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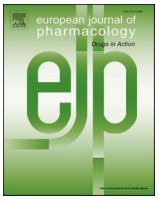

Is there a role for biogenic amine receptors in mediating β-phenylethylamine and RO5256390-induced vascular contraction?

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ABSTRACT

Background: Substantial evidence indicates trace amines can induce vasoconstriction independently of noradrenaline release. However, the mechanism underlying noradrenaline-independent vasoconstrictor responses to trace amines has not yet been established. This study evaluates the role of trace amine-associated receptor 1 (TAAR1) and other biogenic amine receptors in mediating β-phenylethylamine and the TAAR-1 selective agonist RO5256390-induced vasoconstriction.

Methods: Vasoconstrictor responses to β-PEA and the TAAR1-selective agonist, RO5256390 were assessed *in vitro* in endothelium-denuded aortic rings and third-order mesenteric arteries of male Sprague Dawley rats.

Results: β-PEA and RO5256390 induced concentration-dependent vasoconstriction of aortic rings but not third-order mesenteric arteries. Vasoconstrictor responses in aortic rings were insensitive to antagonists of 5-HT. The murine-selective TAAR1 antagonist, EPPTB, had no effect on either β-PEA or RO5256390-induced vasoconstriction. The α_1-adrenoceptor antagonist, prazosin, and the α_2-adrenoceptor antagonist, yohimbine, induced a shift of the β-PEA concentration response curve too small to be ascribed to antagonism of α_1-or α_2-adrenoceptors, respectively. The α_2-adrenoceptor antagonist atipamezole had no effect on β-PEA or RO5256390-induced vasoconstriction.

Conclusion: Vasoconstrictor responses to trace amines are not mediated by classical biogenic amine neurotransmitter receptors. Insensitivity of β-PEA vasoconstrictor responses to EPPTB, may be explained by its low affinity for rat rather than murine TAAR1. Therefore, TAAR1 remains the most likely candidate receptor mediating vasoconstrictor responses to trace amines and that prazosin and yohimbine have low affinity for TAAR1.

1. Introduction

Originally, it was thought that vascular responses to the trace amines, including *p*-tyramine, tryptamine, β-phenylethylamine (β-PEA) and *p*-octopamine, were a result of indirect sympathomimetic activity, promoting the efflux of noradrenaline from sympathetic neurones, that activate adrenoceptors (Broadley, 2010; Day, 1967). However, residual tyramine pressor responses are observed following noradrenaline depletion by reserpine in cats (Burn and Rand, 1958), rabbit (Hudgins and Fleming, 1966), rat aorta (Maling et al., 1971) and atria (Rice et al., 1987). Structural similarities between trace amines and biogenic amines (Flatmark, 2000), raise the possibility that vascular responses to trace amines could be mediated by direct sympathomimetic activity. This hypothesis is supported by the finding that vasoconstrictor responses to tryptamine and octopamine can be abolished by antagonists for 5-HT (Stollak and Furchgott, 1983) and α_1-adrenoceptors (Broadley et al., 2013; Fehler et al., 2010), respectively. However, vasoconstrictor responses to β-PEA, tyramine and tryptamine in rat aortic rings were resistant to blockade of $\alpha_{1/2}$-adrenoceptors, β_2-adrenoceptors, inhibition of neuronal transport and inhibition of monoamine oxidase B (Broadley et al., 2013; Fehler et al., 2010).

The discovery of trace-amine-associated receptors (TAARs) in 2001 (Borowsky et al., 2001; Bunzow et al., 2001) raises the possibility that they may mediate vascular responses to trace amines. In support of this, TAAR1 protein and mRNA is expressed in rat aorta (Fehler et al., 2010). However, the lack of TAAR1-selective pharmacological tools has hampered efforts to investigate its potential involvement in trace amine-induced vasoconstriction. As a result, previous studies have compared the potency orders of trace amines (Broadley et al., 2013;

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Abbreviations

5-HT	5-hydroxytryptamine
β-PEA	β-phenylethylamine
CRC	concentration response curve
DMSO	Dimethyl Sulfoxide
EPPTB	N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide
PE	(R)- (−)-phenylephrine hydrochloride
TAAR	trace amine-associate receptor

Fehler et al., 2010) with those obtained in TAAR1 expressing cell lines (Bunzow et al., 2001). Although a valid method, the potency order for vasoconstriction does not fully recapitulate that of cell lines (Broadley et al., 2013). However, overexpression and artificial coupling of TAAR1 to adenyl cyclase in cell lines (Borowsky et al., 2001; Lindemann and Hoener, 2005) may introduce differences in the relative affinity of the trace amines for TAAR1. TAAR1 displays considerable sequence divergence across species leading to the emergence of species-specific pharmacological profiles (Vallender et al., 2010). Human and rat TAAR1 share 76% sequence homology and a similar pharmacological profile (Wainscott et al., 2007), with β-PEA having similar potency between receptor forms. In recent years, a few selective full and partial TAAR1 agonists and a single TAAR1 antagonist have been developed (Bradaia et al., 2009; Revel et al. 2011, 2012, 2013). The aim of this study is to ascertain whether the trace amine, β-PEA and TAAR1-selective agonist, RO5256390, mediate vasoconstriction in the rat aorta by stimulation of TAAR-1 or other biogenic amine receptors.

2. Materials and methods

2.1. Animals

All animal studies are reported in compliance with ARRIVE guidelines (Percie du Sert et al., 2020). The effect of the oestrous cycle on trace amine-induced vascular responses is unknown. Therefore, to reduce experimental variability male Sprague Dawley rats (350–750 g) obtained from Charles River (Harlow, United Kingdom) were utilised. Rats were maintained in conventional animal housing, subjected to a 12-h light-dark cycle (8am-8pm) at a room temperature of 21°C and humidity of 55 g kg^{-1}. All animal care and experimentation were performed in compliance with the Animals (Scientific Procedures) Act 1986 and were approved by the UK Home Office (licence number: PPL2315370).

