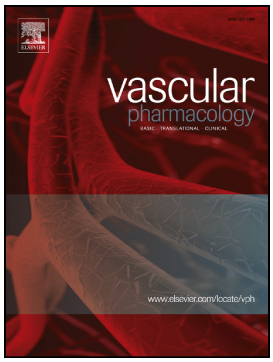

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Neuropeptide Y facilitates P2X1 receptor-dependent vasoconstriction via Y1 receptor activation in small mesenteric arteries during sympathetic neurogenic responses

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ABSTRACT

ATP, norepinephrine and NPY are co-released by sympathetic nerves innervating arteries. ATP elicits vasoconstriction via activation of smooth muscle P2X receptors. The functional interaction between neuropeptide Y (NPY) and P2X receptors in arteries is not known. In this study we investigate the effect of NPY on P2X1-dependent vasoconstriction in mouse mesenteric arteries. Suramin or P2X1 antagonist NF449 abolished α,β-meATP evoked vasoconstrictions. NPY lacked any direct vasoconstrictor effect but facilitated the vasoconstrictive response to α,β-meATP. Mesenteric arteries expressed Y_1 and Y_4 receptors, but not Y_2 or Y_5. Y_1 receptor inhibition (BIBO3304) reversed NPY facilitation of the α,β-meATP-evoked vasoconstriction. L-type Ca^{2+} channel antagonism (nifedipine) had no effect on α,β-meATP-evoked vasoconstrictions, but completely reversed NPY facilitation. Electrical field stimulation evoked sympathetic neurogenic vasoconstriction. Neurogenic responses were dependent upon dual α_1-adrenergic (prazosin) and P2X1 (NF449) receptor activation. Y_1 receptor antagonism partially reduced neurogenic vasoconstriction. Isolation of the P2X1 component by α_1-adrenergic blockade allowed facilitatory effects of Y_1 receptor activation to be explored. Y_1 receptor antagonism reduced the P2X1 receptor component during neurogenic vasoconstriction. α_1-adrenergic and P2X1 receptors are post-junctional receptors during sympathetic neurogenic vasoconstriction in mesenteric arteries. In conclusion, we have identified that NPY lacks a direct vasoconstrictor effect in mesenteric arteries but can facilitate vasoconstriction by enhancing the activity of P2X1, following activation by exogenous agonists or during sympathetic nerve stimulation. The mechanism of P2X1 facilitation by NPY involved activation of the NPY Y_1 receptor and the L-type Ca^{2+} channel.

Keywords: Neuropeptide Y, P2X receptors, vasoconstriction, neurogenic, calcium channel

1. Introduction

The sympathetic nervous system is a potent regulator of vascular resistance and blood pressure. Post-ganglionic sympathetic neurons innervate arteries and stimulate vasoconstriction. Norepinephrine, ATP and NPY are neurotransmitters that are co-released at the sympathetic neuroeffector junctions of arteries [26]. Co-signalling by all three neurotransmitters at vascular smooth muscle cells contributes to the neurogenic contractile response, though the degree of contribution can vary dependent upon vascular bed, stimulus strength and disease state [9,27,47]. Norepinephrine and ATP consistently act as neurotransmitters with primary trophic effects, whilst the effect of neuronally released NPY is highly variable and can have primary trophic or post-junctional modulatory effects [3,10]. Circulating NPY is increased in patients with hypertension, cardiac hypertrophy and heart failure [23,36,50].

Extracellular ATP exerts fast biological effects through the activation of P2X receptors, a family (P2X1-7) of non-selective cation channels [38]. Release of ATP from post-ganglionic sympathetic neurons plays an important role in the neurogenic vasoconstriction of proximal small arteries [47]. The P2X1 receptor is the main post-junctional receptor for ATP in small resistance-sized arteries, though the expression of other vascular smooth muscle P2X receptors have been identified in different vascular beds [18]. The non-selective cation current passed during P2X1 opening in smooth muscle cells can stimulate vasoconstriction via several mechanisms. These include direct elevation in intracellular Ca^{2+} through inward movement of Ca^{2+} through the channel itself, and membrane depolarisation caused by the non-selective cation current and subsequence opening of voltage-gated Ca^{2+} channels [18, 29]. Endothelial P2X1 has also been reported to stimulate vasodilation via Ca^{2+}-activated K^+ channels in some blood vessels [20]. The molecular basis of NPY effects in the vasculature is poorly understood. NPY is a 36 amino acid neuropeptide that activates a family of four G protein-coupled receptors (Y_1, Y_2, Y_4 and Y_5) [6]. The existence of a Y_6 receptor remains controversial and is a pseudogene in humans and primates [8]. Y receptors are coupled predominantly to $G_{i/o}$ signal transduction pathways [45], though signaling pathways insensitive to pertussis toxin have been described [31,36]. The diversity of Y receptor expression and coupling to second messenger pathways in the vasculature may underlie the pleiotropic effects of NPY, but also provides opportunity for physiological and pharmacological fine-tuning of arterial function.

