British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01373.x
www.brjpharmacol.org

RESEARCH PAPER

Characteristics of the actions by which 5-HT affects electrical and mechanical activities in rabbit jugular vein

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Keywords
5-hydroxytryptamine; 5-HT_{2A} subtype receptor; 5-HT_7 subtype receptor; cAMP; endothelium; membrane hyperpolarization; rabbit jugular vein

Received
13 December 2010
Revised
15 February 2011
Accepted
11 March 2011

BACKGROUND AND PURPOSE

5-HT is known to be a potent vasospasmogenic agonist in various arteries. However, in veins the vasomodulating actions of 5-HT, and the underlying mechanisms, remain to be fully clarified. Here, we characterized the actions by which 5-HT affects electrical and mechanical activities in the rabbit jugular vein.

EXPERIMENTAL APPROACH

Membrane potential and isometric tension were measured in endothelium-intact and -denuded preparations. Localization of 5-HT receptor subtypes was examined immunohistochemically.

KEY RESULTS

5-HT induced a transient then a small, sustained smooth muscle cell hyperpolarization in endothelium-intact strips. In endothelium-denuded strips, 5-HT induced only a sustained hyperpolarization, and this was changed to a depolarization by the selective 5-HT_7 receptor inhibitor SB269970. This depolarization was inhibited by the 5-HT_{2A} receptor blocker sarpogrelate. 5-HT induced a relaxation of $PGF_{2\alpha}$-induced contracted strips that was similar in endothelium-intact and -denuded preparations. The latter relaxation was changed to contraction by SB269970 and this contraction was inhibited by sarpogrelate. Immunoreactive responses against endothelial and smooth muscle 5-HT_{2A} receptors and smooth muscle 5-HT_7 receptors were identified in the vein. The 5-HT-induced relaxation of the $PGF_{2\alpha}$ contraction was inhibited by the cAMP-dependent protein kinase inhibitor Rp-cAMPS and by the AC inhibitor SQ22536.

CONCLUSIONS AND IMPLICATIONS

These results indicate that 5-HT activates both smooth muscle 5-HT_7 receptors (to produce relaxation) and smooth muscle 5-HT_{2A} receptors (to produce contraction) in rabbit jugular vein. We suggest that in this particular vein, the 5-HT_{2A} receptor-induced depolarization and contraction are masked by the 5-HT_7 receptor-induced responses, possibly via actions mediated by cAMP.

Abbreviations

AS 19, (2S)-(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin; L-NNA, N^{ω}-nitro-L-arginine; MDL 11939, α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol; Rp-cAMPS, adenosine 3,5-cyclic monophosphorothioate, Rp-isomer; SB200646, N-(1-methyl-1H-indol-5-yl)-N′-3-pyridinylurea; SB269970, (2R)-1-[(3-hydroxyphenyl)sulphonyl]-2-[2-(4-methyl-1-pip eridinyl)ethyl]pyrrolidine; SQ22536, 9-(tetrahydro-2′-furyl)adenine; TCB-2, (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide

Introduction

5-HT displays a variety of electrical and mechanical activities in blood vessels, dependent upon both the region and the species. Various reports indicate that 5-HT induces a contraction accompanied by membrane depolarization in smooth muscle cells both in basilar arteries (dog, Fujiwara *et al.*, 1982; cat, Harder and Waters, 1983; rabbit, Garland, 1987; Nagao

and Suzuki, 1987) and in rabbit coronary artery (Garland, 1985), suggesting that membrane depolarization plays a role in 5-HT-induced contraction in these arteries. In contrast, in the porcine coronary artery 5-HT induces an endothelium-dependent NO-mediated relaxation without a change in the smooth muscle membrane potential (Frieden and Bény, 1995), indicating that in that artery, 5-HT acts on the endothelium to induce vascular relaxation. These findings suggest that in arteries, 5-HT can induce contraction/relaxation through a mechanism either dependent or independent of membrane potential. In veins, however, the actions by which 5-HT affects the electrical activities of smooth muscle have not been fully clarified.

The receptor subtypes mediating the effects of 5-HT on mechanical activities have been characterized in blood vessels as follows: 5-HT induces (i) contraction via smooth muscle 5-HT_{2A} receptors and/or smooth muscle $5\text{-HT}_{1B/1D}$ receptors (Hoyer *et al.*, 1994; Alexander *et al.*, 2009) and (ii) relaxation via smooth muscle 5-HT_{7} receptors and/or two types of endothelial receptors ($5\text{-HT}_{1B/1D}$ and 5-HT_{2B}) that are coupled to the release of endothelial NO (Gupta, 1992; Ellis *et al.*, 1995; Grayson and Gupta, 1995; Glusa and Roos, 1996). However, as these subtypes co-exist in certain types of arteries and veins, the net effect of 5-HT on mechanical activities in a given vessel might be expected to depend on the relative expression levels of subtypes mediating smooth muscle contraction and relaxation. In addition, factors such as changes in endothelial function could affect 5-HT-induced changes in mechanical activities.

