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Neuropeptide Y: Direct vasoconstrictor and facilitatory effects on P2X1 receptor-dependent vasoconstriction in human small abdominal arteries

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ABSTRACT

Neuropeptide Y (NPY) is co-released with norepinephrine and ATP by sympathetic nerves innervating arteries. Circulating NPY is elevated during exercise and cardiovascular disease, though information regarding the vasomotor function of NPY in human blood vessels is limited. Wire myography revealed NPY directly stimulated vasoconstriction (EC_{50} 10.3 ± 0.4 nM; $N = 5$) in human small abdominal arteries. Maximum vasoconstriction was antagonised by both BIBO03304 (60.7 ± 6%; $N = 6$) and BIIE0246 (54.6 ± 5%; $N = 6$), suggesting contributions of both Y_1 and Y_2 receptor activation, respectively. Y_1 and Y_2 receptor expression in arterial smooth muscle cells was confirmed by immunocytochemistry, and western blotting of artery lysates. α,β-meATP evoked vasoconstrictions (EC_{50} 282 ± 32 nM; $N = 6$) were abolished by suramin (IC_{50} 825 ± 45 nM; $N = 5$) and NF449 (IC_{50} 24 ± 5 nM; $N = 5$), suggesting P2X1 mediates vasoconstriction in these arteries. P2X1, P2X4 and P2X7 were detectable by RT-PCR. Significant facilitation (1.6-fold) of α,β-meATP-evoked vasoconstrictions was observed when submaximal NPY (10 nM) was applied between α,β-meATP applications. Facilitation was antagonised by either BIBO03304 or BIIE0246. These data reveal NPY causes direct vasoconstriction in human arteries which is dependent upon both Y_1 and Y_2 receptor activation. NPY also acts as a modulator, facilitating P2X1-dependent vasoconstriction. Though in contrast to the direct vasoconstrictor effects of NPY, there is redundancy between Y_1 and Y_2 receptor activation to achieve the facilitatory effect.

1. Introduction

Post-ganglionic sympathetic nerves are critical regulators of arterial tone, local tissue perfusion and systemic blood pressure. The neurotransmitters norepinephrine, ATP and NPY are co-released at the sympathetic neuroeffector junctions of arteries, acting upon post-junctional receptors of vascular smooth muscle to influence arterial tone. The degree of individual contribution of norepinephrine, ATP and NPY in influencing arterial tone varies between vascular beds, strength of sympathetic outflow and disease state [1–3]. Whilst norepinephrine and ATP have primary trophic effects, stimulating vasoconstriction *via* adrenergic and purinergic receptors, respectively, the effect of NPY is highly variable and dependent on context. NPY is a 36-amino acid peptide that mediates its biological effect through activation of a family of G-protein-coupled receptors, Y_1-Y_5 receptors in humans [4]. NPY has direct vasoconstrictive or post-junctional modulatory effects dependent upon species and vascular bed studied [5–8]. Y receptors couple predominantly to $G_{i/o}$ heteromeric G-proteins [9], though coupling to other signal transduction pathways has also been described [10,11]. A diverse expression pattern of Y receptor subtypes and promiscuous signal transduction coupling provides an opportunity for pharmacological and physiological fine-tuning of NPY vasomotor and modulatory effects in arteries. This may also underlie pleiotropic vascular effects of NPY described in previous studies. Importantly, there are limited studies investigating the vasomotor effects of NPY in human blood vessels, though patients with hypertension, heart failure, and cardiac hypertrophy have increased levels of circulating NPY [10,12,13]. Circulating NPY also increases physiologically during sympathetic activation including exercise, cold exposure and hypoxia [14,15].

Extracellular ATP mediates its fast biological effects through the activation of P2X receptor ligand-gated ion channels [16]. Humans express 7 receptor subtypes (P2X1–7) which assemble as trimers and pass

Abbreviations: αβmeATP, α,β-methylene adenosine 5'triphosphate.

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cations non-selectively when activated by ATP [17]. ATP released from post-ganglionic sympathetic nerves mediates the neurogenic vasoconstriction in small arteries [2,8]. In rodents, the P2X1 receptor has been identified as the major post-junctional receptor for ATP [8,18], though expression of other arterial smooth muscle P2X receptors have been identified in different vascular beds [19,20]. Despite this, there is currently limited information regarding the molecular basis of ATP vasomotor effects in human blood vessels [21]. Activation of arterial smooth muscle P2X receptors can stimulate vasoconstriction *via* several Ca^{2+}-dependent mechanisms. First, an increase in cytoplasmic Ca^{2+} due to Ca^{2+}-entry through P2X receptors, and second, voltage-gated Ca^{2+} opening as a consequence of membrane depolarisation caused by the passage of cations through P2X receptors [18,19].

Here we investigate the vasomotor effects of NPY in human small resistance-sized arteries, and examine the functional interaction with P2X1 receptor-dependent vasoconstriction.