Though ATP and NPY are co-released at sympathetic neuroeffector junctions, their functional interaction in small arteries is poorly understood. Here we investigate the role of NPY in modulating P2X1-dependent vasoconstriction in small mesenteric arteries, studying their interaction following application of exogenous agonists and during sympathetic neurogenic vasoconstriction *in vitro*.

2. Materials and methods

2.1. Animals and study ethics

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the European Communities Council Directive of 24 November, 1986 (86/609/EEC) and were performed at the University of East Anglia. All studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [28]. A total of 102 animals were used in the experiments described here. 8-10 week-old adult male C57BL/6 mice (20-30 g) were used here. Mice were randomly assigned to standard cages, with four to five animals per cage, and housed at 21±2ºC with 55±10 % humidity and 12/12 light cycle per day and food and water available *ad*

libitum. Mice were sacrificed on the day of the experiment by CO_2 asphyxiation prior to tissue isolation.

2.2. Solutions

Physiological saline solution (PSS) composed of (mM): 130 NaCl; 4.7 KCl; 1.18 KH_2PO_4; 1.17 $MgSO_4$; 14.9 $NaHCO_3$; 5.5 glucose; 0.026 EDTA; 1.6 $CaCl_2$. High potassium Physiological saline solution (KPSS) composed of (mM): 74.7 NaCl; 60 KCl; 1.18 KH_2PO_4; 1.17 $MgSO_4$; 14.9 $NaHCO_3$; 5.5 glucose; 0.026 EDTA; 1.6 $CaCl_2$. Hanks balanced salt solution (HBSS) composed of (mM): 137 NaCl; 5.36 KCl; 0.44 KH_2PO_4; 0.34 Na_2HPO_4; 5.5 Glucose; 10 HEPES, pH 7.0 with NaOH if necessary.

2.3. Wire myography

The small intestine was removed and pinned down on a Sylgard-coated plate containing ice-cold HBSS. First order mesenteric arteries (<200μm) were cleaned of connective tissue, cut in rings (~ 2 mm) and mounted on a Mulvany myograph (Danish Myo Technology A/S, Denmark) to measure isometric tension. Data was acquired using LabChart 8 Pro software (AD Instruments Ltd, Oxford, UK). The rings were placed on a chamber filled with PSS and bubbled with 5% CO_2/medical air (21% O_2) mixture at pH 7.4. Before the experiments, the segments were normalized and subjected to an optimal tension (90% of tension equivalent to an intramural pressure of 100 mm Hg) and stabilized for at least 30 min. After the equilibration period, we discarded arterial rings that developed a tension of <1 mN in response to KPSS challenge. The myograph chamber has a final volume of 5mL in every experiment. Small volumes of agonists (5 μL of NPY and α,β-meATP) are added to the chamber to evoke responses. Antagonists are introduced upon total exchange of KPSS. The chamber is repeatedly fully evacuated with 5 mL fresh KPSS between experiments to fully washout drugs and return arterial rings to basal tone. Experiments were performed at 37°C. Arterial contractions were averaged at maximum peaks or areas under the curve–reached with KPSS solution. Functionally intact endothelium was confirmed at the end of experiments by relaxation to acetylcholine.

2.4. Electrical field stimulation

Electrical field stimulation (EFS) was discharged by two platinum electrodes (Danish Myo Technology A/S, Denmark) located either side of the arterial ring. A Grass SD9 stimulator was used to apply EFS with parameters 70V, 50 μs, 2–64 Hz, positive monopolar for 5 s. After the equilibrium period, we discarded arterial rings that developed tension <1 mN in response to KPSS challenge and

that did not develop tensions >1 mN in response to the electrical protocol. Reproducible vasoconstriction was achieved by applying EFS every 5 mins.

