and α,β-meATP on responses to EFS in porcine isolated mesenteric large arteries under basal tone conditions

Under basal tone conditions guanethidine (1 μM), a sympathetic neuron blocker, almost abolished the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=10) (Fig. 5A). Prazosin (0.1 μM) significantly inhibited the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 5B) to a similar extent to that produced by guanethidine. α,β-meATP (1 μM) caused a transient contraction (Table 1) which returned to the baseline before the construction of the next FRC, but had no significant effect on the contractile responses to EFS in porcine mesenteric large arteries (n=8) (Fig. 5C).

Effects of prazosin and α,β-meATP on responses to EFS in porcine mesenteric large arteries under raised tone conditions

U46619 (5-25 nM) contracted the large mesenteric arteries by 20 ± 4% of the KCl response (n=14). Under raised tone conditions, EFS produced contractile responses which were frequency-dependent and slightly larger than under basal tone conditions when investigated before and after U46619 addition in the same arteries ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=14) (data not shown), although this enhancement was not apparent when the responses were not paired (Figures 5 and 6). Under raised tone conditions guanethidine (1 μM), almost abolished the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=4) (Fig. 6A). Prazosin (0.1 μM), significantly inhibited the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 6B), but there was a substantial prazosin-resistant component under raised tone conditions. α,β-meATP (1 μM) caused a transient contraction (Table 1), which returned to the same level of tone before the construction of the next FRC. When assessed

using ANOVA, α,β-meATP was shown to significantly attenuate the contractile responses to EFS. Although the two FRCs were significantly different ($P < 0.05$, ANOVA) (n=12) (Fig. 6C) there was no significant change at any single frequency upon post-hoc analysis. In experiments where prazosin and α,β-meATP were co-applied there was evidence of additivity at all frequencies (data not shown).

Effects of prazosin and α,β-meATP on responses to EFS in porcine mesenteric medium arteries under basal tone conditions

In porcine mesenteric medium arteries, under basal tone conditions, EFS produced contractile responses which were frequency-dependent although large responses were only seen at high frequencies. Under these conditions prazosin (0.1 μM) significantly inhibited the contractile responses to EFS at basal tone ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=6) (Fig. 7A). Further addition of guanethidine (1 μM) reduced the residual response after prazosin but this further reduction was not significantly different from that produced by prazosin alone (n=6) (Fig. 7A). α,β-meATP (1 μM) caused a transient contraction (Table 1) which returned to baseline before the construction of the next FRC, and did not alter the contractile responses under basal tone conditions (Fig. 7B) (n=6).

Effects of prazosin and α,β-meATP on responses to EFS in porcine medium mesenteric arteries under raised tone conditions

U46619 (5-10 nM), contracted the medium arteries by 20 ± 4% of KCl response (n=14). Under raised tone conditions, responses to EFS were slightly larger than under basal tone conditions when investigated before and after U46619 addition in the same arteries ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=5) (data not shown), although this enhancement was not apparent when the responses were not paired (Figure 7). Prazosin (0.1 μM) significantly inhibited the contractile responses to EFS under raised tone conditions ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=6) (Fig. 7C). Subsequent addition of guanethidine (1 μM) reduced the residual response further, at both 16 and 32 Hz ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=6) (Fig. 7C). α,β-meATP (1 μM) caused a transient contraction (Table 1) which returned to the baseline before beginning the next FRC. This contraction was significantly larger than that produced by α,β-methyleneATP (1 μM) under basal conditions ($P < 0.01$, Student's unpaired t-test, n=8) (Table 1). α,β-meATP attenuated the contractile response to EFS, at all frequencies, reaching statistical significance at 32 Hz ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 7D) in porcine mesenteric medium arteries under raised tone conditions.

Effects of prazosin and α,β-meATP in porcine mesenteric small arteries under basal tone conditions

Under basal tone conditions EFS produced contractile responses which were frequency-dependent. α,β-meATP (1 μM) caused a transient contraction (Table 1) which returned to the baseline before the construction of the next FRC, but had no significant effect on the contractile responses to EFS in porcine mesenteric small arteries under basal tone conditions (n=7) (Fig. 8A). Subsequent exposure to prazosin (0.1 μM) significantly inhibited the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 8A).