2.2. Rat isolated aortic rings

Rats were euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbitone (Merial, Woking UK). Death was confirmed by cessation of circulation. The thoracic aorta was removed, and the perivascular adipose tissue removed before cutting into ring sections approximately 3–5 mm in length. A fixed and mobile hook were then passed through the ring sections, the fixed hook was then secured inside a 20 mL organ bath. Organ baths were filled with prewarmed (37°C) Krebs bicarbonate buffer which was continuously gassed with CO_2/O_2 (5%/95%, BOC gasses, Guildford, UK). Krebs bicarbonate buffer was made up in distilled water and comprised of: NaCl (118 mM), KCl (4.7 mM), $NaHCO_3$ (25 mM), $MgSO_4 \bullet 7H_2O$ (1.2 mM), KH_2PO_4 (1.2 mM) $CaCl_2 \bullet 2H_2O$ (2.5 mM) and glucose (10 mM). The mobile hook was connected to an isometric transducer (Dynamometer UF1, 57 g sensitivity range, Pioden Controls, Ltd Canterbury, UK) and a resting tension of 1.5 g was applied. Isometric tension was measured and displayed on a computer using Powerlab chart 5 software (AD instruments, Oxford, UK). For all aortic ring experiments, the endothelium was removed prior to mounting in organ baths by gentle rolling of the aortic ring around a metal wire. After successful mounting, vessels were allowed to equilibrate for 1 h prior to commencing experiments.

2.3. Aortic rings experimental procedure

Following a 1-h equilibration period, aortic rings were washed with prewarmed Krebs bicarbonate solution. Pre-warmed high potassium (60 mM) Krebs bicarbonate solution was then added to organ baths to contract tissue and confirm viability. High potassium Krebs buffer was made by adjusting the NaCl concentration from 118 mM to 62.7 mM and the KCl concentration to 60 mM to maintain iso-osmolarity. At the peak of contraction, the absence of a functional endothelium was confirmed by a lack of relaxation to 100 μM carbachol. After washout and equilibration, cumulative concentration-response curves (CRCs) were obtained for contractile responses to β-PEA or agonists in the absence or presence of antagonists or inhibitors. Antagonists or inhibitors were added 15 min prior to constructing a CRC. To construct a CRC, successive concentrations of agonist were added in half logarithmic increments with each response being allowed to plateau before subsequent bolus addition. Following drug washout, tissue viability was confirmed using high potassium Krebs bicarbonate solution to contract tissue.

2.4. Rat isolated third order mesenteric arteries

Rats were euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbitone (Merial, Woking UK). Following confirmation of death by cessation of circulation, the entire mesenteric bed and attached small intestine was isolated and removed. The mesenteric bed was then pinned out in a petri dish in ice-cold Krebs-bicarbonate solution. Third-order branches of the arterial tree were then identified as per the method by Spiers and Padmanabhan (2005) and cleared of connective tissue and fat under a dissection microscope. Using straight spring scissors (Inter-Focus Ltd, Linton, UK) a small incision was made in the vessels through which the vessel was cannulated with a 40 μM stainless steel wire (Danish Myo Technologies, Denmark). The vessel and wire were then carefully removed and transferred to a petri-dish containing ice-cold Krebs-bicarbonate solution where it was cut to a length of 2 mm. The vessel was then mounted in a Mulvany-Halpern myograph chamber (Models 420 A and 620 M, Danish Myo Technologies, Denmark) containing pre-warmed (37°C) Krebs bicarbonate solution that was continually supplied with a mixture of 95% O_2 and 5% CO_2. At this point, for all experiments, the endothelium was removed by passing a human hair through the lumen of the vessel. Vessels were allowed to equilibrate for 30 min prior to being normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany and Halpern, 1977). The mean vessel diameter under these conditions was 279 ± 6.6 μM (n = 59). Vessels were left for a further 30 min before experiments commenced.

2.5. Thin wire myography experimental protocol

Following a 30-min incubation period, blood vessels were washed with pre-warmed Krebs bicarbonate solution. Pre-warmed high potassium (60 mM) Krebs-bicarbonate solution was then added to organ baths to contract tissue and confirm viability. Following wash out, the tissue was then pre-constricted with 10 μM phenylephrine. At the peak of contraction, the absence of a functional endothelium was confirmed by a lack of relaxation to 10 μM carbachol. Vessels were considered endothelium-denuded following a relaxation of ≤25 % of the contractile response to phenylephrine.

2.6. Data analysis

The plateau for each contraction to each concentration of agonist

was measured from the baseline prior to the CRC. Each contraction was then expressed as a percentage of the maximum contraction observed in response to high potassium (60 mM KCl) Krebs-bicarbonate solution. Contractile responses were plotted as the mean response ± standard error of the mean (S.E.M). Concentration response curves were fitted to the following logistic equation with no constraints:

$$Y = E_{Min} + \frac{(E_{Max} - E_{Min})}{\left(1 + 10^{\left(LogEC_{50} - [A]\right) \times Hill\ Slope}\right)}$$

where Y is the response, E_{Min} is the expected response at a concentration of 0, E_{Max} is the expected response as the concentration goes to infinity and [A] is the concentration of agonist. E_{Max} values are reported as a percentage of the contractile responses to 60 mM KCl ± S.E.M. Curve-