2.5. Immunocytochemistry

Freshly isolated smooth muscle cells were liberated by enzymatic digestion of mesenteric arteries. Arteries were incubated for 30 mins at 37°C in HBSS containing 13 U/mL papain, 150 U/mL collagenase, 0.8 U/mL elastase and 0.75 mg/mL bovine serum albumin (BSA). The enzymatic solution was replaced with fresh HBSS followed by gentle trituration with a fire polished glass Pasteur pipette to liberate smooth muscle cells. Cell suspensions were transferred to poly-L-lysine coated coverslips and left to adhere for 1 hour. Cells were fixed with 4% (w/v) paraformaldehyde for 15 mins at room temperature. Cells were washed with phosphate-buffered saline (PBS) before blocking and permeabilization with PBS containing 1% (w/v) BSA and 0.25% (v/v) triton X-100 at room temperature for 30 mins. Incubation overnight at 4°C with either rabbit polyclonal anti-Y_1 receptor (Alomone Labs Cat# ANR-021, RRID:AB_2040030) or anti-Y_4 receptor (Alomone Labs Cat# ANR-024, RRID:AB_2040034) at a 1:100 dilution in PBS containing 1% (w/v) BSA. Cells without primary antibody were used to control for non-specific binding of the secondary antibody. Cells were washed in PBS followed by incubation for 1 hour at room temperature with donkey anti-rabbit Alexa fluor 488-conjugated secondary antibody (Abcam Cat# ab150073, RRID:AB_2636877) at 1 µg/mL in 1% (w/v) BSA. Cells were thoroughly washed with PBS before mounting with media containing the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) (Abcam). Fluorescence was visualized by a Zeiss ApoTome microscope.

2.6. RNA extraction, cDNA synthesis and RT-PCR

Freshly isolated mesenteric arteries or brains were lysed in Tri-Reagent (Sigma) using a disposable pestle [14]. Phases were separated with 1-bromo-3-chloropropane, total RNA was precipitated with isopropanol and washed with 75% ethanol. Total RNA was treated with DNase I (Invitrogen) before quantification. 0.5 µg total RNA was primed with 100 ng random hexamers (Bioline) and reverse transcribed to cDNA using Superscript III (Invitrogen) for 1 hour at 42°C. Reverse transcriptase was omitted to control for the presence of genomic DNA. The following oligonucleotide primers (shown 5'to 3') were used for PCR detection of receptors: Y_1, sense CCGCTTCAACAGAGGTGAAC, antisense AGCGAATGTATATCTTGAAGTAGCA; Y_2, sense CGCAAGAGTCAATACAGCCA, antisense CACCAAATGGCACAAGACCG; Y_4 sense AGGTCGTCTGCTTTGTGTCC, antisense GGAAAAGCCCAGACACGACT; Y_5 sense GGCATCCCGAGGACTCTAGTA, antisense GGCAGTGGATAAGGGCTCTC. 2 ng/µL cDNA used in PCR reactions containing 1.5 U Taq DNA polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.7 M betaine and 0.2 µM of primers. The thermal

cycling protocol was 94°C for 1 minute for initial denaturation; 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds; 72°C for 5 minutes (final extension).

2.7. Drugs and salts

All basic salts, DMSO, BSA, papain, type I collagenase, elastase and acetylcholine were purchased from Sigma Aldrich. Tetrodotoxin citrate (Abcam); guanethidine sulphate (Cayman); neuropeptide Y, α,β-meATP, NF449, suramin, BIBO3304, BIIE0246 (Tocris).

2.8. Data and statistical analysis

All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 24.0). Data are expressed as mean±SEM where *n* represents the number of animals. For hypothesis testing, a Kruskal-Wallis test followed by a Student Newman-Keuls *t* test, post hoc Tukey, or Mann-Whitney *U* were used where appropriate. Comparison between arterial rings from the same animal were made by paired *t* test or Friedman test followed by a Wilcoxon signed-rank test. The threshold for statistical significance was $P < 0.05$ throughout. Agonist and antagonist concentration-response curves were fitted using a modified Hill equation as below:

$$Y = Start + (End - Start)\frac{X^n}{k^n + X^n}$$

where k = Michaelis constant and *n* number of cooperative sites.

2.9. Nomenclature of targets and ligands

Key protein targets and ligands correspond to entries in **http://www.guidetopharmacology.org**, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [17], and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 [2].