2. Materials and methods

2.1. Human tissue and study ethics

Surgical surplus of abdominoplasty tissue was anonymously donated by female patients undergoing deep inferior epigastric perforators (DIEP) flap surgery for the purpose of breast reconstruction at the Norfolk and Norwich University Hospital. All samples were donated following informed consent. Patient demographic: age 56 ± 1.5 years; weight 63.1 ± 1.3 kg; body mass index 22.5 ± 0.5 kg/m^2; non-diabetic, HbA1c <42 mmol/mol; normotensive, blood pressure ≤ 140/80 mmHg; disease-free and not taking anti-inflammatory medication. Study ethics were approved by NHS Health Research Authority (IRAS: 254610).

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

Small arteries (<150 μm diameter) were dissected free in ice-cold HBSS and cleaned of connective tissue, cut into ring of approximately 2 mm thickness, and mounted on a Mulvany 4-channel wire 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 mmHg) and stabilized for at least 60 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 5 mL 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.

2.4. Immunocytochemistry

Arterial rings 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 h. Smooth muscle cells were identifiable by their characteristic spindle shape. 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; RRID AB_2040030) or anti-Y_2 receptor (Alomone Labs; RRID AB_2040032) at 1:200 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 h at room temperature with donkey anti-rabbit Alexa fluor 488-conjugated secondary antibody (Abcam) at 1 μg/mL in 1% (*w/v*) BSA. Fluorescence was visualized by a Zeiss ApoTome microscope.

2.5. Western blotting

Dissected arteries flushed clear of blood, cut into small sections with a scalpel, and solubilised in 1 mL ice-cold lysis buffer composed of 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2% (*v*/v) Triton X-100, 20 mM Tris-HCl pH 7.6, and supplemented with 1× EDRA-free protease complete inhibitor cocktail (Roche). Samples were solubilised for 2 h at 4 °C on a rotating wheel. 50 μg of total protein was mixed with 4 μL of sample buffer composed of 240 mM Tris-HCl pH 6.8, 12% (*v*/v) sodium dodecyl sulphate (SDS), 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 50 mM dithiothreitol in a final volume of 20 μL. Samples were incubated for 6 mins at 96 °C. Sampled were resolved by electrophoresis of SDS gels (5% stacking, 10% resolving), and transferred to PVDF membrane (0.45 μm pore; Millipore, UK). Membranes were blocked for 1 h at room temperature in PBS containing 0.1% (*v*/v) Tween-20 and 5% (*w/v*) semi-skimmed milk. Primary antibodies against Y_1 receptor (Alomone Labs; RRID AB_2040030) and Y_2 (Alomone Labs; RRID AB_2040032) were used at 1:1000 dilution, and goat anti-rabbit HRP secondary (Fisher Scientific, UK) used at 1:1000 dilution. All antibodies were diluted in blocking solution. Immunoreactivity was detected using ECL Western blotting solution (Pierce).

2.6. RNA extraction, cDNA synthesis and RT-PCR

Arterial rings were lysed in Tri-Reagent (Sigma) using a disposable pestle [22]. 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 h 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 with expected product size shown in parenthesis: P2X1 (341 bp), sense GCTTTCCACGCTTCAAGGTC, antisense GAGGTGACGGTAGTTGGTCC; P2X2 (200 bp), sense GCACAGACGGGTACCTGAAG, antisense GGAGTACTTGGGGTTGCACT; P2X3 (564 bp), sense TGTATCAGACAGCCAGTGCG, antisense CGGATGCCAAAAGCCTTCAG; P2X4 (311 bp), sense AGCAACGGAGTCTCAACAGG, antisense TGGAAACTGTGTCCTGCGTT; P2X5 (263 bp), sense GCAATGTGATGGACGTCAAGG, antisense GTACCCGGAGGAGACAGACT; P2X6 (405 bp), sense GACTTCGTGAAGCCACCTCA, antisense TTGTGGTTCATAGCGGCAGT; P2X7 (414 bp), sense CGGTTGTGTCCCGAGTATCC, antisense AATGCCCATTATTCCGCCCT. 2 ng/μL cDNA used in PCR reactions containing 1.5 U Taq DNA polymerase (Promega), 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 min for initial denaturation; 40

cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 90 s; 72 °C for 5 min (final extension).

2.7. Drugs, salts and enzymes

All basic salts, DMSO, BSA, papain, type I collagenase, elastase and acetylcholine were purchased from Merck. Neuropeptide Y, αβmeATP, 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 human donors. 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 donor 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 ligands and targets

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 [23], and are permanently archived in the Concise Guide to PHARMACOLOGY [24].

