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The involvement of intracellular Ca^{2+} in 5-$HT_{1B/1D}$ receptor-mediated contraction of the rabbit isolated renal artery

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1 5-Hydroxytryptamine$_{1B/1D}$ (5-$HT_{1B/1D}$) receptor coupling to contraction was investigated in endothelium-denuded rabbit isolated renal arteries, by simultaneously measuring tension and intracellular [Ca^{2+}], and tension in permeabilized smooth muscle cells.

2 In intact arterial segments, 1 nM – 10 μM 5-HT failed to induce contraction or increase the fura-2 fluorescence ratio (in the presence of 1 μM ketanserin and prazosin to block 5-HT_2 and α_1-adrenergic receptors, respectively). However, in vessels pre-exposed to either 20 mM K^+ or 30 nM U46619, 5-HT stimulated concentration-dependent increases in both tension and intracellular [Ca^{2+}].

3 1 nM – 10 μM U46619 induced concentration-dependent contractions. In the presence of nifedipine (0.3 and 1 μM) the maximal contraction to U46619 (10 μM) was reduced by around 70%. The residual contraction was abolished by the putative receptor operated channel inhibitor, SKF 96365 (2 μM).

4 With 0.3 μM nifedipine present, 100 nM U46619 evoked similar contraction to 30 nM U46619 in the absence of nifedipine, but contraction to 5-HT (1 nM – 10 μM) was abolished.

5 In permeabilized arterial segments, 10 mM caffeine, 1 μM IP_3 or 100 μM phenylephrine, each evoked transient contractions by releasing Ca^{2+} from intracellular stores, whereas 5-HT had no effect. In intact arterial segments pre-stimulated with 20 mM K^+, 5-HT-evoked contractions were unaffected by 1 μM thapsigargin, which inhibits sarco- and endoplasmic reticulum calcium-ATPases.

6 In vessels permeabilized with α-toxin and then pre-contracted with Ca^{2+} and GTP, 5-HT evoked further contraction, reflecting increased myofilament Ca^{2+}-sensitivity.

7 Contraction linked to 5-$HT_{1B/1D}$ receptor stimulation in the rabbit renal artery can be explained by an influx of external Ca^{2+} through voltage-dependent Ca^{2+} channels and sensitization of the contractile myofilaments to existing levels of Ca^{2+}, with no release of Ca^{2+} from intracellular stores. *British Journal of Pharmacology* (2000) **130,** 835–842

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Introduction

The contractile effects of 5-HT on vascular smooth muscle are mediated by both 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors. However, the second messenger system linking vascular smooth muscle contraction to 5-$HT_{1B/1D}$ receptor stimulation is, at present, not clearly defined. Although in a number of vascular preparations the 5-$HT_{1B/1D}$ receptor has been shown to be negatively linked to the formation of adenosine 3:5′-cyclic monophosphate (cyclic AMP), 5-$HT_{1B/1D}$ receptor-mediated contraction may well occur in a cyclic AMP-independent manner (Movahedi & Purdy, 1997; Randall *et al*., 1996; Sumner *et al*., 1992).

In many blood vessels, the full contractile effect evoked through 5-$HT_{1B/1D}$ receptors is only obtained when the vessels are first prestimulated with another contractile agent. The importance of this prestimulation step appears to vary between different vessels, for example, in the dog saphenous vein, 5-$HT_{1B/1D}$ receptor stimulation evokes contraction in the absence of a prestimulating agent (Sumner *et al*., 1992). In contrast, in other vessels such as the rabbit ear and renal arteries, 5-$HT_{1B/1D}$ receptor-mediated contractions remain 'silent' until the arteries are prestimulated e.g. with either angiotensin II, U46619, histamine, phenylephrine, noradrenaline or by elevations in extracellular K^+ concentration (Choppin & O'Connor, 1993; 1994; Yildiz & Tuncer, 1995). Although the mechanism responsible for this 'unmasking' or 'augmentation' of 5-$HT_{1B/1D}$ receptor-mediated contraction is unclear, the wide variety of contractile agents that are effective in enabling the contraction suggests a mechanism which is common to each of the agents used as prestimulants.

Smooth muscle contraction is determined by both the free intracellular [Ca^{2+}] and the sensitivity of the contractile myofilaments to Ca^{2+}. Again, the precise role of Ca^{2+} in 5-$HT_{1B/1D}$ receptor-mediated contraction is controversial and indeed may vary between vessels. In the dog saphenous vein, 5-$HT_{1B/1D}$ receptor-mediated contraction is abolished by the removal of extracellular Ca^{2+} and inhibited by the voltage-operated Ca^{2+} channel blocker, verapamil, suggesting a crucial role for Ca^{2+} influx through these Ca^{2+} channels (Sumner *et al*., 1992). Similarly, in the rabbit isolated ear artery 5-$HT_{1B/1D}$ receptor-mediated contraction following prestimulation with phenylephrine was inhibited by nifedipine and completely abolished by the removal of extracellular Ca^{2+} (Movahedi & Purdy, 1997). In contrast, 5-$HT_{1B/1D}$ receptor-mediated contractions in the rabbit saphenous vein appeared to be insensitive to the voltage-operated Ca^{2+} channel blocker, nifedipine (Razzaque *et al*., 1995).

The ability of 5-$HT_{1B/1D}$ receptor stimulation to induce Ca^{2+} release from intracellular stores also appears to be a controversial issue. In primary cultures of vascular smooth muscle cells from the bovine basilar artery, 5-$HT_{1B/1D}$ receptor stimulation induced an increase in intracellular calcium

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concentration. A proportion of this response remained in the absence of extracellular Ca^{2+} indicating that Ca^{2+} release could occur from intracellular stores (Ebersole *et al*., 1993). In contrast, 5-HT failed to evoke contraction in permeabilized strips of rabbit mesenteric artery in the absence of Ca^{2+}, indicating an absence of any Ca^{2+} release from intracellular stores (Seager *et al*., 1994).