3. Results

3.1. α,β–meATP evokes P2X1 receptor-dependent vasoconstriction in small mesenteric arteries

Application of α,β-meATP at 20 min intervals evoked reproducible transient vasoconstriction in rings of small mesenteric arteries (**Fig. 1A**). α,β-meATP evoked vasoconstriction in a concentration-dependent fashion (EC_{50} 226±34 nM; *N*=5, 5 arterial rings exposed to water (vehicle) *vs*. 5 arterial

rings to α,β–meATP) (**Fig. 1B**). Responses were fully inhibited by the broad spectrum purinergic receptor antagonist suramin (IC_{50} 16±3 μM; *N*=7, 7 arterial rings exposed to water (vehicle) *vs*. 7 arterial rings to suramin) (**Fig. 1C, D**), and by NF449 (IC_{50} 466±59 nM; *N*=5) (**Fig. 1E, F**), a P2X1 receptor antagonist [22].

3.2. NPY facilitates P2X1 receptor-dependent vasoconstriction

Application of up to 100 – 300 nM NPY had no effect on the basal tone of arterial rings (*N*=8, 8 rings exposed to water (vehicle) *vs*. 8 rings in NPY) (**Fig. 2A**). To investigate the modulatory effects of NPY on P2X1-dependent vasoconstriction, we evoked an initial vasoconstriction with 100 nM α,β-meATP (an approximate EC_{30}; **Fig. 1B**) followed by exposure to NPY, and made a pairwise comparison with a subsequent α,β-meATP-evoked vasoconstriction. In these experiments NPY had a graded faciliatory effect on the vasoconstriction evoked by α,β-meATP (**Fig. 2B**), increasing the peak response (**Fig. 2C**) and total contractile response (area under the curve) (**Fig. 2D**). 10nM NPY facilitated both peak and the total response (**Fig. 2C, D**) and was used for further investigation (*N*=8, 8 rings exposed to water (vehicle) *vs.* 3 groups of 8 rings exposed to NPY 1, 10 or 30nM NPY). We next examined the effect of NPY on the α,β-meATP concentration-response relationship. NPY increased the efficacy of α,β-meATP in the log-linear phase of the concentration-response curve for response peak but had no effect on response maxima (**Fig. 2E**), whereas NPY enhanced the maxima of the total response (**Fig. 2F**) (*N*=7, 7 rings exposed to water (vehicle) *vs*. 7 rings to 10nM NPY). These data demonstrate that NPY facilitates and strengthens P2X1-dependent vasoconstriction. In an effort to understand the molecular basis of NPY facilitation, we sought to determine the expression of Y receptors known to be functionally expressed by mammals, namely Y_1, Y_2, Y_4 and Y_5. mRNA transcripts for all receptors were detectable in brain but only Y_1 and Y_4 receptor transcripts were expressed by mesenteric arteries (**Fig. 3A**). The expression of Y_1 (**Fig. 3B**) and Y_4 (**Fig. 3C**) at the protein-level was confirmed by immunocytochemistry in smooth muscle cells freshly isolated from mesenteric artery (*N*=5). Next we explored the role of Y_1 by using the selective antagonist BIBO3304 [51]. BIBO3304 (10 nM; 15 min) had no effect on basal tension in arterial rings (*P*>0.05; *N*=6). BIBO3304 inhibited NPY facilitation of α,β-meATP-evoked vasoconstriction (**Fig. 3D**), completely suppressing the faciliatory effect of NPY (**Fig. 3E**; *P*<0.05, *N*=6, 6 rings exposed to DMSO (BIBO3304 vehicle) and water (NPY vehicle) *vs.* 6 rings exposed to NPY and DMSO, and 6 rings exposed to BIBO3304 and water (NPY vehicle) *vs.* 6 rings to BIBO3304 and NPY) . In control experiments we tested the effect of BIIE0246 (200 nM, 15 min) a selective Y_2 receptor antagonist [12], as the Y_2 receptor is not expressed by mesenteric artery (**Fig. 3A**). BIIE0246 had no effect on the faciliatory effect of NPY (**Fig. 3F, G**; *P*<0.05; *N*=6, 6 rings exposed to DMSO (BIIE0246 vehicle) and water (NPY vehicle) *vs.* 6 rings exposed to NPY and DMSO, and 6 rings exposed to BIIE0246

and water (NPY vehicle) *vs.* 6 rings to BIIE0246 and NPY)). BIIE0246 had no effect on baseline tension (P>0.05; N=6, 6 rings exposed to DMSO (vehicle) vs. 6 rings in BIIE0246). Unfortunately, the role of Y_4 could not be investigated as commercially available selective antagonists are not available. However, the complete reversal of NPY facilitation by BIBO3304 suggests either Y_1 and Y_4 are mutually redundant, or Y_4 is not involved.

