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5-HT moduline: an endogenous inhibitor of 5-$HT_{1B/1D}$-mediated contraction in pulmonary arteries

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1 5-HT moduline (5-HTm) is tetrapeptide (Leu-Ser-Ala-Leu) previously shown to act as a specific endogenous antagonist to central 5-$HT_{1B/1D}$ receptors. Its effects were investigated in rat and rabbit pulmonary arteries (PAs).

2 In rabbit PAs, contractile responses to the 5-$HT_{1B/1D}$ receptor agonist 5-carboxamidotryptamine (5-CT) were inhibited by 1 and 10 μM 5-HTm in a non-competitive fashion with the maximum contractile response (E_{max}, per cent of response to 50 mM KCl) being reduced from $65.6 \pm 7\%$ ($n=6$) to $39.7 \pm 6.5\%$ ($n=6$) and 25.2 ± 7.9 ($n=4$), respectively. The ability of 5-HTm to inhibit responses to 5-CT was increased by the aminopeptidase inhibitor bestatin (10 μM).

3 In the rabbit PAs, the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methylester (L-NAME) potentiated responses to 5-CT (E_{max}: 106 ± 22.5 ($n=4$)) and this response was also inhibited by 10 μM 5-HTm (E_{max}: $38 \pm 13\%$ ($n=8$)).

4 5-HTm (10 μM) inhibited responses to 5-CT in rat PAs, the E_{max} being reduced from $24.8 \pm 4.1\%$ ($n=7$) to $15.5 \pm 3.7\%$ ($n=9$). 5-HTm induced relaxation of 5-CT-pre-constricted rat PAs with a pIC_{50} of 9.0 ± 0.6 ($n=9$).

5 In PAs from chronic hypoxic, pulmonary hypertensive rats, the maximum response to 5-CT was increased to $80 \pm 8.5\%$ ($n=11$). 5-HTm reduced this response to $34.4 \pm 6.3\%$ ($n=12$). L-NAME markedly inhibited the ability of 5-HTm to inhibit responses to 5-CT (E_{max} before 5-HTm: $100.5 \pm 16\%$ ($n=5$), E_{max} after 5-HTm: $107 \pm 11.3\%$ ($n=4$)).

6 In conclusion we show here for the first time that 5-HTm is a non-competitive inhibitor of 5-$HT_{1B/1D}$ receptor-mediated constriction in PAs. In rat PAs, L-NAME can inhibit this effect of 5-HTm.

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Abbreviations: 5-CT, 5-carboxamidotryptamine; 5-HTm, 5-hydroxytryptamine moduline; LV, left ventricular; L-NAME, N^{ω}-nitro-L-arginine methylester; LSAL, Leu-Ser-Ala-Leu; PAs, pulmonary arteries; RV, right ventricular; TV, total ventricular

Introduction

5-HT moduline (5-HTm, Leu-Ser-Ala-Leu) was originally isolated from rat brain (Rousselle *et al*., 1996) and acts as a specific endogenous antagonist to central 5-$HT_{1B/1D}$ receptors with a high affinity ($K_D = 0.2–0.8$ nM) (Massot *et al*., 1996). Centrally, immunocytochemical studies have shown that 5-HTm is heterogenously distributed in neuronal structures of the brain and is associated with the 5-$HT_{1B/1D}$ receptor (Grimaldi *et al*., 1997). Binding of 5-HTm is absent in the brains of 5-HT_{1B} knockout mice (Cloez-Tayarani *et al*., 1997). 5-HTm acts as an allosteric modulator of the 5-HT_{1B} receptor and its inactivation is through a bestatin-sensitive aminopeptidase and an endoprotease cleaving 5-HTm into Leu-Ser and Ala-Leu (Plantefol *et al*., 1999a). The release of 5-HTm from crude synaptosomal preparations from rat brains was shown to be *via* a calcium-dependent K^+ stimulated mechanism (Massot *et al*., 1996). To date, there is only one publication assessing the activity of 5-HTm in the periphery, showing that 5-HTm, likely originating from the adrenal medulla and released after acute stress, interacts with the 5-HT_{1B} receptors on immunocompetent cells (Grimaldi & Fillion, 2000). However, no research has been conducted to address the concept that it may regulate peripheral vascular 5-HT_{1B} receptors and this is the concept that was tested here.