In the present study, we have examined the role of both Ca^{2+} and myofilament sensitization in 5-HT$_{1B/1D}$ receptor-mediated contraction in the rabbit isolated renal artery. This artery requires prestimulation to enable contraction *via* 5-HT$_{1B/1D}$ receptor stimulation (Choppin & O'Connor, 1993, 1994; Hill *et al*., 1997).

Methods

Contractile studies in intact arterial segments

Female New Zealand white rabbits (1.75–2.5 kg) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.v.) and killed by rapid exsanguination. The main renal arteries were removed and placed in Krebs buffer bubbled with 95% O_2/5% CO_2. Isolated arteries were cleared of connective tissue, cut into segments approximately 2 mm in length and mounted in a Mulvany-Halpern myograph containing Krebs buffer bubbled with 95% O_2/5% CO_2, at 37°C. The endothelium was removed by gently rubbing the intimal surface of the vessel with a human hair. Vessels were set at a resting tension of 9.1 mN (Choppin & O'Connor, 1994) and allowed to equilibrate for 1 h. Arterial segments were first stimulated with phenylephrine (100 μM) followed by acetylcholine (10 μM) to test the viability of smooth muscle and endothelial cells. Only those tissues able to develop a sustained contraction to phenylephrine and failing to relax to acetylcholine were used. In all experiments, artery segments were incubated with ketanserin and prazosin (both 1 μM) for 30 min before and during agonist application, in order to block 5-HT$_2$ and α1-adrenoceptors, respectively.

Simultaneous measurements of tension and fura-2 fluorescence

Artery segments were mounted in a Mulvany-Halpern myograph as described above. Vessels were then loaded with the fluorescent Ca^{2+} indicator fura-2 AM (Jensen *et al*., 1992, 1993). Briefly, vessels were incubated for 2 h at room temperature in Krebs buffer containing 10 μM fura-2 AM and bovine serum albumin (1 mg ml^{-1}), 0.02% Pluronic F-127, 0.1% cremaphor EL and 0.5% DMSO to facilitate fura-2 loading. After thorough washing, the tissues were placed on the stage of an Axiovert 35 microscope (Carl Zeiss, Germany) connected to a Deltascan D101 high intensity dual wavelength excitation source spectrofluorometer (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) which provided dual excitation fluorescent illumination at 340 and 380 nm. The output beam from the Deltascan was conducted to the microscope by quartz fibre optics. Excitation wavelengths were then reflected up by a dichroic mirror (FT395nm, Carl Zeiss, Germany) and focused onto the tissue with a quartz objective (Ultrafluor $\times$10 magnification, 0.2 numerical aperture, Carl Zeiss). Epifluorescence was collected *via* the objective and passed through the dichroic mirror. The emitted light was then filtered by a band filter (BP 510$\pm$5 nm, Carl Zeiss) en route to the photomultiplier tube (PMT). A camera output port with an adjustable aperture allowed the field of view to be adjusted so that only the vessel was included. Isometric force and fluorescence signal were captured at 4 Hz using A/D interface (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) and an IBM AT PC using commercially available software (Delta, Photon Technology International Inc., NJ, U.S.A.).

Both changes in tension and intracellular [Ca^{2+}] were normalized for each individual vessel by expressing them as a percentage of the control responses to 60 mM K^+

Permeabilized vessels

Isolated segments (2 mm) of rabbit renal artery were permeabilized by incubation in oxygenated 10 mM EGTA mock intracellular solution (MIS) containing *Staphylococcus aureus* α-toxin (2500 haemolytic units ml^{-1}) for 30 min. Permeabilized segments were then mounted in a myograph and bathed in 10 mM EGTA MIS bubbled with 95% O_2/5% CO_2, at room temperature (20–23°C). Vessels were maintained at a predetermined optimal tension of 5 mN throughout the duration of the experiment. Permeabilization was assessed by the ability of Ca^{2+} to stimulate contraction at concentrations (80 nM–10 μM) which had no effect on non-permeabilized preparations. The free [Ca^{2+}] of the MIS was controlled by mixing quantities of MIS containing either 10 mM EGTA or 10 mM Ca^{2+}-EGTA. Free metal ion concentrations were calculated using a computer programme adapted from (Fabiato & Fabiato, 1979) by Dr Godfrey Smith (University of Glasgow). Affinity constants for H^+, Ca^{2+} and Mg^{2+} binding to EGTA were taken from Smith & Miller (1984).

In all permeabilization experiments the endothelium was removed by rubbing the intimal surface of the vessels with a human hair. Any surviving endothelial cells were rendered non-functional by the permeabilization process (Laher *et al*., 1995).

Drugs

The composition of the Krebs buffer was (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.6, $NaHCO_3$ 25, glucose 11.7. The composition of the MIS was (mM): potassium propionate 100, $MgCl_2$ 7, HEPES 25, Na_2ATP 5, creatine phosphate 15. Creatine phosphate, inositol 1,4,5-trisphosphate and thapsigargin were obtained from Calbiochem (Nottingham, U.K.). Disodium-ATP, caffeine, GTP, 5-HT creatine sulphate, phenylephrine hydrogen chloride and prazosin HCl, were obtained from Sigma Chemicals Co. (Poole, Dorset, U.K.). Ketanserin tartrate was obtained from RBI. Fura-2 AM was obtained from Molecular Probes (U.K.). Potassium proprionate was obtained from Phase Separations Ltd. N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) was purchased from Melford's Laboratories Ltd. (Ipswich, Suffolk, U.K.).