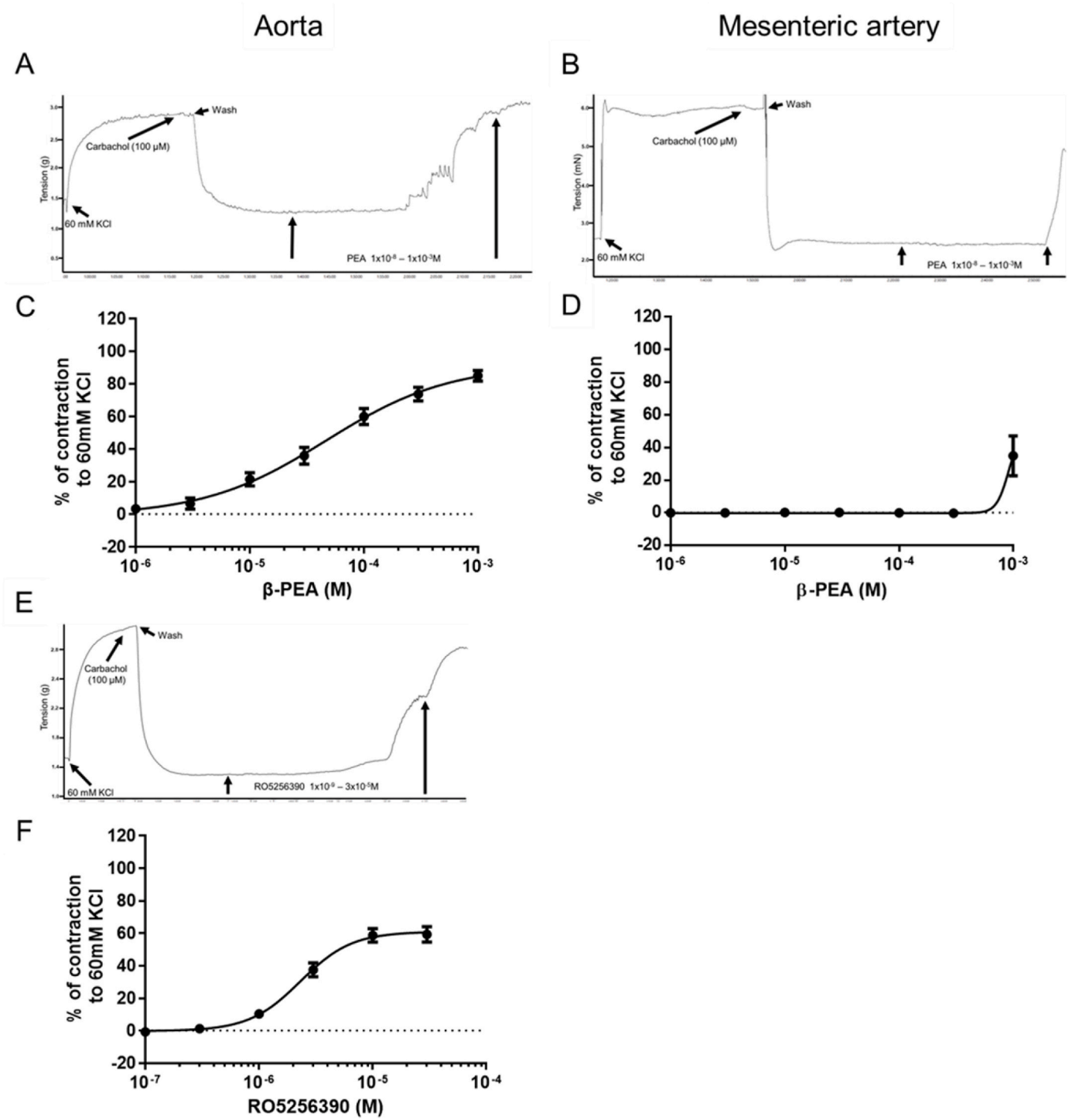

Fig. 1. A comparison of β-PEA CRCs in rat aortic rings and third order mesenteric arteries and RO5256390 in rat aortic rings. Representative trace of β-PEA-induced contractile responses in (A) aortic rings and (B) third-order mesenteric arteries. Representative trace of RO5256390-induced contractile responses in (E) aortic rings. β-PEA CRCs were obtained (C, n = 31) in rat aortic rings and (D, n = 7) third-order mesenteric arteries. RO5256390 CRCs were obtained (F, n = 31) in rat aortic rings Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60 mM KCl.

fitting was carried out using Graphpad prism software (Graphpad software, San Diego). Negative logEC_{50} values were obtained for each individual curve. EC_{50} values were calculated from the -logEC_{50} and are reported as the mean value with 95% confidence intervals. Mean EC_{50}, and E_{Max} values were compared by unpaired, two-tailed Student's t-test or one-way ANOVA with Dunnett's multiple comparison post-hoc test as appropriate. P-values $\leq$0.05 were considered statistically significant. Sample sizes subjected to statistical analysis contain at least 5 animals per group where n represents the number of rats providing aortas. The degree of shift or dose ratio induced by antagonists was calculated using the following equation:

$$Dose\ ratio = \frac{EC_{50}\ with\ antagonist}{EC_{50}\ no\ antagonist}$$

where a maximum contractile response was not achieved the EC_{20} (20% of the contractile response to 60 mM KCl) was used to calculate the dose ratio. P $\leq$ 0.05 was accepted as being statistically significant.

2.7. Drugs and solutions

(R)- (−)-phenylephrine hydrochloride (PE), serotonin hydrochloride (5-hydroxytryptamine, 5-HT), cinanserin hydrochloride, carbamoylcholine chloride (carbachol) atipamezole hydrochloride, medetomidine hydrochloride and clonidine hydrochloride were purchased from Tocris Bioscience (Abingdon, United Kingdom). Prazosin hydrochloride, yohimbine hydrochloride, (S)-4-((S)-2-phenyl-butyl)-4,5-dihydro-oxazol-2-ylamine (RO5256390), N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB) and β-phenylethylamine hydrochloride (β-PEA) were purchased from Sigma Aldrich (Poole, United Kingdom). All chemicals for Krebs-bicarbonate buffer were of analytical grade and purchased from Fischer Scientific (Loughborough, United Kingdom). All drugs were dissolved in Krebs-bicarbonate buffer with the exception of prazosin hydrochloride, RO5256390 and EPPTB which were dissolved in 100% (vol/vol) dimethyl sulfoxide (DMSO; Fischer Scientific, United Kingdom) then diluted to desired concentration with Krebs bicarbonate buffer. For the TAAR1-selecive antagonist EPPTB, the concentration of DMSO in organ baths did not exceed 0.25%. For all other antagonists, the concentration of DMSO did not exceed 0.05%. Due to the insoluble nature of the TAAR1-selective antagonist EPPTB, the maximum achievable concentration in which the antagonist remained in solution once added to organ baths was 5 μM.