Effects of prazosin and α,β-meATP in porcine mesenteric small arteries under raised tone conditions

U46619 (0.5-2 nM) contracted porcine mesenteric small arteries by 20 ± 3% of KCl response (n=14). Under raised tone conditions, EFS produced contractile responses which were frequency-dependent and larger than under basal tone conditions at all frequencies reaching statistical significance at 16 Hz ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (see also Table 1). α,β-meATP (1 μM), a P2X receptor desensitizing agent, caused a transient contraction (Table 1) which returned to the baseline before the construction of the next FRC. α,β-meATP inhibited the responses to nerve stimulation (e.g. at 8 Hz by 41 ± 11% and at 16 Hz by 36 ± 8%) (Fig. 8B) in porcine mesenteric small arteries under raised tone conditions, in contrast to its lack of effect under basal tone conditions. Further addition of prazosin (0.1 μM) reduced the response to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 8B).

Discussion

This study has presented evidence of purinergic neurotransmission in porcine mesenteric arteries using a perfused mesenteric arterial bed, modified from the method of McGregor (1965), as well as in porcine isolated mesenteric arteries of different sizes. At basal tone, the electrically-evoked contractile responses in the mesenteric arterial bed and isolated arteries were mediated by noradrenaline since they were inhibited by prazosin. However, under conditions of raised tone, the electrically-evoked responses were enhanced and a role for ATP was evident since these responses were reduced by the P2X receptor desensitizing agent α,β-meATP. These observations suggest that, under conditions of partial vascoconstriction, which mimic more closely conditions found *in vivo*, ATP acts as a functional sympathetic neurotransmitter in the porcine mesentery. These results extend our previous observations in rat mesenteric arteries (Pakdeeechote et al., 2007; Rummery et

al., 2007) to porcine tissue.

In the porcine mesenteric arterial bed, blockade of the frequency-dependent contractile responses to EFS by the sympathetic nerve blocker guanethidine confirmed the neurogenic nature of these responses. The porcine isolated perfused mesenteric arterial bed can thus be used as a model for the study of sympathetic neurotransmission and/or receptor pharmacology. Under basal tone conditions, responses of the porcine mesenteric arterial bed to nerve stimulation were susceptible to α_1-adrenoceptor blockade by prazosin, but were resistant to P2X receptor desensitization with α,β-meATP, indicating that they were mediated by activation of postjunctional α_1-adrenoceptors, as observed in the rat mesenteric arterial bed by us and by others (Eikenburg, 1984; Kong et al., 1994; Williams and Clarke, 1995; Pakdeechote et al., 2007). The sympathetic neurogenic responses were enhanced under conditions of raised tone induced by U46619, as we and others have observed in the rat isolated mesenteric arterial bed (Tabuchi et al., 1990; Pakdeeechote et al., 2007). Under these conditions, the neurogenic responses were partly susceptible to blockade by α,β-meATP, indicating the increased involvement of a purinergic component in sympathetic neurotransmission. These neurogenic responses are likely to involve ATP activation of P2X1 receptors, since P2X1 receptors are desensitized by α,β-meATP (North, 2002; Roberts et al., 2006; Saul et al., 2013). There is compelling evidence for a role of P2X1 receptors in sympathetic cotransmission in a number of different animal species, using pharmacological, electrophysiological and immunohistochemical approaches, and P2X1 receptor knockout mice (Vial and Evans, 2002). The present study is the first full report of purinergic cotransmission in a porcine blood vessel and is important because of the similarities between the porcine and human cardiovascular systems (Swindle et al., 2011), and because of the dearth of studies on purinergic neurotransmission in human blood vessels (see Burnstock and Ralevic, 2014). Measurements of sympathetic nerve activity in humans show that these fire at much lower frequencies (< 1Hz) (Montano et al., 2009; Malpas 2010) than those used to stimulate sympathetic nerves in the present study and in other isolated blood vessels, identifying differences between the *in vivo* recordings and the *in vitro* studies.