Staphylococcus aureus α-toxin (Haemolysin) was obtained from RBI Ltd (U.S.A.). Each vial contained 5000 haemolytic units and was dissolved in 1 ml of 10 mM EGTA MIS. Fura-2 AM, nifedipine and thapsigargin were dissolved in DMSO (0.1%), all other drugs were dissolved in MIS or Krebs buffer.

Data analysis

Data are expressed as the mean$\pm$s.e.mean, with n referring to the number of animals used in each case. Comparison between means was made using the Student's t-test and significance assumed at $P<0.05$.

Results

Effect of 5-HT on tension and fura-2 fluorescence ratio; influence of 20 mM K^+ or U46619

Sixty mM K^+ caused an increase in artery tension of 28.9 ± 3.6 mN ($n=16$) which is approximately 75% of the maximal contraction of the artery (increase evoked with 100 μM phenylephrine, 37.8 ± 4.0 mN, $n=16$).

In arterial segments pre-incubated with ketanserin and prazosin (both 1 μM), 5-HT (1 nM – 10 μM) failed to evoke a contractile response or to increase fura-2 fluorescence ($n=4$; Figure 1A).

Twenty mM K^+ evoked a small increase in both tension and fura-2 fluorescence ratio, equivalent to $9.1 \pm 3.2\%$ and $22.7 \pm 6.2\%$ of the response to 60 mM K^+, respectively. The subsequent application of 5-HT (1 nM – 10 μM) now evoked concentration-dependent increases in both tension and fura-2 fluorescence ratio ($n=4$; Figures 1B and 2). Ten μM 5-HT evoked increases in tension and fura-2 fluorescence ratio equivalent to $94.3 \pm 7.0\%$ and $37.4 \pm 3.4\%$ of the response to 60 mM K^+, respectively.

U46619 (30 nM) evoked an increase in tension and fura-2 fluorescence ratio equivalent to $51.5 \pm 28.4\%$ and $19.6 \pm 6.7\%$ of the response to 60 mM K^+, respectively. The subsequent application of 5-HT (1 nM – 10 μM) evoked a concentration-dependent increase in both tension and fura-2 fluorescence ratio ($n=4$; Figures 1C and 2), with 10 μM 5-HT evoking increases equivalent to $94.2 \pm 23.5\%$ and $50.2 \pm 12.8\%$, respectively of the response to 60 mM K^+.

Effects of nifedipine and SKF 96365 on contraction to U46619

The cumulative addition of U46619 (1 nM – 10 μM) to segments of renal artery evoked sustained, concentration-dependent contraction. Following incubation of segments with 300 nM nifedipine, the contractions to U46619 were inhibited in a non-competitive manner. The maximal contraction to U46619 (10 μM) was significantly reduced from $84.6 \pm 10.1\%$ to only $15.9 \pm 2.3\%$ of the reference contraction to 100 μM phenylephrine ($P<0.05$; $n=4$; Figure 3). One μM nifedipine failed to inhibit further the U64419-evoked contractions, reducing the contraction to 10 μM U46619 by a similar extent to 300 nM ($16.1 \pm 5.4\%$; $n=4$). Three hundred nM nifedipine completely abolished contractions to 60 mM extracellular K^+ ($n=4$). Co-incubation of arterial segments with nifedipine (300 nM) and the receptor-operated Ca^{2+} channel blocker, SKF 96365 (2 μM), almost totally abolished the U46619-evoked contractions, reducing the maximal contraction obtained with 10 μM U46619 in the presence of nifedipine to $4.6 \pm 4.8\%$ ($P<0.05$; $n=4$).

Effect of nifedipine against contraction to 5-HT in the presence of U46619

The application of 5-HT (1 nM – 10 μM) to renal artery segments pre-exposed to 30 nM U46619 (pre-contraction equivalent to $11.9 \pm 2.3\%$ of the maximal contraction to phenylephrine, 100 μM; $n=4$) evoked concentration-dependent contraction. In this series of experiments, 10 μM 5-HT evoked

