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5-Hydroxytryptamine stimulation of phospholipase D activity in the rabbit isolated mesenteric artery

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1 The involvement of phospholipase D (PLD) in the 5-hydroxytryptamine 5-HT_{1B}/5-HT_{1D}-signalling pathway was assessed in the rabbit isolated mesenteric artery.

2 RT–PCR analysis of mesenteric smooth muscle cells revealed a strong signal corresponding to mRNA transcript for the 5-HT_{1B} receptor. The PCR fragment corresponded to the known sequence for the 5-HT_{1B} receptor. No signal corresponding to 5-HT_{1D} mRNA was detected.

3 Neither 5-HT (3 μM) nor KCl (45 mM) individually stimulated any significant increase in the smooth muscle concentration of [^{33}P]-PtdBut to reflect PLD activity. However, in the presence of KCl (45 mM), 5-HT evoked a concentration-dependent increase in [^{33}P]-PtdBut, to a maximum of 84% with 5-HT (3 μM).

4 [^{33}P]-PtdBut accumulation evoked by 5-HT in the presence of KCl was abolished in nominally calcium-free Krebs-Henseleit Buffer (KHB) or with the selective protein kinase C inhibitor, Ro-31 8220 (10 μM, 20 min).

5 5-HT (3 μM) in the presence of KCl (45 mM) failed to increase either the accumulation of [^{33}P]-phosphatidic acid in the presence of butanol, or total [^{3}H]-inositol phosphates ([^{3}H]-InsP) in the presence of LiCl (10 mM).

6 5-HT (0.1–1 μM) abolished forskolin (1 μM) stimulated increases in cyclic AMP (15 fold increase), an action which was pertussis toxin-sensitive.

7 Therefore, in the presence of raised extracellular potassium 5-HT can stimulate PLD *via* 5-HT_{1B} receptors in the rabbit mesenteric artery. This action requires extracellular calcium and the activation of protein kinase C. These characteristics are identical to the profile for 5-HT_{1B}/5-HT_{1D}-receptor evoked contraction in vascular smooth muscle cells, suggesting a role for PLD in this response to 5-HT.

Keywords: 5-HT; vascular smooth muscle; 5-HT_1 receptors; phospholipase D; smooth muscle contraction

Abbreviations: 5-HT, 5-hydroxytryptamine; InsP, inositol phosphates; KHB, Krebs-Henseleit Buffer; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidylbutanol; PtdOH, phosphatidic acid; PTx, pertussis toxin

Introduction

5-hydroxytryptamine (5-HT)-induced contraction in vascular smooth muscle cells is mediated by both 5-HT_2 and 5-HT_1 receptors (Martin, 1994). The most widespread are 5-HT_2 receptors, which are linked by G-proteins to an increase in the activity of the enzyme phosphoinositidase C and the generation of IP_3 and diacylglycerol (Martin, 1994). However, 5-HT_1 receptors stimulate smooth muscle contraction in a number of preparations, including the rabbit renal, mesenteric and coronary arteries, and they may also be of increased predominance in small resistance arteries (Choppin & O'Connor, 1993; 1995; Ellwood & Curtis, 1997). In general, the contraction appears to be mediated by what were defined as 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$-like receptors, which have recently been redefined as 5-HT_{1B}/5-HT_{1D} receptors (Hartig *et al*., 1996).

The cellular signalling pathways activated by 5-HT_1 receptors have not been elucidated in any great detail. The only clear observation is that the activation of 5-HT_1 receptors is negatively coupled to adenylyl cyclase *via* a pertussis toxin (PTx) sensitive G-protein (Dickenson & Hill, 1995; Sumner & Humphrey, 1990). However, the reduction in the cytoplasmic concentration of cyclic AMP which follows receptor stimulation may not be responsible for the ensuing smooth muscle contraction. In the dog saphenous vein, reductions in cyclic AMP were only observed if adenylyl cyclase was prestimulated with forskolin, and appeared not to be responsible for contraction *per se* (Sumner & Humphrey, 1990). Furthermore, evidence against smooth muscle contraction occurring as a direct result of a reduction in cyclic AMP concentration has also recently been provided in the rabbit femoral artery (Randall *et al*., 1996). In contrast, in the bovine pulmonary artery, contraction to the 5-HT_{1B}/5-HT_{1D} selective agonist sumatriptan did appear to correlate well with the concomitant reductions in cyclic AMP (Sweeney *et al*., 1995).

Although the importance of cyclic AMP in smooth muscle contraction is unclear, it is apparent that the contraction mediated by 5-$HT_{1B/1D}$ receptors requires co-stimulation with another contractile agonist (Choppin & O'Connor, 1995; Sweeney *et al*., 1995) or partial depolarization by elevations in the extracellular concentration of potassium (Choppin & O'Connor, 1994; Plane *et al*., 1995).

