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5-HT_{2B} receptor-mediated calcium release from ryanodine-sensitive intracellular stores in human pulmonary artery endothelial cells

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1 We have characterized the 5-hydroxytryptamine (5-HT)-induced calcium signalling in endothelial cells from the human pulmonary artery. Using RT-PCR we show, that of all cloned G-protein coupled 5-HT receptors, these cells express only 5-$HT_{1D\beta}$, 5-HT_{2B} and little 5-HT_4 receptor mRNA.

2 In endothelial cells 5-HT inhibits the formation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) via 5-$HT_{1D\beta}$ receptors but fails to activate phosphoinositide (PI) turnover. However, the latter pathway is strongly activated by histamine.

3 Despite the lack of detectable inositol phosphate (IP) formation in human pulmonary artery endothelial cells, 5-HT ($pD_2 = 5.82 \pm 0.06$, $n=6$) or the selective 5-HT_2 agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) ($pD_2 = 5.66 \pm 0.03$, $n=7$) elicited transient calcium signals comparable to those evoked by histamine ($pD_2 = 6.44 \pm 0.01$, $n=7$). Since 5-HT_{2A} and 5-HT_{2C} receptor mRNAs are not detectable in pulmonary artery endothelial cells, activation of 5-HT_{2B} receptors is responsible for the transient calcium release. The calcium transients are independent of the inhibition of adenylate cyclase, since DOI does not stimulate 5-$HT_{1D\beta}$ receptors.

4 Both, the 5-HT- and histamine-stimulated calcium signals were also observed when the cells were placed in calcium-free medium. This indicates that 5-HT triggers calcium release from intracellular stores.

5 Heparin is an inhibitor of the IP_3-activated calcium release channels on the endoplasmic reticulum. Intracellular infusion of heparin through patch pipettes in voltage clamp experiments failed to block 5-HT-induced calcium signals, whereas it abolished the histamine response. This supports the conclusion that the 5-HT-induced calcium release is independent of IP_3 formation.

6 Unlike the histamine response, the 5-HT response was sensitive to micromolar concentrations of ryanodine and, to a lesser extent, ruthenium red. This implies that 5-HT_{2B} receptors trigger calcium release from a ryanodine-sensitive calcium pool.

7 It has been postulated that cyclic ADP-ribose (cADPR) is a soluble second messenger which activates ryanodine receptors. However, calcium signals similar to the 5-HT response could not be elicited by intracellular infusion with cADPR. Furthermore, the subsequent application of 5-HT or DOI elicited a calcium signal that was not affected by the above pretreatment.

8 We conclude that human 5-HT_{2B} receptors stimulate calcium release from intracellular stores through a novel pathway, which involves activation of ryanodine receptors, and is independent of PI-hydrolysis and cADPR.

Keywords: Human pulmonary artery endothelial cells; 5-HT_{2B} receptor; heparin; ryanodine receptor

Introduction

It has been known for many years that 5-hydroxytryptamine (5-HT) stimulates contractions of the rat isolated stomach fundus (Vane, 1957). The rat stomach fundus 5-HT receptor was pharmacologically characterized (Cohen & Fludzinski, 1987; Baxter *et al.*, 1994) and found not to be identical to the similar 5-HT_{2C} receptor (Baez *et al.*, 1990). Subsequently, the rat stomach fundus 5-HT receptor was cloned and further characterized (Foguet *et al.*, 1992a; Kursar *et al.*, 1992; Wainscott *et al.*, 1994). This receptor was then named 5-HT_{2B} (Hoyer *et al.*, 1994), because its pharmacology, DNA sequence, and genomic organization are closely related to the 5-HT_{2A} and even more to the 5-HT_{2C} receptor (Foguet *et al.*, 1992b). Subsequent cloning of the human homologue of the rat receptor (Schmuck *et al.*, 1994; Kursar *et al.*, 1994) revealed high levels of mRNA expression in human peripheral tissues. Low expression levels were seen in brain and blood (Schmuck *et al.*, 1994). Since 5-HT_{2C} receptor mRNA expression has only been detected in the central nervous system and the choroid plexus (Julius *et al.*, 1988), several peripheral functions that were thought to be mediated by 5-HT_{2C} receptors may rather be caused by 5-HT_{2B} receptors.

We recently reported that human 5-HT_{2B} receptors are expressed in various rat and porcine blood vessels and human endothelial cells (Ullmer *et al.*, 1995). This localization as well as the pharmacology of 5-HT_{2B} receptors implicates an involvement in the endothelium-dependent relaxation of various blood vessels, like rat jugular vein (Leff *et al.*, 1987; Bodelsson *et al.*, 1993; Ellis *et al.*, 1995) and pig pulmonary artery (Glusa & Richter, 1993) via activation of the endothelial Ca^{2+}-calmodulin-dependent nitric oxide synthase. Rat 5-HT_{2B} receptors activate calcium-dependent chloride channels when expressed in *Xenopus* oocytes (Foguet *et al.*, 1992a). The mouse and human homologues were shown to stimulate phosphoinositide (PI) turnover in transformed embryonic teratocarcinoma cells, 1C11, (Loric *et al.*, 1995) or in transfected HEK 293 cells, respectively (Schmuck *et al.*, 1994). In contrast, PI hydrolysis could not be found in association with 5-HT_{2B} receptor-mediated contractions of the rat stomach

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fundus (Secrest *et al.*, 1989). However, the contraction was partially inhibited after blocking L-type calcium-channels. The remaining contraction could be mildy reduced by ryanodine (Cox & Cohen, 1995). Similar studies have not been performed with blood vessels that elicit a 5-HT$_{2B}$-like endothelium-dependent relaxation.