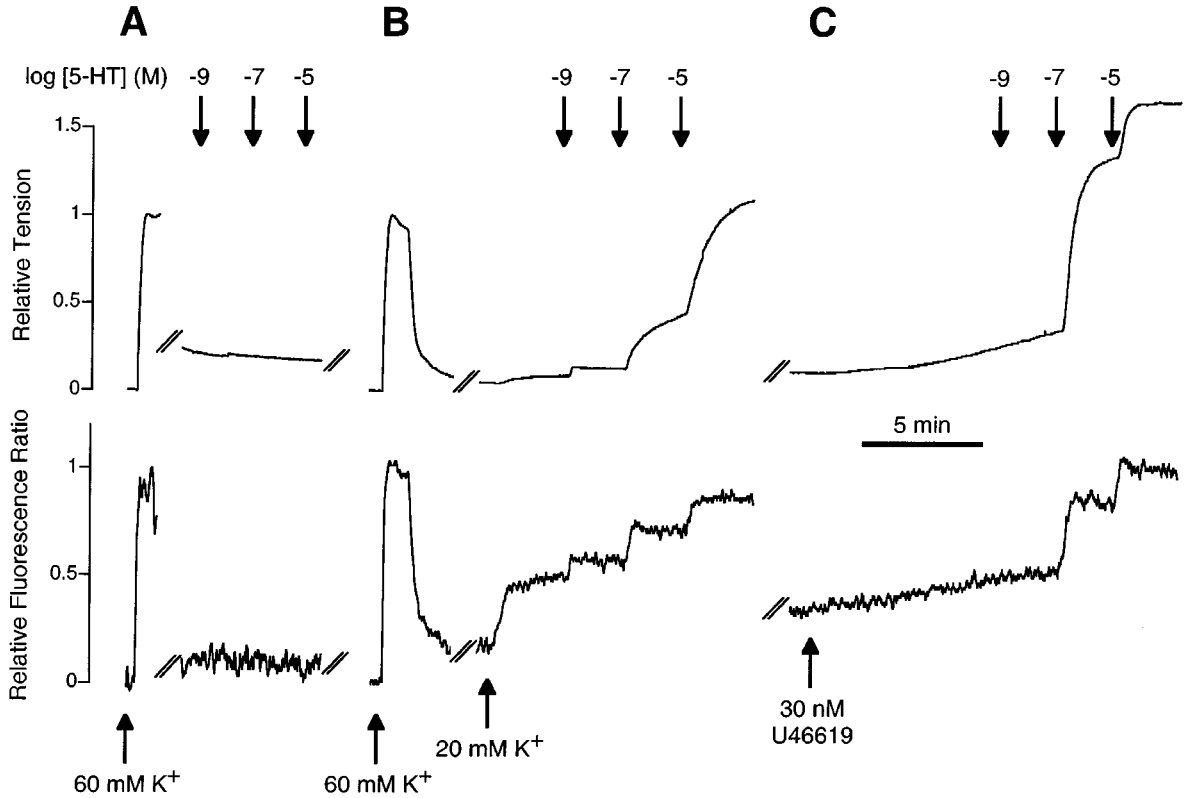

Figure 1 The effect of 5-HT on tension and intracellular [Ca^{2+}] in isolated segments of rabbit renal artery in the absence of prestimulation (A), after prestimulation with 20 mM K^+ (B), or after prestimulation with 30 nM U46619 (C). Responses are compared with responses to 60 mM K^+ which evoked a maximal contraction and increase in fura-2 fluorescence ratio. Arterial segments were incubated with ketanserin and prazosin (both 1 μM) prior to and throughout the cumulative addition of 5-HT.

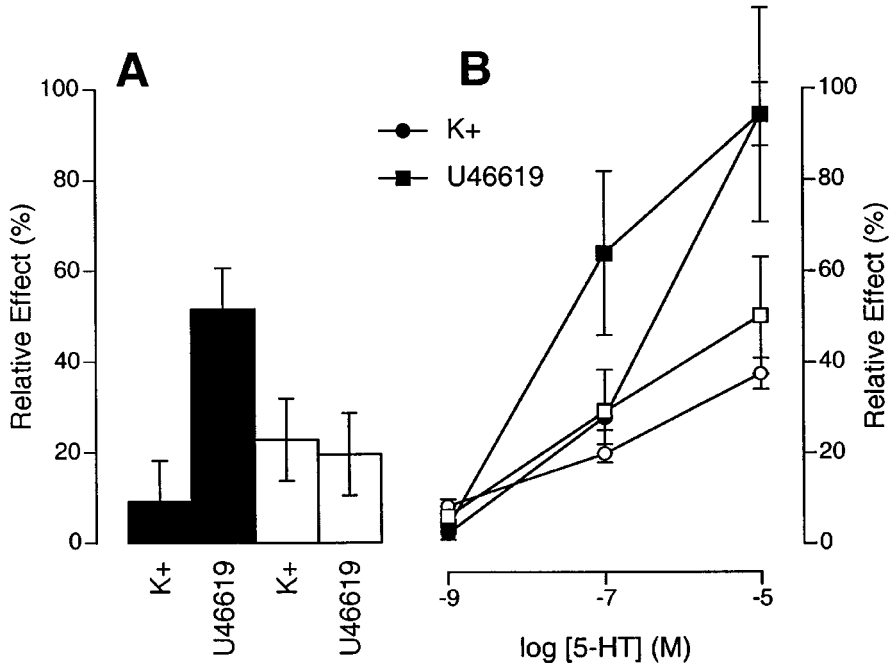

Figure 2 Mean effect of 5-HT on tension and intracellular [Ca^{2+}] after prestimulation with either 20 mM K^+ or 30 nM U46619 in isolated segments of rabbit renal artery. Closed columns and symbols represent tension recordings. Open columns and symbols represent fura-2 fluorescence ratio. (A) Effect of either K^+ or U46619 alone. (B) Additional responses to 5-HT. Responses are expressed as a percentage of the mean contraction or increase in fura-2 fluorescence ratio evoked by 60 mM K^+ ± s.e.mean, $n=4$ observations.

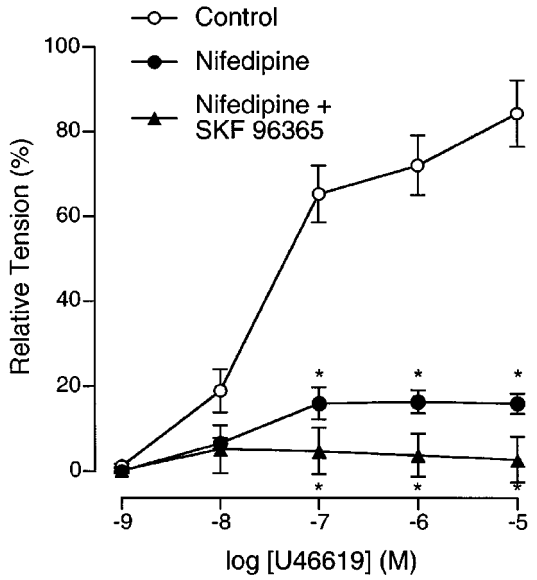

Figure 3 Effect of nifedipine and SKF 96365 against contraction to U46619 in isolated segments of rabbit renal artery. Arterial segments were incubated with vehicle (0.1% DMSO, Control), nifedipine (300 nM), or nifedipine (300 nM) and SKF 96365 (2 μM) for 30 min prior to and throughout the cumulative application of U46619. Responses are expressed as a percentage of the contraction to 100 μM phenylephrine (mean ± s.e.mean; $^*P<0.05$; $n=4$).