3. Results

3.1. Contractile effects of β-PEA and RO5256390 in rat aortic rings and isolated third-order mesenteric arteries

In endothelium-denuded rat third-order mesenteric arteries, a contractile response to β-PEA was only observed at the maximum concentration of β-PEA (1 mM, Fig. 1B–D). The mean contractile response at this concentration was 35 ± 12 %. A total of 2/7 vessels failed to contract to β-PEA even at the maximum concentration of β-PEA. As a complete CRC was not obtained in third-order mesenteric arteries neither an EC_{50} nor E_{Max} were calculated. As it is clear that β-PEA-induced contraction is substantially different between aorta and mesenteric vessels, all further experiments were carried out using rat aortic rings

β-PEA-induced concentration-dependent contraction of endothelium-denuded aortic rings (Fig. 1A–C). In aortic rings β-PEA had an EC_{50} 42 (24–74) μM and E_{Max} of 86 ± 4%. The selective TAAR1 agonist, RO5256390 also induced concentration-dependent contraction of endothelium-denuded aortic rings (Fig. 1). The EC_{50} for this response was 2.7 (1.9–3.7) μM with an E_{Max} of 61 ± 4%.

3.2. Sensitivity of β-PEA and RO5256390 to TAAR1 antagonism by EPPTB

EPPTB had no significant effect on the TAAR1 selective agonist, RO5256390, CRCs (Fig. 2D). In the presence of EPPTB, the EC_{50} (0.7 (0.2–2.8) μM and $E_{Max,}$ (82 ± 10%) were not significantly different (one-way ANOVA) from those obtained in the absence of EPPTB (0.9 (0.4–2.3) μM, 75 ± 8%). DMSO (0.25% vol/vol) used as a vehicle control induced a leftward shift of the RO5256390 CRC (Fig. 2C). However, the EC_{50} (0.9 (0.4–2.3) μM and E_{Max} (75 ± 8%) of RO5256390 in the presence of DMSO were not found to be significantly different (one-way ANOVA) from those obtained in the absence of DMSO of (2.7 (1.9–3.7) μM, 61 ± 4%).

Addition of the TAAR1-selective antagonist EPPTB (5 μM) had no significant effect on the CRC for β-PEA compared with the vehicle control (Fig. 2B). However, DMSO (0.25% vol/vol), used as a vehicle control for EPPTB, appeared to reduce β-PEA-induced contractile responses and shifted the CRC to the right (Fig. 2A) In the presence of EPPTB or DMSO (0.25% vol/vol) vehicle control a maximal response was not obtained therefore an accurate EC_{50} value was not obtained. At the maximum concentration of β-PEA (1 mM) used, the mean contractile response to β-PEA was 57 ± 7% in the presence of DMSO vehicle control. This was not found to be significantly different from the corresponding response in the presence of EPPTB of 79 ± 11%.

3.3. Sensitivity of β-PEA-induced contractile responses to antagonism of 5-HT receptors

The 5-HT_2 receptor antagonist, cinanserin (100 nM), induced a significant 14-fold rightward shift of 5-HT-induced CRC in endothelium-denuded aortic rings (Fig. 3A). Cinanserin significantly (P $\leq$ 0.001) increased the EC_{50} from 0.7 (0.4–1.2) μM to 5.8 (2.5–14.0) μM. The observed degree of shift in the EC_{50} was approximately 14-fold. By contrast, cinanserin had no significant effect on the CRC for β-PEA (Fig. 3B). The EC_{50} and E_{Max} of β-PEA in the presence of cinanserin were 37 (7–200) μM and 84 ± 5 %, respectively. Neither were significantly different from the EC_{50} (42 (24–74) μM and E_{Max} (86 ± 4%) obtained in the absence of cinanserin.

As 5-HT receptors had no involvement in β-PEA-induced contraction, the role of 5-HT receptors in RO5256390-induced contraction was not assessed.

3.4. Sensitivity of β-PEA-induced contractile responses to antagonism of α_1 -adrenoceptors

The α_1-adrenoceptor antagonist prazosin (1 μM) induced a large significant rightward shift of the phenylephrine CRC (Fig. 4A). In the absence of prazosin, the EC_{50} and EC_{20} (20% of the contractile response to 60 mM KCl) for the phenylephrine CRC were 15 (9–26) nM and 4 (2–8) nM, respectively. As a complete CRC was not obtained in the presence of prazosin, an EC50 was not estimated. To calculate the observed degree of shift the EC_{20} of 6 (4–8) μM was used. Prazosin induced an approximately 6500-fold rightward shift of the phenylephrine CRC.

Prazosin induced a small rightward shift (approximately 5-fold) of the β-PEA CRC (Fig. 4C). The EC_{50} of the β-PEA CRC in the presence of prazosin 366 (264–508) μM was significantly greater (P $\leq$ 0.01) than the EC_{50} obtained in the absence of prazosin 42 (24–74) μM. However, prazosin had no effect on the E_{Max} of β-PEA (90 ± 8 %) compared with the absence of prazosin (86 ± 4%).

There was no observable shift of the RO5256390 CRC in the presence of prazosin (Fig. 4E). The EC_{50} and E_{Max} in the presence of prazosin were 2 (1–4) μM and 67 ± 9%, respectively. Neither were significantly different from the values obtained in the absence of prazosin of 2.7 (1.9–3.7) μM and 61 ± 4%, respectively.