An autologous vein is the optimum graft for surgical treatment in patients suffering from peripheral arterial disease. However, a vein implanted into the arterial side of the circulation is subjected to changes in conditions (such as increased sheer stress) and undergoes changes in its cell population (such as loss of endothelial cells, migration and invasion of inflammatory cells, and migration and proliferation of vascular smooth muscle cells). Studies using rabbit jugular vein have indicated that 5-HT plays a role in the pathogenesis of vein-graft spasms (Makhoul *et al.*, 1987; Radic *et al.*, 1991). Interestingly, 20 years ago in another rabbit study it was found that 5-HT induces endothelium-dependent relaxation in a native jugular vein but contraction in an autologous vein graft (Komori *et al.*, 1990), suggesting an increased sensitivity to the contractile effects of 5-HT in the graft. We recently found that in rabbit vein grafts, the ability of endothelial cells to release NO is lacking (Kodama *et al.*, 2009a, 2009b) and that 5-HT induces constriction through activation of both 5-HT_{1B} and 5-HT_{2A} receptors located on the smooth muscle cells (Kodama *et al.*, 2009b). More importantly, we found that chronic administration *in vivo* of the 5-HT_{2A} receptor antagonist sarpogrelate increases the endothelial 5-HT_{1B} receptor-mediated endothelial NO release in the vein graft, thereby attenuating the contraction induced by 5-HT. Taken together these results suggest that changes in the expressions of 5-HT receptor subtypes in endothelial and smooth muscle cells and changes in the functions of the endothelium may be responsible for modulating the actions of 5-HT, at least in a rabbit jugular vein graft. In addition, it has been suggested that 5-HT may be involved in the genesis of pulmonary hypertension, possibly through an action on voltage-dependent K^+ channels (K_V 1.5) (Cogolludo *et al.*, 2006). A prerequisite for a proper understanding of the role of 5-HT in jugular vein grafts would seem to be a clarification of the actions by which 5-HT affects the electrical and mechanical properties of the native vein under physiological conditions. However, these actions have yet to be fully clarified in rabbit jugular vein.

In this study, using the rabbit native jugular vein, we investigated the role played by the endothelium. 5-HT-induced responses were examined both in endothelium-intact preparations, either untreated or treated with the NO-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA), and in endothelium-denuded preparations. The receptor subtypes responsible for these actions of 5-HT were characterized pharmacologically, and the localization of the receptors was examined immunohistochemically. In addition, the role played by cAMP in the 5-HT-induced responses was examined by using inhibitors of the cAMP/c AMP-dependent PKA signal cascade.

Methods

Animals

All experiments performed in this study conformed to Guidelines on the Conduct of Animal Experiments issued by the Graduate School of Medical Sciences in Nagoya City University and were approved by the Committee on the Ethics of Animal Experiments in that institution. Male Japanese albino rabbits (supplied by Kitayama Labes, Ina, Japan), weighing 2.5–3.0 kg, were used in this study.

Tissue preparation

Rabbits were anaesthetized by injection of pentobarbitone sodium (50 $mg \cdot kg^{-1}$ given i.v.), then killed by exsanguination. The external jugular vein was immediately excised and placed in Krebs solution. Once connective tissue had been carefully removed, each segment was cut open along its long axis and circularly cut preparations were prepared, as described previously (Itoh *et al.*, 1992). In some preparations, the endothelium was removed by gently rubbing the intimal surface with small pieces of razor blade (Itoh *et al.*, 1992). Guanethidine (5 μM, to prevent effects due to release of sympathetic transmitters) and diclofenac (3 μM, to inhibit the production of COX products) were present throughout the experiments.

Electrophysiological study

Membrane potentials were measured in smooth muscle cells using a conventional microelectrode technique, as described previously (Kusama *et al.*, 2005; Watanabe *et al.*, 2008; Kajikuri *et al.*, 2009). 5-HT (10 μM) induced a large transient, followed by a small sustained hyperpolarization in endothelium-intact preparations. When 5-HT (10 μM) was applied for 1.5 min three times (interval between applications, 25 min), the amplitude of the 5-HT-induced transient (but not sustained) response gradually declined until a repeatable 5-HT response was obtained (Figure 1A). Therefore, to obtain reproducible electrical responses, 5-HT (10 μM) was applied for 1.5 min at 25 min intervals. The effects on these responses of the agents of interest (such as glibenclamide, certain 5-HT receptor-subtype antagonists or agonists, Rp-cAMPS) were then examined.

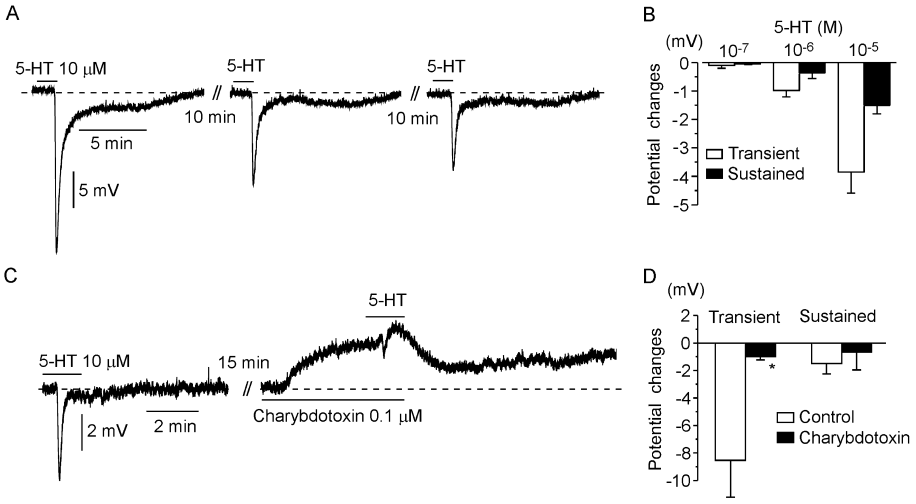

Figure 1

Effects of 5-HT on membrane potential in smooth muscle cells of endothelium-intact rabbit jugular vein. (A) 5-HT (10 μM) was applied for 1.5 min three times (interval between applications, 25 min). Broken line indicates the resting smooth muscle cell membrane potential level. (B) Concentration-dependent effects of 5-HT on smooth muscle cell membrane potential (n = 5–7). (C) Effect of charybdotoxin (0.1 μM) on 5-HT-induced membrane potential changes. (D) Summary of the effects (n = 4). Each record in a given set of three or two was obtained from the same impalement. Data are shown as mean ± SEM. *P < 0.05 versus 'Control'.