We have shown that 5-HT_{1B} receptors mediate contraction to 5-HT in human, rat and rabbit pulmonary vascular beds (MacLean *et al*., 1996a; Morecroft & MacLean, 1998; Morecroft *et al*., 1999; MacLean & Morecroft, 2001) and these receptors also play a key role in the enhanced pulmonary vascular response to 5-HT in pulmonary hypertension (MacLean, 1999; MacLean *et al*., 1996b; 2000). As 5-HT_{1B} receptor antagonists reduce the development of hypoxia-induced pulmonary hypertension in rats (Keegan *et al*., 2001), it is of great interest to investigate the ability of an endogenous 5-HT_{1B} receptor antagonist to inhibit 5-HT_{1B} receptor-mediated constriction in rats pulmonary hypertensive models.

Here we have investigated the influence of 5-HTm on responses to 5-$HT_{1B/1D}$ receptor agonist 5-carboxamydotryptamine (5-CT) in isolated rat and rabbit pulmonary resistance arteries. We also examined the effect of bestatin. We

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investigated the effects of 5-HTm on vessels pre-treated with L-NAME as we have previously shown inhibition of nitric oxide can potentiate responses to 5-HT$_{1B/1D}$ receptor stimulation (MacLean, 1999). To investigate if hypoxia affects the activity of 5-HTm, we have also examined its effect on responses to 5-CT in rats subjected to 2 weeks of chronic hypoxia with ensuing pulmonary hypertension.

Methods

Control rat and rabbit pulmonary arteries

Male New Zealand white rabbits (3.5 kg) were euthanized by i.v. administration of sodium pentobarbitone (100 mg kg^{-1}) with 1000 i.u. heparin into the marginal ear vein and the lungs removed. Wistar rats (28 days) were euthanized with i.p. sodium pentobarbitone (60 mg kg^{-1}). Under dissecting microscope, intralobar pulmonary resistance arteries ($\sim$250–300 μm i.d. rabbits; 150–200 μm i.d. rats) were isolated and mounted as ring preparations in isometric wire myographs. The vessels were maintained in Krebs buffer solution at 37°C and aerated with 16% O_2/5% CO_2 balance N_2. A transmural pressure equivalent to 12–16 mmHg was applied to the tissue to give values similar to those *in vivo*.

Following a 45-min equilibration period the vessels responsiveness to 50 mM KCl was determined followed by washout. This was then repeated and followed by a further equilibration period. Cumulative concentration-dependent response curves (CCRCs) were constructed for 5-CT in the presence and absence of 5-HTm (1 and 10 μM, pre-incubation time of 45 min) and bestatin (10 μM, pre-incubation time of 30 min). The effect of 5-HTm was also studied in vessels pre-incubated with L-NAME (100 μM, 20 min). The effect of the 5-HT$_{1B/1D}$-receptor antagonist SB 224289 (0.2 μM, Price *et al*., 1997) and the 5-HT$_{1D}$-receptor antagonist BRL15572 (0.5 μM, Price *et al*., 1997) on responses to 5-CT (pre-incubation time 45 min) were examined in the rabbit vessels. Additional controls were constructed with tetrapeptide analogues of 5-HTm, (LSAd-Leu, d-LeuSAL, 10 μM), in place of 5-HTm (LSAL) in rat vessels pre-treated with L-NAME to examine for specificity of the LSAL sequence. In rat vessels, the concentration-dependency of 5-HTm was examined by pre-constricting vessels with 5-CT (100 μM) and constructing a cumulative concentration responses curve to 5-HTm itself. Time controls were carried out simultaneously in vessels pre-constricted with 5-CT but with no 5-HTm added. The ability of 10 μM 5-HTm to affect endothelin-1 (1 pmol–0.3 μM)-induced constriction was examined in rabbit PAs.