3.3. Facilitation by NPY requires the L-type Ca^{2+} channel

The L-type Ca^{2+} channel antagonist nifedipine had no effect on α,β-meATP-evoked vasoconstriction (**Fig. 4A**), but nifedipine could completely relax contractions evoked by membrane depolarisation with 60mM K^+ (**Fig. 4B**) (N=5, 5 rings exposed to DMSO (vehicle) *vs.* 5 rings in nifedipine). These data suggest P2X1-dependent vasoconstriction is independent of L-type Ca^{2+} channel activity. Treatment with nifedipine at 100 nM (approx. IC_{80} for L-type: [13,49], could completely reverse NPY facilitation of α,β-meATP-evoked vasoconstriction (**Fig. 4C, D**) (N=9, 9 rings exposed to DMSO, 9 rings exposed to NPY and DMSO, 9 rings exposed to NPY and nifedipine).

3.4. Y1 receptor activation facilitates P2X1-dependent vasoconstriction during sympathetic neurogenic response

Thus far we have investigated the role of NPY in facilitating P2X1-dependent vasoconstriction through the application of exogenous agonists. Next we explored whether Y_1-dependent facilitation of P2X1-dependent vasoconstriction occurred during sympathetic neurogenic responses. Electrical field stimulation (EFS) of arterial rings evoked transient vasoconstrictions (**Fig. 5A**), that were abolished by tetrodotoxin (TTX) (**Fig. 5A**) (N=7, 7 rings exposed to citrate buffer (vehicle) *vs.* 7 rings in TTX) and the sympatholytic agent guanethidine (**Fig. 5A**). EFS evoked vasoconstriction in a frequency-dependent fashion up to a maximal response at 64 Hz (**Fig. 5B**). TTX abolished all responses (**Fig. 5B**) including the 64 Hz response which was used for later mechanistic investigation (**Fig. 6**). These data demonstrate the EFS protocol adopted elicits sympathetic vasoconstriction in small mesenteric arteries. Treatment with 3 μM NF449, which maximally inhibited α,β-meATP-evoked vasoconstriction (**Fig. 1F**), attenuated neurogenic vasoconstriction (**Fig. 5C**). NF449 inhibited the neurogenic response by approximately 50% at maximal frequencies (**Fig. 5D**) (N=6, 6 rings exposed water (vehicle) *vs.* 6 rings to NF449). Antagonism of Y_1 receptors with BIBO3304 caused a significant but partial reduction in the magnitude of neurogenic vasoconstriction (**Fig. 6A**), suggesting Y_1 receptor activation facilitates the neurogenic response. In an effort to isolate the P2X1 component of the neurogenic response so as to test the influence of Y_1 receptor activation, we first applied prazosin at 1 μM to abolish the contribution of α_1-adrenergic receptors. This resulted in a substantial

reduction in the neurogenic vasoconstriction (**Fig. 6B**) that was not inhibited further by 10 μM prazosin (N=5; P>0.05). To reveal the effect of Y_1 antagonism on the P2X1 component of the neurogenic contraction, we applied both prazosin and BIBO3304 (**Fig. 6C**). Quantitative analysis of the pharmacology revealed that activation of α_1-adrenergic receptors (prazosin) contributed approximately 80% of the neurogenic vasoconstriction, and that combined antagonism of α_1-adrenergic receptors and P2X1 abolished neurogenic vasoconstriction (**Fig. 6D**). Antagonism of Y_1 receptors (BIBO3304) reduced the vasoconstriction by approximately 50% (**Fig. 6D**) (N=5, 5 rings exposed to DMSO (vehicle) *vs.* 5 rings with BIBO3304). Importantly we observed that neurogenic vasoconstriction in the presence of prazosin and BIBO3304 was significantly smaller (N=5; P<0.05) than in the presence of prazosin alone (**Fig. 6D**). These data suggest the P2X1-dependent component is smaller when Y_1 receptors are antagonised, supporting a faciliatory role of Y_1 receptor activation on P2X1 during sympathetic neurogenic vasoconstriction.