The only other functional evidence relating to the second messenger pathways which couple 5-HT_1 receptors to contraction was obtained in the rabbit mesenteric artery (Parsons *et al*., 1996; Plane *et al*., 1995; Seager *et al*., 1994). In these resistance arteries, contraction to 5-HT required extracellular calcium and the sensitization of the contractile myofilaments to calcium. Myofilament sensitization to 5-HT was blocked with inhibitors of either protein kinase C or phospholipase A_2 and mimicked by arachidonic acid. A role for arachidonic acid in myofilament

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sensitization supports the suggestion that this effect may, at least in part, reflect an inhibition of myosin light chain phosphatase by arachidonic acid (Somlyo & Somlyo, 1994)

Although protein kinase C activation appeared to make an important contribution to the contraction which followed 5-HT_1 receptor stimulation in the mesenteric artery, this was not associated with any increase in the specific accumulation of IP_3 or the release of calcium from internal stores (Seager *et al*., 1994). These latter observations raise the possibility that the diacylglycerol which is presumably responsible for protein kinase C activation, may be derived from phosphatidylcholine by the sequential action of phospholipase D (PLD) and phosphatidate phosphohydrolase. It is known that protein kinase C regulates PLD activity, as does cytoplasmic calcium (Huang *et al*., 1991; Balboa *et al*., 1994; Yeo & Exton, 1995), and human, murine and rat forms of PLD have recently been cloned (Hammond *et al*., 1995; Colley *et al*., 1997; Park *et al*., 1997). In vascular smooth muscle cells, PLD activity has been reported to be increased by a number of different agonists (Malarkey *et al*., 1996).

The present study investigated the potential involvement of PLD in the contractile pathway activated by 5-HT_{1B}/5-HT_{1D} receptors in the rabbit mesenteric artery. Some of these experiments have been described in a preliminary report to the British Pharmacological Society (Adams & Garland, 1995).

Methods

Tissue preparation

Female, New-Zealand White rabbits were anaesthetized with sodium pentabarbitone (60 mg kg^{-1}, i.v), and killed by rapid exsanguination. The mesenteric bed was removed and maintained in ice-cold, oxygenated Krebs-Henseleit Buffer (KHB), composition (mM); NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 1.3, $MgSO_4$ 1.2, HEPES 10 and glucose 10, pH 7.4. Third order mesenteric arteries were then dissected free from connective tissue, removed and incubated for 20 h in KHB in the presence or absence of radiolabel, ([^{33}P]-orthophosphate, 10 μCi ml^{-1}, *myo*-[^{3}H]-inositol, 1 μCi ml^{-1} where appropriate) and, where stated, 100 ng ml^{-1} PTx at 37°C in an oxygenated, humidified chamber. Variability in labelling was limited as far as possible by carrying out paired experiments, although the short-half life of the isotope did mean that some variability was unavoidable. Alternatively, arterial segments were prepared for RNA extraction.

Extraction of cellular RNA

Arteries isolated as described, were opened laterally and the endothelium gently rubbed off. The tissues were then washed twice with ice-cold phosphate buffered saline (PBS). Total RNA was extracted from 30–50 mg of tissue using Trizol reagent (Life Technologies, U.K.) according to the manufacturer's protocol. Extracted RNA was taken up into RNase/DNase free H_2O and used immediately or stored at −70°C. Samples were treated by RNase-free DNaseI for 1 h at 37°C to ensure extracted RNA was free of genomic DNA.

Oligonucleotide synthesis and design

Primers were designed to the published genetic sequences for the rabbit 5-HT_{1B} and 5-HT_{1D} receptors (Harwood *et al*., 1995), (GenBank No. Z50162, rabbit 5-$HT_{1D\alpha}$/5-HT_{1D} receptor; GenBank No. Z50163, rabbit 5-$HT_{1D\beta}$/5-HT_{1B} receptor). The following oligonucleotide primers were synthesized by the Molecular Recognition Unit (Department of Biochemistry, University of Bristol): $5HT_{1B}$ receptor, sense primer, 5′-gctgtcgtcggatatcacct-3′, antisense primer, 5′-cccaccgtggagtagacagt-3′; $5HT_{1D}$ receptor, sense primer, 5′-catgtgtggagtgttccag-3′, antisense primer, 5′-agatagttggcaggggtgtg-3′.

PCR analysis

A one tube RT–PCR system was used following the manufacturers protocol (Boehringer Mannheim). All reaction reagents, except the template RNA, were prepared as master mixes and then aliquoted to each tube to provide uniform reaction conditions and minimize intertube variation. Positive and negative controls were performed for each reaction. Negative controls had the reverse transcription step omitted, while positive controls used genomic clones for the $5HT_{1B}$ and $5HT_{1D}$ receptors (generous gifts from Glaxo-Wellcome) and RNA extracted from the rabbit renal artery which we have previously shown to contain mRNA for both the 5-HT_{1B} and 5-HT_{1D} receptors (Hinton *et al*., 1998; Hill *et al*., 1998). Following reverse-transcription at 50°C for 30 min, each reaction cycle consisted of; denaturation for 30 s at 94°C; primer annealing for 30 s at 55°C and template extension for 45 s at 68°C for 35 cycles, the final primer extension step was 7 min. This was performed in a PTC-200 Peltier Thermal Cycler, M.J. Research (Genetic Research Instrumentation Ltd. U.K.). Upon completion, 10 μl of the reaction product was analysed on a 2% agarose gel in Tris-HCl (100 mM), boric acid (90 mM) and EDTA (pH 8.4) (1 mM) containing ethidium bromide (1 μg ml^{-1}). The gel was viewed under UV light and photographed. The identity of the PCR products was confirmed by sequencing. Briefly, PCR bands were purified and subcloned using the pGEM T-vector (Promega). The inserts were then sequenced commercially and compared with the published 5-HT_{1B} receptor subtype sequence (Harwood *et al*., 1995).

Measurement of PLD activity

In the presence of a saturating concentration of butanol, PLD-mediated hydrolysis of phosphatidylcholine leads to the formation of phosphatidylbutanol rather than phosphatidic acid (PtdOH). This transphosphatidylation reaction is considered to be unique to PLD and can be used to quantify PLD activity. Under these conditions, any significant accumulation of PtdOH would be most likely to indicate an action of diacylglycerol kinase on diacylglycerol derived from the hydrolysis of phosphatidylinositol (Boarder & Purkiss, 1993).