3. Results

3.1. P2X1 receptor-dependent vasoconstriction

60 mM K^+ produced sustained vasoconstriction (Fig. 1A) whilst αβme-ATP elicited a more transient vasoconstriction (Fig. 1B) in human arterial rings. Aβme-ATP elicited vasoconstriction with an EC_{50} of 282 ± 32 nM ($N = 5$; Fig. 1C) and a maximal magnitude 81 ± 4% ($N = 8$) of the response to 60 mM K^+. We subsequent applied 300 nM αβme-ATP in further experiments when investigating modulatory effects of NPY. The magnitude of response to αβme-ATP was reproducible following washout and a 20 min application interval. Endothelial denudation, as determined by loss of vasodilatory response to acetylcholine in arterial rings pre-constricted with phenylephrine (Fig. 1D), significantly enhanced αβme-ATP-evoked vasoconstriction (Fig. 1E). RT-PCR analysis of P2X receptor mRNA transcripts revealed expression of P2X1, P2X4 and P2X7 in arteries (Fig. 2A), of which P2X1 and P2X4 have been shown previously to be activated by αβme-ATP [16]. All P2X receptor mRNA transcripts were detectable when using human brain cDNA (data not shown). Next, we took a pharmacological approach to determine which purinergic receptor was responsible for mediating vasoconstriction in response to αβme-ATP. Vasoconstriction was inhibited by the broad-spectrum purinergic antagonist suramin (Fig. 2B) with an IC_{50} of 825 ± 45 nM ($N = 5$; Fig. 2C). αβme-ATP-evoked vasoconstriction was also abolished by the P2X1 receptor selective antagonist NF449 (Fig. 2D). NF449 inhibited the response with an IC_{50} of 24 ± 5 nM ($N = 5$; Fig. 2E).

3.2. NPY-evoked vasoconstriction is dependent upon Y_1 and Y_2 receptors

NPY could directly elicit vasoconstriction in human arterial rings (Fig. 3A). At higher concentrations of NPY, the vasoconstrictive response was significantly more sustained than that of αβme-ATP, but could be rapidly reversed upon washout (Fig. 3A). NPY elicited vasoconstriction with an EC_{50} of 10.3 ± 0.4 nM ($N = 6$) (Fig. 3B). We

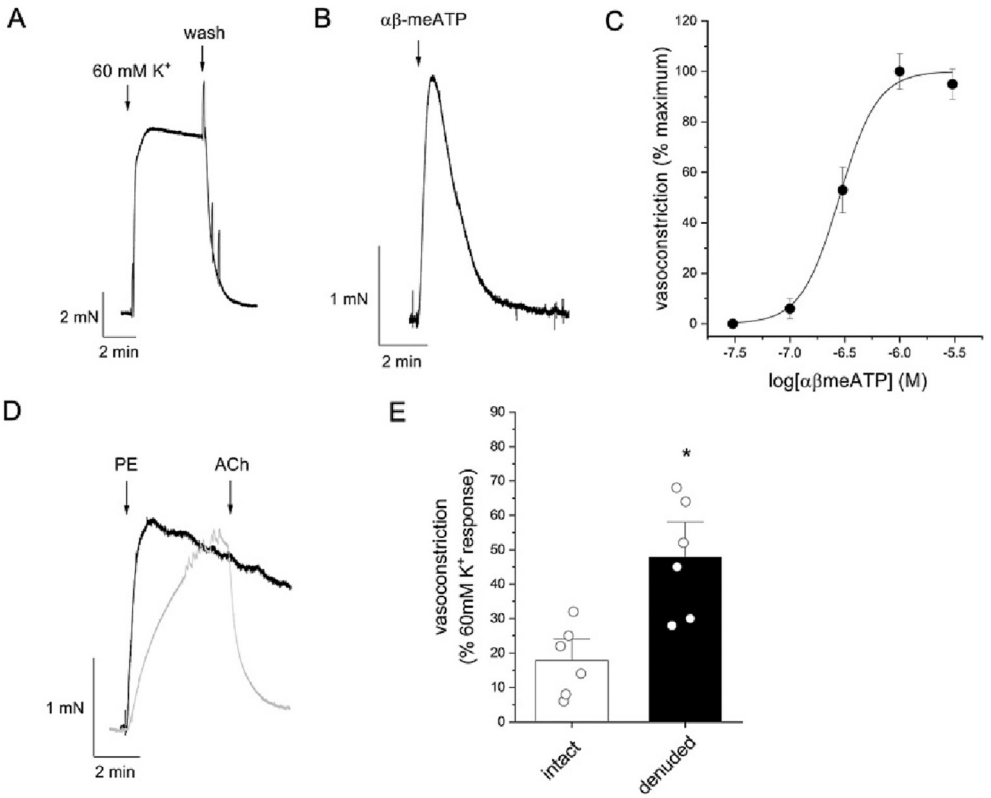

Fig. 1. αβme-ATP-evoked vasoconstriction in human small abdominal arteries. (A) representative trace showing sustained vasoconstriction evoked by 60 mM K^+ and subsequent washout and recovery. (B) Representative trace showing transient vasoconstriction evoked by 1 μM αβme-ATP. (C) Concentration-response relationship for αβme-ATP and magnitude of vasoconstriction. Data normalized to maximal response to αβme-ATP. (D) Representative trace showing functional assessment of endothelial denudation ($N = 6$). A control arterial ring (*black*) and denuded ring (*grey*) are constricted with phenylephrine (PE; 1 μM) followed by application of the endothelium-dependent vasodilator acetylcholine (ACh, 10 μM). (E) Magnitude of vasoconstriction in response to αβme-ATP (1 μM) in intact and endothelium denuded arterial rings. *$P <$ 0.05. Averaged data given as mean ± SEM with biological replicates.