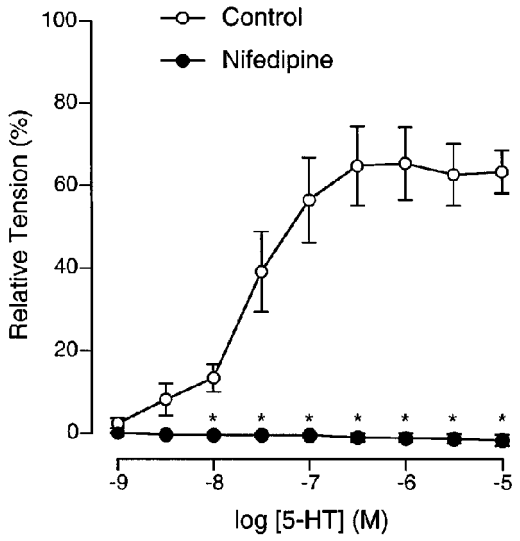

Figure 4 Effect of nifedipine on contraction to 5-HT after prestimulation with U46619 in isolated segments of rabbit renal artery. Arterial segments were incubated with ketanserin and prazosin (both 1 μM) and either vehicle (0.1% DMSO, Control) or nifedipine (300 nM) before the addition of 5-HT. The level of prestimulation was matched in both sets of experiments (U46619 increased from 30–100 nM in the additional presence of nifedipine). Responses are expressed as a percentage of the maximal contraction to 100 μM phenylephrine (mean ± s.e.mean; $^*P<0.05$; $n=4$).

a contraction equivalent to 63.28 ± 5.28% of the maximal phenylephrine-evoked contraction ($n=4$; Figure 4).

In the presence of 300 nM nifedipine, the concentration of U46619 used for prestimulation was increased to 100 nM. This concentration of U46619 evoked a contraction equivalent to 16.9 ± 4.2% of the contraction to 100 μM phenylephrine, and was therefore not significantly different to contraction with 30 nM U46619 in the absence of nifedipine. The subsequent application of 5-HT (1 nM – 10 μM) now failed to evoke any measurable contraction ($n=4$; Figure 4).

Intracellular Ca^{2+} release with caffeine, IP_3 and phenylephrine but not 5-HT in permeabilized arteries

Ten mM caffeine evoked reproducible, transient contractions in permeabilized arterial segments incubated in 80 nM Ca^{2+}/200 nM EGTA MIS for 10 min between each application of caffeine. These reproducible contractions were abolished if the artery segments were incubated in Ca^{2+} free (200 nM EGTA)

MIS to prevent refilling of the caffeine sensitive Ca^{2+} stores. Contractions returned following re-incubation of the segments in 80 nM Ca^{2+}/200 nM EGTA MIS for 10 min. ($n=4$: Figure 5A). Incubation of the permeabilized artery segments with thapsigargin (1 μM; 30 min) abolished the caffeine-evoked contractions.

In all subsequent experiments, permeabilized arterial segments were first challenged with caffeine to establish the viability of the Ca^{2+} stores. Only arteries in which reproducible caffeine responses occurred were used.

IP_3 (1 μM) also evoked reproducible, transient contractions ($n=4$) in permeabilized arterial segments maintained in 80 nM Ca^{2+}/200 nM EGTA MIS and pre-incubated with GTP (10 μM; 5 min). Exogenous IP_3 did not evoke contraction without the additional presence of GTP ($n=2$). As with the contraction evoked by caffeine, an incubation period of 10 min was sufficient to allow the IP_3 sensitive Ca^{2+} stores to refill. Incubation in Ca^{2+} free (200 nM EGTA) MIS abolished IP_3-evoked contractions, and these could be re-established by incubation in 80 nM Ca^{2+}/200 nM EGTA MIS for 10 min ($n=4$; Figure 5B).

In 80 nM Ca^{2+}/200 nM EGTA MIS containing GTP (10 μM; 5 min), the application of phenylephrine but not 5-HT (10 μM) evoked a transient contraction attributable to Ca^{2+} release from intracellular stores Figure 5C.

Lack of effect of thapsigargin on 5-HT-evoked contractions in arterial segments prestimulated with 20 mM K^+

At a concentration which abolished caffeine (10 mM)-evoked contraction in both intact and permeabilized artery segments, thapsigargin (1 μM; 30 min; $n=4$) failed to alter the 5-HT (1 nM – 10 μM)-evoked contractions in intact vessels incubated with ketanserin and prazosin (1 μM) and prestimulated with 20 mM K^+ ($n=4$; $P<0.05$; Figure 6).

5-HT and phenylephrine-evoked Ca^{2+} myofilament sensitization in permeabilized arterial segments

Ten μM 5-HT and 100 μM phenylephrine each evoked contractions equivalent to 20.2 $\pm$ 4.4% and 26.9 $\pm$ 6.1% of the maximal Ca^{2+}-induced contraction in permeabilized arterial segments sub-maximally contracted with 175 nM Ca^{2+} MIS and GTP (10 μM; $n=4$; Figure 7), respectively.