Chronic hypoxic rats

Male Wistar rats were placed in a hypobaric chamber. This was depressurized over the course of 2 days to 550 mbar (equivalent to 10% O_2). Temperature was maintained at 21–22°C and the chamber was ventilated with air at approximately 45 l min^{-1}. The duration of hypoxia was 14 days. The rats were age-matched with the controls used in this study. Vessels were dissected out and studied as above except that a transmural pressure of 33–35 mmHg was applied as described previously (MacLean & Morecroft, 2001; MacLean *et al*., 1996b). As an index of pulmonary hypertension (Herget *et al*., 1978), right ventricular hypertrophy was assessed by measuring the right ventricular free wall (RV) and left ventricle together with the septum (LV+S) separately. Total ventricular weight (TV) was calculated as RV+(LV+S) and the ratio RV/TV calculated.

Analysis

Responses to 5-CT were expressed as a per cent response to 50 mM KCl. Responses to 5-HTm are expressed as a percentage of 5-CT-induced pre-constriction. pEC_{50} values were only calculated where maximum responses were achieved within the concentration range of 5-CT studied. Statistical comparisons were made using Student's *t*-test where $P<0.05$ was taken as the level of statistical significance.

Drugs and solutions

5-CT maleate was purchased from Tocris Cookson (U.K.). L-NAME and bestatin were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). All peptides were made by Thistle Peptides, Scotland. All drugs were dissolved in distilled water.

Results

Rabbit pulmonary arteries

5-CT induced a vasoconstriction with a pEC_{50} value of 5.56 ± 0.23 ($n=6$) and the response curve for 5-CT was biphasic in nature. The high affinity phase was completely inhibited by the 5-HT$_{1B}$ receptor antagonist SB224289 and partially inhibited by the 5-HT$_{1D}$-receptor antagonist BRL 15572 at 0.3 μM 5-CT. Both antagonists inhibited the lower affinity phase of the CCRC with SB224289 being more potent (Figure 1). 5-HTm (1 μM and 10 μM) inhibited the responses to 5-CT, reducing the maximum contractile response (E_{max}) from $65.6\pm7\%$ ($n=6$) to $39.7\pm6.5\%$ ($n=6$, $P<0.05$) and 25.2 ± 7.9 ($n=4$, $P<0.01$), respectively (Figure 2). From Figure 2, it is also apparent that 5-HTm has a more profound effect on the high affinity phase of the CCRC, with 10 μM 5-HTm totally abolishing this phase whilst only partially inhibiting the lower affinity phase. The pEC_{50} value was not significantly changed being 5.7 ± 0.38 ($n=6$) and 5.2 ± 0.35 ($n=4$) respectively. The ability of 1 μM 5-HTm to inhibit responses to 5-CT was increased in the presence of bestatin (e.g. $P<0.05$ at 30 μM, Figure 2). In the presence of L-NAME, the maximum contractile response to 5-CT was increased to $106\pm22.5\%$ ($P<0.05$) (pEC_{50} value of 5.98 ± 0.29 ($n=5$)). 5-HTm (10 μM) was still able to inhibit this potentiated response to 5-CT, reducing the maximum response to $38\pm13\%$ ($n=8$, $P<0.01$) (pEC_{50} value of 4.3 ± 0.09 ($n=8$), Figure 3). 5-HTm (10 μM) did not affect endothelin-1 induced constriction (pEC_{50}, of controls: 7.9 ± 0.2 ($n=4$), plus 5-HTm: 7.8 ± 0.3 ($n=4$); E_{max}, of controls: 93.7 ± 9.6, plus 5-HTm: 94.1 ± 8.9).

Rat pulmonary arteries

Control rats 5-CT produced a small contractile response in control rat pulmonary arteries (pEC_{50} value of 6.1 ± 0.1