4. Discussion

Our data demonstrates that NPY does not directly elicit vasoconstriction in small mesenteric arteries, either when applied exogenously or via sympathetic neurogenic vasoconstriction, and instead elicited a faciliatory effect on P2X1-dependent vasoconstriction. The incidence of NPY acting directly as a vasoconstrictor is highly variable across species and vascular beds studied. NPY has direct vasoconstrictor effects on human forearm arteries [21], canine coronary arteries [46] and porcine superior retinal arteries [10], but not human pulmonary arteries [11]. Mixed response have been reported for rat mesenteric arteries [3, 40]. The expression of Y receptor subtypes (Y_1, Y_2, Y_4 and Y_5) also varies between blood vessels, though the Y_1 receptor is consistently observed [1,4,11], which is consistent with this study. In some arteries and veins, activation of Y_1 causes direct vasoconstriction [32,33]. It is not fully understood why NPY lacks vasoconstrictor effects in some blood vessels, despite expression of Y receptors known to mediate direct vasoconstriction. Studies on isolated vascular smooth muscle cells have demonstrated that Y_1 receptor activation elevates cytoplasmic Ca^{2+} [39,42]. The signal transduction mechanisms underlying Y_1 receptor-mediated elevation in cytoplasmic Ca^{2+} are poorly defined, though a study in porcine aortic smooth muscle cells suggests that the response is due to atypical activation of phospholipase C (PLC) via the βγ subunits of heterotrimeric G protein [43]. The degree of PLC activation is dependent upon both the types of PLC isoenzyme and βγ subunit [5], and thus may explain the heterogeneity observed in the vasoconstrictive effects of NPY in different vascular beds. Alternative splice variants of the Y_1 receptor have also been suggested to contribute to the heterogeneity in NPY signalling [37]. Our study demonstrates that Y_2 receptors were not present in mouse mesenteric arteries, though in rat mesenteric arteries both post-junctional Y_1 and Y_2 receptors contribute to neurogenic vasoconstriction [16]. The

findings of this study and others demonstrate the highly heterogenous manner of NPY signalling within arteries. NPY is widely accepted to facilitate α_1-adrenergic vasoconstriction [52], but this is the first demonstration of NPY facilitating purinergic vasoconstriction in small arteries.

Our work supports an important role of P2X1 in mediating sympathetic neurogenic contraction of small arteries [29,48]. P2X1 is also involved in pressure-induced autoregulatory vasoconstriction in renal afferent arterioles [24,25], and intravenous infusion of the P2X1 agonist α,β-meATP causes a strong pressor effect *in vivo* [30]. P2X receptor subtypes display varying degrees of desensitisation, and P2X1 undergoes rapid desensitisation in the presence of agonist [41]. Concordantly, a study by Harhun et al. [19] demonstrated that P2X1 activation caused transient membrane depolarisation in smooth muscle cells isolated from the renal artery. In this study the associated P2X1-dependent elevation in intracellular Ca^{2+} was sensitive to nicardipine and suggested an involvement of L-type Ca^{2+} channels in the P2X1 response without prior exposure to NPY [19]. Such electrophysiological and calcium measurements in isolated smooth cells are at odds with the functional contractile responses observed in this study, suggesting no role of the L-type Ca^{2+}channels before NPY facilitation. Our findings that P2X1-dependent vasoconstriction did not involve the L-type Ca^{2+} channel is supported by previous work in rat mesenteric arteries [15]. In neurons, Y_1 receptor activation causes suppression of voltage-gated Ca^{2+} currents, though this action is via inhibition of N-type Ca^{2+} channels [35,53]. L-type Ca^{2+} channel activity is inhibited by NPY in cardiac myocytes [7], neuroendocrine cells [34] and neurons [41], although the molecular identity of the Y receptor mediating these effects is not always apparent. Interestingly, a study in vascular smooth muscle cells demonstrated that NPY could potentiate voltage-gated Ca^{2+} currents [54]. In this study by Xiong et al. [54], NPY potentiated Ca^{2+} currents by causing a hyperpolarising shift in the steady-state activation curve of the L-type Ca^{2+} channel. This mechanism may be helpful in understanding why P2X1 receptor activation only engages with the L-type Ca^{2+} channel following Y_1 receptor activation, assuming a small transient depolarisation evoked by P2X1 [19] under normal conditions is not sufficient to activate the L-type Ca^{2+} channel. The signal transduction mechanism by which this is achieved in the study by Xiong et al. [54] is unclear.