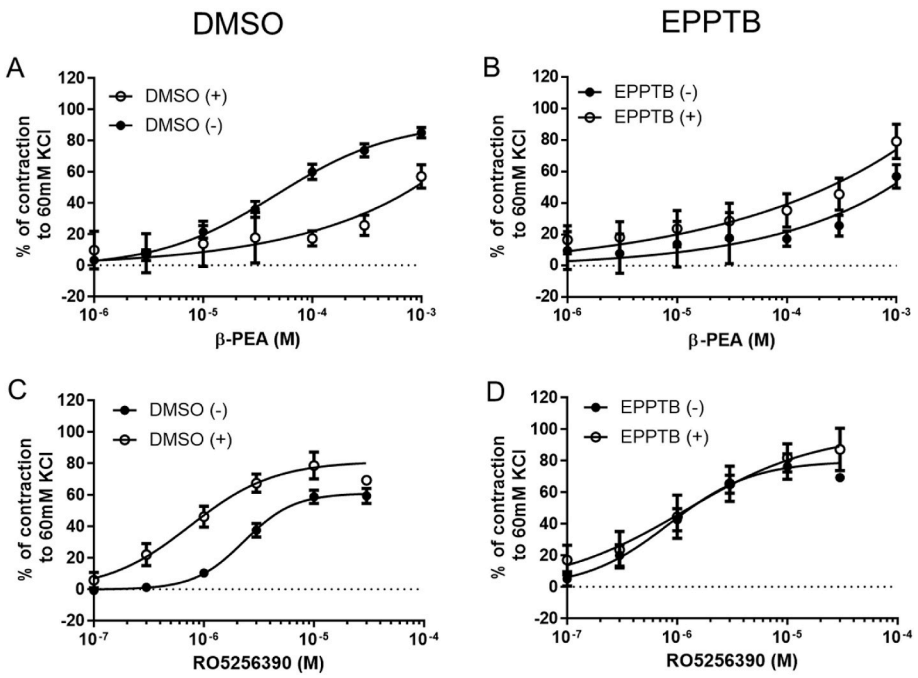

Fig. 2. The effect of DMSO (0.25% vol/vol, A,C) or the murine selective TAAR1 antagonist, EPPTB (B, D) on β-PEA (A, B) and RO5256390 (C, D) CRC's. β-PEA CRCs were obtained (A) in the absence (● n = 23) or presence of DMSO (○ n = 6). β-PEA CRCs were obtained (B) in the absence (● n = 6, 0.25% DMSO vehicle control)) or presence (○ n = 7) of 5 μM EPPTB. RO5256390 CRCs were obtained (C) in the absence (● n = 31) or presence (○ n = 12) of DMSO. RO5256390 CRCs were obtained (D) in the absence (● n = 12, 0.25% DMSO control) or presence (○ n = 7) of 5 μM EPPTB. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60 mM KCl.

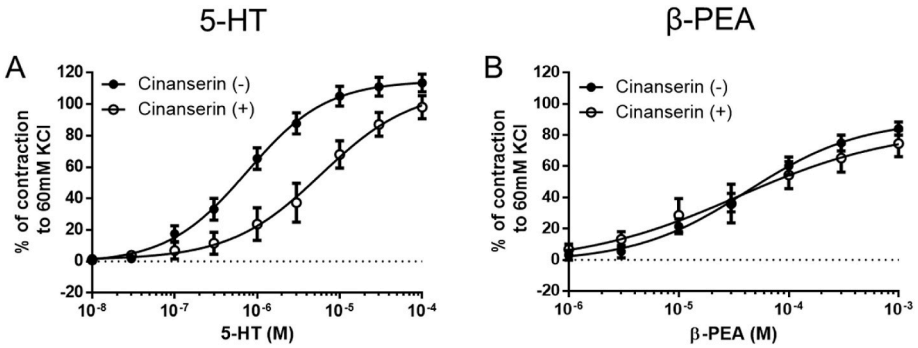

Fig. 3. The effect of 5-HT receptor antagonism (A, B) on 5-HT and β-PEA CRCs5-HT CRCs were obtained (A) in the absence (● n = 10) or presence (○ n = 6) of cinanserin. β-PEA CRCs were obtained (B) in the absence (● n = 18) or presence (○ n = 5) of 100 nM cinanserin. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60 mM KCl.

3.5. Sensitivity of β-PEA-induced contraction to antagonism of α_2-adrenoceptors

The α_2-adrenceptor antagonist yohimbine (1 μM) induced an approximately 7-fold rightward shift of the clonidine CRC (Fig. 4B). Clonidine had an EC_{50} and E_{Max} of 12 (7–22) μM and 68 ± 7 %, respectively. In the presence of yohimbine, the EC_{50} of clonidine was significantly increased ($P \leq 0.001$) to 85 (54–134) μM whereas the E_{Max} was not significantly affected (78 ± 12 %).

Yohimbine induced an approximately 7-fold rightward shift of the β-PEA CRC (Fig. 4D). In the presence of yohimbine the EC_{50} of β-PEA was 63 (12–333) μM. This was significantly greater ($P \leq 0.01$) than the EC_{50} obtained in the absence of yohimbine 14 (4–50) μM. The E_{Max} of β-PEA in the presence of yohimbine (98 ± 17 %) was not significantly different from that obtained in the absence of yohimbine (86 ± 5 %).

No statistically significant shift of the RO5256390 CRC was observed with yohimbine (Fig. 4F). The EC_{50} and E_{Max} in the presence of yohimbine were 3 (2–5) μM and 73 ± 8%, respectively. Neither were significantly different from the values obtained in the absence of yohimbine of 2 (1–3) μM and 73 ± 7%, respectively.

As yohimbine induced an equal shift of the β-PEA and clonidine CRCs, the role of α_2-adrenoceptors was further investigated using an alternative α_2-agonist (medetomidine) and α_2-antagonist (atipmezole).

The α_2-adrenceptor antagonist atipamezole (1 μM) induced an approximately 3-fold rightward shift of the medetomidine CRC (Fig. 5A). Medetomidine had an EC_{50} and E_{Max} of 0.1 (0.09–0.2) μM and 78 ± 10 %, respectively. In the presence of atipamezole, the EC_{50} of medetomidine was significantly increased ($P \leq 0.001$) to 0.4 (2.6–5.4) μM whereas the E_{Max} was not found to be significantly reduced (52 ± 7 %).