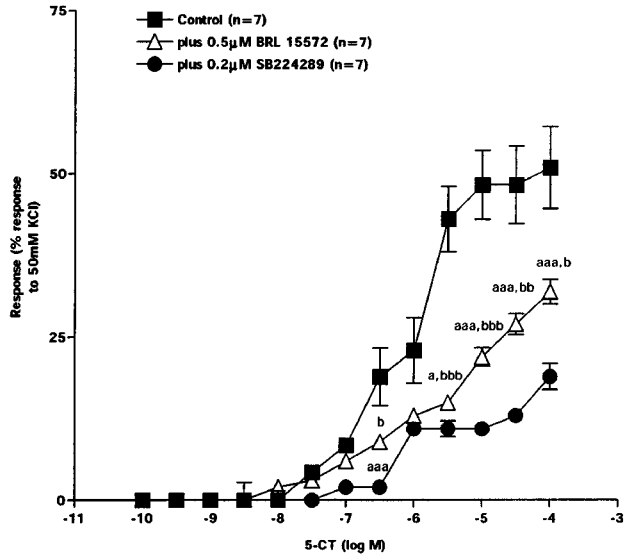

Figure 1 Effect of the 5-HT$_{1D}$-receptor antagonist BRL 15572 and the 5-HT$_{1B}$-receptor antagonist SB224289 on responses to 5-carboxamidotryptamine (5-CT) in rabbit pulmonary arteries. n = number of animals. Data is shown as mean ± s.e.mean. Statistical difference (Student's t-test) from 'plus SB224289': a $P<0.05$, aaa $P<0.001$, from 'control': b $P<0.05$, bb $P<0.01$, bbb $P<0.001$.

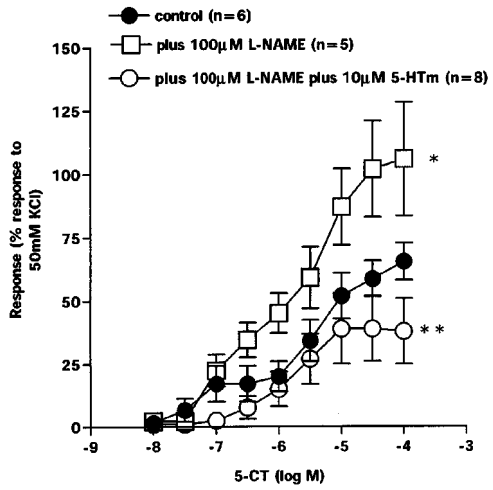

Figure 3 Effect of 5-HT moduline (5-HTm), in the presence and absence of N$^{\omega}$-nitro-L-arginine methylester (L-NAME), on responses to 5-carboxamidotryptamine (5-CT) in rabbit pulmonary arteries. n = number of animals. Data is shown as mean ± s.e.mean. Statistical differences (Student's t-test) from control (*$P<0.05$) and from plus L-NAME (**$P<0.01$).

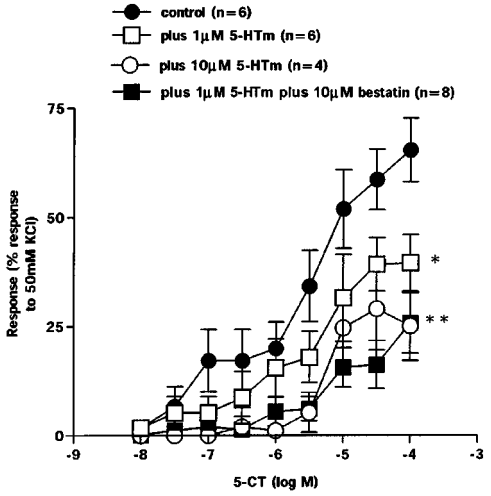

Figure 2 Effect of 5-HT moduline (5-HTm), in the presence and absence of bestatin, on responses to 5-carboxamidotryptamine (5-CT) in rabbit pulmonary arteries. n = number of animals. Data is shown as mean ± s.e.mean. Statistical difference (Student's t-test) from control (*$P<0.05$, **$P<0.01$).

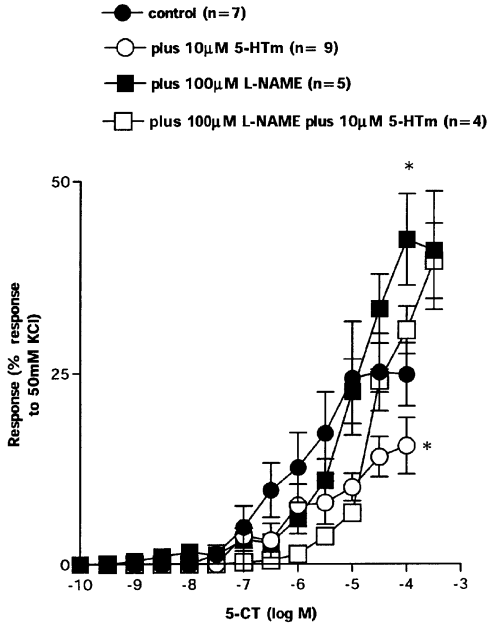

Figure 4 Effect of 5-HT moduline (5-HTm), in the presence and absence of N$^{\omega}$-nitro-L-arginine methylester (L-NAME), on responses to 5-carboxamidotryptamine (5-CT) in control rat pulmonary arteries. n = number of animals. Data is shown as mean ± s.e.mean. Statistical difference from control (Student's t-test) *$P<0.05$.