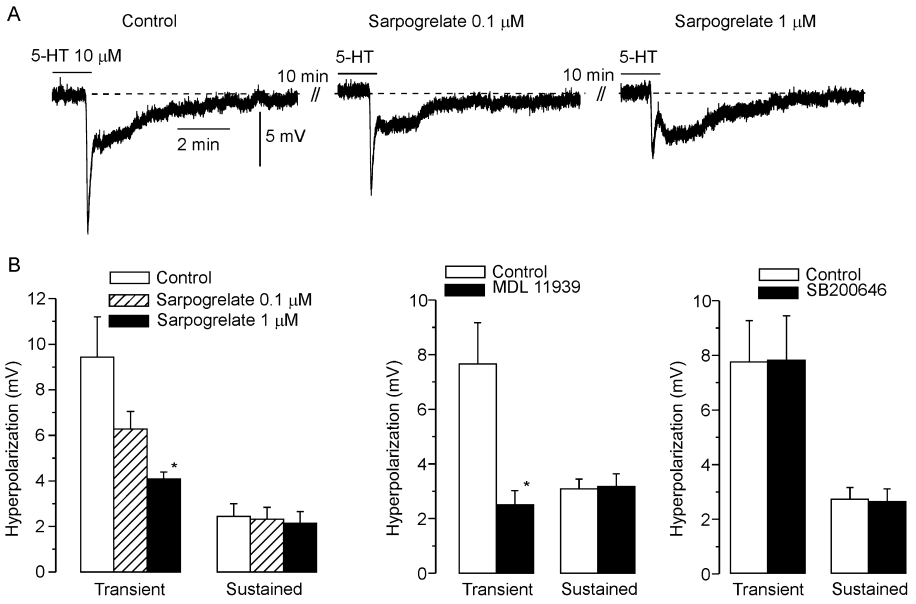

Figure 2

Effects of the 5-HT_{2A} receptor antagonists (sarpogrelate and MDL 11939) and the 5-$HT_{2B/2C}$ receptor antagonist SB200646 on 5-HT-induced changes in smooth muscle cell membrane potential in endothelium-intact preparations. (A) After recording the control 5-HT (10 μM) response, sarpogrelate (0.1 μM) was pretreated for 5 min and 5-HT was again applied in the presence of 0.1 μM sarpogrelate. After a 20 min interval, sarpogrelate (1 μM) was then pretreated for 5 min, and 5-HT was finally applied in the presence of 1 μM sarpogrelate. (B) Summary of the effects of sarpogrelate (0.1 and 1 μM, left panel), MDL 11939 (1 μM, middle panel) and SB200646 (1 μM, right panel) on 5-HT (10 μM)-induced transient and sustained hyperpolarizations.

Isometric tension measurement

Using the circularly cut strips mentioned above (4 mm long, 0.7–0.8 mm wide), isometric tension was measured as described previously (Kusama *et al.*, 2005; Watanabe *et al.*, 2008; Kajikuri *et al.*, 2009). Resting tension was adjusted to obtain maximum contraction in high-K^+ solution (128 mM).

To observe the concentration-dependent effects, 5-HT (10^{-9}–10^{-5} M) was cumulatively applied for 2 min at each

concentration in an ascending order during the contraction induced by $PGF_{2\alpha}$ in endothelium-intact or -denuded strips. This protocol was repeated at 25–30 min intervals to obtain reproducible responses.

To examine the roles played by the endothelium in 5-HT-induced responses, the effect of 5-HT was examined in the absence and presence of the endothelial NOS (eNOS) inhibitor L-NNA. L-NNA (0.1 mM) was applied as pretreatment for 60 min and was present thereafter. As L-NNA enhanced the contraction induced by $PGF_{2\alpha}$, the concentration of $PGF_{2\alpha}$ applied in the presence of L-NNA was reduced so as to obtain matched amplitudes of contraction. When the effect of the PKA inhibitor Rp-cAMPS or that of the adenylate cyclase inhibitor SQ22536 was to be examined on the response induced by 5-HT, the appropriate inhibitor was applied as pretreatment for 30 min and was present thereafter.

Immunohistochemical staining

Segments of external jugular vein were immersion-fixed in 4% paraformaldehyde in 10 mM phosphate buffer and embedded in OCT compound (Tissue Tek; SAKURA Finetechnical, Tokyo, Japan), then frozen and stored at –80°C. Sections were cut at 6 μm thickness on a cryostat, then mounted on MAS-coated glass slides (Matsunami Glass, Kishiwada, Japan), as described previously (Kusama *et al.*, 2005).