Atipamezole had no significant effect on either the β-PEA or RO5256390 CRC (Fig. 5B and C). In the presence of atipamezole the EC_{50} of β-PEA and RO5256390 were 45 (19–108) μM and 1.8 (0.6–5.5) μM, respectively. Neither were significantly different from the values

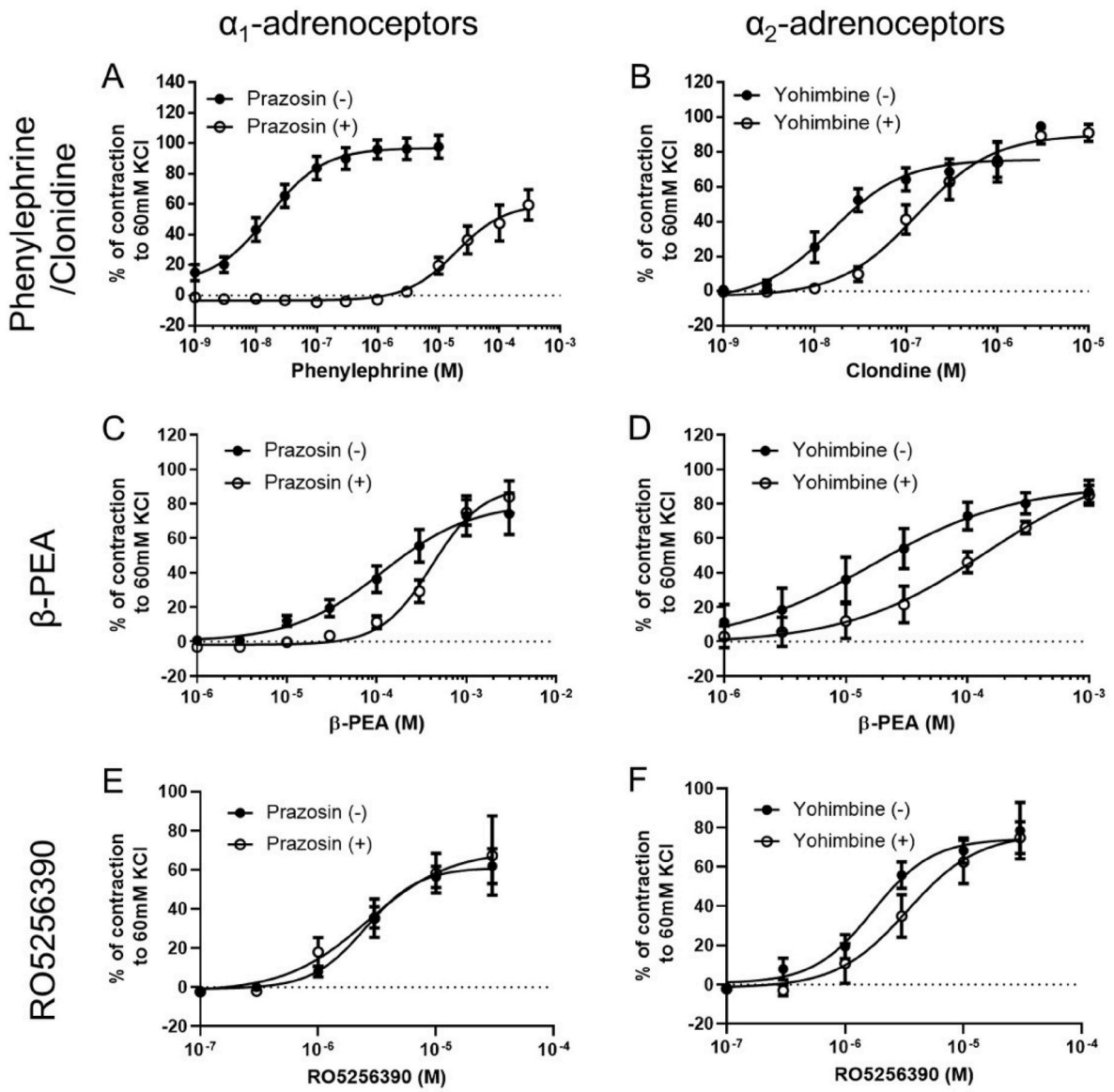

Fig. 4. The effect of 1 μM prazosin (A,C,E) or 1 μM yohimbine (B,D,F) on phenylephrine- (A), clonidine- (B), β-PEA- (C,D) and RO5256390-induced (E,F) contraction. Phenylephrine CRCs were obtained (A) in the absence (● n = 9) or presence (○ n = 5) of prazosin. Clonidine CRCs (B) were obtained in the absence (● n = 5) or presence (○ n = 5) of yohimbine. β-PEA CRCs were obtained (C) in the absence (● n = 5) or presence (○ n = 6) of 1 μM prazosin. β-PEA CRCs were obtained (D) in the absence (● n = 5) or presence (○ n = 5) of 1 μM yohimbine. RO5256390 CRCs were obtained (E) in the absence (● n = 31) or presence (○ n = 11) of 1 μM prazosin. RO5256390 CRCs were obtained (F) in the absence (● n = 11) or presence (○ n = 8) of 1 μM yohimbine. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60 mM KCl.

obtained in the absence of atipamezole of 42 (24–74) μM and 2.7 (1.9–3.7) μM, respectively. Atipamezole had no significant effect on the E_{Max} of β-PEA (82 ± 2) or RO5256390 (83 ± 3%), compared with its absence (86 ± 4% and 61 ± 4%, respectively).

4. Discussion

4.1. Differences between β-PEA-induced contraction in aortic rings and mesenteric arteries

β-PEA induced contractile responses of rat aortic rings across a concentration range (1 μM to 1 mM), consistent with that of previous investigations (Broadley et al., 2013; Fehler et al., 2010). However, in 3rd-order mesenteric arteries, β-PEA-induced contractile responses were only observed at a concentration of 1 mM corroborating our previous finding in the perfused rat mesenteric vascular bed (Anwar et al., 2012) that β-PEA is a poor vasoconstrictor of rat mesenteric vasculature. As the aim of this study was to characterise contractile responses to β-PEA, 3rd order mesenteric artery responses were not studied further.