After labelling, individual arteries were equilibrated for 30 min in oxygenated KHB, in a shaking water bath maintained at 37°C. Arteries were then exposed to *n*-butanol (100 mM final concentration) followed by agonist application 5 min later. Reactions were terminated with 250 μl of ice-cold, 10% perchloric acid, and the arteries were transferred into vials containing acidified chloroform/methanol (1/2, v v^{-1}). Lipid extraction was carried out on ice by the method of Bligh & Dyer (1959).

Separation and identification of [^{33}P]-phosphatidylbutanol ([^{33}P]-PtdBut) and [^{33}P]-phosphatidic acid ([^{33}P]-PtdOH) was achieved according to the methods of Purkiss & Boarder (1993). Briefly, chloroform-extracted lipids were dried under nitrogen and redissolved in 50 μl chloroform/methanol (19/1, v v^{-1}). Samples (40 μl) were loaded onto heat-treated, silica gel thin layer chromatography (TLC) plates pre-treated with 1% potassium oxalate. Separation was achieved through one dimensional TLC performed in a TLC tank, pre-equilibrated

with a developing solvent mixture of ethyl acetate/acetic acid/2,2,4-trimethylpentane (9/2/5, v v^{-1}). [^{33}P]-PtdBut and [^{33}P]-PtdOH bands were identified by autoradiography and comparison with iodine stained standards. Sample bands were then scraped and lipid levels quantified by scintillation counting. Initial experiments revealed the presence of a radioactive metabolite that co-eluted with [^{33}P]-PtdBut. Consequently, in all experiments lipid extracts were also run from tissue samples which had not been exposed to butanol and the radioactivity subtracted from all [^{33}P]-PtdBut samples. The level of the unidentified metabolite was not altered during stimulation with 5-HT and raised potassium (5413 ± 1754 d.p.m. mg^{-1} control $n=4$, 4244 ± 665 d.p.m. mg^{-1} stimulated $n=3$, $P>0.05$)

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

Mesenteric arteries were prepared as described above, and labelled with *myo*-[^{3}H]-inositol. Experiments were performed on individual arteries at 37°C in 500 μl KHB containing LiCl (10 mM) and 1 μCi ml^{-1} *myo*-[^{3}H]-inositol. Reactions were terminated after 30 min exposure to agonist challenge by the addition of 500 μl of ice-cold perchloric acid. Samples were then placed on ice for 30 min. This was followed by acid extraction and [^{3}H]-InsP separated by anion exchange chromatography on Dowex 1-X8 (chloride form, 200–400 mesh) according to the methods described by Challiss *et al*. (1994).

Measurement of cyclic AMP mass

Arterial tissue was incubated overnight in standard KHB, in the absence or presence of PTx, then equilibrated for 30 min in KHB (final volume 150 μl; 37°C) and stimulated with forskolin (1 μM) for 5 min prior to stimulation with either 5-HT or vehicle. Reactions were terminated after a further 3 min by the addition of 10 μl HCl (10 M). Samples were placed on ice for 30 min prior to neutralization with 100 μl, pre-titrated 1 M NaOH. Cyclic AMP mass was measured with a competitive binding assay utilizing a binding protein prepared from bovine adrenal glands, essentially as described by Brown *et al*. (1971).

Materials

All chemical reagents were of the highest available standard. KHB; all reagents were obtained from BDH. TLC; samples were run on Whatman LK6D TLC plates and developed in acetic acid/ ethyl acetate (both Sigma)/2,2,4 trimethylpentane (BDH) solvent mixture. All radiochemicals were obtained from Amersham International. Ro 31-8220 (Calbiochem). Phosphatidylbutanol (Lipid Products, South Nutfield, Surrey). All remaining reagents unless otherwise stated were obtained from Sigma.

Data analysis

All data are given as mean ± s.e.mean from triplicate determinations in three separate experiments, unless otherwise stated. Statistical analysis of comparative values was performed using unpaired Students *t*-test.

Results

Reverse transcription-polymerase chain reaction

RT–PCR of RNA extracted from mesenteric vascular smooth muscle cells demonstrated the presence of mRNA transcript for the 5-HT_{1B} receptor ($n=6$). In contrast, no detectable RT–PCR product was observed for the 5-HT_{1D} receptor ($n=6$) Figure 1. The lack of signal for the negative control (omission of reverse transcription) demonstrated the amplification of mRNA only and the absence of contaminating genomic DNA. Positive control reactions were performed using 5-HT_{1B} and 5-HT_{1D} receptor cDNAs and rabbit renal artery RNA. The location of the bands for each of the receptors corresponded to the expected amplified cDNA fragment size. Further evidence that the observed band corresponded specifically to the 5-HT_{1B} receptor subtype, was obtained after cloning the PCR products into a plasmid vector (pGEM T-vector, Promega). The fragment was sequenced and found to be identical to the published sequence for the 5-HT_{1B} receptor subtype (Harwood *et al*. 1995).

5-HT stimulated accumulation of [^{33}P]-PtdBut in the mesenteric artery

In the presence of butanol (100 mM) neither 5-HT (3 μM), nor KCl (45 mM) individually evoked any measurable increase in the accumulation of [^{33}P]-PtdBut over basal values ($26{,}240 \pm 1642$ d.p.m. mg^{-1}). 100 mM butanol is sufficient completely to divert the formation of PtdOH by PLD to PtdBut (Boarder & Purkiss, 1993). However, in tissue depolarized with KCl (45 mM), and then stimulated with 5-HT, a clear concentration-dependent accumulation of [^{33}P]-PtdBut was obtained. Maximal stimulation occurred in the presence of 3 μM 5-HT, and was associated with a 1.8 fold increase in basal values up to $48{,}390 \pm 4742$ d.p.m. mg^{-1} protein ($n=4$) Figure 2.