5. Conclusions

Our data support a modulatory role of NPY in small mesenteric arteries, whereby activation of the Y_1 receptor facilitates P2X1 receptor-dependent vasoconstriction through engagement of the L-type Ca^{2+} channel.

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Author contributions

Samuel J. Fountain Conceptualization; Funding acquisition; Supervision; Writing - original draft; Writing - review & editing.
Maria del Carmen Gonzalez-Montelongo Data curation; Formal analysis; Writing - review & editing.

Declaration of Competing Interest

Authors declare no conflict of interest.

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Fig. 1. α,β-methylene-ATP evoked contractions of mesenteric arteries are mediated by P2X1. (A) Reproducible contraction with 1 μM α,β-methylene-ATP (α,β-meATP) applied at 20 min intervals. (B) α,β-meATP evokes concentration dependent contractions (EC_{50} 226±34 nM; *N*=5). (C) Representative paired contraction demonstrating suramin (300 μM) antagonism of α,β-meATP (1 μM) evoked contraction (*N*=5). (D) Concentration dependent antagonism by suramin (IC_{50} 16±3 μM; *N*=7) of contractions evoked by 1 μM α,β-meATP. (E) Representative paired contraction demonstrating inhibitory effect of P2X1 antagonist NF449 (3 μM) of α,β-meATP (1 μM) evoked

contractions. (F) Concentration dependent antagonism by NF449 (IC_{50} 466±59 nM; *N*=5) of contractions evoked by 1 μM α,β-meATP.

Fig. 2. Neuropeptide Y potentiates P2X1-dependent evoked contractions of mesenteric artery. (A) NPY application has no effect on basal tone. Representative trace showing no effect following NPY (100 nM) application, followed by contraction in the presence of 60 mM KCl (*N*=8). (B) Representative paired contractions showing effect of 10 min preincubation of varying NPY concentrations on response to 100 nM α,β-meATP. Traces in the presence of vehicle control are *grey* and traces in the presence of NPY are *black*. Average data (*N*=8) of paired contractions showing effect of NPY on the peak of the contractile response (C) and area of response (D) evoked by 100 nM α,β-meATP. 1st response in the presence of vehicle alone and 2nd response after 10 min exposure to 1, 10 or 30 nM NPY. Effect of NPY (*open* circles; 10 nM 10 min, *N*=7) on concentration-response curve for α,β-meATP for peak of contractile response (E) and area of contractile response (F) compared to vehicle control (*closed circles*; *N*=7). *ns*, not significant; * $p<0.05$ by Friedman and Wilcoxon signed-rank test for data in panels C, E and F; $p<0.05$ by Student-Newman-Keuls *t* test followed by post hoc Tukey for data in panel D. Concentration-response curve for peak of contractile response (E) and area of contractile response (F) to 100 nM α,β-meATP in the presence of vehicle control (*closed circles*; *N*=7) or 10 nM NPY (*open* circles; 10 nM, 10 min; *N*=7). *ns*, not significant; * $p<0.05$ by Friedman and Wilcoxon signed-rank test for data in panels C, E and F; $p<0.05$ by Student-Newman-Keuls *t* test followed by post hoc Tukey for data in panel D.

Fig. 3. Facilitation of P2X1-dependent contractions by neuropeptide Y is mediated by Y_1 receptor activation. (A) RT-PCR analysis of NPY receptors Y_1, Y_2, Y_4 and Y_5 in mesenteric arteries and brain (positive control). Predicted band sizes: Y_1 894, 968, 1117 or 1138 bp dependent on transcript variant, Y_2 422 bp, Y_4 554 bp, Y_5 1407 bp. +/- RT (reverse transcriptase). Representative images showing immunocytochemical analysis of Y_1 receptor (B) and Y_4 receptor (C) in isolated smooth muscle cells from mesenteric artery. Images are overlays of green channel fluorescence arising from secondary antibody and blue channel arising from DAPI (nuclear stain). *Control* indicates experiments performed in the absence of primary antibody. Scale bar is 25 μm. (D) Representative contractile response to 100 nM α,β-meATP showing facilitation by NPY (10 nM, 10 min) and reversal by selective Y_1 receptor antagonist BIBO3304 (10 nM, 15 min). (E) Average data showing effect of BIBO (10 nM, 15 min) on contraction evoked by 100 nM α,β-meATP with and without facilitation by NPY (10 nM, 10 min). *N*=6 for all. * $p<0.05$ comparison within a paired experiment (Friedman, Wilcoxon signed-rank test). ** $p<0.05$ comparison between unpaired experiments (Kruskal-Wallis and Mann-Whitney U); *ns*, not significant. (F) Representative contractile response to 100 nM α,β-meATP showing facilitation by NPY (10 nM, 10 min) and lack of reversal by