Discussion

The present study provides the first direct demonstration that smooth muscle intracellular [Ca^{2+}] is elevated specifically in response to 5-HT$_{1B/1D}$ receptor stimulation. Both the 5-HT-evoked increase in smooth muscle intracellular Ca^{2+} and contraction required prestimulation with either raised extracellular K^+ or U46619. The sensitivity of the ensuing contraction to nifedipine indicated that Ca^{2+} influx occurred

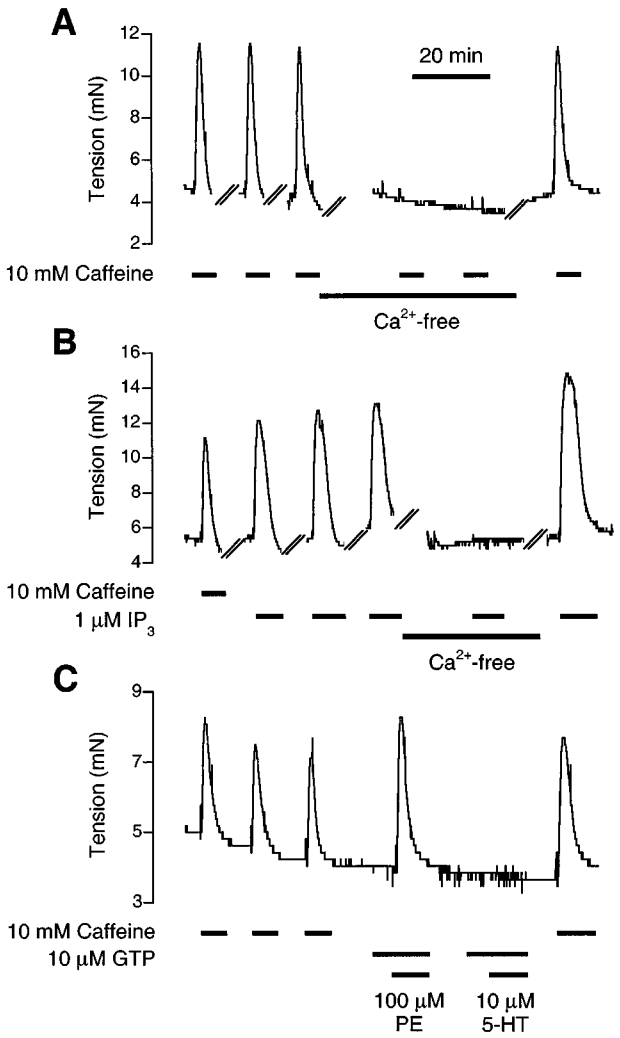

Figure 5 Effect of 5-HT on the release of intracellular Ca^{2+} stores in α-toxin permeabilized segments of rabbit renal artery. Permeabilized arterial segments were incubated in 80 nM Ca^{2+}/200 nM EGTA or Ca^{2+}-free (10 mM EGTA) mock intracellular solution. Lines under the trace indicate the period of application. Both caffeine (10 mM, A) and IP_3 (1 μM in the presence of 10 μM GTP, B) were able to evoke transient, reproducible contractions which were abolished under Ca^{2+}-free conditions. PE (100 μM) but not 5-HT (10 μM) (both in the presence of 10 μM GTP) was able to evoke contractions by releasing intracellular stored Ca^{2+} (C).

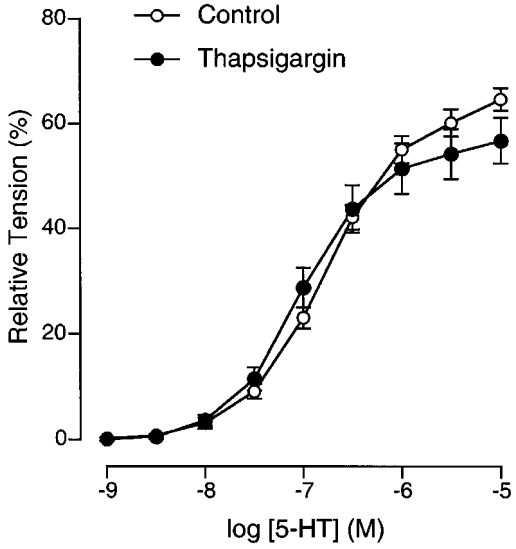

Figure 6 Lack of any effect of thapsigargin on 5-HT-induced contraction in isolated segments of rabbit renal artery prestimulated with 20 mM K^+. Arterial segments were incubated with ketanserin and prazosin (both 1 μM) and either vehicle (0.1% DMSO, control) or thapsigargin (1 μM). Responses are expressed as a percentage of the contraction to 100 μM phenylephrine and are shown as the mean $\pm$ s.e.mean, $n=4$.