5-HT stimulated accumulation of [^{33}P]-PtdBut in the presence of the protein kinase C inhibitor, Ro 31-8220 and modified extracellular calcium concentrations

The accumulation of [^{33}P]-PtdBut stimulated by 5-HT was significantly reduced by prior incubation with the specific

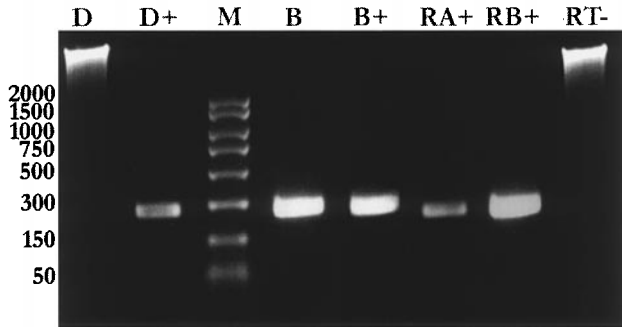

Figure 1 RT–PCR analysis of 5-HT_{1B} and 5-HT_{1D} mRNA expression in rabbit mesenteric artery vascular smooth muscle cells. Primers specific for the rabbit 5-HT_{1B} and 5-HT_{1D} receptor genes were used to amplify PCR products of 265 and 270 base pairs, respectively from RNA extracted from mesenteric vascular smooth muscle cells. D: Absence of any detectable RT–PCR product corresponding to the mesenteric artery 5-HT_{1D} receptor mRNA; D+: Positive control corresponding to the amplification of 5-HT_{1D} receptor cDNA; M: Molecular weight markers as labelled; B: RT–PCR product corresponding to the amplification of mRNA isolated from the mesenteric artery for the 5-HT_{1B} receptor; B+: Positive control corresponding to the amplification of 5-HT_{1B} receptor cDNA; RD+: RT–PCR product corresponding to the amplification of mRNA for the 5-HT_{1D} receptor isolated from the rabbit renal artery; RB+: RT–PCR product corresponding to the amplification of mRNA for the 5-HT_{1B} receptor isolated from the rabbit renal artery. RT–: Negative control with mesenteric arterial tissue (omission of reverse transcription).

protein kinase C inhibitor, Ro 31-8220 or by the removal of external calcium. Additionally, in the presence of 10 μM Ro 31-8220, applied for 20 min before tissue stimulation, there was a significant reduction in the basal accumulation of [^{33}P]-PtdBut, which was reduced from 15,478 $\pm$ 5096 to 5766 $\pm$ 1943 d.p.m. mg^{-1} ($n=3$). In the presence of Ro 31-8220, the accumulation of [^{33}P]-PtdBut stimulated by 3 μM 5-HT (in the presence of KCl (45 mM)) was reduced to 7526 $\pm$ 656 d.p.m. mg^{-1} ($n=3$), compared to a control level of 35,971 $\pm$ 3751 d.p.m. mg^{-1} (Figure 3a).

A similar inhibitory effect was obtained by removing external calcium from the KHB. In nominally calcium free KHB, there was a dramatic reduction in the accumulation of [^{33}P]-PtdBut stimulated by 5-HT (Figure 3b). Interestingly, unlike Ro 31-8220, calcium removal did not significantly alter the basal accumulation of [^{33}P]-PtdBut (basal in KHB 8254 $\pm$ 2310 d.p.m. mg^{-1}, in nominally calcium-free KHB, 6952 $\pm$ 4108 d.p.m. mg^{-1}, $n=3$).

Measurement of phosphatidic acid-[^{33}P]-PtdOH and total inositol phosphate-[^{3}H]-InsP accumulation

Figure 4 demonstrates the effect of stimulation with 5-HT on the levels of [^{33}P]-PtdOH in mesenteric arteries incubated in the absence or presence of butanol (100 mM).

In the presence of butanol, a basal accumulation of [^{33}P]-PtdOH was obtained (38,576 $\pm$ 4466 d.p.m. mg^{-1}, $n=3$). However, in contrast to the experiments measuring [^{33}P]-PtdBut, neither membrane depolarization (45 mM potassium) nor combined stimulation with 5-HT had any effect on the basal accumulation of [^{33}P]-PtdOH in the mesenteric artery. The absence of any measurable increase in [^{33}P]-PtdOH with 5-HT, which had stimulated an 84% increase in [^{33}P]-PtdBut accumulation (Figure 2), is confirmation that the concentration of butanol used in these experiments was sufficient completely to divert the PLD-mediated lipid hydrolysis of PtdOH to PtdBut.