selective Y_2 receptor antagonist BIIE0246 (200 nM, 15 min). (G) Average data showing lack of effect of BIIE0246 (200 nM, 15 min) on contraction evoked by 100 nM α,β-meATP with and without facilitation by NPY (10 nM, 10 min). *N*=6 for all. * $p<0.05$, Friedman, Wilcoxon signed-rank test.

Fig. 4. Facilitation of P2X1-dependent contractions by neuropeptide Y requires L-type Ca^{2+} channel activity. (A) Contractions evoked by 100 nM α,β-meATP are insensitive to L-type Ca^{2+} channel inhibitor nifedipine (1 – 100 nM, 15 min). Data is for peak contraction. (B) Representative trace showing reversal of 60 mM KCl-evoked contraction by 100 nM nifedipine. Representative of 5 independent experiments. (C) Representative contractile response to 100 nM α,β-meATP showing facilitation by NPY (10 nM, 10 min) and reversal by nifedipine (100 nM, 15 min). (D) Average data showing effect of nifedipine (100 nM, 15 min) on contraction evoked by 100 nM α,β-meATP with and without facilitation by NPY (10 nM, 10 min). *N*=9 for all. * $p<0.05$ comparison within a paired experiment (Friedman, Wilcoxon signed-rank tes); ** $p<0.05$ comparison between unpaired experiments (Kruskal-Wallism Mann-Whitney U); *ns*, not significant.

Fig. 5. Contribution of post-junctional P2X1 receptors to sympathetic neurogenic responses in isolated mesenteric arteries. (A) Representative contractions evoked by electrical field stimulation. Frequency-dependent contractions (*upper trace*), are sensitive to tetrodotoxin (TTX; 1 μM, 30 min) (*middle trace*) and guanethidine (10 μM, 30 min). Representative of 7-10 independent experiments. (B) Average data showing frequency-dependent contractions in the presence of vehicle control (*closed circles*) and in the presence of TTX (1 μM, 30 min) (*open circles*). *N*=7 for all; * $p<0.05$ (Kruskal-Wallis, Mann-Whitney U). (C) Representative responses showing frequency-dependent contractions in the presence of vehicle control (*upper trace*) and P2X1 receptor antagonist NF449 (3 μM, 30 min) (*lower trace*). (D) Average data showing frequency-dependent contractions in the presence of vehicle control (*closed circles*) and NF449 (3 μM, 30 min) (*open circles*). *N*=6 for all; * $p<0.05$ (Friedman, Wilcoxon signed-rank).

Fig. 6. Y_1 receptor activation facilitates P2X1-dependent contraction during sympathetic neurogenic responses. (A) Representative trace showing inhibitory effect of Y_1 receptor antagonist BIBO3304 (10 nM, 30 min) on neurogenic contractile response (*N*=5). (B) Representative trace showing pharmacological strategy of isolating P2X1 component of neurogenic response. Antagonism of alpha-adrenergic response with prazosin (1 μM; 30 min) reveals small residue component abolished by P2X1 receptor antagonist NF449 (3 μM, 30 min) (*N*=5). (C) Representative trace showing effect of BIBO3304 (10 nM, 30 min) and prazosin (1 μM, 30 min) combined, revealing inhibition of purinergic component (*N*=5). (D) Average data showing effects of prazosin, BIBO3304,

prazosin and NF449 combined, and prazosin and BIBO3304 combined. * p<0.05 compared to control group, ** p<0.05 compared to another test group (Kruskal-Wallis, Mann-Whitney U).

APPENDICE

Eq. (A.1)

$$Y = Start + (End - Start)\frac{X^n}{k^n + X^n}$$

where k = Michaelis constant and *n* number of cooperative sites.

Graphical abstract:

Highlights

- NPY facilitates purinergic vasoconstriction via Y1 receptor activation.
- NPY facilitates purinergic vasoconstriction via engagement of the L-type Ca^{2+} channel.
- P2X1 receptors are an effector of NPY action in small arteries.