($n=7$), E_{max}: 24.8 ± 4.1%). This was inhibited by 10 μM 5-HTm (pEC_{50} value of 5.81 ± 0.2 ($n=6$)), with the maximum response being reduced by ~40% to 15.5 ± 3.7% ($n=9$), $P<0.05$ (Figure 4). In the presence of L-NAME, the maximum response to 5-CT was increased from 24.8 ± 4.1 ($n=7$) to 42.4 ± 6 ($n=5$, $P<0.05$) whilst potency was decreased (pEC_{50} value was 5.0 ± 0.13 ($n=5$), $P<0.001$ vs control (Figure 4)). In the presence of L-NAME, 5-HTm no longer reduced the maximum response to 5-CT (pEC_{50} value was 4.46 ± 0.08 ($n=4$) in the presence of 5-HTm) but the responses to 0.3 – 10 μM were reduced ($P<0.05$) (Figure 4)).

The tetrapeptide analogues Leu-SerAla-d-Leu and d-Leu-Ser-Ala-Leu did not inhibit responses to 5-CT (pEC_{50} values were 4.87 ± 0.1 ($n=4$) and 4.92 ± 0.12 ($n=4$) respectively, Figure 5). In vessels pre-constricted with 5-CT (in the presence of L-NAME) the induced tone was 33 ± 6% of the response to 50 mM KCl. 5-HTm reversed the pre-constriction to 5-CT in rat vessels in a concentration-dependent fashion with a pIC_{50} of 9.0 ± 0.6 (Figure 6). The relaxation to 5-HTm was very gradual, taking 4 – 5 min to reach maximum effect for each concentration added. The maximum reversal of tone at 10 μM 5-HTm, taking into account the time control effects, was

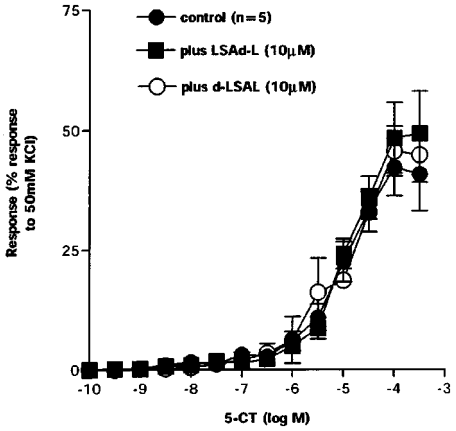

Figure 5 Effect of 5-HT moduline analogues Leu-Ser-Ala-d-Leu (LSAd-L) and d-Leu-Ser-Ala-Leu (d-LSAL) on responses to 5-carboxamidotryptamine (5-CT, in the presence of 100 μM N$^{\omega}$-nitro-L-arginine methylester) in control rat pulmonary arteries. n = number of animals. Data is shown as mean ± s.e.mean.

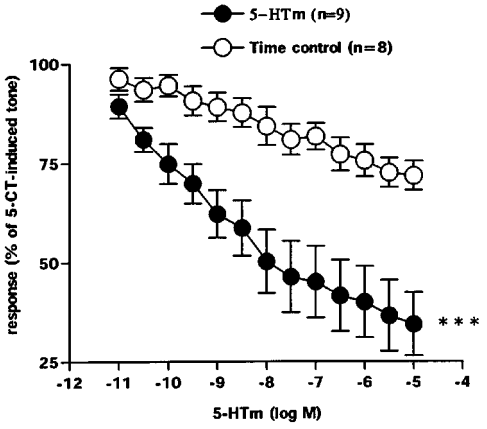

Figure 6 Cumulative concentration response curve to 5-HT moduline (5-HTm) in control rat pulmonary arteries pre-constricted with 30 μM 5-carboxamidotryptamine (5-CT) in the presence of 100 μM N$^{\omega}$-nitro-L-arginine methylester. Time controls were run simultaneously with no 5-HTm added. Responses are expressed as the per cent relaxation of the 5-CT-induced tone. n = number of animals. Data is shown as mean ± s.e.mean. Statistical difference (Student's t-test) from time control ***$P<0.001$.