In the present study, we analyzed calcium mobilization, PI-turnover and adenylate cyclase activity in endothelial cells from human pulmonary arteries. We found that the 5-HT$_{2B}$ receptor triggers calcium release from intracellular stores in the absence of detectable IP formation through a pathway which is insensitive to heparin but blocked by ryanodine and ruthenium red.

Methods

Cells and culture conditions

Endothelial cells from human pulmonary artery were purchased from Clonetics and grown according to the supplier's instructions. Cells were used for up to 15 passages.

Oligonucleotides

The following oligonucleotides (ON) which are specific for the different cloned human 5-HT receptor subtypes (data not shown) were used as primers for RT-PCR (accession numbers and positions of the PCR product within the coding sequence in parentheses).

human 5-HT$_{1A}$ (M83181, position (-133) - 197)
ON 1: CCT GCT TGG GTC TCT GCA TTC C
ON 2: GGA GCG CTC CAA GGG GAT GGC A

human/rat 5-HT$_{1D\alpha}$ (M81589, position 272-852)
ON 3: TCA TGC CCA TCA GCA T(AC)G CC
ON 4: GAA AAT CAA GCT TGC TGA (CT)AG

human/rat 5-HT$_{1D\beta}$ (M75128, position 140-742)
ON 5: CCT GGA AAG T(AC)C TGC TGG T
ON 6: CGG TC(CT) TGT TGG G(TC)G TCT GT

human 5-HT$_{1E}$ (Z11166, position 134-629)
ON 7: AAG CAC AGA TAG CCA GAA TT
ON 8: ACG GTG TAG ATG CTC AGA CC

human/rat 5-HT$_{1F}$ (L05596, position 138-503)
ON 9: TGC (AG)AT (TC)AT TGT GAC (CT)CG GA
ON 10: C(GT)G CTA (TG)TT CCT TGG TGC CTC

human 5-HT$_{2A}$ (M36966, position 560-837)
ON 11: ACT CCA GAA CTA AGG CAT TT
ON 12: AGC TAA TTT GGC CCG TGT GCC

human 5-HT$_{2B}$ (X77307, position 404-835)
ON 13: ACG TTC TCT TTT CAA CCG CA
ON 14: CCG GTG ACG AGC AAG GTG TT

human 5-HT$_{2C}$ (M81778, position 323-611)
ON 15: CCC TGT CTC TCC TGG CAA TC
ON 16: TTG TTC ACG AAC ACC TTT TC

human 5-HT$_4$ (Z48150, length of PCR-product 397 bp)
ON 17: CTG GAT AGG TAT TAC GCC ATC T
ON 18: GTG CTG TGC TGG TCA GCT GTC T

human 5-HT$_{5A}$ (X81482, position 306-662)
ON 19: CTG GGC G(CA)C G(CT) T GGC AGC T
ON 20: TCC AGT A(CG)A C(AG)A AGA GCA CCA C

human 5-HT$_6$ (Z49119, length of PCR product 200 bp)
ON 21: GCT GCT GAT CGT GCT CAT TTG CA
ON 22: CTG TCA TGC GCA GCT TGT AGC GCA

human 5-HT$_7$ (L21195, position 704-1035)
ON 23: AGG ACT TTG GCT ATA CGA TT,
ON 24: GAG GAA AAA TGG CAG CCA G;

Oligonucleotides were synthesized on an Applied Biosystems 380A synthesizer and purified by gel filtration through Sephadex G 25 columns (Boehringer).

RT-PCR

RNA was prepared from 5×10^6 cells and residual DNA was digested with DNase as described by Ullmer *et al.* (1995). For the RT-PCR, 1 μg of each RNA was reverse transcribed in a final volume of 20 μl using M-MLV reverse transcriptase (BRL) in PCR-Buffer (Boehringer), 10 mM DTT, 5 μM hexanucleotides (Pharmacia), 0.2 mM of each dNTP, 40 u rRNasin (Promega) for 1 h at 37°C. PCRs were performed in a final volume of 50 μl with 10% of the reverse transcription mixture, 10 pmol of each primer, 1 u Taq-DNA-polymerase (Boehringer), 0.25 mM of each dNTP (Cetus) and 2 μCi [α-^{32}P]-dCTP under the following conditions: 30 s at 55°C, 1 min at 72°C, 30 s at 94°C for 38 cycles. The ^{32}P-labelled PCR-products were separated on 4% agarose gels (NuSieve, FML). The gels were dried and exposed to X-ray films.

Measurement of [^{3}H]-inositol phosphate formation

For the measurement of the [^{3}H]-inositol phosphate formation 2×10^6 cells were split into a 24 well plate and labelled to equilibrium with *myo*-[2-^{3}H]-inositol (3 μCi ml^{-1}) for an additional 24 h in growth medium. The medium was aspirated and cells were washed once with 500 μl HBS-buffer (NaCl 130 mM, NaH_2PO_4 900 μM, $MgSO_4$ 800 μM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, glucose 25 mM in HEPES 20 mM pH 7.4). Two min after applying 20 mM Li^+, the cells were stimulated by addition of the agonist in HBS-buffer. The measurement of total [^{3}H]-inositol phosphate formation was performed as described by Seuwen *et al.* (1988).