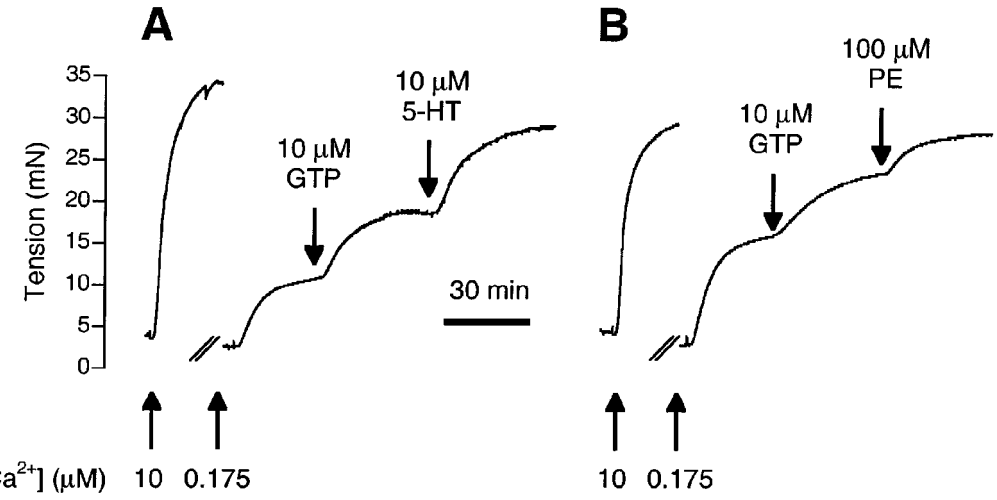

Figure 7 Sensitization to 5-HT (A) and PE (B) in α-toxin permeabilised segments of rabbit renal artery contracted with a fixed concentration of Ca^{2+}. Permeabilized arterial segments were precontracted with 175 nM Ca^{2+} and 10 μM GTP in the presence of thapsigargin (1 μM). Ca^{2+} was buffered by 10 mM EGTA.

through voltage-operated Ca^{2+} channels, while the ability of 5-HT to stimulate contraction in permeabilized cells in the presence of a clamped concentration of calcium suggested that the contraction also involved a sensitization of the contractile myofilaments to Ca^{2+}. No evidence was obtained in the permeabilized preparations to suggest that 5-HT could release Ca^{2+} from intracellular stores.

The majority of blood vessels in which 5-HT$_{1B/1D}$ mediated smooth muscle contraction has been described require prestimulation, either with another agonist or with a slight increase in the extracellular [K^+], before 5-HT can evoke contraction (Choppin & O'Connor, 1993; Movahedi *et al*., 1997; Yildiz *et al*., 1995). The suggestion has been that these manoeuvres each serve, in some way, to increase the intracellular [Ca^{2+}] to some 'threshold value' such that the contractile action of 5-HT is facilitated. However, no direct measurements of [Ca^{2+}] have been reported to help to define this process. We have now demonstrated that in the renal artery the 5-HT-evoked contraction is associated with increases in intracellular Ca^{2+} levels, and that these increases also require prestimulation with another contractile agent. Since 5-HT-evoked contractions appear to be mediated largely by Ca^{2+} influx through voltage-operated Ca^{2+} channels, and not release from intracellular stores, the process of prestimulation must in some way enable the 5-HT$_{1B/1D}$ receptor to stimulate Ca^{2+} influx through these channels. Although it is unclear how such an interaction may occur, the diversity of contractile agents which have been used to unmask or augment 5-HT$_{1B/1D}$ receptor-mediated contractions suggests that prestimulating agents mediate the augmentation through a common step.

One possibility is that other agonists facilitate calcium entry to the subsequent addition of 5-HT by activating the phospholipase C signalling pathway. It seems unlikely that the agonist-evoked release of stored Ca^{2+} by IP_3 is responsible, because this might actually be expected to decrease I_{Ca} (Klockner & Isenberg, 1991). However, the formation of diacylglycerol and the subsequent activation of protein kinase C (PKC) does represent a possible way to enhance Ca^{2+} entry. The activation of PKC stimulates L-type Ca^{2+} channels in vascular smooth muscle cells (McHugh & Beech, 1997). This action of PKC may reflect an influence on the gating of the channels rather than of single channel conductance (Obejero-Paz *et al*., 1998). Alternatively or additionally, enhanced Ca^{2+} current may follow the activation of PKC by the $\beta\gamma$ subunits released from heterotrimeric G-proteins (Zhong *et al*., 1999). However, in the portal vein this effect appeared to be mediated by the Ca^{2+}-independent isoform of PKC, PKCε (Zhong *et al*., 1999). This isoform is absent from the rabbit renal artery, although another Ca^{2+}-independent isoform, PKCδ, is present, and appears to be aligned with the contractile myofilaments (Hinton *et al*., 1999b). Perhaps the primary role of prestimulation is to activate Ca^{2+} channels by depolarizing the smooth muscle cell membrane, and then 5-HT increases the Ca^{2+} current through a pathway which involves the release of $\beta\gamma$ subunits from G_i, which is linked to 5-HT$_{1B/1D}$ receptors. The $\beta\gamma$ subunits may then activate subsequent steps which include the activation of one or more of the PKC isoforms which have been identified in the renal artery (Hinton *et al*., 1999b). One of these steps may be the activation of phospholipase D, the activity of which can be increased by the stimulation of 5-HT$_{1B/1D}$ receptors in vascular smooth muscle cells (Hinton *et al*., 1999a).

The possibility that the prestimulating agents augment or unmask 5-HT$_{1B/1D}$ receptor-mediated contractions by membrane depolarization would then explain the ability of raised extracellular K^+ also to act as a prestimulant. As well as raising intracellular [Ca^{2+}], each of the contractile agents which have been used to reveal 5-HT$_{1B/1D}$ receptor-mediated contraction can also cause membrane depolarization (Lalanne *et al*., 1984; Nilsson *et al*., 1998; Tosun *et al*., 1998; Yamakawa *et al*., 1997; Haeusler *et al*., 1989). Although 5-HT$_{1B/1D}$ receptor stimulation may not activate voltage-operated Ca^{2+} channels in quiescent vessels, stimulation may occur in depolarized arteries when the channel open probability has been increased. A similar type of effect has been reported in cardiac cells, where isoprenaline potentiates Ca^{2+} current in electrically prestimulated cells (Reuter *et al*., 1982).