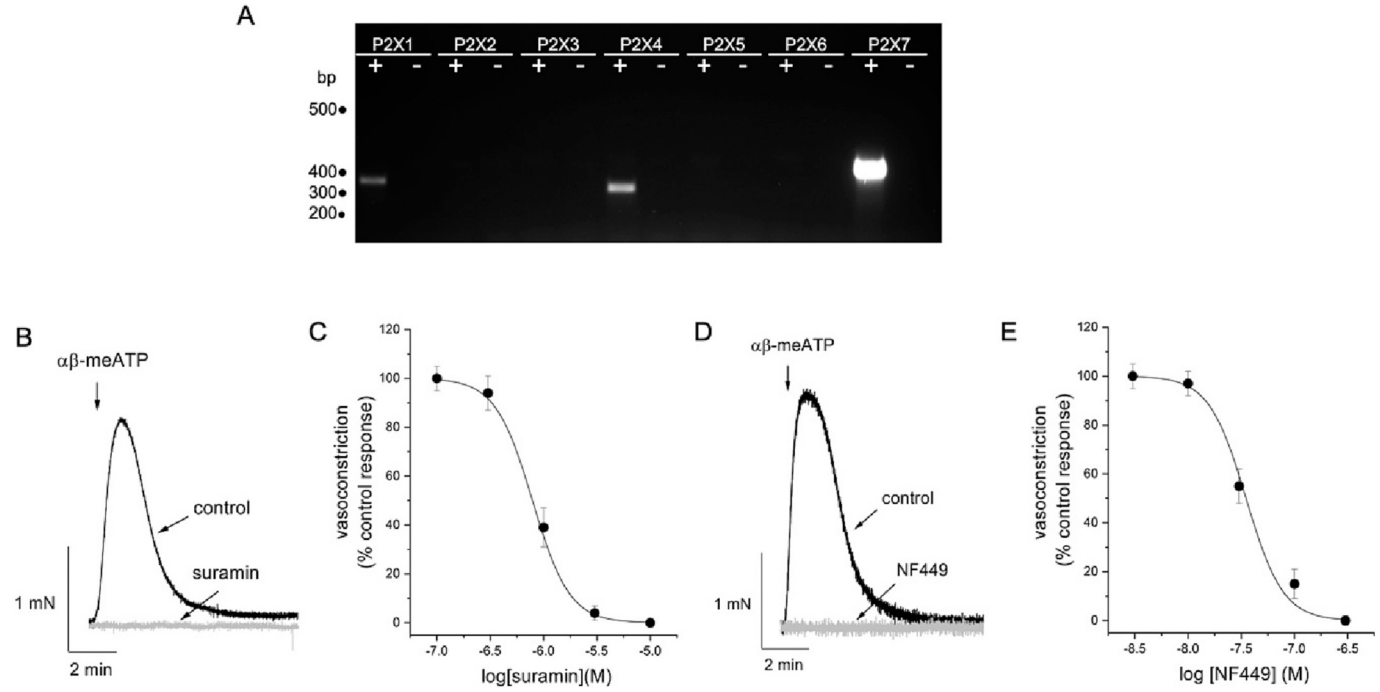

Fig. 2. αβme-ATP evoked vasoconstriction is mediated by P2X1 receptors. (A) RT-PCR analysis of P2X receptor mRNA transcripts expressed by human small abdominal arteries ($N = 6$). (+) with reverse transcriptase, (−) without reverse transcriptase to control for genomic DNA. (B) Representative trace showing vasoconstriction to αβme-ATP (300 nM) in the presence of vehicle control (*black*) or suramin (10 μM, 20 mins) (*grey*) ($N = 6$). (C) Concentration-inhibition curve for suramin and vasoconstriction evoked by αβme-ATP ($N = 6$). (D) Representative trace showing vasoconstriction to αβme-ATP (300 nM) in the presence of vehicle control (*black*) or NF449 (100 nM, 20 mins) (*grey*) ($N = 6$). (E) Concentration-inhibition curve for NF449 and vasoconstriction evoked by 1 μM αβme-ATP ($N = 6$). Averaged data given as mean ± SEM.