Determination of cyclic AMP

To measure cyclic AMP production, 2×10^6 cells were split 48 h prior to experiments in 24 well plates. Cells were prelabelled with [2-^{3}H]-adenine at 5 μCi ml^{-1} for 2 h in growth medium, washed and incubated in HBS containing 1 mM isobutylmethylxanthine (IBMX), 1 μM forskolin and the indicated agonist at 37°C for 15 min. The cells were then extracted with 5% ice-cold trichloracetic acid (TCA). [^{3}H]-ATP and [^{3}H]-cyclic AMP were separated by sequential chromatography on Dowex and alumina columns as described by Salomon (1979). All pD_2 values were calculated with the SigmaPlot 4.1 software package.

Combined patch-clamp and [Ca^{2+}]$_i$ measurements in single cells

Cells seeded on glass coverslips were loaded for 45 min with 5 μM fura-2 acetoxymethylester prior to experiments. Coverslips were mounted on a Zeiss Axiovert-135M microscope and perfused with oxygenated buffer (composion, mM: NaCl 140, KCl 5, $CaCl_2$2, $MgCl_2$1, glucose 5.6, HEPES 5, Ph 7.4).

Whole cell recordings were performed with a List LM-PC amplifier. Patch electrodes were prepared from thin walled capillary borosilicates glass. The intracellular pipette solution contained (mM): K-gluconate 150, $MgCl_2$ 2, $CaCl_2$ 0.2, EGTA 0.1, Mg-ATP 5, Li-GTP 0.1 and HEPES 5, adjusted to pH 7.2 with KOH. Where indicated, this solution was supplemented with 45 USP units ml^{-1} of heparin which corresponds to an approximate concentration of 0.1 mM, 10 μM ryanodine, 10 μM ruthenium red or 100 μM cADPR. Pipette resistances were 3–10 MΩ. Calcium responses were recorded from cells in current-clamp mode (Boddeke *et al.*, 1993). Substances were applied by low pressure (3–4 psi, 3s) from a micropipette

placed 5–10 μm from the cell. Changes in fura-2 fluorescence were determined using a Centronics photodiode array mounted on the inverted microscope and connected to a set of amplifiers. Excitation was alternated at 340 and 380 nm, emission was recorded at 509 nm. Fluorescence ratios R = F_{340}/F_{380} were calculated on line. Calibration was performed with 5 μM ionomycin and EGTA at the end of each experiment as described above. The calcium responses were recorded and analyzed using pClamp (Axon Instruments) and IDL Interactive data language (Creaso) software. The pK_B value was calculated according to the formula: pK_B = log (A'/A - 1) - log [B], where A'/A is the ratio of the agonist concentrations (EC_{50} in the presence / EC_{50} in the absence of antagonist) and [B] the concentration of antagonist. The calculated calcium concentrations, pD_2 and pK_B values are given as means ± s.e.

Chemicals

Cyclic adenosine diphosphate ribose (cADPR), [2-^{3}H]-adenine and *myo*-[2-^{3}H]-inositol were purchased from Amersham, Dübendorf, Switzerland. Histamine dihydrochloride, methysergide maleate, 5-HT hydrochloride, 5-carboxamidotryptamine maleate (5-CT) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) were obtained from Research Biochemicals Inc., Natick, U.S.A. Forskolin, IBMX, fura-2, ruthenium red (oxychloride), heparin (low molecular weight, sodium salt) and ryanodine were obtained from Sigma, Buchs, Switzerland. Human total brain and human total intestine mRNA were purchased from Clontech, Palo Alto, U.S.A. 8-Bromo-cADPR was kindly provided by Prof. W. Wadman, Institute of Zoology, University of Amsterdam, Netherlands.

Results

Human pulmonary artery endothelial cells (PAE cells) express 5-HT$_{1D\beta}$, 5-HT$_{2B}$ and little 5-HT$_4$ receptor mRNA

We first analyzed mRNAs for 5-HT receptor subtypes expressed in PAE cells using RT-PCR with primers for all known human G-protein coupled 5-HT receptors. Contamination with genomic DNA was excluded in all RNA preparations by performing the same RT-PCR procedure without adding reverse transcriptase.

Of all the known G-protein coupled 5-HT receptors, human PAE cells express only 5-HT$_{1D\beta}$, 5-HT$_{2B}$, and to a small extent 5-HT$_4$ receptors (Figure 1). Levels of mRNA expression can be roughly estimated by comparing the density of the PCR-signals with the positive controls, tissues known to express those receptors, which are human total brain for the 5-HT$_{1D\beta}$ and 5-HT$_4$ receptors and human total intestine for the human 5-HT$_{2B}$ receptor. Other 5-HT receptor mRNAs (5-HT$_{1A}$, 5-HT$_{1D\alpha}$, 5-HT$_{1E}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_{5A}$, 5-HT$_6$, 5-HT$_7$) are not expressed in PAE cells (data not shown).