The sections were incubated overnight at 4°C with anti-$5HT_{2A}$ or anti-$5HT_7$ polyclonal antibody (1:100 dilution; ImmunoStar, Hudson, WI, USA) as the primary antibody. After the sections had been rinsed with PBS, they were incubated for 1 h at room temperature with the second antibody (Alexa Fluor 488 anti-rabbit IgG antibody; 1:1000 dilution; Molecular Probes, Eugene, OR, USA), followed by a wash with PBS. The fluorescence of Alexa Fluor 488 was then detected by confocal laser-scanning microscopy, under identical conditions in each case.

Solutions

The composition of the Krebs solution was as follows (mM): NaCl, 122; KCl, 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 15.5; KH_2PO_4, 1.2; glucose, 11.5. It was bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). The PBS solution contained 2.9 mM NaH_2PO_4, 9 mM Na_2HPO_4 and 137 mM NaCl, and its pH was 7.2–7.4.

Drugs

The drugs used were as follows: L-NNA (Peptides Institute Inc., Osaka, Japan); 5-HT hydrochloride, glibenclamide and diclofenac sodium (Sigma Chemical Co., St. Louis, MO, USA); SB269970 hydrochloride, SB 200646 hydrochloride, MDL 11939, TCB-2 and AS 19 (Tocris Bioscience, Ellisville, MO,

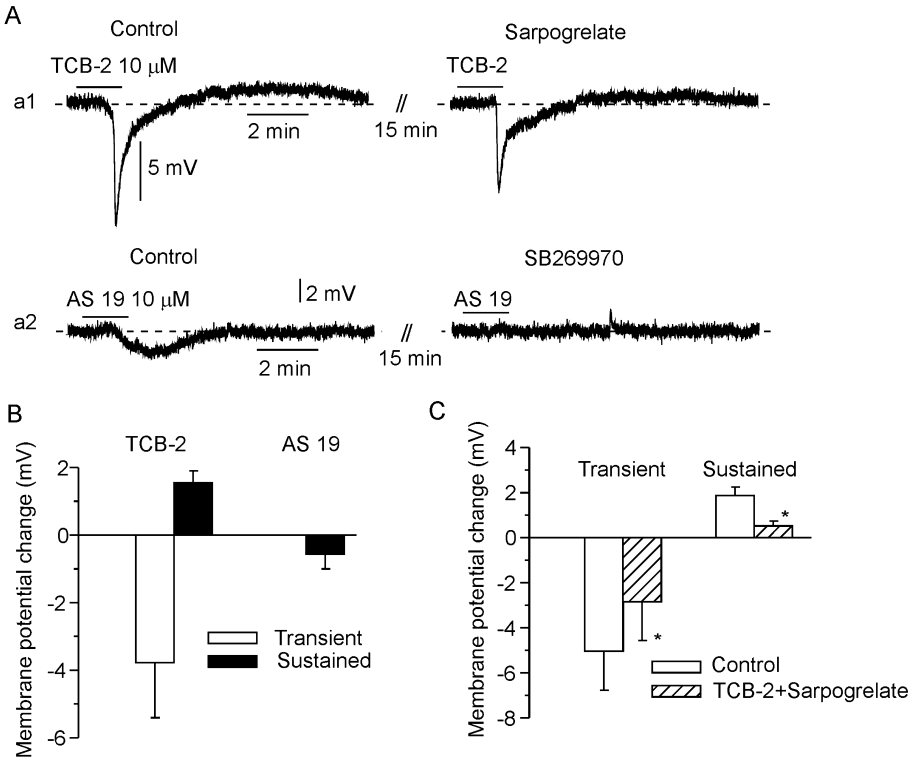

Figure 3

Effects of the 5-HT_{2A} receptor agonist TCB-2 and the 5-HT_7 receptor agonist AS 19 on smooth muscle cell membrane potential in endothelium-intact preparations. (Aa1) Effect of TCB-2 (10 μM) in the absence ('Control') and presence of 1 μM sarpogrelate ('Sarpogrelate'). (Aa2) Effect of AS 19 (10 μM) in the absence ('Control') and presence of the 5-HT_7 receptor antagonist SB269970 ('SB269970'). Each record in a given set of two was obtained from the same impalement. (B) Summary of the effects of TCB-2 and AS 19 (n = 4). (C) Effects of sarpogrelate (1 μM) on TCB-2-induced membrane potential changes (n = 4). Data are shown as mean ± SEM. *P < 0.05 versus 'Control'.

USA); Rp-cAMPS triethylammonium salt (Merck Japan Ltd, Tokyo, Japan); SQ22536 (Enzo Life Sciences Inc., Farmingdale, NY, USA); guanethidine (Tokyo Kasei, Tokyo, Japan). $PGF_{2\alpha}$ was kindly provided by Ono Pharmaceutical Co. (Osaka, Japan) and sarpogrelate hydrochloride by Mitsubishi Tanabe Pharma (Osaka, Japan).

Stock solutions were made of glibenclamide, SB200646, AS 19 (each in DMSO) and $PGF_{2\alpha}$ (in ethanol). All other drugs were dissolved in ultrapure Milli-Q water (Japan Millipore Corp., Tokyo, Japan). The above stock solutions were stored at –80°C and diluted in Krebs solution just before use.

Statistical analysis

All results are expressed as mean ± SEM, with *n* values representing the number of rabbits used (each rabbit provided only one segment for a given experiment). A one-way or two-way repeated-measures ANOVA, with *post hoc* comparisons made using the Scheffé procedure or Student's unpaired *t*-test, was used for the statistical analysis. The level of significance was set at $P < 0.05$.