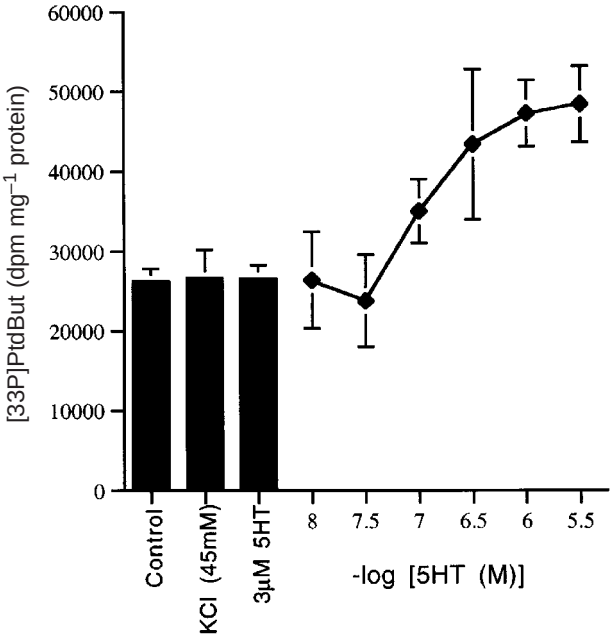

Figure 2 Effect of 5-HT on the accumulation of [^{33}P]-PtdBut in rabbit mesenteric arteries in the presence of KCl (45 mM). Artery segments were pre-labelled with 10 μCi ml^{-1} ^{33}P-P$_i$ for 20 h then stimulated with increasing concentrations of 5-HT or vehicle for 3 min in the presence of KCl (45 mM) and butanol (100 mM). Points are mean $\pm$ s.e.mean of four separate experiments. Increases in the accumulation of [^{33}P]-PtdBut in response to 0.1 μM 5-HT and all higher concentrations were significant, $P<0.01$.

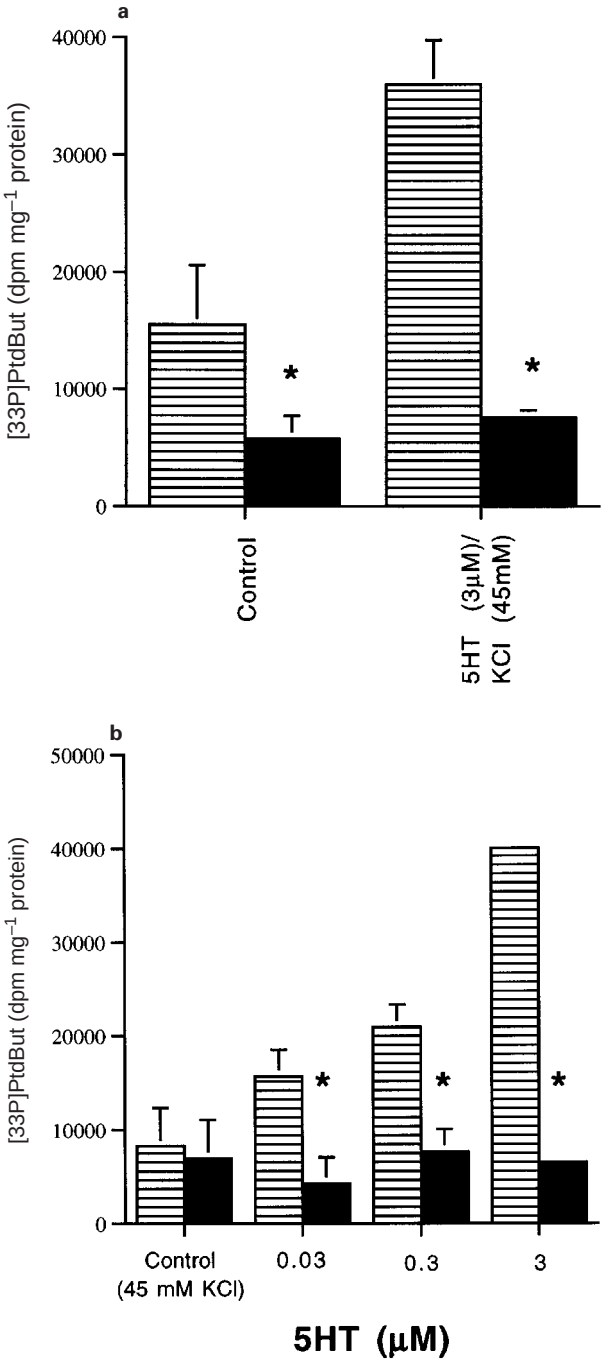

Figure 3 (a) Attenuation of basal and stimulated [^{33}P]-PtdBut accumulation with the protein kinase C inhibitor, Ro 31-8220. ^{33}P-P$_i$-labelled mesenteric arteries were incubated for 20 min with 10 μM Ro 31-8220 prior to stimulation with 5-HT (3 μM)/KCl (45 mM). The basal (control, left lined column) accumulation of [^{33}P]-PtdBut, was inhibited in the presence of Ro 31-8220 (left solid columns, $P<0.01$). A similar profile was obtained with arteries stimulated with 5-HT/KCl (right hatched column) which were also reduced (right solid column). Mean $\pm$ s.e.mean $n=3$ in each case. (b) Effect of reducing extracellular calcium on the accumulation of [^{33}P]-PtdBut following stimulation with 5-HT/KCl in mesenteric arteries. Individual arteries were incubated for 30 min in standard (lined columns) or nominally-calcium free KHB (filled columns) before stimulation with increasing concentrations of 5-HT and KCl (45 mM) which blocked accumulation ($P<0.01$). Mean $\pm$ s.e.mean $n=3$.

In the absence of butanol, both the basal (70,602 ± 14,487 d.p.m. mg^{-1}) and the 5-HT/KCl-stimulated accumulation of [^{33}P]-PtdOH (105,218 ± 16,622 d.p.m. mg^{-1}) were larger than the respective, butanol-containing, controls (38,576 ± 4466 and 44,351 ± 8735 d.p.m. mg^{-1}). This indicates that a component of the basal, PLD-dependent synthesis of [^{33}P]-PtdOH, was sensitive to block with butanol (100 mM). The increased accumulation of [^{33}P]-PtdOH in the presence of 5-HT/KCl failed to reach significance, which presumably reflects the greater metabolic instability of [^{33}P]-PtdOH compared with [^{33}P]-PtdBut.

