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Involvement of 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors in 5-HT-induced contraction of endothelium-denuded rabbit epicardial coronary arteries

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1 The receptors responsible for 5-hydroxytryptamine (5-HT)-mediated contraction of rabbit isolated epicardial coronary artery denuded of endothelium was examined by bioassay.

2 A variety of 5-HT mimetics caused concentration-dependent contractions. The rank order of agonist potency was 5-carboxamidotryptamine (5-CT) > 5-HT > (±)-α-methyl-5-hydroxytryptamine ((±)-α-me-5-HT) = sumatriptan. This was not consistent with relative potencies at any single recognized 5-HT receptor, suggesting the presence of a mixed receptor population. In one subset of preparations precontracted with U46619 (10–30 nM) with the endothelium intact, none of the agonists caused a relaxation.

3 Contractions to 5-HT were antagonized by ketanserin, a 5-HT_{2A}-selective antagonist, but the displacement of concentration-response curves was inconsistent with an interaction between 5-HT and a single receptor population; the slope of regression between antagonist log M concentration and agonist log (concentration-ratio −1) was shallow (0.57). Responses to 5-HT were also antagonized by the 5-$HT_{1B/1D}$-receptor antagonist GR127935 and, again, the slope of regression was shallow (0.68). These data suggest a possible involvement of 5-HT_{2A} and 5-HT_{1B} or 5-HT_{1D} receptors in the response to 5-HT.

4 Contractions to (±)-α-me-5-HT, which is selective for 5-HT_{2A} over 5-HT_{1B} and 5-HT_{1D} receptors, were competitively antagonized by low concentrations of ketanserin. The regression between antagonist log M concentration and agonist log (concentration-ratio −1) fitted the Schild equation with a slope that was not significantly different from unity (0.95), giving a pA_2 value of 9.0. GR127935 (3–30 nM), had no effect on the contractile response to (±)-α-me-5-HT. These data establish, unequivocally, the presence of 5-HT_{2A} receptors in the tissue.

5 Sumatriptan, a relatively selective 5-$HT_{1B/1D}$-receptor agonist, induced contractions that were antagonized competitively by GR127935 (3–30 nM), although there was a reduction in the maximum response when concentrations of GR127935 exceeded 3 nM. The apparent pA_2 (estimated by imposing a unit slope on the log agonist (concentration-ratio −1) value in the presence of 3 nM GR127935) was 8.92. Contractions to sumatriptan were not affected by low (5-HT_{2A} receptor-selective) concentrations of ketanserin, but were antagonized in a competitive manner at higher concentrations (pA_2 6.5). These data appear to confirm the presence of 5-HT_{1B} and/or 5-HT_{1D} receptors in the tissue.

6 Antagonism of 5-HT responses by GR127935 was reassessed after blockade of 5-HT_{2A} receptors with 1 μM ketanserin. Under these conditions, GR127935 was able to antagonize 5-HT-induced contractions fully. The slope of regression between log M antagonist concentration and log agonist (concentration-ratio −1) fitted the Schild equation with a slope not significantly different from unity (1.1) (albeit there was still a reduction in maximum response when GR127935 concentration exceeded 3 nM). The apparent pA_2 value was 8.8. This reinforces the evidence that 5-HT_{1B} and/or 5-HT_{1D} receptors contribute to the effects of 5-HT in the tissue.

7 In conclusion, in endothelium denuded rabbit epicardial coronary arteries, 5-HT activates 5-HT_{2A} and 5-HT_{1D} and/or 5-HT_{1B} receptors to cause contraction. This appears to be similar to the situation in man.

Keywords: 5-HT, 5-HT_{1D} receptor; 5-HT_{1B} receptor; 5-HT_{2A} receptor; 5-carboxamidotryptamine; (±)-α-methyl-5-hydroxytryptamine; coronary artery; endothelium; GR127935; sumatriptan

Introduction

5-Hydroxytryptamine (5-HT) has been shown to play an important role in pathogenic coronary vasoconstriction (Hillis & Lange, 1991; Golino *et al*., 1991); there is a conversion of mild dilatation of human coronary arteries *in vivo* to constriction in the presence of atherosclerotic disease (Chester *et al*., 1993) and the coronary vasoconstriction is mediated probably by 5-HT_{1B} (formerly 5-HT_{1DB}) receptors (Kaumann *et al*., 1993). This alteration in response to 5-HT is probably due to a malfunction or loss of responsiveness of the vascular endothelium to 5-HT and hence a diminished release of NO (Heistad *et al*., 1984; Stewart *et al*., 1987). The possibility also exists that following endothelial injury, 5-HT constrictor receptors on the coronary vascular smooth muscle become unopposed, unmasking a pathogenic coronary vasoconstriction hitherto limited by the presence of endothelium (McFadden *et al*., 1993).

Activation of 5-$HT_{1B/1D}$ receptors causes a constriction in human (Kaumann *et al*., 1993; 1994), dog (Fenuik *et al*., 1989) and guinea-pig (Ellwood & Curtis, 1996a, b) coronary arteries. Howevver, endothelium-dependent relaxation has also been demonstrated in response to activation of 5-$HT_{1B/1D}$ receptors in porcine coronary artery (Schoeffter & Hoyer, 1990), guinea-pig jugular vein (Gupta, 1992) and porcine pulmonary artery (Glusa & Richter, 1993). In rabbit isolated perfused heart 5-

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HT causes a coronary vasodilation which is converted into a vasoconstriction following removal of the endothelium (Ellwood & Curtis, 1994). In addition, in isolated hearts from hypercholesterolaemic rabbits the vasodilator response to 5-HT is replaced by a vasoconstriction which is sensitive to 5-HT_{2A} receptor blockade (Vrints *et al*., 1990; Verbeuren *et al*., 1991). However, in rabbit isolated epicardial coronary arteries 5-HT causes a constriction even in the presence of endothelium and this is augmented by endothelium denudation (Awano *et al*., 1989). Feletou *et al*. (1994) proposed that this response is mediated by the 5-HT_{1B} or 5-HT_{1D} receptor.

The difference in the response of rabbit perfused isolated heart and rabbit isolated epicardial coronary artery to 5-HT may reflect heterogeneity in the distribution of receptors that mediate vasodilatation and vasoconstriction throughout the coronary bed. Epicardial arteries are capacitance vessels, whereas coronary flow (i.e., the variable that is measured in perfused hearts) is dominated by the effects of resistance vessels. We have previously characterized the profile of receptors mediating coronary vascular responses to 5-HT in the guinea-pig endothelium-intact and denuded isolated, perfused heart (Ellwood & Curtis, 1996b, c). Both 5-HT_1 and 5-HT_2 receptors appear to be involved in a complex response profile. The question that arises therefore is: what is the nature of the response profile to 5-HT agonism in epicardial coronary arteries?