~38%. This is consistent with its effect (~40%) against the maximum response to 5-CT illustrated in Figure 4.

Chronic hypoxic rats The RV/TV of control rats was 0.256 ± 0.008 (n = 9) and of the chronic hypoxic rats was 0.388 ± 0.008 (n = 11, $P<0.001$), indicative of pulmonary hypertension. Responses to 5-CT were increased in vessels from CH rats. The maximum contraction increased from 25 ± 4% (n = 7, Figure 4) to 80 ± 8.5% ($P<0.001$) with a pEC$_{50}$ value of 5.22 ± 0.04 (n = 11) $P<0.001$ vs control (Figure 7). Responses to 5-CT were markedly inhibited by 5-HTm which reduced the maximum contractile response to 34.4 ± 6.3 (n = 12, $P<0.001$, Figure 7). Responses to 5-CT were not significantly enhanced by L-NAME, but surprisingly, in the presence of L-NAME, responses to 5-CT were not inhibited by 5-HTm (Figure 7).

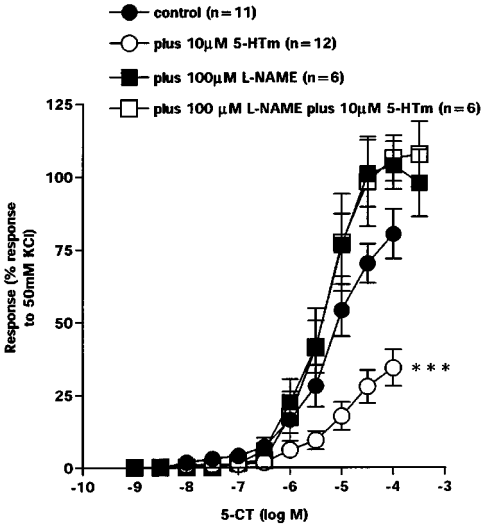

Figure 7 Effect of 5-HT moduline (5-HTm), in the presence and absence of N$^{\omega}$-nitro-L-arginine methylester (L-NAME), on responses to 5-carboxamidotryptamine (5-CT) in chronic hypoxic, pulmonary hypertensive rat pulmonary arteries. n = number of animals. Data is shown as mean ± s.e.mean. Statistical difference (Student's t-test) from control ***$P<0.001$.

Discussion

We have shown that 5-HTm is an endogenous non-competitive antagonist against responses to a 5-HT$_{1B/1D}$ agonist in the rat and rabbit pulmonary artery. This is the first time that such an effect of 5-HTm has been demonstrated in arterial preparations.

CCRCs to 5-CT in the rabbit PAs were biphasic in nature. It is known that 5-CT can activate 5-HT$_{2A}$ receptors in the rabbit aorta (Feniuk *et al*., 1985) and so to verify that 5-HT$_{1D}$ and/or 5-HT$_{1B}$ receptors were contributing to both phases of the CCRC to 5-CT in the rabbits PAs, we investigated the effects of the 5-HT$_{1B}$ receptor antagonist SB224289 and the 5-HT$_{1D}$-receptor antagonist BRL 15572. The high affinity phase was completely inhibited by the 5-HT$_{1B}$ receptor antagonist and partially inhibited by the 5-HT$_{1D}$-receptor antagonist at 0.3 μM 5-CT. Both antagonists inhibited the lower affinity phase of the CCRC, with the 5-HT$_{1B}$-receptor antagonist being most effective. This suggests that both 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors contribute to both phases of the CCRC to 5-CT, with 5-HT$_{1B}$ receptors predominating in the first phase. We cannot rule out, however, the possibility that 5HT$_{2A}$ receptors may contribute to responses to higher concentration of 5-CT. We demonstrated that responses to 5-CT in the rabbit PAs were inhibited by 5-HTm in a concentration-dependent fashion. The effect of 5-HTm was more profound against the first high affinity phase of the CCRC to 5-CT. One interpretation of this is that this is the component of the CCRC mediated mainly by the 5-HT$_{1B}$ receptor and 5-HTm is 10 fold more potent against 5-HT$_{1B}$ receptor coupling than 5-HT$_{1D}$ receptor coupling (Rousselle *et al*., 1998). The effect of 5-HTm was specific to 5-CT, having no effect on responses to endothelin-1.