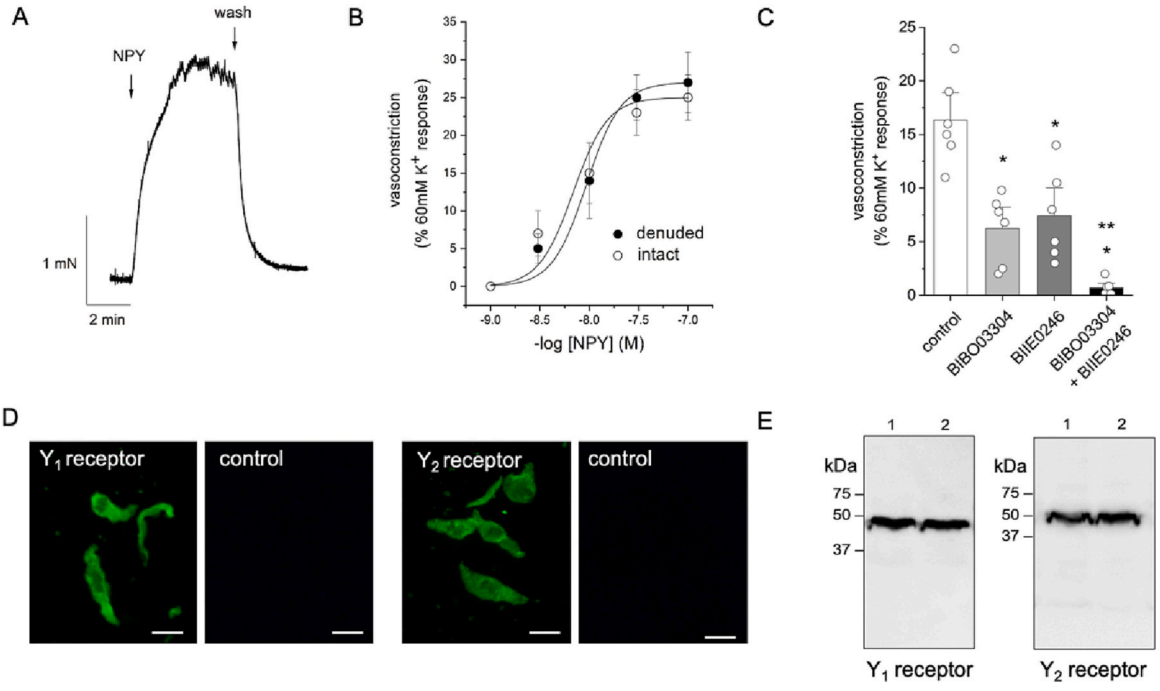

Fig. 3. NPY evokes vasoconstriction *via* activation of Y_1 and Y_2 receptors. (A) Representative trace showing sustained vasoconstrictive response to NPY (100 nM) followed by recovery to baseline after wash-out ($N = 6$). (B) Concentration-response curve for NPY and vasoconstriction in intact and endothelium-denuded arterial rings ($N = 6$). (C) Effect of BIBO03304 (10 nM, 20 min) and BIIE0246 (200 nM, 20 min), separate and in combination, on vasoconstriction evoked by NPY (100 nM) ($N = 6$). *$P < 0.05$ *vs* control, ** $P < 0.05$ *vs* single treatment groups. Averaged data given as mean ± SEM with biological replicates. (D) Detection of Y_1 and Y_2 receptor expression by immunocytochemistry in freshly isolated smooth muscle cells of human small abdominal arteries. Representative images are given ($N = 6$) with experiments performed in the absence of fluorophore-conjugated secondary antibody shown as *control*. Scale bar is 25 μm. (E) Representative Western blotting using abdominal artery lysates from two patients (1 + 2). Blotting using rabbit polyclonal antibodies against NPY Y_1 receptor (predicted M_w 44 kDa) and against NPY Y_2 receptor (predicted M_w 43 kDa).

subsequently applied 10 nM NPY when investigating modulatory effects of NPY on the αβme-ATP response. The maximal response to NPY was not significantly different to αβme-ATP, 21 ± 5% 60 mM K^+ response for αβme-ATP compared to 25 ± 4% 60 mM K^+ response for NPY ($N = 6$; $P > 0.05$ *vs* αβme-ATP). Unlike the vasoconstrictive response to αβme-ATP, endothelial denudation had no effect on the response to NPY (Fig. 3B). There was no significant difference in the sensitivity of denuded rings to NPY (EC_{50} 16.9 ± 0.6 nM, $N = 6$; $P > 0.05$ denuded *vs* intact rings) or magnitude of response at maximal NPY concentrations (25 ± 4% 60 mM K^+ response for intact rings; 27 ± 5% 60 mM K^+ response for denuded rings; $N = 6$, $P > 0.05$). The vasoconstrictive response to NPY was sensitive to both BIBO3304 (60.7 ± 6% inhibition, $N = 6$; Fig. 3C), a selective Y_1 receptor antagonist, and BIIE0246 (54.6 ± 5% inhibition, $N = 6$; Fig. 3C), a selective Y_2 receptor antagonist. BIBO03304 and BIIE0246 abolished the response to NPY (Fig. 3C), suggesting both Y_1 and Y_2 receptors contributed to NPY-evoked vasoconstriction in these arteries. Expression of Y_1 and Y_2 receptors was confirmed by immunocytochemistry of enzymatically isolated smooth muscle cells (Fig. 2E), and by western blotting of arterial lysates (Fig. 2F). Western blotting produced immunoreactive bands of the predicted size of approximately 44 kDa and 43 kDa for Y_1 and Y_2 receptors, respectively.

3.3. NPY facilitates P2X1 receptor-dependent vasoconstriction

We have previously identified a functional interaction between P2X1 and NPY in mouse resistance arteries, though the existence of similar mechanisms in human arteries is unknown. To this end, we examined the effect of pre-exposure to submaximal concentrations of NPY on a subsequent vasoconstrictive response to αβme-ATP. In these experiments, NPY potentiated the magnitude of vasoconstriction elicited by αβme-ATP (1.6 ± 0.2-fold increase, $N = 5$; Fig. 4A). Endothelial denudation removed the potentiating effect of NPY (Fig. 4B). In contrast to the direct vasoconstrictive response to NPY, there was apparent functional redundancy between Y_1 and Y_2 receptors regarding the facilitatory effect of NPY on αβme-ATP-evoked vasoconstriction. BIBO03304 or BIIE0246 applied separately had no significant effect on the facilitatory effect of NPY (Fig. 5A & B; $P > 0.05$ *vs* control response). However, when applied in combination BIBO03304 and BIIE0246 abolished the facilitatory effect of NPY (Fig. 5C; $P < 0.05$ *vs* control response).