The accumulation of total [^{3}H]-inositol phosphates was measured in mesenteric arterial tissue pre-labelled with *myo*-[^{3}H]-inositol and stimulated in the presence of the uncompetitive inhibitor of inositol monophosphatase, LiCl (10 mM). Stimulation with 5-HT (10 μM) for 30 min, either in the presence or absence of KCl (45 mM), failed to elicit any increase in basal [^{3}H]-InsP, 8052 ± 530 d.p.m. mg^{-1}, $n = 4$. In contrast, noradrenaline stimulated a significant increase in the accumulation of [^{3}H]-inositol phosphates, to 16,516 ± 2993 d.p.m. mg^{-1} ($P < 0.01$)

Influence of pertussis-toxin against the stimulation of PLD activity and the inhibition of cyclic AMP levels with 5-HT in the mesenteric artery

Basal levels of cyclic AMP were increased from 15 ± 11.8 to 182 ± 24 pmol mg^{-1} protein in the presence of 1 μM forskolin (8 min). In the final 3 min of forskolin-stimulation, 5-HT (0.3 – 1 μM) was added. 5-HT completely abolished the formation of cyclic AMP ($n = 3$). The inhibitory action of 5-HT was abolished by pre-treatment of the mesenteric tissue with 100 ng ml^{-1} PTx for 20 h. This suggests that the inhibitory effect of 5-HT on adenylyl cyclase is probably mediated through a G-protein of the G_i/G_o class. The effect of PTx on adenylyl cyclase accumulation is illustrated in Figure 5.

Both basal and 5-HT-stimulated accumulation of [^{33}P]-PtdBut was abolished in mesenteric arteries labelled with ^{33}P-P_i in the presence of PTx. Although these data suggest that PLD may be regulated through G_i measurement of the incorporation of [^{33}P]-P_i into the total cellular lipid pool revealed that PTx pre-treatment inhibited the incorporation of radiolabel by approximately 90% (Table 1). Such an effect would decrease the specific activity of the lipid substrate for PLD, and

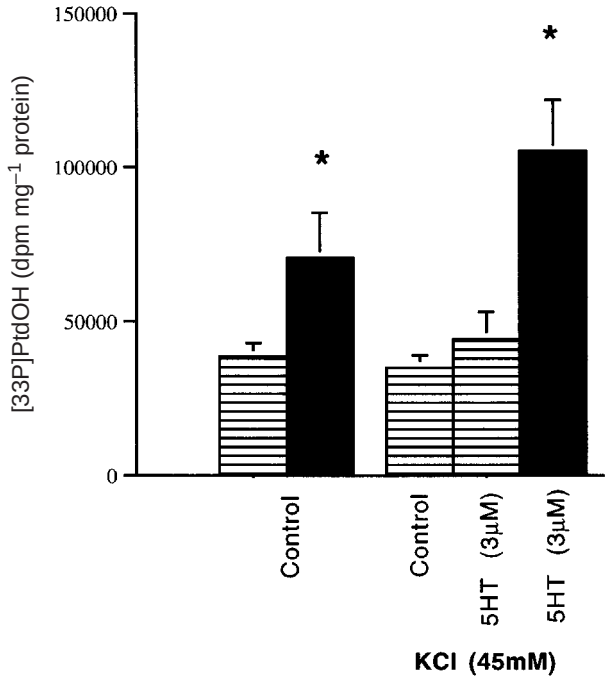

Figure 4 The effect of butanol on the accumulation of phosphatidic acid in mesenteric arteries stimulated with 5-HT (3 μM) or vehicle. Pre-labelled arteries were incubated in the presence (lined columns) or absence (filled columns) of butanol and stimulated with 3 μM 5-HT in the presence of elevated (45 mM) levels of KCl. The two columns on the left show the basal accumulation of phosphatidic acid, the columns on the right show accumulation in the presence of 5-HT and potassium relative to potassium alone (control). Statistically significant increases were only obtained in the arteries which were not incubated in butanol, mean ± s.e.mean $n = 3$, *$P < 0.01$.

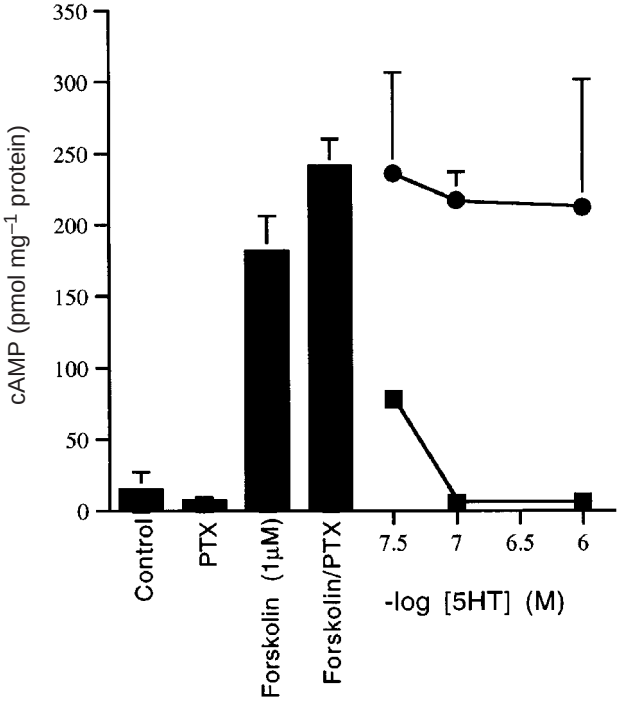

Figure 5 Inhibition of forskolin-stimulated cyclic AMP accumulation by 5-HT is blocked with pertussis toxin. Mesenteric arteries were incubated for 20 h in the presence (solid circles) or absence (solid squares) of 100 ng ml^{-1} PTx. 1 μM forskolin was then applied for 5 min to elevate the cellular concentration of cyclic AMP. This effect of forskolin was not modified by prior incubation with PTx. However, PTx blocked the reduction in cyclic AMP in response to 5-HT. Points are the mean ± s.e.mean of three experiments.