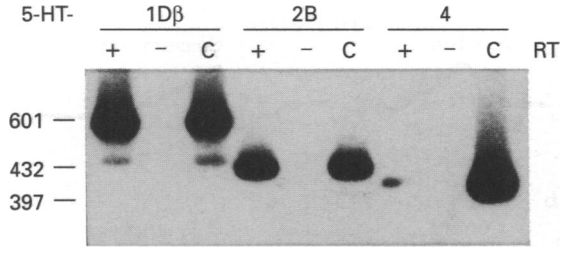

Figure 1 Agarose gel electrophoresis of PCR-amplified products derived from cDNA of cultures of human endothelial cells from pulmonary artery (PAE cells). As controls for the absence of DNA contaminations, all RT-PCRs were performed with or without reverse transcriptase (+ or −RT) added to the cDNA synthesis reaction. All PCR-products were identified by direct DNA-sequencing. The following primers and positive controls (C) were used (in parentheses: respective size of the PCR-product and tissue source): 5-HT$_{1D\beta}$ ON5/ON6 (601 bp, human total brain), 5-HT$_{2B}$ ON13/ON14 (432 bp, human total intestine), 5-HT$_4$ receptor ON17/ON18 (397 bp, human total brain).

5-HT inhibits cyclicAMP formation through activation of 5-HT$_{1D\beta}$ on PAE cells

Multiple publications indicate that receptor-effector coupling may differ for receptors in experimental expression systems and in their natural environment. Non-transformed PAE cells endogenously expressing 5-HT receptors seemed to be a good model to study receptor coupling since adaptive changes which cells may undergo through extended cultivation should be more limited in finite than in immortalized cultures.

Heterologously expressed 5-HT$_{1D\beta}$ receptors inhibit forskolin-stimulated cyclic AMP accumulation (Weinshank *et al.*, 1992). In human PAE cells, 5-HT inhibited adenylate cyclase activity in a concentration-dependent manner. The 5-HT$_1$ receptor agonist, 5-CT, exhibited the same efficacy but a higher potency of inhibition than 5-HT (respective pD_2 ± s.e.mean: 8.13 ± 0.23 and 7.4 ± 0.15; n = 4, Figure 2). Furthermore, the 5-HT$_2$ receptor-selective agonist, DOI was ineffective, indicating that the observed activity resulted from activation of 5-HT$_{1D\beta}$ and not 5-HT$_{2B}$ receptors.

Effects of 5-HT and histamine on inositol phosphate formation

Since it was found that 5-HT$_{2B}$ receptors stimulate PI hydrolysis in various experimental expression systems (Foguet *et al.*, 1992a; Schmuck *et al.*, 1994; Loric *et al.*, 1995), we investigated whether 5-HT or, for comparison, histamine stimulated the hydrolysis of phosphatidylinostol in PAE cells. In three independent experiments we did not detect a statistically relevant increase of IP production in 5-HT-treated cells whereas histamine clearly stimulated this second messenger pathway with a pD_2 ± s.e.mean of 5.15 ± 0.08 (n = 3, Figure 3a). We carried out time course experiments in which the cells were incubated with high concentrations of 5-HT (0.1 mM) and histamine (1 mM) for prolonged time intervals. There was no detectable increase in IP even after incubating with 5-HT for up to 40 min (Figure 3b).

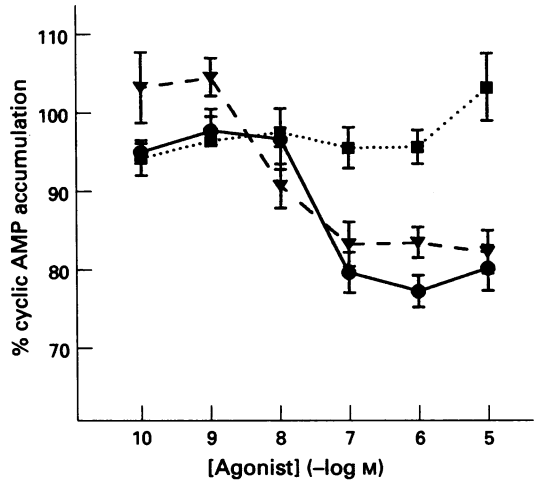

Figure 2 Concentration-response curve of 5-HT (▼), 5-CT (●) and DOI (■) for the inhibition of forskolin-stimulated cyclic AMP accumulation in PAE cells. Data are expressed as percentage of the cyclic AMP concentration found in parallel culture wells which were stimulated with 0.1 mM forskolin. Points are the means ± s.e.mean of triplicate determinations from 4 independent experiments.

5-HT stimulates transient calcium release through activation of 5-HT$_{2B}$ receptors

Measuring fluorescence in fura-2 loaded single cells we found that 5-HT stimulated a transient increase in calcium in human PAE cells, despite undetectable PI turnover (Figure 4a). A similar calcium signal was observed after application of histamine (Figure 4b). The maximal amplitude of the 5-HT response corresponded roughly with that obtained with histamine. The 5-HT$_1$-selective agonist, 5-CT (10 μM) and the 5-HT$_{1D}$-selective agonist, sumatriptan (10 μM) were ineffective (data not shown). DOI, an agonist acting on the three 5-HT$_2$ receptor types, evoked responses which were similar to those of 5-HT. Therefore, it must be concluded that the calcium release is triggered by the 5-HT$_{2B}$ receptor, the only 5-HT$_2$ receptor type expressed in PAE cells. Figure 4 shows typical recordings with increasing concentrations of 5-HT or histamine. The resulting dose-response curves for 5-HT, DOI and histamine were calculated from average peak changes in $[Ca^{2+}]_i$ (Figure 5a). The pD_2 values for 5-HT and DOI-induced calcium signalling were 5.82 ± 0.06 ($n=6-8$) and 5.66 ± 0.03 ($n=7-9$), respectively, which are considerably lower than the pD_2 of 5-HT observed for PI formation (8.04, Schmuck *et al.*, 1994) or the pK_D measured in ligand binding experiments (8.35, own unpublished experiment) in HEK 293 cells transiently transfected with the human 5-HT$_{2B}$ receptor clone. The calcium release triggered by histamine revealed a pD_2 of 6.44 ± 0.01 ($n=7-8$), which was higher than the pD_2 calculated from the PI-hydrolysis experiment (5.15) in the same cells. Pretreatment with 1 nM methysergide, a highly potent antagonist for the human 5-HT$_{2B}$ receptor (own unpublished results), shifted the 5-HT concentration-response curve to the right. The calculated pK_B value was 9.6 ± 0.42 ($n=5$, Figure 5b).