4. Discussion

Our data reveals that NPY has a dual role in human small abdominal arteries, a direct vasomotor function, and a modulatory effect on the vasomotor action of P2X1 receptors. These results differ from our recent work on mouse small mesenteric arteries, where NPY has no direct vasoconstrictor function [8]. NPY has been shown to have direct vasoconstrictor effects in other human vascular beds including forearm arteries [25], but lacks direct vasomotor effects in pulmonary arteries [26]. This variability in the direct vasomotor effects of NPY extends to other species and different vascular beds [5,6,27,28]. Our findings suggest the direct vasoconstrictor effect of NPY in human small abdominal arteries is mediated by both Y_1 and Y_2 receptors, and that activation of both are required for maximal vasoconstriction. Both Y_1 and Y_2 receptors were expressed by smooth muscle cells of the human arteries studied, and the vasoconstrictor response of NPY was independent of functional endothelium. The expression of the Y_1 receptor is consistently observed in blood vessels [26,29,30], though Y_2 receptor expression is more variable. Though previously not described in humans as having vasoconstrictor function, the Y_2 receptor has been identified in mediating the post-junctional effects of sympathetic nerve stimulation in rat mesenteric arteries [31]. The signal transduction mechanisms by with Y receptor activation in vascular smooth muscle causes vasoconstriction are poorly defined, though some studies have revealed this is mediated *via* atypical activation of phospholipase C (PLC) *via* heteromeric G-protein βγ subunits [32]. In this scenario, the degree of PLC activation is dependent upon PLC and βγ subunit isoform [33], and may underlie the disparity of direct vasoconstrictor effects of NPY observed between species and vascular bed studied. In this study we used BIBO03304 and BIIE0246 to identify functional roles of Y_1 and Y_2 receptors, respectively. BIBO03304 is a non-peptide selective antagonist of the Y_1 with an approximate IC_{50} of approximately 1 nM at the human orthologue [34]. [^{125}I]NPY binding studies in cells overexpressing NPY

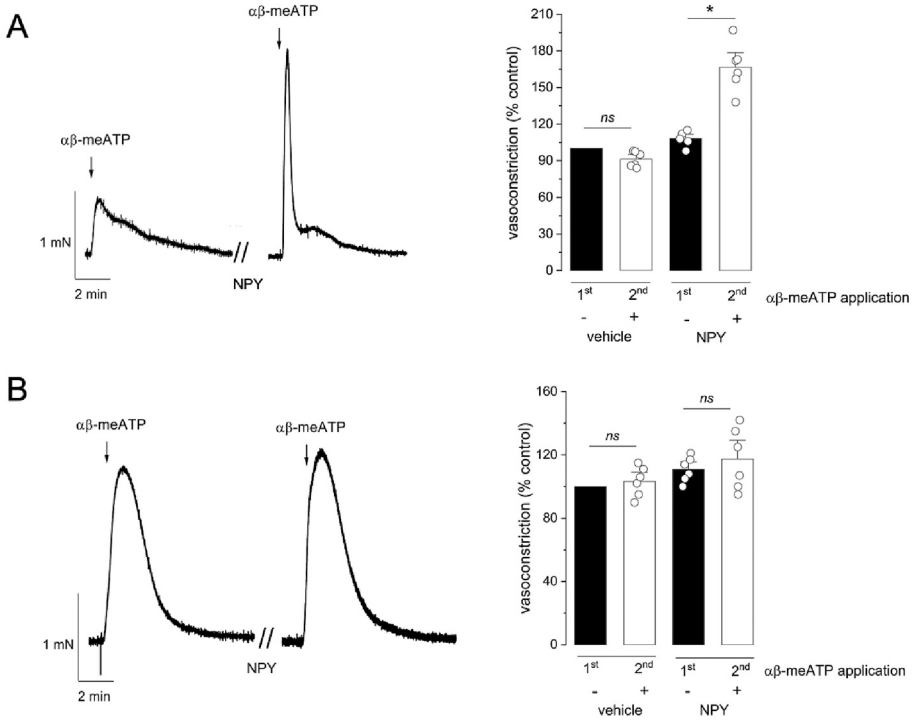

Fig. 4. Modulatory effects of NPY in intact and endothelium-denuded arterial rings. (A) The left panel shows representative traces of paired vasoconstriction response to αβme-ATP (300 nM) with exposure to NPY (10 nM, 15 min) between applications in arterial rings with intact endothelium. The right panel shows averaged magnitudes of paired (1st response, 2nd response) vasoconstrictor responses to αβme-ATP with exposure to vehicle control or NPY. (B) The left panel shows representative traces of paired vasoconstriction response to αβme-ATP (300 nM) with exposure to NPY (10 nM, 15 min) between applications in arterial rings where endothelium has been denuded. The right panel shows averaged magnitudes of paired (1st response, 2nd response) vasoconstrictor responses to αβme-ATP with exposure to vehicle control or NPY. *$P < 0.05$, *ns* denotes no significant difference. Averaged data given as mean ± SEM with biological replicates.