Results

Effects of 5-HT on smooth muscle cell membrane potential

The smooth muscle cells of the endothelium-intact jugular vein were electrically quiescent and the resting membrane potential was –50.3 ± 1.7 mV ($n = 7$). Application of 5-HT (10 μM) for 1.5 min induced a two-phase hyperpolarization: a transient hyperpolarization followed by a small, sustained hyperpolarization. When 5-HT (10 μM) was repeatedly applied at 25 min intervals the transient response gradually declined but the sustained response did not (within three trials) (Figure 1A). Thereafter, the responses to 5-HT were

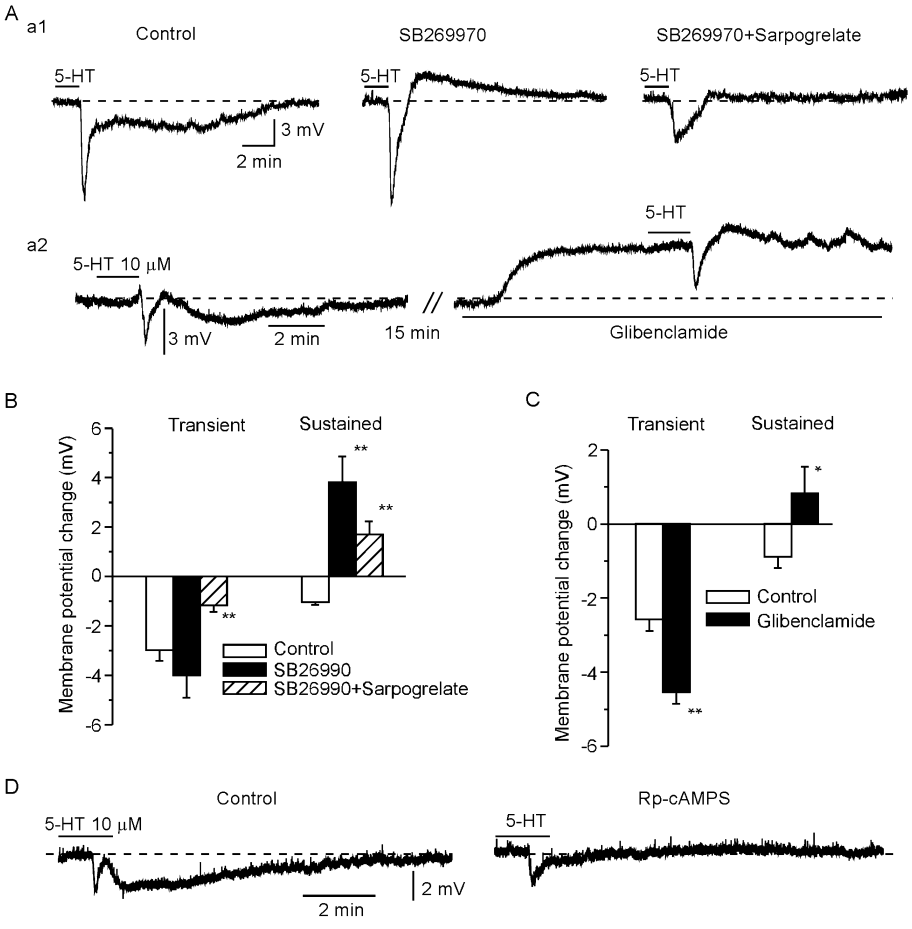

Figure 4

Effects of SB269970, glibenclamide and Rp-cAMPS on 5-HT-induced changes in smooth muscle cell membrane potential in endothelium-intact preparations. (Aa1) After recording the control response, 5-HT (10 μM) was applied in the presence of SB269970 (0.3 μM) and then in the presence of SB269970 together with sarpogrelate (3 μM). (Aa2) After recording the control response, 5-HT (10 μM) was applied in the presence of glibencamide (10 μM). (B) Summary of the effects of SB269970 without and with sarpogrelate ($n = 5$). (C) Summary of the effects of glibenclamide ($n = 4$). Data are shown as mean ± SEM. *$P < 0.05$ **$P < 0.01$ versus 'Control'. (D) Effects of Rp-cAMPS on 5-HT-induced hyperpolarization. Rp-cAMPS (0.1 mM) was pretreated for 30 min and was present thereafter. Similar effects were obtained in two other preparations. Each record in a given set of two or three was obtained from the same impalement.

repeatable. After the 3rd application of 10 μM 5-HT, each concentration of 5-HT (10^{-7}–10^{-5} M) was intermittently applied for 1.5 min at 25 min intervals to observe the concentration-dependent effects of 5-HT on the smooth muscle cell membrane potential (Figure 1B).

Charybdotoxin [an inhibitor of large-conductance calcium-activated K^+ channels (BK_{Ca}) and intermediate-conductance calcium-activated ones (IK_{Ca}), 0.1 μM] depolarized the membrane (3.7 ± 0.7 mV, $n = 4$; $P < 0.01$; Figure 1C) and inhibited the 5-HT-induced transient hyperpolarization (Figure 1D).