5-HT stimulates release of calcium from intracellular stores

To determine whether the 5-HT-induced calcium signal was caused by release from intracellular stores and/or by stimulated influx across the plasma membrane, we performed the same single cell measurements of 5-HT-induced fura-2 fluorescence in the presence or absence of calcium in the extracellular medium. In Figure 6 typical examples of calcium transients induced by 5-HT (10 μM) and DOI (10 μM) in the presence or absence of extracellular calcium are shown, indicating that calcium is released from intracellular stores. The calcium responses (nM) to 5-HT or DOI in the presence or absence of extracellular calcium were 314 ± 38 ($n=5$), 282 ± 24 ($n=7$), 349 ± 29 ($n=5$) and 312 ± 33 ($n=8$), respectively.

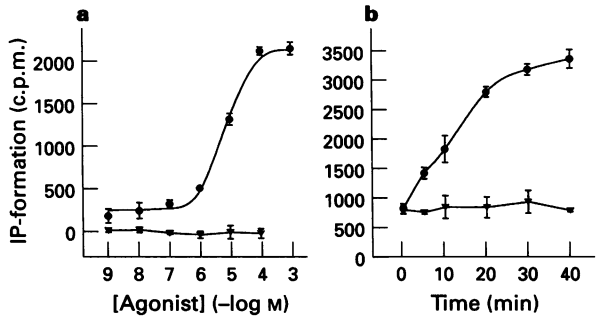

Figure 3 Stimulation of phosphatidylinositol hydrolysis in human endothelial cells from pulmonary artery. (a) Cells were stimulated for 20 min with increasing concentrations of the agonists 5-HT (▼) and histamine (●). (b) Time course of IP formation following stimulation of cells with 0.1 mM 5-HT (▼) and 1 mM histamine (●). The curves shown are representatives from three independent experiments. The means of triplicate determinations per data point ± s.e.mean are presented.

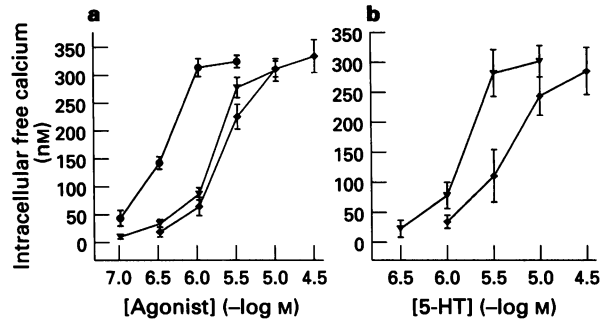

Figure 5 Concentration-response curves of the intracellular calcium in PAE cells. PAE cells were induced by 5-HT (▼), DOI (■) or histamine (●), $n=6-9$ (a). The 5-HT-induced dose-response curve (▼) was shifted to the right when PAE cells were pretreated with 1 nM methysergide (◆) for 15 min, $n=5$ (b). The data represent mean values ± s.e.

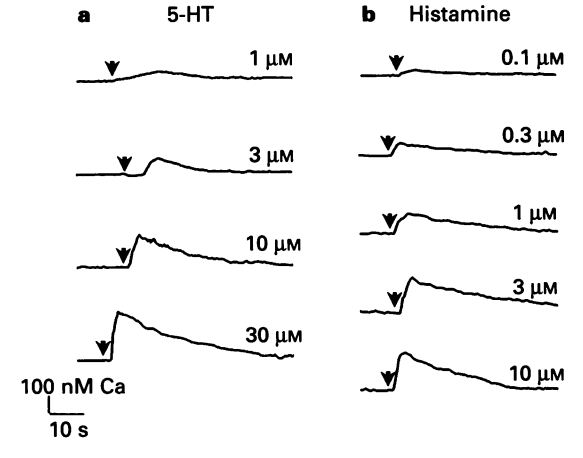

Figure 4 Increases in intracellular calcium following administration of the indicated concentrations of 5-HT (a) or histamine (b) to PAE cells. The traces shown are representatives of 6–9 independent recordings.

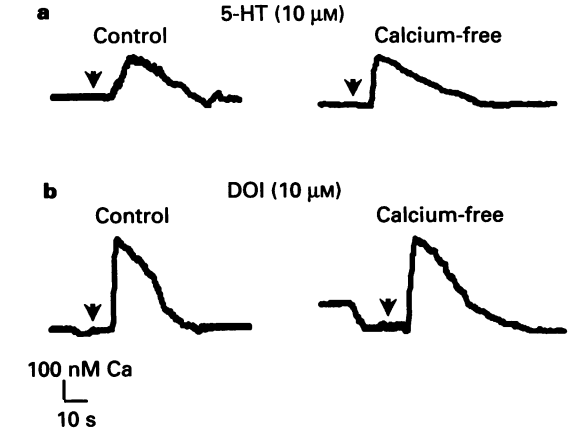

Figure 6 Effect of pretreatment for 10 min of cells with EGTA (3 mM) on the calcium response to 10 μM 5-HT (a) or 10 μM DOI (b). The traces shown are representatives of 5–8 recordings.