Involvement of 5-HT receptor subtypes in mediating responses can be probed by use of selective agonists and antagonists. Selectivity is never complete over the broad concentration range required for estimating affinity and potency orders, so that examination of drugs in different combinations is desirable. GR127935, is an antagonist selective for 5-$HT_{1B/1D}$ over 5-HT_2 receptors (Skingle *et al*., 1996) and, therefore, can be used to investigate the presence of 5-$HT_{1B/1D}$ receptors. To probe for the presence of 5-HT_{2A} receptors, (±)-α-methyl-5-HT ((±)-α-me-5-HT) and ketanserin may be used. Ketanserin, at nanomolar concentrations, is a selective 5-HT_{2A} antagonist (Van Nueten *et al*., 1981; Humphrey *et al*., 1982; Leff & Martin, 1986; Mylecharane, 1990). Ketanserin does have 5-$HT_{1B/1D}$ receptor antagonist activity, but only in the micromolar range (Kaumann *et al*., 1993; 1994). Its use in combination with the agonist (±)-α-me-5-HT (Ismaiel *et al*., 1990) therefore allows clear identification of the participation of the 5-HT_{2A} receptor in responses.

The tendency of rabbit isolated coronary arteries to constrict to 5-HT may make them useful for assay to investigate the role of 5-HT in pathogenic vasoconstriction and the receptors that mediate this response. The aim of this study was therefore to assess the response to 5-HT agonists in rabbit isolated epicardial coronary arteries in the presence and absence of endothelium, by use of the antagonists described above as tools, to characterize the receptors that mediate the responses.

It should be noted that 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors have been recently renamed (Hartig *et al*., 1996). The two variants of the 5-HT_{1D} receptor described by Weinshank *et al*. (1992), namely 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$, the latter being the subtype homologue of the rodent 5-HT_{1B} receptor (Hamblin *et al*., 1992; Adham *et al*., 1992), are now called 5-HT_{1D} and 5-HT_{1B} receptors, respectively (Hartig *et al*., 1996). The new nomenclature is used throughout the present paper.

Methods

Preparation of rabbit isolated coronary artery

All experiments were performed in accordance with the United Kingdom Home Office 'Guide to the Operation of the Animals (Scientific Procedures) Act 1986'. New Zealand White rabbits (Froxfield, 2.5–3.0 kg), were terminally anaesthetized and treated with heparin (pentobarbitone 50 mg kg^{-1}, i.v., sodium heparin, 250 i.u., i.v.). Once reflex responses to paw pinch or corneal touching had ceased, the chest was opened and the heart was quickly excised and placed in ice-cold Krebs solution. Approximately 50 mm of the left epicardial coronary artery was carefully dissected under a binocular microscope (Weild, model MGD17, Herburg, Switzerland). Dissected coronary arteries were cleared of excess adipose and connective tissue and divided into 3 mm wide rings (a maximum of 4 per heart). The endothelium was removed by inserting a length (1 mm) of fine bore polythene tubing (outside diameter 400 μm, Portex, Hythe, U.K.) connected to a syringe into the left coronary artery and slowly perfusing the vessel lumen with 2–3 ml of air (Eskinder *et al*., 1990; Liu *et al*., 1994) before dissection. The coronary artery rings were then mounted horizontally on 2 parallel tungsten wire triangles (100 μm in diameter), in tissue baths containing Krebs solution (37°C) of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1, gassed with 95% O_2 and 5% CO_2 to achieve a pH of 7.4. In addition, 1 μM imipramine and 10 μM corticosterone were added to the Krebs solution to prevent neuronal and extraneuronal (endothelial/smooth muscle) uptake of 5-HT, respectively (Apperly *et al*., 1976; Grant & Zuker, 1979).

Experimental protocols

Rings with and without endothelium were allowed to equilibrate for 90 min under a resting tension of 1 g, during which time the tissues were washed 4 times and tension was reapplied as necessary. During this stabilization period, tissues were exposed to pargyline (500 μM) for 30 min to inhibit irreversibly momoamine oxidase (Taylor *et al*., 1974). Tension in the preparation and isometric contractions were recorded via force displacement transducers (Mayward Instruments, model 49043, Cambridge, U.K.) which were connected to an 8 channel preamplifier (Gould, model 660, Cleveland, MA, U.S.A.) and recorded on a computer (Mac Lab, model MCIII, AD Instruments, Sydney, Australia). A reference contraction to KCl (60 mM) was produced in all tissues. This concentration of KCl was maximally effective in preliminary experiments.

Preparations were then washed with Krebs solution and 60 min later were exposed to two or three separate challenges with the stable thromboxane A_2-mimetic, U46619 (10–30 nM), administered at 30 min intervals until the magnitude of peak contraction had stabilized (steady state). The presence or absence of functional endothelium was then assessed with ACh (1 μM) in vessels precontracted with U46619. Vessels responding to ACh with a relaxation (indicative of functional endothelium) were discarded.

Measurement of agonist potency

Following exposure to ACh, the tissues were washed and left for 60 min. Agonist-evoked contractile responses were then produced by cumulative addition of increasing agonist concentrations. Each successive agonist concentration was administered after the response to the previous concentration had reached a steady state (2 to 4 min after administration). In separate relaxation studies, tissues were precontracted with U46619 (30 nM) and left for 30 min to allow tension to stabilize. Attempts were then made to evoke relaxations by cumulative addition of agonists.

Measurement of antagonist potency

Concentration-response curves to 5-HT, 5-carboxamidotryptamine (5-CT) a selective 5-HT_1 receptor agonist (Hoyer *et al*., 1994), sumatriptan, a selective 5-$HT_{1B/1D}$ receptor agonist (Humphrey *et al*., 1988), or (±)-α-methyl-5-hydroxytryptamine ((±)α-me-5-HT), a selective 5-HT_2 agonist (Ismaiel *et al*., 1990) were constructed in the presence of antagonist or antagonist vehicle. Separate preparations were used for individual concentration-response curves because preliminary studies revealed that successive concentration-response curves in a single preparation were not reproducible. Antagonists

were examined at a minimum of three concentrations (one concentration per tissue). Tissues were bathed in the vehicle- or antagonist-containing solution for 60 min before construction of agonist concentration-response curves.

Agonist concentration-ratios were plotted by the method of Arunlakshana & Schild (1959). Slopes were determined by linear regression by use of individual antagonist concentration and agonist log (concentration-ratio -1) values. Measured slopes are presented in order to illustrate whether agonist-antagonist interactions are consistent with an involvement of one or more receptor in mediating the response of the agonist. Only those regressions with slopes not different from unity have been described as Schild plots. When GR127935 was used, the antagonist potency was determined from the displacement of the agonist concentration-response curve observed in the absence and presence of (in separate coronary rings) one concentration of the antagonist (3 nM), by applying the following relationship: $pA_2 = \log_{10}$ (concentration-ratio -1) $-\log_{10}$ (molar concentration of antagonist), and expressed as the apparent pA_2 value (Gupta, 1992). This was because GR127935 at concentrations greater than 3 nM caused a reduction in the maximum response to agonists. These values should not be regarded as truly meaningful in a quantitative sense. The reduction in maximum could be interpreted as grounds for uncertainty over the assumption of simple competition; the apparent pA_2 is a preferable term to apparent pK_B in these circumstances.