Table 1 Effect of pertussis toxin (PTx, 100 ng ml^{-1}) on the incorporation of [^{33}P]-P_i and *myo*-[^{3}H]-inositol into the total cellular lipid pool

Incorporation of radiolabel	*Incubation buffer* *Control*	*+ PTx* (100 ng ml^{-1})
[^{33}P]-P_i (d.p.m. mg^{-1})	2,452,292 ± 52,877	252,327 ± 12,379*
myo-[^{3}H]-inositol (d.p.m. mg^{-1})	20,821 ± 3533	31,555 ± 3525

Rabbit mesenteric arteries were dissected and labelled for 20 h at 37°C with the appropriate isotope in the absence or presence of PTx. Lipids were extracted and incorporation quantified by scintillation counting (d.p.m. mg^{-1}). Each number is the mean ± s.e.mean of three separate experiments. *Incorporation reduced $P < 0.001$.

inevitably lead to a reduction in the specific accumulation of PtdBut.

Similar experiments performed on [^{3}H]-inositol incorporation, demonstrated that co-incubation with PTx had no effect on the incorporation of this radiolabel (Table 1).

Discussion

These data provide the first indication that a link might exist between 5-HT_{1B}/5HT_{1D} receptor induced smooth muscle contraction and activation of the enzyme PLD. Furthermore, the detection of mRNA transcript for the 5-HT_{1B} but not the 5-HT_{1D} receptor indicates that the action of 5-HT in the mesenteric artery is mediated by the former. The fact that 5-HT is able to stimulate a large, concentration-dependent increase in PLD activity in cells prestimulated with potassium, is identical to the profile for 5-HT-induced contraction in smooth muscle cells in this artery (Choppin & O'Connor, 1994; Plane *et al*., 1995). This suggests that PLD may play a part in the contractile response. These results also support our previous evidence obtained in this artery, indicating that 5-HT_{1B}/5-HT_{1D} receptor-induced contraction is independent of the activation of PLC and does not require the release of calcium from intracellular stores (Seager *et al*., 1994).

Stimulation of 5-HT_1 receptors leads to a decrease in cellular cyclic AMP concentration *via* G_i/G_o linked G-proteins. In the case of smooth muscle cells, this decrease has been suggested to be linked to smooth muscle contraction (Sweeney *et al*., 1995). However, this evidence is not substantive. It is equally possible that changes in adenylyl cyclase activity may be involved in smooth muscle mitotic responses to 5-HT (Pauwels *et al*., 1996). In the present study, 5-HT decreased cyclic AMP following forskolin stimulation, and without the need to raise external potassium. These observations argue against a major role for cyclic AMP in contraction. We have previously demonstrated that the contractile response induced by 5-HT in mesenteric resistance arteries requires the activation of protein kinase C, and is independent of the specific formation of IP_3 (Seager *et al*., 1994). The present study confirms the latter observations. 5-HT, in contrast to noradrenaline, did not stimulate any accumulation of [^{3}H]-InsP in the presence of lithium, employed to block the enzyme inositol monophosphatase. These observations suggest that the diacylglycerol, which is presumably necessary to activate protein kinase C, must therefore be derived from another membrane phospholipid.

A possible source for diacylglycerol is phosphatidylcholine, which could be hydrolyzed either by a specific PLC or by PLD to form phosphatidic acid and subsequently hydrolyzed to diacylglycerol by phosphatidate phosphohydrolase. Both of these pathways have been found in vascular smooth muscle cells (Malarkey *et al*., 1996). PLD activation has been suggested to explain the temporal dissociation between the accumulation of Ins(1,4,5)P_3 and diacylglycerol (Plevin *et al*., 1992). Failure to detect any [^{33}P]-PtdOH in our experiments, in the presence of butanol, suggests that diacylglycerol is not formed by a phosphatidylcholine specific PLC. Such activity would have led to [^{33}P]-PtdOH accumulation, following the conversion of diacylglycerol by diacylglycerol kinase. However, whether or not a functional phosphatidylcholine-specific phospholipase C does exist in the mesenteric artery would require direct investigation of, for example, PLC-substrate specificity (Wolf & Gross, 1985). Failure to detect [^{33}P]-PtdOH also indicates that the concentration of butanol employed in our experiments was sufficient completely to divert PLD activity to the formation of phosphatidylbutanol.

Although vasoconstrictor agonists have been shown to increase PLD activity in vascular smooth muscle cells, it is not clear whether PLD activity was an absolute requirement for smooth muscle contraction in these studies. However, in the present study, the profile of PLD activity appeared to be effectively identical to the profile described in the same artery for contraction to 5-HT (Choppin & O'Connor, 1995; Plane *et al*., 1995). The clearest correlation was that 5-HT alone did not stimulate any contraction or increase in PLD activity. However, under depolarizing conditions, 5-HT stimulated both smooth muscle contraction and PLD activity. In addition, both parameters displayed a major requirement for external calcium and for protein kinase C activity (Choppin & O'Connor, 1995; Plane *et al*., 1995).