As in the perfused mesenteric arterial bed, sympathetic neurogenic contractile responses generated in porcine isolated large, medium and small mesenteric arteries at basal tone were abolished by prazosin and were unaffected by α,β-meATP. At raised tone, there was evidence of a purinergic component of neurotransmission since there was significant blockage of the neurogenic contractile response after exposure to α,β-meATP. It has been shown that in rat mesenteric artries, purinergic neurotransmission increases with decreasing size of the vessel (Gitterman and Evans, 2001). The

relative level of P2X1 receptor immunoreactivity was higher in small and medium rat arteries than in large arteries (Lewis and Evans, 2001) and small arteries are generally more densely innervated than large conduit arteries (Burnstock, 1975). Thus, our hypothesis was that a greater purinergic component of sympathetic neurotransmission would be observed in the small and medium sized arteries than in the large arteries. However, we did not see clear differences in the size of the purinergic component among the different artery sizes in the porcine mesenteric vasculature.

The mechanism by which raised tone uncovers a nerve-mediated purinergic response is unknown, although it is not specific to U46619, because we also observed this using endothelin-1 in the rat mesenteric arterial bed (Pakdeechote et al., 2007). It is likely to occur through a postjunctional mechanism, since contractile responses to α,β-meATP were also enhanced under conditions of U46619-induced raised tone. In the porcine splenic artery U46619 has similarly been shown to enhance contractile responses to α,β-meATP (by a mechanism independent of ERK, Rho kinase and p38 MAP kinase) (Roberts, 2012). Neither is the mechanism specific to P2X receptors, since precontraction with U46619 enhanced contraction of the porcine ear artery mediated via α_2-adrenoceptors (Bhattacharya and Roberts, 2003). Both U46619 and endothelin-1 have been shown to cause vascular smooth muscle depolarization and to increase calcium levels (Van et al., 1988; Rubanyi and Polokoff, 1994; Shaw et al., 2004; Crane and Garland, 2004). In their presence, the more positive resting membrane potential may increase the open probability of the voltage gated calcium channels that are opened subsequent to P2X1 receptor activation. Alternatively, U46619 (and endothelin) may increase the production of second messengers which may alter the contractile machinery and allow responses mediated by P2X receptors to become apparent. Indeed, downstream of P2X receptor activation there may be activation of Rho kinase and Ca^{2+} sensitization of contractile myofilaments (Yeoh and Brock, 2005; Inscho et al., 2009). α-adrenoceptors may also be sensitive to these potentiating effects, but the relative effect may be smaller against a background of adrenergic neurotransmission already evident at basal and raised tone. The data raise the possibility that any mechanism that increases vascular smooth muscle excitability will increase the contribution of ATP as a sympathetic neurotransmitter. Clearly this is relevant to physiological conditions, where blood vessels would be exposed to the influence of circulating and locally released agents, or in the case of smaller arteries myogenic-induced depolarisation (Rummery et al., 2007).

In summary, we have shown that at raised tone ATP is a cotransmitter with noradrenaline in porcine mesenteric arteries, extending our previous findings in rat arteries. The data suggest that the relative importance of ATP as a cotransmitter in sympathetic nerves may have been underestimated

in blood vessels studied at relatively low tone/pressure.

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Table 1

Size of artery	Contraction (g) to 16 Hz in the absence of U46619	Contraction (g) to 16 Hz in the presence of U46619	Contraction (g) to α,β-methyleneATP (1 μM) in the absence of U46619	Contraction (g) to α,β-methyleneATP (1 μM) in the presence of U46619
Large	3.32 ± 1.5 (n=12)	3.93 ± 1.89 (n=12)	0.72 ± 0.6 (n=8)	4.6 ± 1.8 (n=8)***
Medium	1 ± 0.4 (n=10)	1.2 ± 0.7 (n=10)	0.76 ± 0.57 (n=8)	1.7 ± 0.79 (n=8)**
small	0.22 ± 0.06 (n=5)	0.31 ± 0.06 (n=5)***	0.54 ± 0.43 (n=6)	0.96 ± 0.26 (n=6)**

** P < 0.01; *** P < 0.001 (Student's t test)

Figure legends

Figure 1. The porcine isolated mesenteric arterial bed shows: (1) superior mesenteric artery, (2) large mesenteric artery, (3) medium mesenteric arteries, (4) medium mesenteric artery (used in the present study), (5) terminal small mesenteric arteries, (6) small intestine and (7) part of the tissue covering the mesenteric bed, which has been dissected and reflected to expose the underlying vascular tree. A mesenteric vascular arcade (which included the arteries indicated by the dotted lines) was cut away from the rest of the mesenteric arterial bed and placed in a perfusion apparatus where it was perfused with warmed oxygenated Krebs solution via a large mesenteric artery (2) (see methods).