If the slope of regression between log M antagonist concentration and log agonist (concentration-ratio -1) was significantly less than unity, we refrained from describing the antagonist log M intercept value for the linear relationship between antagonist log M and agonist log (concentration ratio -1) as a pA_2.

Drugs

The following drugs were used: imipramine hydrochloride, corticosterone, pargyline hydrochloride, 5-hydroxytryptamine creatinine sulphate, U46619 (9,11-dideoxy-11α,9α-expoxy-methanoprostaglandin F_{2a}) and acetylcholine hydrochloride (all purchased from the Sigma Chemical Company, St Louis, MO, U.S.A.); mesulergine, 5-carboxamidotryptamine, ketanserin tartrate, and (±)-α-methyl-5-hydroxytryptamine (from RBI Inc. Natick, MA, U.S.A.); sumatriptan, and GR127935 (N-[4-methoxy-3-(4-methyl-piperazinyl)phenyl]-2″-methyl-4-(5-methyl-1,2,4,-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide) were gifts from Pfizer Research (Sandwich, Kent).

GR127935 was dissolved in 1% w/v citric acid diluted in distilled water. The maximum bath concentration of citric acid was 0.1 nM. Corticosterone and pargyline were dissolved in dimethylsulphoxide (DMSO) and diluted in distilled water, giving a maximum bath DMSO concentration of 1 nM. U46619 was dissolved in absolute ethanol and diluted in distilled water, giving a maximum ethanol bath concentration of 0.1 nM. All other drugs were made up in distilled water and diluted in Krebs solution.

Statistical analysis and calculations

Agonist-evoked contractile responses are expressed as arithmetic mean ± s.e.mean of the % of the KCl response in each corresponding tissue. Each agonist-antagonist combination was examined in preparations from 5–8 different rabbits, where the *n* number represents single tissues from individual animals. Data were analysed by use of a logistic non-linear curve fitting programme (Microcal Origin, version 4.0) from the following equation:

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^P} + A_2$$

where X_0 is the centre of the curve fit (equivalent to EC_{50}), P is the midpoint slope parameter (equivalent to the slope of the concentration-response curve), A_1 is the initial y value (the response in the absence of agonist - zero), A_2 is the final y value (the maximum effect), and the y value at x_0 is halfway between two limiting values A_1 and A_2.

The EC_{50} values (the concentrations of agonists required to produce 50% of the calculated maximum response for the agonist) were used to determine pEC_{50} values (the negative $\log_{10}$ of the EC_{50} value). Agonist log concentration-ratios were determined by subtracting the pEC_{50} value of the agonist in the presence of the antagonist in the test preparation from the pEC_{50} in the control preparation, by use of test and control preparations obtained from the same animal to calculate individual values; the data are expressed as mean log concentration-ratios (with 95% confidence interval). Increments of concentration ratio of ten fold with a ten fold increase in antagonist concentration were regarded as indicative of a competitive interaction at a single receptor (increments become less than ten fold when non-blocked lower affinity receptors predominate in mediating a response to high concentrations of agonist). Statistical comparisons of pEC_{50} values were made by use of Dunnett's test for multiple comparisons. P values < 0.05 were considered significant.

Results

Assessment of endothelial integrity: responses to acetylcholine

ACh (1 μM) caused a relaxation of precontracted coronary rings in the presence of intact endothelium, whereas, following removal of the endothelium, ACh either had no effect or caused a contraction (data not shown).

Effect of 5-HT mimetics

5-CT (1 nM – 10 μM), 5-HT (1 nM – 10 μM), (±)-α-me-5-HT (1 nM – 1 mM) and sumatriptan (1 nM – 300 μM) caused concentration-dependent contractions in coronary rings denuded

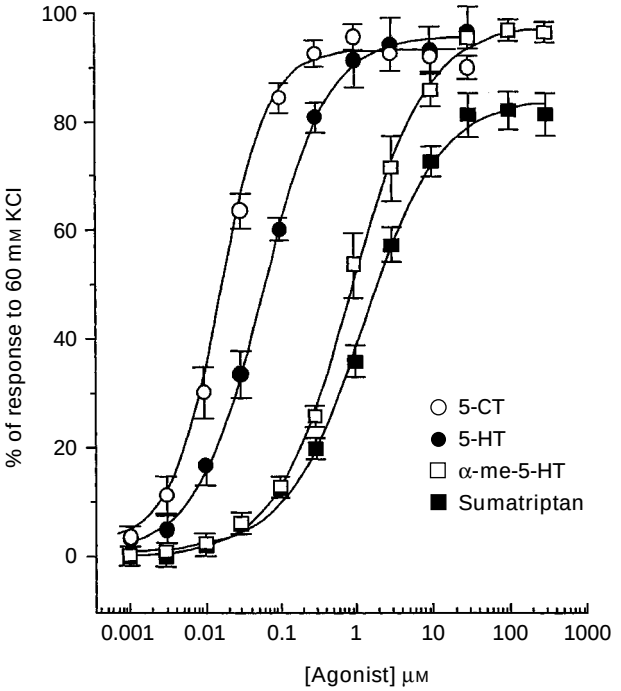

Figure 1 Concentration-response curves to 5-CT ($n = 6$), 5-HT ($n = 8$), (±)-α-me-5-HT ($n = 6$) and sumatriptan ($n = 7$). Data are expressed as the % of response to 60 mM KCl; vertical lines show s.e.mean.