This comparable profile of results suggests a fundamental role for PLD in the vasoconstrictor response to 5-HT in the mesenteric artery. Functional responses attributable to PLD activity could be mediated in a number of ways by phosphatidic acid and/or one or more of its derivatives, such as lysophosphatidic acid. For example, phosphatidic acid has been implicated in mitogenesis and the activation of protein kinase C, both directly and after conversion to diacylglycerol (Boarder, 1994; Lang *et al*., 1995). Lysophosphatidic acid has also been implicated in mitogenesis possibly because, in common with phosphatidic acid, it prolongs the activation of GTP-bound *Ras* proteins (Tsai *et al*., 1989). This may favour sustained smooth muscle contraction through the subsequent activation of *Raf* and MAP kinase (Malarkey *et al*., 1996).

How 5-HT receptors in the mesenteric artery are coupled to the activation of PLD is not clear, but the activity of PLD may be downstream of PLC, the enzyme may be linked to a tyrosine protein kinase and modulated by guanine nucleotides, protein kinase C and calcium (see Exton, 1997 for review). The former seems unlikely in the mesenteric artery, because 5-HT did not stimulate the accumulation of either IP_3, or release calcium from IP_3 sensitive stores in this artery (Seager *et al*., 1994). The involvement of a tyrosine kinase linked phosphorylation mechanism was not investigated, although 5-HT_{1B}/5-HT_{1D} receptor-mediated contraction in the rabbit renal artery was blocked by inhibitors of tyrosine kinase activity so a similar link may exist (Hill & Garland, 1996). PLD might be activated by the $\beta\gamma$ subunits of G_i. $\beta\gamma$ subunits, liberated from high capacity G-proteins such as G_i have a direct modulatory effect on a range of cellular effectors. These include the regulation of adenylyl cyclase activity (Tang & Gilman, 1991), and the stimulation of isoforms of phospholipase C (Camps *et al*., 1992) and phosphoinositide 3-kinase (Zhang *et al*., 1995). We were unable to establish whether PLD activity was mediated by a PTx sensitive G-protein. This was due to the fact that PTx inhibited the incorporation of [^{33}P]-P_i into the cellular lipid pool in the mesenteric smooth muscle cells. Why PTx had such an effect is unclear and was not investigated further in the present study. Whatever the precise explanation, under similar experimental conditions labelling of cellular lipids with myo-inositol was unaffected.

A role for protein kinase C in the contractile response of the mesenteric artery, was indicated by the observed inhibition of 5-HT-induced contraction and myofilament sensitization with inhibitors for this enzyme (Seager *et al*., 1994; Parsons *et al*., 1996). In the present study, the selective protein kinase C inhibitor, Ro 31-8220, dramatically reduced the basal activity of PLD, and totally blocked subsequent stimulation with 5-HT/KCl. Protein kinase C activity could therefore be permissive and/or involved in upstream stimulation of PLD.

Certainly, protein kinase C activated by the stimulation of either growth factor or G-protein linked receptors has been shown to stimulate PLD activity in other cell types (Balboa & Insel, 1998; Balsinde *et al*., 1997; Yeo & Exton, 1995). There are a number of ways that protein kinase C could be activated independently of diacylglycerol generated from phosphatidylinositol. Phosphatidic acid could activate protein kinase C, either directly or after being broken down to diacylglycerol by phosphomonoesterases (Cockcroft, 1992). The activity of protein kinase C could also be enhanced in the presence of lysophosphocholine and arachidonic acid. Arachidonic acid had been suggested to be intimately involved in the 5-HT-induced sensitization of myofilaments to calcium in the mesenteric artery (Parsons *et al*., 1996). Possibly, the basal activation of PLD by protein kinase C 'primes' the cell, so that once calcium levels rise on depolarization with potassium, 5-HT can enhance PLD activity, generating a positive feedback stimulation through phosphatidic acid and diacylglycerol.

Although it has been suggested that calcium in the micromolar range will directly stimulate PLD activity (Huang *et al*., 1991), this does not necessarily appear to be true in vascular smooth muscle, where PLD is not stimulated by simply elevating the intracellular concentration of calcium (LaBelle *et al*., 1996). This observation is supported in the present study, where the basal activity of PLD was unaffected by removing extracellular calcium, and was not stimulated by raising extracellular potassium which will increase calcium influx. Protein kinase C activity provides a synergistic interaction by reducing the calcium requirement of PLD (Cockcroft, 1992). Calcium was required for 5-HT to stimulate PLD activity in the mesenteric artery, as this effect was abolished in the absence of calcium.

Finally, it is possible that PLD activity might be regulated by changes in the cytoplasmic concentration of cyclic AMP. In isolated neutrophils, cyclic AMP inhibits the activation of PLD which is stimulated by the tripeptide fMetLeuPhe (Agwu *et al*., 1991). As 5-HT_{1B}/5-HT_{1D} receptors are negatively coupled to adenylyl cyclase *via* a pertussis toxin-sensitive G-protein (Dickenson & Hill, 1995), it remains possible that a reduction in cyclic AMP might lead to the activation of PLD. If that was the case, this could represent the link between cyclic AMP and contraction, and explain the correlation reported by Sweeney *et al*. (1995).

In summary, in mesenteric resistance arteries we have found that 5-HT can stimulate the enzyme PLD in the smooth muscle cells. Our data obtained with RT–PCR indicate that the receptor responsible is the 5-HT_{1B} receptor. The conditions and characteristics required for PLD activation mirror those for contraction to 5-HT which follows 5-HT_{1B}/5-HT_{1D} receptor stimulation in this artery. So overall, these data provide indirect evidence to suggest that PLD may represent a component of the link between 5-HT_1 receptor activation and smooth muscle contraction in the mesenteric artery.

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