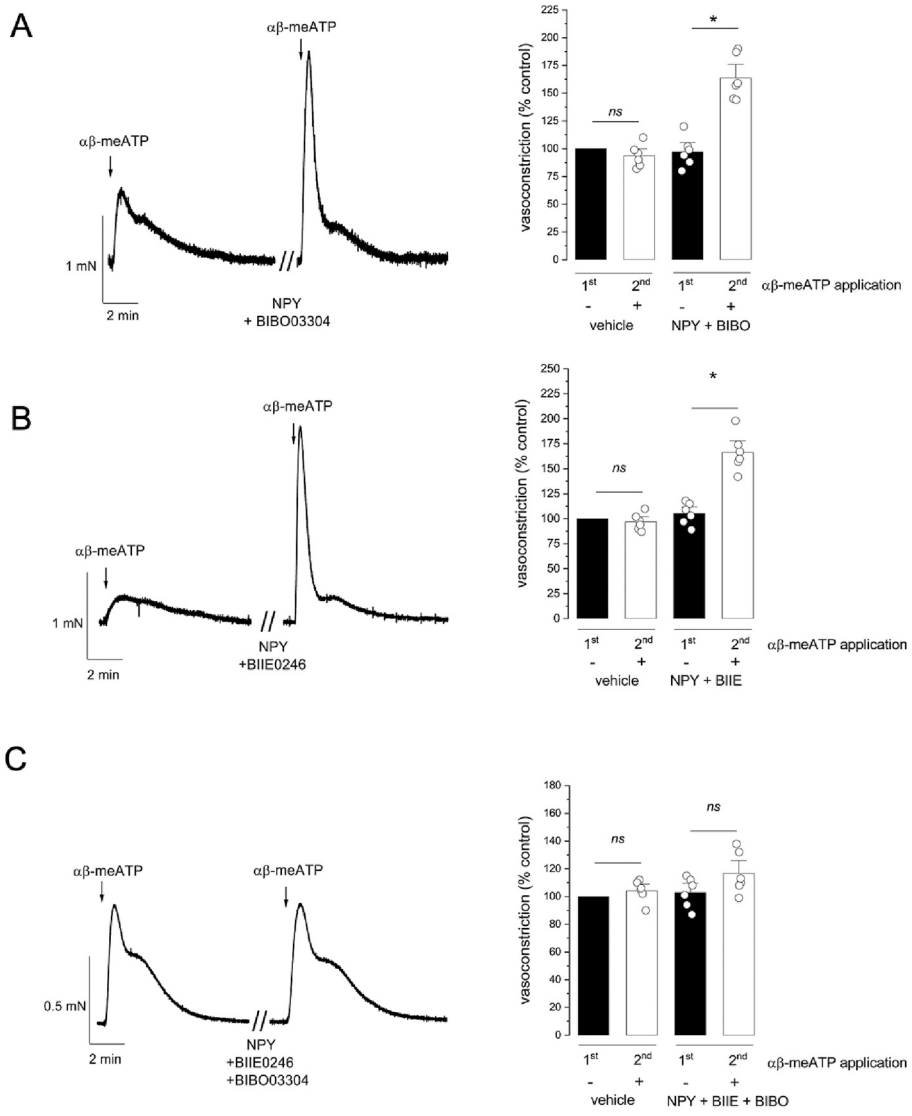

Fig. 5. NPY facilitates P2X1 receptor-dependent vasoconstriction through activation of Y_1 or Y_2 receptors. (A) The left panel shows representative traces of paired vasoconstriction response to αβme-ATP (300 nM) within exposure to BIBO03304 (10 nM, 20 min) and NPY (10 nM, 15 min) between applications in arterial rings. The right panel shows averaged magnitudes of paired (1st response, 2nd response) vasoconstrictor responses to αβme-ATP with exposure to vehicle control or NPY plus BIBO03304 ($N = 6$). (B) The left panel shows representative traces of paired vasoconstriction response to αβme-ATP (300 nM) with exposure to BIIE03304 (200 nM, 20 min) and NPY (10 nM, 15 min) between applications in arterial rings. The right panel shows averaged magnitudes of paired (1st response, 2nd response) vasoconstrictor responses to αβme-ATP with exposure to vehicle control or NPY plus BIIE03304 ($N = 6$). (C) The left panel shows representative traces of paired vasoconstriction response to αβme-ATP (300 nM) with exposure to BIIE03304 and BIBO03304 (200 nM and 10 nM, respectively, 20 min), and NPY (10 nM, 15 min) between applications in arterial rings. The right panel shows averaged magnitudes of paired (1st response, 2nd response) vasoconstrictor responses to αβme-ATP with exposure to vehicle control or NPY plus BIIE03304 and BIBO03304 ($N = 6$). *$P < 0.05$, *ns* denotes no significant difference. Averaged data given as mean ± SEM with biological replicates.