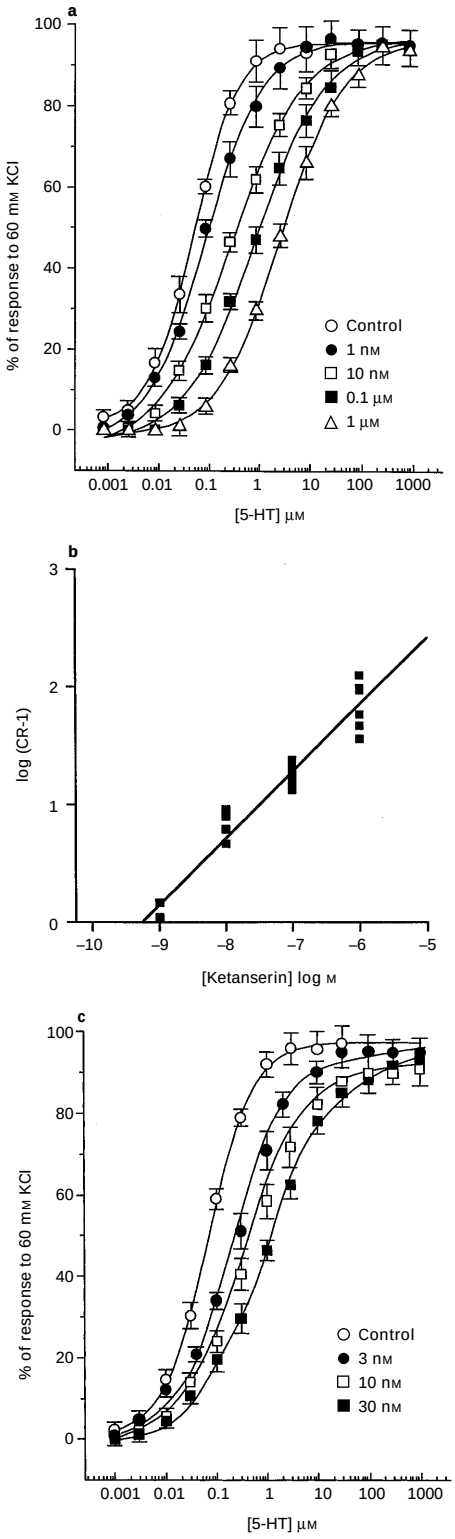

Figure 2 (a) Concentration-response curves to 5-HT alone, (control, $n=6$) and in the presence of ketanserin 1 nM ($n=6$), 10 nM ($n=6$), 0.1 μM ($n=6$) or 1 μM ($n=6$). Data are expressed as % of the response to 60 mM KCl; vertical lines show s.e.mean. (b) Regression between log M ketanserin concentration and log 5-HT (concentration-ratio -1) in rabbit coronary rings ($n=6$ for each concentration). Each point represents data obtained from a separate preparation. The gradient of best fit was determined by linear regression. The slope was 0.57 (0.54–0.61), $r=0.974$, $P<0.05$). The ketanserin log M intercept value for the regression (with 95% confidence limits) was 9.5 (9.2–9.7) (c) Concentration-response curves to 5-HT alone, (control, $n=7$) and in the presence of GR127935 3 nM ($n=7$), 10 nM ($n=7$) or 30 nM ($n=6$). Data are expressed as % of the response to 60 mM KCl; vertical lines show s.e.mean.

Table 1 Characteristics of the contractile responses to 5-HT mimetics in coronary artery rings denuded of endothelium

Agonist	*Maximum* (%)	*pEC_{50}*	*Midpoint slope*	n
5-HT	97±4	7.27±0.05	0.91±0.05	8
5-CT	93±7	7.86±0.08	0.91±0.04	6
Sumatriptan	84±5	5.95±0.04	0.89±0.06	8
(±)-α-me-5-HT	94±4	6.03±0.08	0.92±0.05	6

Data are expressed as arithmetic mean±s.e.mean. Maximum (%) represents the maximum contraction developed to an agonist expressed as percentage response to 60 mM KCl. Abbreviations: 5-HT = 5-hydroxytryptamine, 5-CT = 5-carboxamidotryptamine and (±)-α-me-5-HT = (±)-α-methyl 5-hydroxytryptamine.

of endothelium (Figure 1). The following rank order of potency was obtained on the basis of the calculated pEC_{50} values (Table 1): 5-CT > 5-HT > (±)-α-me-5-HT = sumatriptan. The maximum contractile response to sumatriptan was significantly less ($P<0.05$) than that of the other agonists (Table 1).

None of the agonists tested caused a relaxation of coronary rings precontracted with U46619 (10–30 nM) even in the presence of endothelium (data not shown).

All subsequent experiments were carried out with endothelium denuded preparations. 5-HT, sumatriptan and (±)-α-me-5-HT were used in conjunction with selective antagonists to investigate further the receptor subtypes involved in the contractile response. Since 5-CT is less selective for $5\text{-HT}_{1B/1D}$ receptors than sumatriptan (versus, e.g., 5-HT_{1A} receptors; Hoyer *et al*., 1994) this agonist was not used.

Responses to 5-HT

Ketanserin (1 nM–1 μM) caused concentration-dependent rightward shifts of the concentration-response curve to 5-HT (Figure 2a). There was no change in the maximum response to 5-HT in the presence of ketanserin. The pEC_{50} value in the presence of 5-HT alone was 7.29±0.08, and in the presence of 1 nM, 10 nM, 0.1 μM and 1 μM, the pEC_{50}s were 6.72±0.14, 6.38±0.08, 5.69±0.05 and 5.42±0.06, respectively (all $P<0.05$ versus 5-HT alone). The shift in the response to 5-HT in the presence of ketanserin was not as great as expected for an interaction between 5-HT and a single receptor. This was reflected in the log concentration ratios, at the 50% response level, in the presence of 1 nM, 10 nM, 0.1 μM and 1 μM ketanserin of 0.36 (0.29–0.43), 0.89 (0.77–1.01), 1.28 (1.18–2.06) and 1.84 (1.67–2.06), respectively (means with 95% confidence limits) that clearly failed to increase ten fold with a ten fold increase in ketanserin concentration. In accordance with this, the interaction between ketanserin and 5-HT did not fit the Schild equation and the slope of the linear regression between ketanserin log M and log 5-HT (concentration-ratio -1) was 0.57 (0.54–0.61), significantly less than 1 ($P<0.05$; Figure 2b). The antagonist log M axis intercept value for this regression was 9.5 (9.2–9.7). 5-HT therefore appeared to activate at least two populations of receptors, higher concentrations activating a population resistant to block by ketanserin.

The lowest concentration of GR127935 used (3 nM) caused a rightward shift in the concentration-response curve to 5-HT without a reduction in the maximum response (Figure 2c). The 5-HT pEC_{50} was increased from 7.27±0.06 to 6.44±0.13 ($p<0.05$), and the log concentration-ratio was 0.47 (0.3–0.57). Higher concentrations of GR127935 (10 and 30 nM) caused shifts in the concentration-response relationship that were no different from one another, and reduced the maximum response to 5-HT (Figure 2c). Therefore, the apparent pA_2 was calculated from the 3 nM GR127935 data. This was 8.84 (8.75–8.93). The relationship between GR127935 log M and 5-HT log (concentration-ratio -1) for the full data set (3, 10 and

30 nM GR127935) was linear ($P<0.05$) with a slope of 0.68, consistent with activation by 5-HT of two populations of receptors, one sensitive and another insensitive to GR127935.

Proof of the presence of 5-HT$_{2A}$ receptors: responses to (±)-α-me-5-HT

Ketanserin, even at nanomolar concentrations, caused a rightward shift of the concentration-response curves to (±)-α-me-5-HT (Figure 3a). This was concentration-dependent. Ketanserin (10 nM – 1 μM) had no effecft on the maximum response to (±)-α-me-5-HT (Figure 3a). The pEC$_{50}$ to (±)-α-me-5-HT alone was 6.04±0.08, and in the presence of 10 nM, 0.1 μM and 1 μM ketanserin, the pEC$_{50}$ values were reduced to 5.06±0.08 ($P<0.05$), 4.04±0.12 ($P<0.05$), and 3.23±0.04 ($P<0.05$), respectively. The log concentration-ratios increased ten fold with ten fold increases in ketanserin concentration: 0.95 (0.79 – 1.13), 1.93 (1.78 – 2.09) and 2.8 (2.68 – 2.94), respectively. Accordingly, the relationship between log M ketanserin concentration and log (±)-α-me-5-HT (concentration-ratio −1) fitted the Schild equation with a slope no different from unity (0.95 (0.93 – 0.97)), and the Schild plot (Figure 3b) gave a pA$_2$ value (with 95% confidence limits) of 9 (8.9 – 9.2).