4.2. Involvement of TAAR1 in β-PEA and RO5256390-induced vasoconstriction

RO5256390 was 10-fold more potent at inducing contraction in rat aortic rings compared to β-PEA. However, its efficacy was significantly lower suggesting that it might act as a partial agonist or that the response to β-PEA involved activation of receptors in addition to TAAR1. A difference in efficacy between β-PEA and RO5256390 is not apparent in a cell-based study (Revel et al., 2013). However in this study the TAAR1 was genetically manipulated to artificially coupled to adenyl cyclase as a method to assess receptor activation and this may have influenced the observed agonist efficacies. It is also possible that the

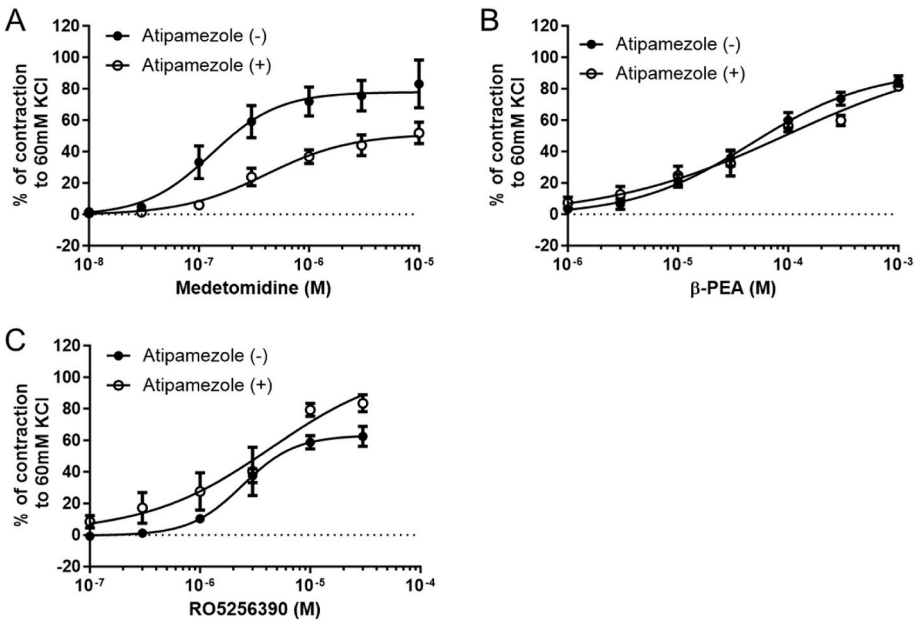

Fig. 5. The effect of 1 μM atipamezole (A-C) on medetomidine- (A), β-PEA- (B) and RO5256390-induced (C) contraction. Medetomidine CRCs were obtained (A) in the absence (● n = 5) or presence (○ n = 5) of atipamezole. β-PEA CRCs were obtained (B) in the absence (● n = 23) or presence (○ n = 5) of atipamezole. RO5256390 CRCs were obtained (C) in the absence (● n = 31) or presence (○ n = 5) of atipamezole. Contractile responses are reported as the mean percentage (±SEM) of the maximum contractile response to 60 mM KCl.

population of TAAR activated by RO5256390 and β-PEA differ. β-PEA, along with tryptamine, is a known agonist of TAAR4 (Borowsky et al., 2001), whereas RO5256390 lacks agonistic activity at TAAR4 (Revel et al., 2013). Activation of TAAR4 by β-PEA could account for the unexpected increase in efficacy compared with RO5256390. Previous studies have demonstrated that TAAR1 but not TAAR4 is expressed in rat aorta (Fehler et al., 2010). However, due to a lack of TAAR4-selective reagents this was not confirmed at the protein level. Unfortunately, since its discovery TAAR4 has largely been ignored and there are no selective pharmacological tools available to test this possibility.

EPPTB is a selective antagonist with high affinity ($K_i = 0.9–4$ nM) for murine TAAR1 but much lower affinity for rat TAAR 1 with a K_i of 0.9–1 μM (Bradaia et al., 2009; Stalder et al., 2011). Compared to vehicle controls, 5 μM EPPTB had no significant effect on CRCs to either β-PEA or RO5256390. Using the receptor theory equation (DR-1 = antagonist concentration * K_B^{-1}) derived by Arunlakshana and Schild (1959), based on the K_i (0.9 μM) and concentration (5 μM) used, there should theoretically be a 6.3-fold rightward shift of a TAAR1 CRC by 5 μM EPPTB. We were unable to use concentrations in excess of 5 μM due to the limit of solubility for EPPTB in Krebs bicarbonate solution. A concentration of 5 μM in the organ bath required a DMSO concentration of 0.25% which itself had significant effects on the CRCs to both RO5256390 and β-PEA. Previous studies have reportedly used higher concentrations with rat tissue (Batista-Lima et al., 2018; Koh et al., 2019). However, the effects of the solvent on trace amine CRCs were not reported in either study. It is therefore possible that these studies, using higher concentrations of RO5256390, are reporting a vehicle effect rather than a pharmacological one.

4.3. Biogenic amine receptors are not involved β-PEA and RO5256390-induced vasoconstriction

The chemical structure of trace amines is closely related to that of biogenic amines (McGeer, 2013) leading to the hypothesis that trace amines may be agonists of receptors for biogenic amines. Therefore we studied the sensitivity of trace amine-induced vasoconstriction to antagonists selective for biogenic amine receptor types known to mediate vasoconstrictor responses.

In vasculature, 5-HT causes vasoconstrictor responses by activation of 5-HT_{2A} receptors (Watts et al., 2012). Indeed, we found that the contractile CRC to 5-HT was significantly shifted 14-fold to the right by the 5-HT_2 receptor antagonist, cinanserin. However, the CRC for vasoconstriction induced by β-PEA was not significantly affected by cinanserin and therefore 5-HT_2 receptors do not mediate this response. As cinanserin had no effect on the CRC to β-PEA we did not investigate its effect against RO5256390.