The 5-HT_{2A} receptor antagonist sarpogrelate (0.1 and 1 μM) did not modify the resting membrane potential of the smooth muscle cells (–0.1 ± 0.2 mV for 0.1 μM and –0.2 ± 0.3 mV for 1 μM; $n = 5$; $P > 0.1$ in each case). Sarpogrelate (1 μM) significantly inhibited the 5-HT (10 μM)-induced transient, but not sustained, hyperpolarization (Figure 2). Neither MDL 11939 (a selective 5-HT_{2A} receptor antagonist, 1 μM) nor SB200646 (a 5-$HT_{2B/2C}$ receptor antagonist, 1 μM) modified the resting membrane potential ($n = 4$; $P > 0.1$ in each case). MDL 11939 inhibited the 5-HT (10 μM)-induced transient, but not sustained, hyperpolarization, whereas SB200646 did not modify either phase of the 5-HT-induced hyperpolarization (Figure 2B).

The selective 5-HT_{2A} receptor agonist TCB-2 (10 μM) induced a transient hyperpolarization, then a small sustained depolarization (Figure 3Aa1). Sarpogrelate (1 μM) attenuated both phases of the TCB-2-induced response (Figure 3Aa1 and C). The selective 5-HT_7 receptor agonist AS 19 (10 μM) induced a sustained hyperpolarization. The 5-HT_7 receptor antagonist SB269970 (0.3 μM) did not modify the resting smooth muscle cell membrane potential ($n = 4$; $P > 0.1$) but it inhibited the hyperpolarization induced by AS 19 (Figure 3Aa2).

In the presence of SB269970, 5-HT (10 μM) induced a transient hyperpolarization followed by a depolarization. This depolarization was inhibited by the 5-HT_{2A} receptor blocker sarpogrelate (Figure 4Aa1 and B). The K_{ATP} channel inhibitor glibenclamide (10 μM) depolarized the smooth muscle cell membrane (4.6 ± 0.6 mV, $n = 4$, $P < 0.05$). This enhanced the 5-HT-induced transient hyperpolarization but blocked the sustained hyperpolarization (Figure 4C). The PKA inhibitor Rp-cAMPS (0.1 mM) did not modify the resting membrane potential ($n = 3$, $P > 0.1$). However, it appeared to inhibit the 5-HT-induced sustained (but not transient) hyperpolarization (Figure 4D).

The resting membrane potential in smooth muscle cells in endothelium-denuded preparations (–49.9 ± 1.9 mV, $n = 3$) was not significantly different from that in endothelium-intact preparations ($P > 0.5$). Although ACh (3 μM) induced a hyperpolarization in endothelium-intact preparations (23.4 ± 3.1, $n = 3$), this response was almost non-existent in endothelium-denuded preparations (2.9 ± 0.2, $n = 3$; $P < 0.01$). In endothelium-denuded preparations, small membrane-potential oscillations were occasionally observed in irregular patterns, and whether or not such oscillations were present 5-HT (10 μM) induced only a sustained (i.e. no transient) hyperpolarization. Under these conditions, SB269970 (0.3 μM) did not modify the resting membrane potential ($n = 3$; $P > 0.1$); yet, 5-HT induced a depolarization in the presence of SB269970. The depolarization observed in the presence of SB269970 was attenuated by sarpogrelate ($n = 3$; $P < 0.01$) (Figure 5).

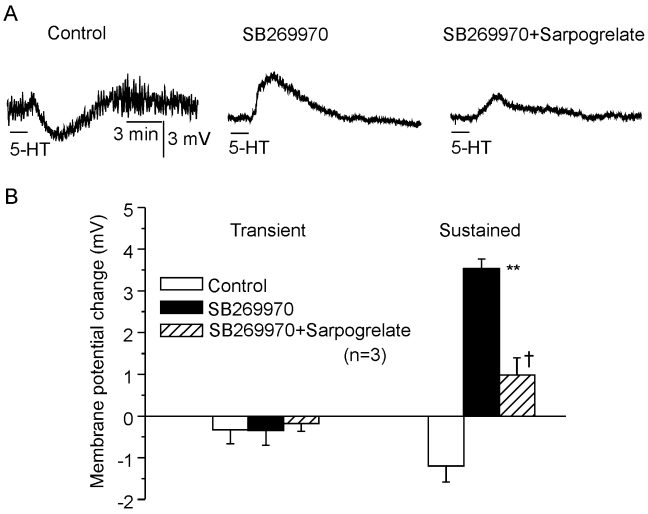

Figure 5

Effects of SB269970 with or without sarpogrelate on 5-HT-induced changes in smooth muscle cell membrane potential in endothelium-denuded preparations. (A) After recording the control 5-HT (10 μM) response, the preparation was pretreated with SB269970 (0.3 μM) for 5 min and 5-HT was applied in the presence of SB269970. After a 20 min interval, the preparation was pretreated with sarpogrelate (3 μM) together with SB269970 for 5 min, then, 5-HT was applied in the presence of these antagonists. Each record in the set of three was obtained from the same impalement. (B) Summary of the effects ($n = 3$). Data are shown as mean ± SEM. **$P < 0.01$ versus 'Control'. †$P < 0.05$ versus 'SB269970'.