Calcium signalling by 5-HT is not affected by intracellular application of heparin

The polyanion heparin is an inhibitor of IP_3-activated calcium release channels on the endoplasmic reticulum (Ghosh *et al.*, 1988). In order to check the possibility that the 5-HT-induced calcium signal was due to the formation of small quantities of IP_3, below the limit of detection of our biochemical assay, we tested whether heparin interfered with calcium signalling. Since cell membranes are impermeable to heparin, it was applied to single cells through the pipette using whole cell patches in which heparin was included in the patch pipette solution. Figure 7 shows typical examples of these experiments. Application of 5-HT (10 μM) induced calcium signals that were unaffected after infusion of heparin (336±41 nM, $n=8$) compared to a physiological salt solution (304±28 nM, $n=8$, Figure 7a). By comparison, histamine (10 μM) induced calcium responses (342±24 nM, $n=9$) that were blocked under identical conditions (47±16 nM, $n=5$, Figure 7b). These results support the conclusion that the 5-HT-induced calcium signalling is independent of phosphatidylinositol hydrolysis, while this second messenger system is activated by the histamine receptor.

Calcium signalling by 5-HT is sensitive to ryanodine and ruthenium red, but is not mediated by cyclic ADP-ribose

A different class of intracellular calcium channels consists of the ryanodine receptors, which are structurally related to the IP_3 receptors (Coronado *et al.*, 1994). Calcium release through these channels can be blocked with ruthenium red and high concentrations of the plant alkaloid, ryanodine. The DOI-induced calcium release (337±32 nM, $n=6$) was abolished after treatment with 10 μM ryanodine (64±39 nM, $n=19$). Ruthenium red (10 μM) was less effective but it still significantly reduced the signal (125±26 nM, $n=12$, Figure 8a). The histamine-induced calcium response (359±44 nM, $n=5$), however, was not affected after the same treatment with either ryanodine (344±38 nM, $n=6$) or ruthenium red (316±22 nM, $n=5$, Figure 8b).

In sea urchin eggs, cyclic adenosine diphosphate ribose (cADPR), which is also present in many mammalian tissues (Rusinko & Lee, 1989), triggers calcium release through ryanodine receptors in an IP_3-independent manner (Galione *et al.*, 1991). To test whether cADPR could be involved as a second messenger or modulator of this ryanodine-sensitive calcium release it was added to the solution of the patch pipette at 100 μM. Breaking the seal and dialyzing the cells with the pipette solution did not elicit a calcium signal (Figure 9). It also did not influence a subsequent calcium response to 5-HT (10 μM) or DOI (10 μM), which still amounted to 326±19 nM ($n=9$) and 343±41 nM ($n=12$) calcium, respectively. The

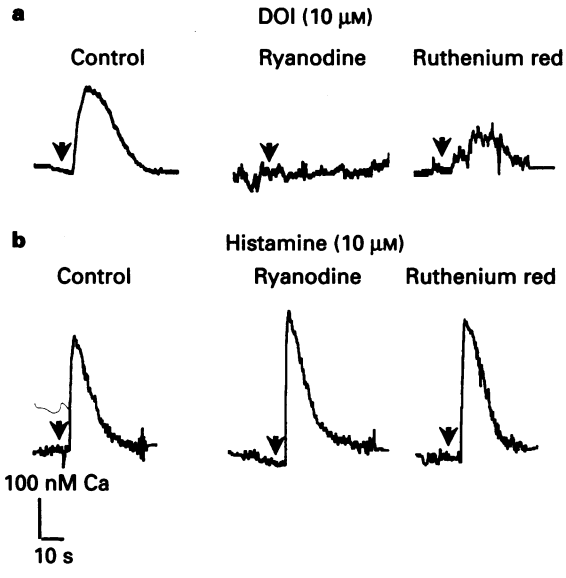

Figure 8 Calcium measurements in single cells before and after intracellular application of 10 μM ryanodine or 10 μM ruthenium red through the patch pipette under current clamp. The calcium response to 10 μM DOI was suppressed by both ryanodine and ruthenium red (a). The response to 10 μM histamine was neither affected by ryanodine nor by ruthenium red (b). The traces shown are representatives of 5–19 experiments.

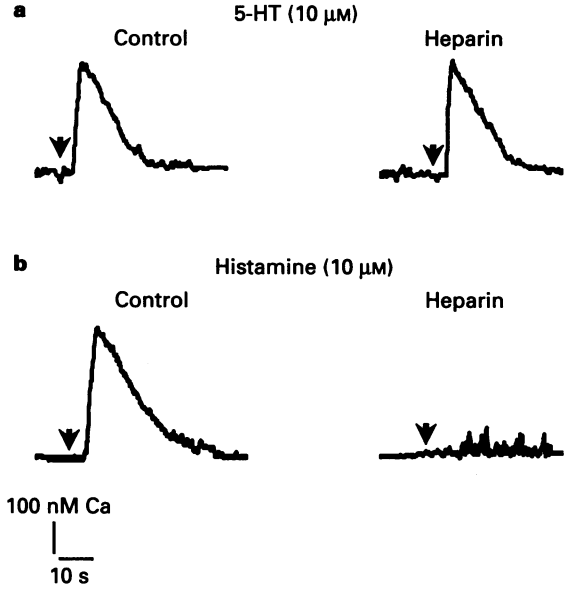

Figure 7 Recordings of cells, which were current-clamped and dialized with control buffer or with buffer containing heparin (100 μM). The calcium response to 10 μM 5-HT was resistant to treatment with heparin (a). The calcium response to 10 μM histamine was inhibited in cells treated with heparin (b). The traces shown are representatives of 5–9 recordings.