Figure 2. Porcine perfused mesenteric arterial bed under basal tone conditions. A) Effects of prazosin (0.1 μM) alone and in combination with guanethidine (1 μM) (n=12), on vasoconstrictor responses to to electrical field stimulation (EFS, 2-16 Hz, 1ms, 90 V, 30 s). ** P < 0.01 vs. control, *** P < 0.001 vs. control (ANOVA followed by Bonferroni post-hoc test). B) Effect of α,β-methyleneATP (1 μM) (n=9) on vasoconstrictor responses to EFS. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error.

Figure 3. The effect of raising tone on responses to electrical field stimulation in the porcine perfused mesenteric arterial bed. A) Representative trace showing effects of pre-constriction with U46619, on vasoconstrictor responses to electrical field stimulation (EFS, 2-16 Hz, 1 ms, 90 V, 30 s) in the porcine perfused mesenteric arterial bed under basal and raised tone conditions. B) Effects of U46619 (n=8) on vasoconstrictor responses to EFS in the porcine mesenteric arterial bed. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. *P < 0. 05 vs. control (ANOVA followed by Bonferroni post-hoc test).

Figure 4. Porcine perfused mesenteric arterial bed under U46619-induced raised tone conditions. Effects of A) guanethidine (1 μM) (n=4), B) prazosin (0.1 μM) (n=12), and C) α,β-methyleneATP (1 μM) (n=5), on vasoconstrictor responses to electrical field stimulation (EFS, 2-16 Hz, 1 ms, 90 V, 30 s) under raised tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. **P < 0.01 vs. control, ***P < 0.001 vs. control (ANOVA followed by Bonferroni post-hoc test). D) Representative trace showing effects of α,β-methyleneATP (1 μM) on vasoconstrictor responses to EFS in the porcine isolated mesenteric arterial bed under raised tone conditions.

Figure 5. Porcine mesenteric large artery under basal tone conditions.Effects of A) guanethidine (1 μM) (n=10), B) prazosin (0.1 μM) (n=5), or C) α,β-methyleneATP (1 μM) (n=8) on contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V) in porcine mesenteric large arteries under basal tone conditions. Each bar represents mean ± standard error. ** $P < 0.01$, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

Figure 6. Porcine mesenteric large artery under U46619 raised tone conditions. Effects of A) guanethidine (1 μM) (n=4), B) prazosin (0.1 μM) (n=12), and C) α,β-methyleneATP (1 μM) (n=12), on contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V) in porcine mesenteric large arteries under U46619 raised tone conditions. Each bar represents mean ± standard error. ** $P < 0.01$, *** $P < 0.001$ vs. control, + shows a significant difference between curves ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test).

Figure 7. Porcine mesenteric medium artery under basal (A,B) and raised (C,D) tone conditions. Basal tone conditions: effects of A) the sequential addition of prazosin (0.1 μM), and guanethidine (1 μM) (n=6), or B) α,β-methyleneATP (1 μM) (n=6), on responses to electrical field stimulation (EFS, 2-32 Hz, 1ms, 30s, 90 V) in porcine medium mesenteric arteries under basal tone conditions. Raised tone conditions: effects of C) the sequential addition of prazosin (0.1 μM), and guanethidine (1 μM) (n=6), or the addition of D) α,β-methyleneATP (1 μM) (n=7), on responses to EFS in porcine medium mesenteric arteries. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control, # $P < 0.001$ vs. prazosin (ANOVA followed by Bonferroni post-hoc test). Each bar represents mean ± standard error.

Figure 8. Porcine mesenteric small artery under basal (A) and raised (B) tone conditions. A) Effects of the sequential addition of α,β-methyleneATP (1 μM) followed by prazosin (0.1 μM) (n=7) on responses to electrical field stimulation (EFS, 2-16 Hz, 1 ms, 30 s, 10 V) in porcine mesenteric small arteries under basal tone conditions. *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test). B) Effects of the sequential addition of α,β-methyleneATP (1 μM) followed by prazosin (0.1 μM) (n=7), on responses to EFS in porcine mesenteric small arteries under U46619 raised tone condtions. * $P < 0.05$, *** $P < 0.001$ vs. control, # $P < 0.001$ vs. α,β-methyleneATP (ANOVA followed by Bonferroni post-hoc test). Each bar represents mean ± standard error.