Both α_1-and α_2-adrenoceptors can mediate contractile responses (Martinotti, 1991; Docherty, 2010) and could potentially be involved in the mechanism of β-PEA-induced vasoconstriction. Therefore, we tested the sensitivity of β-PEA-induced vasoconstriction to antagonists selective for α_1-or α_2-adrenoceptors. The selective α_1-adrenoceptor antagonist, prazosin (1 μM) caused a 4-fold rightward shift of the β-PEA CRC. However, 1 μM prazosin caused a 6500-fold shift of the CRC for phenylephrine, the α_1-adrenoceptor agonist. Based on the K_D of prazosin for α_1-adrenoceptors being 0.3 nM (Aboud et al., 1993), receptor theory (Arunlakshana and Schild, 1959) predicts that there should be a 2800-fold shift of a CRC mediated by α_1-adrenoceptors. This is a similar order of magnitude observed with shift of the phenylephrine CRC by 1 μM prazosin. The degree of shift induced by prazosin on the β-PEA CRC was approximately 1000-fold less and therefore the response is not mediated by activation of α_1-adrenoceptors. Unlike β-PEA, vasocostrictor responses to the selective TAAR1 antagonist, RO5256390, were not affected by prazosin evidencing the lack of role for α_1-adrenoceptors in the mechanism of trace amine-induced vasoconstriction.

The selective α_2-adrenoceptor antagonist, yohimbine (1 μM), caused a 7-fold rightward shift of the CRC to β-PEA and 2-fold rightward shift of the CRC to RO5256390. The K_D of yohimbine for α_2-adrenoceptors is 2.6 nM (Hikasa et al., 2013), based on receptor theory, 1 μM yohimbine should cause a rightward shift of a CRC mediated by α_2-adrenoceptors of 387-fold. As the degree of shift of an α_2-adrenoceptor mediated CRC calculated using receptor theory is orders of magnitude less than that for CRCs generated by β-PEA or RO5256390, their responses would not appear to involve α_2-adrenoceptors. However, yohimbine only shifted the CRC for the α_2-adrenoceptor agonist, clonidine by 7-fold, similar to that observed with β-PEA and RO5256390 and less predicted by receptor theory for an α_2-adrenoceptor mediated CRC. This raises the intriguing

possibility that both yohimbine and clonidine mediate their effects on TAAR rather than postsynaptic α_2-adrenoceptors in rat aorta. Other studies have demonstrated that α_2-agonists, including clonidine, are agonists of TAAR1 in certain species including mouse and humans TAAR1 (Hu et al., 2009; Cichero et al., 2023). TAAR1 is a well-known modulator of monoaminergic neurotransmission with significant impact on mood and cognitive function (Dedic et al., 2021). It is possible that neuropsychiatric effects associated with therapeutic use of clonidine may be a result of TAAR1 agonism (Huffman and Stern, 2007). Studies have also demonstrated TAAR1 agonism attenuates yohimbine-induced reinstatement of cocaine seeking behaviours in rat models (Liu et al., 2020). Although to date no studies have demonstrated a clear molecular interaction between yohimbine and TAAR1.

To further investigate the role of α_2-adrenoceptors the selective α_2-adrenoceptor agonist and antagonist, medetomidine and atipamezole respectively, were examined. Interestingly atipamezole (1 μM) induced a smaller than expected (3-fold) shift of the medetomidine CRC. Based on the published K_b value of 1.7 nM (Jurgens et al., 2007), receptor theory predicts a rightward shift of a CRC mediated by α_2-adrenoceptors of 589-fold. However, atipamezole (1 μM) had no effect on the CRC to either β-PEA or RO5256390. This confirms that β-PEA- and RO5256390-induced contractions are unlinkely to be mediated by α_2-adrenoceptors.

4.4. Study limitations

The major limitation of this study is the lack of commercially available TAAR-selective reagents, particularly in the form of species specific TAAR1-selective antagonists. The current study made use of the only currently available TAAR1 antagonist EPPTB which has very low affinity for rat TAAR1 (Bradaia et al., 2009; Stalder et al., 2011). Use of this antagonist at concentrations above 5 μM were limited by its poor solubility in Krebs Bicarbonate solution requiring a DMSO concentration of 0.25%. DMSO itself had significant effects on both β-PEA and RO5256390-induced concentration response curves causing a leftward and rightward shift, respectively. We currently have no explanation for this difference except to comment that RO5256390 is insoluble whereas β-PEA is soluble in water.

This study also was limited to the study of classical biogenic amine receptors and TAAR1. It is known that β-PEA is an agonist of TAAR4 whereas RO5256390 is not a TAAR4 agonist (Borowsky et al., 2001; Revel et al., 2013). Currently there are no TAAR4-selective reagents available and previous studies suggest that is not expressed in rat aorta the level of mRNA (Fehler et al., 2010). Therefore it was not possible to expand the study to consider TAAR4's involvement.

4.5. Conclusion

β-PEA- and RO5256390-induced contractile responses of rat aorta are not mediated by classical biogenic amine receptors. Despite the lack of antagonism associated with EPPTB, the lack of biogenic amine receptor involvement coupled to the response observed with the selective TAAR1 agonist RO5256390, indicates that TAAR1 is the most likely receptor involved in the mechanism of vasoconstriction in rat aorta induced by trace amines. Despite being a full agonist of TAAR1 RO5256390 displayed reduced efficacy compared with β-PEA indicating other TAARs such as TAAR4 may account for the increased efficacy of β-PEA.

Data availability statement

Data will be made available upon acceptance via DOI. The data is curated by Cardiff University.

CRediT authorship contribution statement

Alexander C. Voisey: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Harrison D. Broadley:** Data curation. **Kenneth J. Broadley:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **William R. Ford:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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