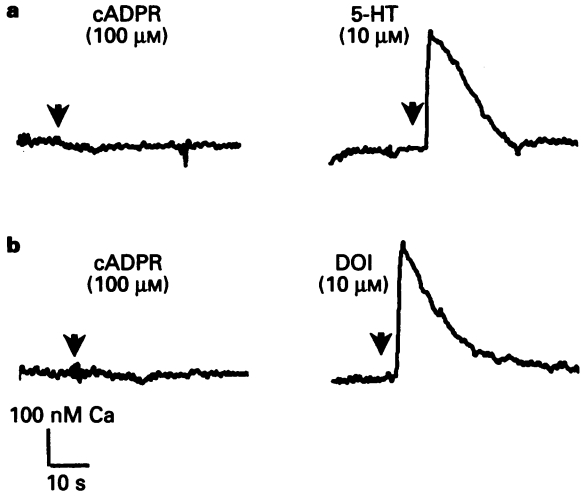

Figure 9 Recordings of cells current-clamped and dialyzed through the patch pipette with buffer containing cADPR (100 μM). cADPR did not affect the intracellular calcium concentration after breaking the patch-seal. Cells dialyzed with cADPR still responded to 10 μM 5-HT (a) and to 10 μM DOI (b) 5–10 min after breaking the seal. The traces shown are representatives of 9–12 recordings.

conclusion that cADPR is not involved in the 5-HT-induced calcium release was confirmed by the observation that bath application of the competitive antagonist 8-bromo-cADPR (0.1 mM, Walseth & Lee, 1993) also did not affect the 5-HT-induced signal (data not shown).

Discussion

Using RT-PCR we demonstrated that three different 5-HT receptor mRNAs, 5-HT$_{1D\beta}$, 5-HT$_{2B}$, and little 5-HT$_4$ are expressed in human PAE cells. An investigation of the second messenger systems activated by these receptors revealed that the 5-HT$_{1D\beta}$ receptor inhibits adenylate cyclase as expected based on studies in which the cloned receptor was expressed in cell lines (Weinshank *et al.*, 1992). Activation of human 5-HT$_{2B}$ receptors, which was shown to trigger phosphatidylinositol (PI) hydrolysis when expressed in HEK 293 cells (Schmuck *et al.*, 1994), did not stimulate the formation of inositol phosphate in cultured PAE cells. Although these cells provide a functional PI system which is activated by histamine H$_1$ receptors, 5-HT$_{2B}$ receptors do not utilize this pathway.

Nevertheless, in human PAE cells calcium release from intracellular pools was triggered after stimulation with 5-HT, the 5-HT$_2$-selective agonist DOI, or histamine. Intracellular calcium pools in endothelial cells play an important role in the rapid $[Ca^{2+}]_i$ rise seen on exposure to several physiological agonists such as adenine nucleotides (Pirotton *et al.*, 1987), bradykinin (Lambert *et al.*, 1986) and thrombin (Jaffe *et al.*, 1987). Like histamine (Lo & Fan, 1987), these agents mediate calcium release via the generation of inositol 1,4,5-trisphosphate (IP$_3$). The IP$_3$-receptor is the best characterized and thought to be the major functional calcium release channel involved in pharmacomechanical coupling. However, we can rule out IP$_3$ as the calcium mobilizing second messenger for the action of 5-HT since intracellular application of heparin, a potent inhibitor of the IP$_3$ receptor, did not block the response, whereas that to histamine was completely blocked by this treatment. We further demonstrated that the calcium release induced by 5-HT, but not that elicited by histamine, was sensitive to ryanodine and ruthenium red. Thus, we conclude that the rapid 5-HT-induced increase in $[Ca^{2+}]_i$ in human PAE cells occurs through an intracellular calcium mobilizing pathway which is independent of IP$_3$ and sensitive to ryanodine.

The presence of ryanodine receptors in endothelial cells was first shown by immunoblotting of bovine aortic endothelial cells and immunofluorescence staining of guinea-pig aorta, where ryanodine receptors are spread over the ER network (Lesh *et al.*, 1993). Functional ryanodine-sensitive intracellular calcium stores were subsequently demonstrated in various endothelial cells. In rat aortic endothelial cells (Ziegelstein *et al.*, 1994) and in mink epithelial cells pretreated with TGFβ (Giannini *et al.*, 1992) ryanodine appeared to modify bradykinin-induced calcium release.

The situation in PAE cells is different from that in HEK 293 cells in which the human 5-HT$_{2B}$ receptor was expressed. In these cells, the human 5-HT$_{2B}$ receptors were found to couple to PI-hydrolysis (Schmuck *et al.*, 1994). The closely related 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors also exert their functions through stimulation of PI-hydrolysis in cortex (Conn & Sanders-Bush, 1986) and smooth muscle (Roth *et al.*, 1984), or choroid plexus (Conn *et al.*, 1986), respectively.