Bestatin has been shown to inhibit endoproteolytic activity in the brain thought to cleave 5-HTm into dipeptides

(Plantefol *et al*., 1999a). The effectiveness of 5-HTm was enhanced in the presence of bestatin and so this may indicate the presence of the endoprotease within the vascular wall. Responses to 5-CT were enhanced in the presence of L-NAME. The effect of L-NAME is thought to synergize with the 5-HT$_{1B}$-mediated effects and such an effect may contribute to the enhanced responsiveness of PAs to 5-HT in pulmonary hypertensive models (MacLean, 1999; MacLean & Morecroft, 2001). 5-HTm was still able to antagonize responses to 5-CT in the presence of L-NAME in the rabbit vessels.

We next studied rat PAs in order to examine the effectiveness of 5-HTm in control rat vessels and then to compare responses in vessels removed from rats after 2 weeks of exposure to chronic hypoxia which induced pulmonary hypertension. 5-HTm induced a concentration-dependent vasorelaxation of 5-CT pre-constricted vessels with a pIC_{50} of $\sim$9.0. In control rat vessels, as reported previously, responses to 5-CT are extremely small (MacLean *et al*., 1996b; MacLean & Morecroft, 2001). They were, however inhibited by 5-HTm. In the presence of L-NAME, responses to 5-CT were enhanced. 5-HTm no longer inhibited responses to 5-CT in a non-competitive fashion although responses to 0.3–10 μM 5-CT were inhibited. The 5-HTm analogues LSAd-L and d-LSAL did not inhibit responses to 5-CT at all, indicating the specificity of the LSAL sequence in this preparation.

Responses to 5-CT were enhanced in rats with pulmonary hypertension as previously described (MacLean *et al*., 1996b; MacLean & Morecroft, 2001). This is believed to be due to the synergistic effects of increased vascular tone and reduced cGMP levels (MacLean, 1999; MacLean & Morecroft, 2001). These enhanced responses were markedly inhibited by 5-HTm, indicating that this peptide could counteract enhanced responses in this model of pulmonary hypertension. L-NAME potentiated responses to 5-CT. In the presence of L-NAME, 5-HTm did not inhibit responses to 5-CT. Hence, in both control and chronic hypoxic rat vessels, L-NAME inhibited the ability of 5-HTm to act as a non-competitive inhibitor of 5-HT$_{1B/1D}$ receptors. This effect of L-NAME has not previously been described.

It is possible that nitric oxide or cGMP in some way interacts with the 5-HT$_{1B}$ receptor to keep it in a conformation or state that enables 5-HTm to bind. Indeed, the 5-HT$_{1B}$ receptor has been shown to activate endothelial nitric oxide synthase and nitric oxide production (McDuffie *et al*., 1999; Ishida *et al*., 1998) which may normally facilitate binding of 5-HTm. It has been shown that there are two interacting sites for 5-HTm, probably corresponding to different conformations of the 5-HT$_{1B}$ receptor. The peptide is thought to bind first to a low-affinity state of the receptor and then induces a high affinity complex (Plantefol *et al*., 1999b). By inhibiting nitric oxide synthase, it may be that 5-HTm cannot induce a high affinity complex but this is purely speculative and would require intensive further investigation.

In conclusion, we have shown that 5-HTm is an endogenous non-competitive antagonist of 5-HT$_{1B/1D}$ receptor-mediated contractile responses in a vascular preparation, the pulmonary artery. As human pulmonary (MacLean *et al*., 1996a; Morecroft *et al*., 1999), radial (Chester *et al*., 2000), cerebral (Nilsson *et al*., 1999), temporal, brachial (de Hoon *et al*., 2000) and coronary arteries have all been shown to constrict to 5-HT$_{1B/1D}$ receptor stimulation, this peptide could have widespread effects on the human vasculature.

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