Y receptors demonstrates BIBO03304 is a highly selective antagonist of the Y_1 receptor, with no effects of BIBO03304 up to 10 μM at Y_2, Y_4 or Y_5 receptors [34]. BIIE0246 is also a non-peptide antagonist [35], selectively antagonising Y_2 receptors with reported IC_{50} values between 3 and 15 nM [36,37]. Binding assays with [^{125}I]$PYY_{3–36}$ in HEK293 cells overexpressing Y_1, Y_2, Y_4 or Y_5 receptors revealed nanomolar potency and high selectivity for Y_2 receptors, with no effects of BIIE0246 on other receptor observed up to 10 μM [37].

Therefore, the concentrations of BIBO03304 and BIIE0246 applied in this study will fully antagonise Y_1 and Y_2 receptors, respectively.

This study demonstrates a key role for the P2X1 receptor in mediating the vasoconstrictor responses to αβmeATP in human arteries. αβmeATP infusion is known to cause a strong pressor effect *in vivo* in rodents [38]. Our previous work has also demonstrated that P2X1 receptors are the post-junctional receptors mediating the vasoconstrictor response to sympathetic nerve stimulation in mouse mesenteric arteries [8]. Work by others in rodents also supports a post-junctional role of P2X1 [18,39,40]. However, our findings represent the first demonstration that P2X1 receptors mediate vasoconstriction in human abdominal arteries. Previous work in human omental arteries have demonstrated that αβmeATP-evoked vasoconstrictions are sensitive to NF449, suggesting a predominant role of P2X1 [21]. Whilst vasoconstrictive responses to 60 mM K^+ in this study were sustained before washout, responses to αβmeATP were transient and decayed back to baseline in the presence of agonist. P2X receptor subtypes delays varied rates of desensitisation in electrophysiological recordings [17], with P2X1 displaying the most rapid rate of desensitisation. The profile of desensitisation is therefore in-keeping with a role of P2X1 in mediating αβmeATP-evoked vasoconstriction in these vessels. The half-maximal concentration of suramin is very close to that reported for P2X1 [41]. The differential sensitivity to suramin and NF449 is also observed during αβme-ATP-evoked vasoconstriction in rat pulmonary arteries [42]. The findings of this study also demonstrate the expression of P2X4 and P2X7 in human small abdominal arteries. Whilst αβmeATP is a partial agonist at human P2X4, αβmeATP does not activate human P2X7 [16], and NF449 does not antagonise human P2X7 at the concentrations used in this study [43]. Therefore, it is not anticipated that P2X7 does not contribute to αβmeATP-evoked vasoconstriction. P2X4 has been reported as a vascular smooth muscle ion channel previously in rodent cerebral arteries [19], though it is reported to have a predominant role in vascular endothelium during flow-dependent control of vascular tone [44]. It is interesting to compare the endothelial-dependency of αβmeATP- and NPY-evoked vasoconstriction, as the effect of αβmeATP is enhanced by removal of the endothelial whereas the response to NPY is not. One possible interpretation is that the activity of smooth muscle P2X1 is under the negative influence of endothelial-derived vasodilatory

substances, which has been shown for other vasoconstrictor responses in human blood vessels including cerebral arteries [45]. However, other studies have suggested a vasodilatory role of P2X1 receptors expressed by vascular endothelium [46]. We currently cannot discriminate between these potential two mechanisms in understanding why removal of the endothelium enhances P2X1 receptor-dependent vasoconstriction.

In addition to a direct role in stimulating vasoconstriction, our data demonstrate a role for NPY in facilitating P2X1 receptor-dependent vasoconstriction. Unlike the vasoconstrictor response to NPY that requires activation of both Y_1 and Y_2 receptors, our data suggests redundancy between Y_1 and Y_2 receptor activation in the facilitation of P2X1 receptor-dependent vasoconstriction. The mechanism by which NPY facilitates P2X1 receptor-dependent vasoconstriction is also not clear in human arteries. Possible mechanisms include involvement of L-type Ca^{2+} channels as described previously [8]. However, it may also involve promiscuous coupling of Y receptors to G_i heteromeric G-proteins. This has been described previously in amygdala neurons as a mechanism by which NPY modulates the activity of $GABA_A$ and NMDA receptors [47], though such signal transduction is unclear for human arteries. Interestingly, enhanced G_i-dependent signalling would be predicted to reduce Epac (exchange protein directly activated by cAMP) activity, and recently Epac activity has been shown in heterologous expression systems to negatively regulate P2X1 [48]. However, such processes required further investigation in human arteries and vascular smooth muscle.

5. Conclusions

NPY has a direct vasoconstrictor effect in human small abdominal arteries mediated by both Y_1 and Y_2 receptors to achieve maximal responses. P2X1 mediates vasoconstriction in human arteries, and NPY facilitates this *via* Y_1 and Y_2 receptors, however there is redundancy between Y_1 and Y_2 receptor activation to achieve facilitation.

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CRediT authorship contribution statement

Maria del Carmen Gonzalez-Montelongo: Data curation, Formal analysis, Writing – review & editing. **Jessica Lauren Meades:** Data curation, Formal analysis, Writing – review & editing. **Anna Fortuny-Gomez:** Data curation, Formal analysis, Writing – review & editing. **Samuel J. Fountain:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

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