The different coupling of the 5-HT$_{2B}$ receptor in HEK 293 and PAE cells may depend on the distribution and abundance of corresponding G-protein subtypes which funnel the signal into different pathways. In this context, it is interesting to note the low potency that 5-HT displays in the calcium release assay (pD$_2$ = 5.82). After expression of the cloned human 5-HT$_{2B}$ receptor in HEK 293 cells we found that 5-HT binds to the receptor with a pK$_D$ of 8.35 and activates PI-hydrolysis with a pD$_2$ of 8.04 (Schmuck *et al.*, 1994). Nevertheless, the PCR data, the effect of DOI, the high sensitivity to methysergide (pK_B = 9.6), and the lack of an effect of 5-CT argues strongly that the calcium signal is indeed caused by 5-HT$_{2B}$ receptors. The low affinity for 5-HT may be caused by coupling to G-proteins that mediate the ryanodine-sensitive calcium release pathway. It seems reasonable that the pK_D of an agonist to a receptor that is located on the blood vessel endothelium is such that the average agonist concentration in the blood (30–150 nM, Frattini *et al.*, 1976) does not permanently activate the receptor.

Another possible explanation for the unusual coupling of 5-HT$_{2B}$ receptors in PAE cells may be an interaction with the 5-HT$_{1D\beta}$ receptors expressed in these cells. Such an interaction has been proposed to explain the unusual pharmacology of the 5-HT$_{1D\beta}$ receptor-stimulated, endothelium-dependent vessel relaxation (Ullmer *et al.*, 1995). However, the pharmacological data presented here are inconsistent with an involvement of 5-HT$_{1D\beta}$ receptors in the mobilization of $[Ca^{2+}]_i$. There was neither an inhibition of adenlyate cyclase activity by DOI, nor a stimulation of $[Ca^{2+}]_i$ release by the 5-HT$_1$ receptor-selective agonist, 5-CT. Thus, an activation of 5-HT$_{2B}$ receptors results in the release of $[Ca^{2+}]_i$ without an interaction with 5-HT$_{1D\beta}$ receptors.

5-HT was shown to induce an endothelium-dependent relaxation through stimulation of 5-HT$_{1C}$-like receptors in porcine pulmonary artery (Glusa & Richter, 1993). This relaxation could be blocked with L-NAME (N^G-nitro-L-arginine methylester), an inhibitor of the endothelial nitric oxide synthase (eNOS). Since eNOS activity is dependent on calcium-calmodulin, calcium release upon stimulation with 5-HT may result in vessel relaxation via the diffusible relaxant, nitric oxide. 5-HT$_{2B}$ receptors are pharmacologically very similar to 5-HT$_{2C}$ receptors (formerly called 5-HT$_{1C}$; Foguet *et al.*, 1992b). Since 5-HT$_{2C}$ mRNA does not occur in PAE cells, it can be concluded that the 5-HT$_{1C}$-like relaxation is mediated by 5-HT$_{2B}$ receptors. This is in agreement with a functional analysis of the rat jugular vein, in which this receptor was found to mediate relaxation (Ellis *et al.*, 1995). Coupling of the 5-HT$_{2B}$ receptor to calcium release as demonstrated here is therefore consistent with the nitric oxide dependence of the vessel relaxation.

In addition to the endothelium-dependent relaxation of vascular tissues, the 5-HT$_{2B}$ receptor elicits strong contractile responses of the rat stomach fundus. In agreement with our results, no activation of PI-hydrolysis could be shown in this smooth muscle tissue (Secrest *et al.*, 1989). Nevertheless, the contractile response to 5-HT was partly dependent on intracellular calcium release (Cox & Cohen, 1995). A candidate mechanism for IP$_3$-independent intracellular calcium release is the calcium-induced calcium release (CICR) involving non skeletal muscle type ryanodine receptors (Sorrentino & Volpe, 1993). The contraction of the stomach fundus was modestly inhibited by bath application of ryanodine, indicating an involvement of ryanodine-sensitive calcium pools (Cox & Cohen, 1995). However, infusion of micromolar concentrations of ryanodine completely blocked the 5-HT-induced calcium signal in PAE cells. A similar receptor-effector coupling mechanism has been described in rat lacrimar cells where a PI-independent calcium mobilization induced by phenylephrine could be completely blocked with ryanodine (Gromada *et al.*, 1995). It was speculated that cyclic ADP-ribose (cADPR) might be the soluble regulator of ryanodine-sensitive calcium channels in sea urchin eggs (Galione *et al.*, 1991). Since PAE cells infused with cADPR were still responsive to 5-HT$_{2B}$ receptor-mediated calcium release and the response was insensitive to 8-bromo-cADPR, the participation of this second messenger can be excluded. Thus, the mediator of the signal between the 5-HT$_{2B}$ receptor and the ryanodine receptor remains to be found. Derivatives of sphingolipids have been proposed as second messengers inducing heparin-insensitive calcium mobilization (Ghosh *et al.*, 1990). A recent report demonstrated that sphingosylphosphorylcholine induced ryanodine-sensitive calcium release from brain microsomes (Dettbarn *et al.*, 1995).

Given the multitude of functions exerted by calcium, several other systems may exist which control fluctuations in intracellular calcium. Here we have provided evidence for the existence of a novel, ligand-activated pathway controlling intracellular calcium release.

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