The concentration-response curve to (±)-α-me-5-HT was not affected by GR127935 (3 – 30 nM) (data not shown), indicating a lack of effect of this agonist on 5-HT$_{1B/1D}$ receptors.

Exploration of the presence of 5-HT$_{1B/1D}$ receptors: responses to sumatriptan

Ketanserin, at nanomolar concentrations (which had been sufficient to antagonize responses to (±)-α-me-5-HT) had no effect on responses to sumatriptan (Figure 4a). Ketanserin caused a parallel rightward shift of the concentration-response curve to sumatriptan only at the two highest concentrations used, 1 and 10 μM (Figure 4a), whereby pEC$_{50}$s were 5.27±0.06 and 4.49±0.05, respectively ($P<0.05$), and the log concentration-ratios were 0.64 (0.59 – 0.07) and 1.43 (1.36 – 1.5), respectively. An apparent pA$_2$ value, estimated for the antagonism of sumatriptan by 1 μM ketanserin was 6.53 (6.47 – 6.58). A similar value (6.5) was found with the 0.1, 1 and 10 μM ketanserin data and plotting log M ketanserin concentration against log sumatriptan (concentration-ratio −1) and imposing a unity slope (data not shown). Both estimates are consistent with an interaction at 5-HT$_{1B/1D}$ rather than at 5-HT$_{2A}$ receptors.

GR127935 (3 – 30 nM), caused a rightward shift in the concentration-response curve to sumatriptan (Figure 4b). There was no significant change in the maximum response when the lowest concentration of GR127935 was used (3 nM, Figure 4b), but at higher concentrations (10 and 30 nM), GR127935 caused a large and significant reduction in the maximum response to sumatriptan ($P<0.05$, Figure 4b). There was also a significant change in the pEC$_{50}$ values: 5.94±0.07 for sumatriptan alone and 5.41±0.06, 4.88±0.05 and 4.39±0.06 (all $P<0.05$) in the presence of 3, 10 and 30 nM GR 127935, respectively. The log concentration-ratios in the presence of 3, 10 and 30 nM GR127935 were 0.53 (0.45 – 0.61), 1.02 (0.94 – 1.11), and 1.5 (1.45 – 1.56), respectively (increasing ten fold with a ten fold increase in GR127935 concentration). An apparent pA$_2$ value was estimated from the effect of the lowest concentration of GR127935 (3 nM). This was 8.92 (8.86 – 9.01), and is consistent with an interaction at 5-HT$_{1B/1D}$ receptors.

Antagonism of responses to 5-HT by GR127935 and ketanserin combined

As neither GR127935 nor ketanserin alone were able to antagonize fully the response to 5-HT, a further study was done with a combination of GR127935 and ketanserin (0.1 or 1 μM) to seek further support for the evidence that responses to 5-HT are mediated by a combination of 5-HT$_{1B/1D}$ and 5-HT$_{2A}$ receptors.

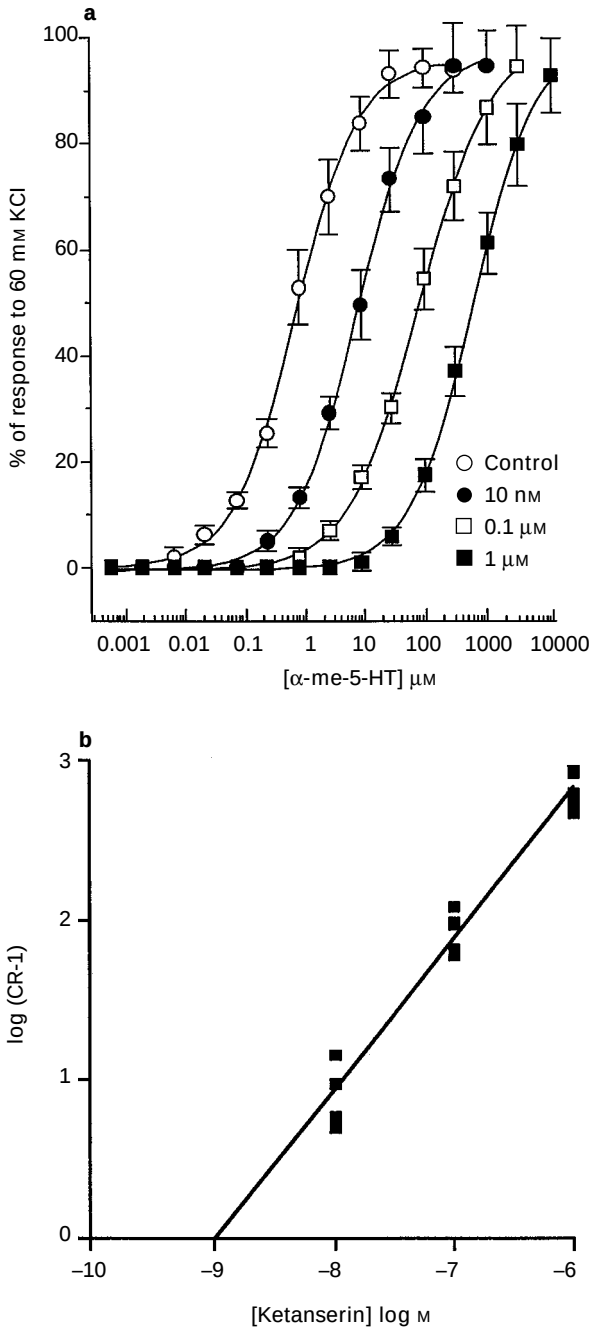

Figure 3 (a) Concentration-response curves to (±)-α-me-5-HT alone, (control, $n=6$) and in the presence of ketanserin 10 nM ($n=6$), 0.1 μM ($n=5$) or 1 μM ($n=6$). Data are expressed as % of the response to 60 mM KCl; vertical lines show s.e.mean. (b) Schild plot for ketanserin (1 nM – 1 μM) versus (±)-α-me-5-HT ($n=6$ for each concentration). CR = concentration-ratio. Each point represents data obtained from a separate preparation. The gradient of best fit was determined by linear regression and the slope was 0.95 (0.93 – 0.97), $r=0.986$; $P<0.05$. The pA$_2$ value (with 95% confidence limits) was 9 (8.0 – 9.2).

In the presence of 0.1 μM ketanserin, 3 nM GR127935 caused a rightward shift in the concentration-response curve to 5-HT, with no significant reduction in the maximum response, and 5-HT had a pEC$_{50}$ value of 5.59±0.11. In the presence of 0.1 μM ketanserin, 10 and 30 nM GR127935 caused a reduction in the maximum response and a rightward displacement of the 5-HT concentration-response curve ($P<0.05$), the magnitudes of which were similar for both concentrations of GR127935 (as occurred in the absence of ketanserin).