Effects of 5-HT on mechanical activities

Under basal conditions, 5-HT (10 μM) did not induce contraction or relaxation in endothelium-intact strips. $PGF_{2\alpha}$ (3 μM) induced a maintained contraction in endothelium-intact strips and 5-HT (10^{-8}–10^{-5} M) induced a concentration-dependent reversible relaxation of this contraction (Figure 6). As L-NNA enhanced the $PGF_{2\alpha}$ contraction in endothelium-intact strips, the concentration of $PGF_{2\alpha}$ was lowered to 1 μM in the presence of L-NNA so as to obtain matched amplitudes of contraction (tension = 0.88 ± 0.11 mN with 1 μM $PGF_{2\alpha}$ in the presence of L-NNA versus 0.75 ± 0.27 mN with 3 μM $PGF_{2\alpha}$ in the absence of L-NNA; each, $n = 5$; $P > 0.5$). In endothelium-intact strips treated with L-NNA, 5-HT (10^{-8}–10^{-5} M) induced a relaxation similar to that seen in L-NNA-untreated strips (Figure 6, $n = 5$; $P > 0.1$).

In endothelium-denuded strips, 5-HT (10 μM) did not modify the basal tension but induced a relaxation of the contraction induced by 1 μM $PGF_{2\alpha}$. The 5-HT (10^{-8}–10^{-5} M)-induced relaxation of the $PGF_{2\alpha}$ contraction was not modified by sarpogrelate (1 μM, $n = 5$; $P = 0.991$), by MDL 11939 (1 μM, $n = 5$; $P = 0.10$ by two-way repeated-measures ANOVA) or by SB200646 (1 μM, $n = 7$; $P = 0.42$) (Figure 7). However, SB269970 (0.3 μM) blocked the 5-HT-induced relaxation (Figure 8A and B). Actually, in the presence of SB269970 the $PGF_{2\alpha}$ contraction was enhanced by 5-HT ($n = 5$; $P < 0.001$ vs.

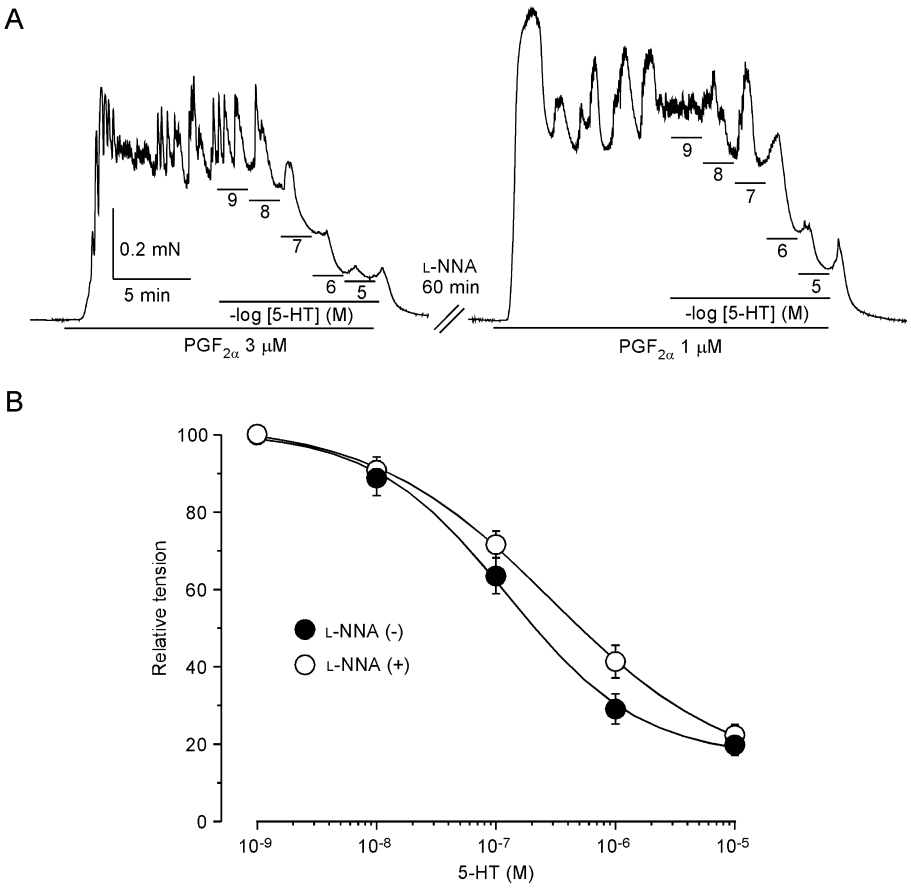

Figure 6

Effects of L-NNA on 5-HT-induced relaxation in endothelium-intact strips. (A) Actual tracings of the effects of 5-HT. 5-HT (10^{-9}–10^{-5} M) was cumulatively applied during the contraction induced by $PGF_{2\alpha}$. L-NNA (0.1 mM) was given as a pretreatment for 60 min and was present thereafter. The concentrations of $PGF_{2\alpha}$ used were 3 μM and 1 μM before and after application of L-NNA, respectively, so as to obtain matched amplitudes of contraction. (B) Summary of the effects of 5-HT on $PGF_{2\alpha}$ contraction in the presence and absence of L-NNA ($n = 5$; $P > 0.1$). Data are shown as mean ± SEM.

control). This 5-HT-induced increase in the $PGF_{2\alpha}$ contraction in the presence of SB269970 was inhibited by sarpogrelate ($n = 5$, $P < 0.05$ vs. in the presence of SB269970 without sarpogrelate) (Figure 8). The tension induced by 1 μM $PGF_{2\alpha}$ was 0.56 ± 0.12 mN in control, 0.54 ± 0.08 mN in the presence of SB269970 and 0.51 ± 0.07 mN in the presence of SB269970+sarpogrelate ($n = 5$, $P > 0.05$ for each comparison).

Localized immunoreactive responses against the 5-HT_7 and 5-HT_{2A} receptor subtypes were identified in the vascular wall (Figure 8D).