The sensitivity of 5-HT evoked contraction to nifedipine indicates that the 5-HT$_{1B/1D}$ receptor mediates contraction by causing Ca^{2+} influx though voltage-operated Ca^{2+} channels. These findings are in line with data from the rabbit ear artery following prestimulation with phenylephrine. In this artery, nifedipine reduced 5-HT-evoked contraction by 55% (Movahedi & Purdy, 1997). These data are also in line with those from the dog saphenous vein, where 5-HT$_{1B/1D}$ receptor-mediated contractions were abolished by the removal of

extracellular Ca^{2+} and inhibited by verapamil (Sumner *et al*., 1992). The saphenous vein is unusual, in that 5-HT$_{1B/1D}$ receptor-mediated contractions do not require any prestimulation of the vessel, a characteristic that facilitates this type of assessment. However, if 5-HT evoked contraction is only revealed in the presence of a prestimulating agent, simply removing extracellular Ca^{2+} may well modify or abolish that initial step and hence prevent contraction. For this reason, we used the constrictor agent U46619 and the putative receptor-operated Ca^{2+} channel blocking agent, SKF 96365. In the rat aorta, U46619-evoked contraction has been shown to occur by Ca^{2+} influx through both L-type and non-L-type Ca^{2+} channels (possibly receptor operated channels, Tosun *et al*., 1998). In the present study, nifedipine abolished all contraction to 60 mM K^+, and inhibited U46619-evoked contractions in a non-competitive manner. The combined application of nifedipine and SKF 96365 completely abolished U46619-evoked contractions. Therefore, by stimulating renal arteries with a marginally increased concentration of U46619, we were able to prestimulate these vessels in the presence of nifedipine, and thus reveal the ability of nifedipine to block selectively the action of 5-HT.

Agonist-evoked increases in intracellular Ca^{2+} can also follow release from intracellular stores. In the present study, the role of intracellular Ca^{2+} release in 5-HT-evoked contractions was studied in arteries permeabilized with α-toxin. In the permeabilized arteries in the presence of GTP (to counter the possible loss of endogneous GTP from the cells, Buus *et al*., 1998), 5-HT failed to evoke a response, in spite of the fact that phenylephrine could evoke a transient contraction. This indicates that the 5-HT$_{1B/1D}$ receptor is not functionally linked to Ca^{2+} release in this vessel. However, in intact arterial segments, 5-HT-evoked increases in intracellular [Ca^{2+}] could only be detected in prestimulated vessels. As it is not possible to prestimulate permeabilized vessels in this way, it remained possible that the inability of 5-HT to induce Ca^{2+} release in the permeabilized vessels reflected the absence of any prestimulation. We addressed this possibility with the Ca^{2+}-ATPase inhibitor thapsigargin, which was used to prevent the refilling of intracellular stores, and thereby render them non-functional. The fact that thapsigargin failed to inhibit 5-HT-evoked contractions in non-permeabilized arteries, provided further evidence that Ca^{2+} release was not contributing to the 5-HT$_{1B/1D}$ receptor-mediated contractions, even in prestimulated vessels. These observations are consistent with those in the rabbit isolated mesenteric artery, where 5-HT failed to induce contraction in permeabilized strips or to stimulate any increase in IP_3 accumulation (Seager *et al*., 1994). However, these observations contrast with those of Ebersole *et al*. (1993) in the bovine basilar artery, where 5-HT$_{1B/1D}$ receptor stimulation in zero extracellular Ca^{2+} was associated with transient increases in intracellular Ca^{2+}, suggesting release of Ca^{2+} from intracellular stores.

In addition to inducing influx through voltage-operated Ca^{2+} channels, experiments carried out in permeabilized renal arteries indicated that the 5-HT$_{1B/1D}$ receptor also mediates contraction through a sensitization of the contractile myofilaments to Ca^{2+}. In the presence of a fixed concentration of calcium (175 nM), 5-HT evoked an additional contraction of around 20% of the tissue maximal contraction. Under these experimental conditions, such contraction must reflect an increase in myofilament sensitivity to calcium. Myofilament sensitization represents an important component of smooth muscle contraction evoked by a number of G-protein linked receptors. It can occur through both PKC-dependent and independent pathways, although it has recently been suggested that the PKC-dependent pathways have a minimal role under physiological conditions (Somlyo & Somlyo, 1998). The available evidence suggests that the non-PKC pathways may involve the activation of $p21^{rhoA}$ and that this pathway and PKC may each inhibit the dephosphorylation of myosin light chain by an inhibitory action on the trimeric phosphatase, SMPP-1M (Akopov *et al*., 1998; Gong *et al*., 1997). In the mesenteric artery, 5-HT$_{1B/1D}$ receptor activation causes myofilament sensitization to calcium, which can be blocked with an inhibitor of phospholipase A_2 (and PKC) and mimicked by arachidonic acid (Parsons *et al*., 1996). Arachidonic acid has also been shown directly to inhibit myosin light chain phosphatase which may explain this effect (Gong *et al*., 1992).

In conclusion, results from the present study indicate that, in the rabbit isolated renal artery, 5-HT$_{1B/1D}$ receptor stimulation evokes both smooth muscle contraction and an increase in the intracellular [Ca^{2+}], but only in vessels prestimulated with either 20 mM K^+ or U46619. The finding that 5-HT-evoked contractions were abolished by the voltage-operated Ca^{2+} channel blocker, nifedipine, yet unaffected by thapsigargin, indicates that 5-HT$_{1B/1D}$ receptor-mediated contractions occur through Ca^{2+} influx through voltage-operated Ca^{2+} channels rather than Ca^{2+} release from intracellular stores. In addition to increasing the intracellular concentration of calcium, 5-HT$_{1B/1D}$ receptor stimulation also induces sensitization of the contractile filaments to Ca^{2+}.

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