However, in the presence of 1 μM ketanserin, the rightward displacement of the 5-HT concentration-response curve caused by 30 nM GR127935 became greater than that caused by 10 μM GR127935 (Figure 5). The log concentration-ratios with 3, 10 and 30 nM GR127935 were 0.44 (0.31–0.6), 0.9 (0.79–1.02) and 1.44 (1.33–1.56), increasing ten fold with a ten fold increase in GR127935 concentration. Accordingly, the slope of regression between log M GR127935 concentration and log 5-HT (concentration-ratio −1), which was 1.1, was not significantly different from unity ($P<0.05$ compared with the value, 0.68, in the absence of ketanserin). The apparent pA_2 value for GR127935 was 8.8 (8.7–9). This was almost identical to the value of the apparent pA_2 (8.7) in tissues pretreated with the lower concentration of ketanserin (0.1 μM), which was determined less reliably by imposing a slope of unity on the log 5-HT (concentration-ratio −1) values in the presence of 3 nM GR127935. These data lend further support to the accumulated evidence that 5-HT interacts with both 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors to constrict rabbit epicardial coronary arteries.

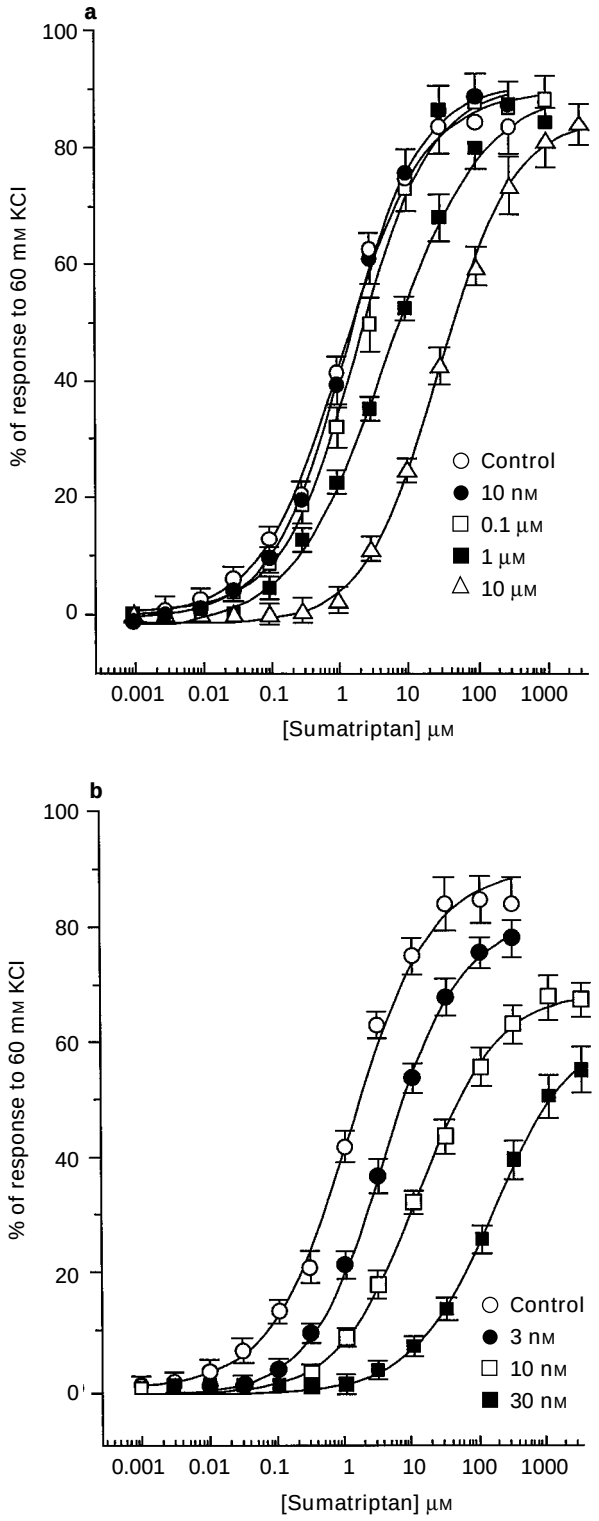

Figure 4 (a) Concentration-response curves to sumatriptan alone, (control, $n=7$) and in the presence of ketanserin 10 nM ($n=7$), 0.1 μM ($n=7$) or 1 μM ($n=6$) and 10 μM ($n=6$). (b) Concentration-response curves to sumatriptan alone, (control, $n=7$) and in the presence of GR127935 3 nM ($n=7$), 10 nM ($n=6$) or 30 nM ($n=6$). Data are expressed as % of the response to 60 mM KCl; vertical lines show s.e.mean.

Discussion

Actions of 5-HT mimetics

We have explored the identity of the receptors mediating contractile responses to 5-HT in rabbit epicardial coronary capacitance arteries. In precontracted endothelium-intact coronary artery rings 5-HT, and a range of mimetics, did not cause a relaxation. This is consistent with published data (Awano *et al.*, 1989). In contrast, following removal of the endothelium, all 5-HT mimetics produced a concentration-dependent coronary artery constriction.

An agonist potency order of 5-CT > 5-HT > sumatriptan > (±)-α-me-5-HT has been used as a fingerprint to define 5-HT_1-like receptors (Martin, 1994). An agonist potency order of 5-HT > (±)-α-me-5-HT > 5-CT is likewise a fingerprint for 5-HT_2 receptors (Martin, 1994). In the present study, the rank order of agonist potency was 5-CT > 5-HT > sumatriptan = (±)-α-me-5-HT. This does not fit exactly with the potency order for any single population of 5-HT receptor but is consistent in many respects with the presence of 5-$HT_{1B/D}$ and 5-HT_{2A} receptors. For example, the pEC_{50}s for sumatriptan (6.0) and (±)-α-me-5-HT (6.0) are similar to those found in tissue (human saphenous vein) where both 5-HT_{1D}-like and 5-HT_2 receptors are believed to be present (Bax *et al.*, 1992). Agonist potency orders in tissues possibly expressing 5-$HT_{1B/}$

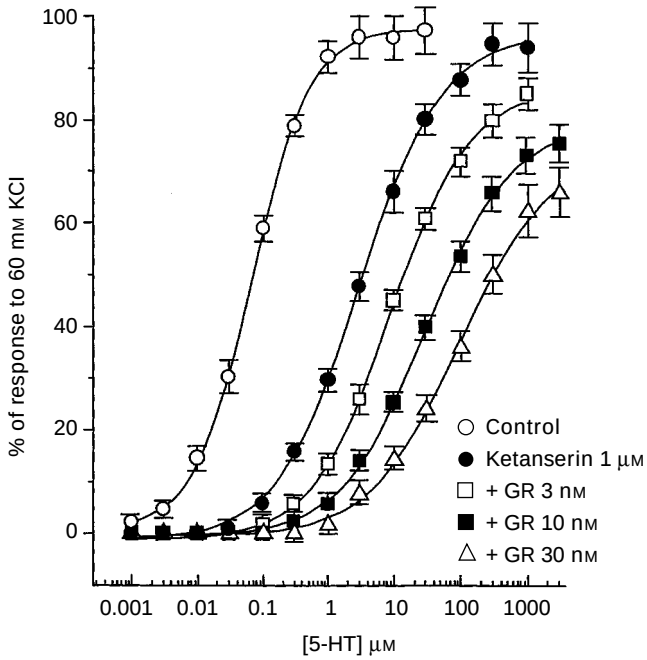

Figure 5 Concentration-response curves to 5-HT alone, (control, $n=7$) and in the presence of 1 μM ketanserin ($n=7$), or 1 μM ketanserin combined with GR127935 3 nM ($n=7$), 10 nM ($n=7$) or 30 nM ($n=6$). Data are expressed as % of the response to 60 mM KCl; vertical lines show s.e.mean.

$_{1D/2A}$ receptors such as rabbit cerebral arteries (5-CT > 5-HT > sumatriptan > (±)-α-me-5-HT) (Deckert *et al*., 1994), and rabbit jugular vein (5-CT > 5-HT > (±)-α-me-5-HT) (Martin *et al*., 1987), is broadly similar to that found in the present study, and differences may simply reflect variation in the relative preponderance of these receptors in different tissues. The profile is certainly very different from the order (±)-α-me-5-HT > sumatriptan > 5-HT > 5-CT and the range of EC_{50} values (4.7 – 5.7) obtained for a rabbit renal artery preparation that expresses a heterogeneous population of receptors believed not to include the 5-HT_{1B} or 5-HT_{1D} subtypes (Tadipatri *et al*., 1991).

To establish the involvement of 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors in the responses observed in the present experiments, combinations of agonists and antagonists with different receptor selectivities were used.

Evidence for the presence of constrictor 5-HT_{2A} receptors

The presence in the preparation of 5-HT_{2A} receptors was established unequivocally by the response to the selective 5-HT_2 receptor agonist (±)-α-me-5-HT (pEC_{50} 6.03) and antagonism of this response by ketanserin ($pA_2 = 9$, unity Schild slope). Ketanserin, at submicromolar concentrations (1 – 100 nM), is selective for 5-HT_{2A} receptors (Van Nueten *et al*., 1981; Mylecharane, 1990; Hoyer *et al*., 1994). Confidence in the relative selectivity of ketanserin for 5-HT_{2A} versus 5-$HT_{1B/1D}$ receptors was established from its lack of effect at submicromolar concentrations ($pA_2 = 6.5$) on contractions caused by sumatriptan, a selective 5-$HT_{1B/1D}$ agonist (Schoeffter & Hoyer, 1989; Peroutka & McCarthy, 1989). Although (±)-α-me-5-HT is known to interact with 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors (Baxter *et al*., 1995), a contributory involvement of 5-HT_{2B} and 5-HT_{2C} receptors to the interaction between (±)-α-me-5-HT and ketanserin can be ruled out on two grounds: the Schild plot indicated an interaction with a single population of receptors and the pA_2 (9) was similar to the pK_i for ketanserin binding with 5-HT_{2A} receptors (8.9) and not 5-HT_{2B} (5.4) or 5-HT_{2C} (7) receptors (Baxter *et al*., 1995).

5-HT_{2A} receptors therefore appeared to be involved in the response to 5-HT, since there were parallel rightward displacements of 5-HT concentration-response curves by ketanserin.

Evidence for the presence of constrictor 5-$HT_{1B/1D}$ receptors

The agonist potency order discussed earlier points to multiple 5-HT receptor involvement in the contractile responses. Independent evidence in support of this was provided by the slope of the linear regression between ketanserin log concentration and 5-HT log (concentration-ratio −1) (0.57), which was significantly less than the slope of the regression for the interaction between (±)-α-me-5-HT and ketanserin (0.95).

Evidence for the presence of 5-$HT_{1B/1D}$ receptors in the tissue was suggested by the lack of effect of ketanserin on the response to sumatriptan. It was confirmed by the ability of GR127935, a selective 5-$HT_{1B/1D}$ receptor antagonist (Skingle *et al*., 1993) to antagonize the constrictor response to sumatriptan. The pA_2 for this interaction could not be determined with certainty owing to a reduction in the maximum response with high antagonist concentrations. The apparent pA_2 value of 8.92 was less than the 9.4 obtained by Razzaque *et al*. (1995) in rabbit saphenous vein, but similar to the 8.9 found by Clitherow *et al*. (1994) in dog saphenous vein. Notwithstanding, these data, taken together, indicate the presence of 5-$HT_{1B/1D}$ receptors in the tissue.

Evidence that 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors jointly contribute to 5-HT-induced contraction

The effect of GR127935 on the contractile responses to 5-HT was assessed to evaluate the involvement of 5-$HT_{1B/1D}$ receptors. The apparent pA_2 (8.8) assessed from the effect of 3 μM GR127935 suggested a high potency effect consistent with an interaction at 5-$HT_{1B/1D}$ receptors. However, an interaction typical of competitive antagonism by GR127935 occurred only with the lowest concentration (3 μM). It was not possible to perform true Schild analysis as the slope of the regression between GR127935 log M concentration and 5-HT log (concentration-ratio −1) with all three concentrations of GR127937 was only 0.68. This was because 30 μM GR127935 was no more effective than 10 μM in antagonizing responses to 5-HT. This is consistent with the emerging evidence that 5-HT activates 5-$HT_{1B/1D}$ receptors and at least one other receptor in the tissue.

Since the presence of 5-HT_{2A} receptors was established in the tissue from the interaction between ketanserin and (±)-α-me-5-HT, it is reasonable to propose that lower affinity activation of 5-HT_{2A} receptors contributed to the effects of 5-HT, as suggested for other tissues (Martin, 1994).

Further evidence in support of the joint presence of constrictor 5-$HT_{1B/1D}$ an 5-HT_{2A} receptors in the tissue was provided by the fact that addition of 1 μM ketanserin enhanced the ability of 30 μM GR127935 to antagonize responses to 5-HT and unmasked a near-competitive interaction between GR127935 and 5-HT (parallel shifts in 5-HT concentration-response curves and a Schild slope close to unity). The estimated apparent pA_2 values for the interaction between 5-HT and GR127935 in the presence and absence of 1 μM ketanserin were similar (in the region of 8.8) despite the necessity of using two different methods to estimate the value in the absence and presence of ketanserin.

Considered together, these data strongly indicate that the mixed receptor population mediating contractile responses to 5-HT includes 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors.

Possible discrimination between the involvement of 5-HT_{1B} and 5-HT_{1D} receptors

In human coronary arteries, 5-HT-induced contraction appears to involve 5-HT_2 and 5-HT_1 receptors (Kaumann *et al*., 1993), as found in rabbit in the present study. In both species, the 5-HT_2 receptor appears to be the 5-HT_{2A} subtype (Kaumann *et al*., 1994 and the present study). However, there appears to be some species-dependent variation in the identity of the type of the 5-HT_1 receptor involved (Magnon *et al*., 1989; Toda & Okamura, 1990; Nyborg, 1991; Feletou & Teisseire, 1992; Cushing & Cohen, 1992). In human coronary arteries the 5-HT_{1B} receptor has been suggested to mediate contraction (Kaumann *et al*., 1994).

In the present study, high concentrations of ketanserin (1 and 10 μM) competitively antagonized the response to sumatriptan, whereas lower concentrations were ineffective. This raises the possibility of the 5-HT_{1D} rather than the 5-HT_{1B} receptor involvement in the response to 5-HT, since it has been suggested that at micromolar concentrations, ketanserin has some affinity for 5-HT_{1D} receptors, but not 5-HT_{1B} receptors (Kaumann *et al*., 1993; 1994). Cloned 5-HT_{1D} receptors from rabbit liver have an 800 fold greater affinity for ketanserin than cloned 5-HT_{1B} receptors (Harwood *et al*., 1985).

However, studies in other species have shown that the ability of ketanserin to differentiate between 5-HT_{1B} and 5-HT_{1D} receptors is questionable. Ketanserin exhibits only a 70 fold affinity difference between human cloned 5-HT_{1D} and 5-HT_{1B} receptors (Zgombick *et al*., 1995; Bard *et al*., 1996). In human isolated coronary arteries, Kaumann *et al*. (1993, 1994) showed that although ketanserin was able to antagonize the contractile responses to 5-HT, ketanserin has no effect on sumatriptan-induced responses. Similar findings have been obtained for non-coronary vessels from the guinea-pig (Sahin Erdmeli *et al*., 1992; Razzaque *et al*., 1995). In dog coronary arteries, ketanserin does not discriminate between 5-HT_{1B} and 5-HT_{1D} receptors (Branchek *et al*., 1995; Terron, 1996).

The apparent pA_2 value estimated for the antagonism of sumatriptan by GR127935 was 8.92. GR127935 has been

shown to have a pA_2 of 9.9 for its interaction with 5-HT_{1B} receptors versus 8.9 for 5-HT_{1D} receptors (Skingle *et al*., 1993). This could be regarded as independent evidence for 5-HT_{1D} (as opposed to 5HT_{1B}) receptor involvement in responses to 5-HT in the present study. Nevertheless, since we were unable to determine a true pA_2 for the GR127935/sumatriptan interaction, it would be unwise to regard this evidence as unequivocal.

Methiothepin, a mixed 5-HT_1/5-HT_{2A} receptor antagonist (Kaumann *et al*., 1994; Deckert *et al*., 1994) has been shown to differentiate between rabbit (although not human; Zgombick *et al*., 1996) cloned 5-HT_{1D} and 5-HT_{1B} receptors, showing a 17 fold greater affinity for 5-HT_{1B} receptors (Bard *et al*., 1996) (Table 2). Therefore, in future studies in rabbit isolated coronary arteries it may be useful to use methiothepin to attempt to discriminate between 5-HT_{1D} and 5-HT_{1B} receptor involvement.

Possible limitations of the study

GR127935 has been shown to be a 5-HT_{1D} receptor partial agonist at concentrations above 10 nM in cultured cell lines (Watson *et al*., 1995; Pauwels *et al*., 1995; Pauwels & Palmier, 1995; Pauwels & Colpaert, 1995) which, if so, would compromise some of our data. However, we saw no evidence of agonist action (contraction) with GR127935 in the present study. A separate difficulty did arise from the observation that the antagonism by GR127935 (at concentrations higher than 3 nM) of the response to 5-HT and sumatriptan was associated with a reduction in the maximum agonist response. Similar observations have been made in dog saphenous vein (Clitherow *et al*., 1994) and rabbit saphenous vein (Razzaque *et al*., 1995), although not in human small intestine (Borman & Burleigh, 1995). It has been argued by Razzaque *et al*. (1995) that the apparent insurmountable action of GR127935 is due to its high lipophilicity and is not necessarily incompatible with a competitive action; the effect is in fact slowly reversible (Skingle *et al*., 1993). We attempted to avoid potential problems associated with possible non-competitive antagonism by focusing on the effects of GR127935 at a very low concentration (3 nM), in order to estimate apparent pA_2 values. Although this made the pA_2 estimates potentially unreliable, it did not suggest interpretations that are incompatible with the conclusions derived from the other data in the study.

The cloning of 5-HT receptors and expression in cell lines may help to resolve species differences (Lucas & Hen, 1995; Hartig *et al*., 1996). However, 5-$HT_{1B/1D}$ receptors display a remarkable variation in drug affinity profile between species despite a high degree of sequences homologies. Although rabbit 5-$HT_{1B/1D}$ receptors show greater than 90% sequence homology to cloned human 5-$HT_{1B/1D}$ receptors (Harwood *et al*., 1995; Bard *et al*., 1996) there are obvious pharmacological differences, such as the different affinities for sumatriptan and ketanserin. Our inability to discriminate between 5-HT_{1D} and 5-HT_{1B} receptor involvement emphasizes the importance of not relying exclusively on the actions of single drugs (or indeed, a single species) for assessing the 5-HT_1 receptor subtypes involved in mediating the actions of 5-HT by pharmacological profiling. Ultimately, this may mean that the only truly clinically relevant means of exploring drug interactions with 5-$HT_{1B/1D}$ receptors is to use human receptors (native or cloned).

Table 2 Affinity constants for 5-HT receptor modulators at cloned receptors

	Rabbit		*Human*		*Dog*	
Drug	*5-HT$_{1D}$*	*5-HT$_{1B}$*	*5-HT$_{1D}$*	*5-HT$_{1B}$*	*5-HT$_{1D}$*	*5-HT$_{1B}$*
5-CT	8.53	8.16	9.15	8.80		
	(9.08)	(8.44)	(9.26)	(8.41)		
5-HT	7.88	8.17	8.41	8.37		
	(8.57)	(8.33)	(8.33)	(8.00)		
Sumatriptan	7.12	6.84	8.34	5.11		
	(7.45)	(6.52)	(8.17)	(7.42)		
Ketanserin	7.66	6.30	7.14	5.28	5.5	5.5
	(7.49)	(5.40)	(7.36)	(5.27)		
Methio-thepin	6.64	7.86	7.96	7.60		

Values are pK_i (and pIC_{50} in parentheses) in log M. Data are from Bard *et al*. (1996), Branchek *et al*. (1995) and Harwood *et al*. (1995).

Conclusion

The present data provide strong evidence for the presence of multiple 5-HT receptors mediating contractile responses to 5-HT in rabbit epicardial coronary artery denuded of endothelium. These are the 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors.

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