5-HT plus SB269970 and the selective 5-HT_{2A} receptor agonist TCB-2 each induced a concentration-dependent small contraction whether applied in the presence or absence of $PGF_{2\alpha}$ ($n = 7$, Figure 9A and B). In the presence of $PGF_{2\alpha}$ and also in its absence, the amplitude of contraction induced by TCB-2 at any given concentration was similar to that induced by 5-HT plus SB269970 ($n = 6$; in each case, $P > 0.1$ by two-way repeated-measures ANOVA). Sarpogrelate (1 μM) significantly attenuated the contraction induced by either agonist (Figure 9A and B).

The selective 5-HT_7 receptor agonist AS 19 (10 μM) attenuated the TCB-2-induced contraction, and this effect of AS 19 was blocked by SB269970 ($n = 4$, Figure 9C and D). The PKA inhibitor Rp-cAMPS (0.1 mM) significantly inhibited the AS 19-induced relaxation during the TCB-2 contraction ($n = 7$; $P < 0.01$; Figure 9E).

Effects of glibenclamide and modulators of the action of cAMP on 5-HT-induced relaxation

Glibenclamide (10 μM) greatly inhibited the contraction induced by 1 μM $PGF_{2\alpha}$ in endothelium-denuded strips (data not shown). It has been suggested that in some arterial preparations, glibenclamide inhibits the mechanical responses to prostaglandins through its action on PG receptors (Cocks *et al.*, 1990; Zhang *et al.*, 1991). Therefore, we studied the effects of glibenclamide on the 5-HT-induced relaxation during the contraction induced by phenylephrine (Figure 10). The amplitude of contraction induced by 3 μM phenylephrine was not significantly different from that induced by 1 μM $PGF_{2\alpha}$ in a given strip ($n = 6$; $P > 0.05$). Glibenclamide (10 μM) tended, non-significantly, to attenuate the phenylephrine-induced contraction ($n = 6$; $P > 0.05$;

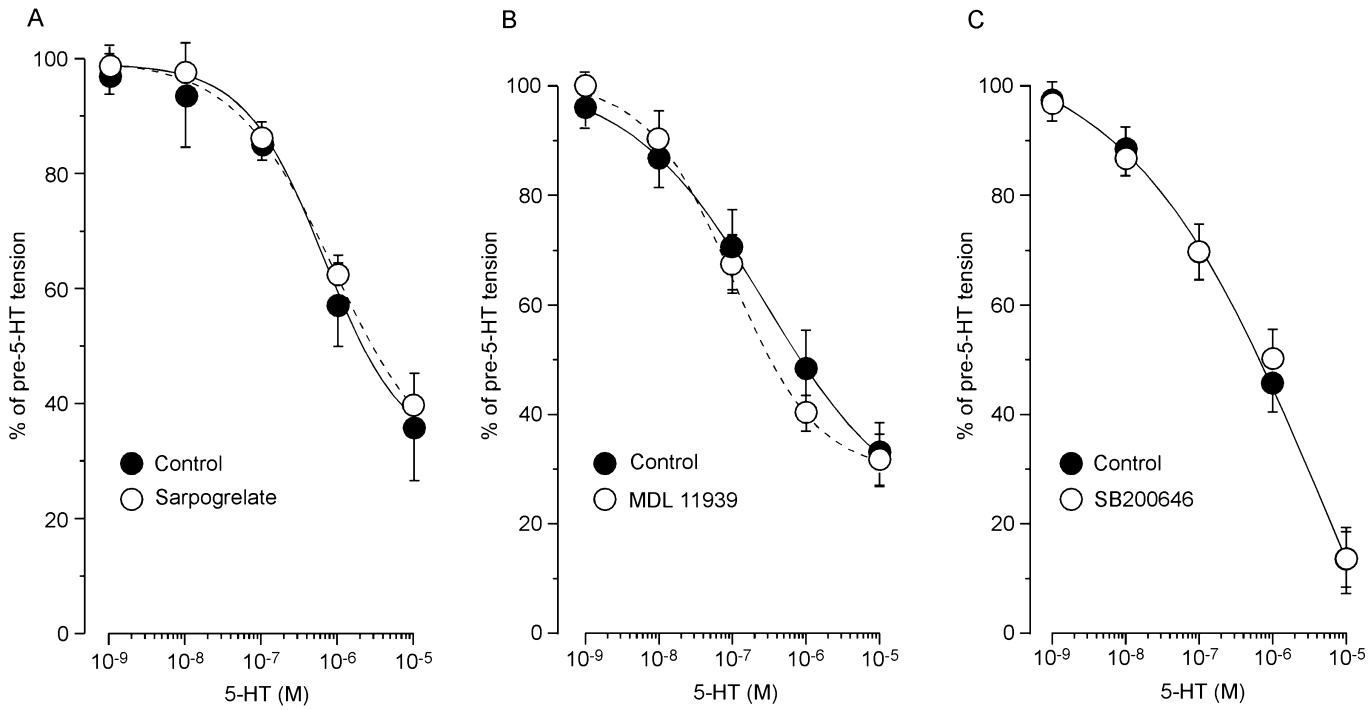

Figure 7

Effects of sarpogrelate, MDL 11939 and SB200646 on 5-HT-induced relaxation in endothelium-denuded strips. Effects of sarpogrelate (1 μM, left panel), MDL 11939 (1 μM, middle panel) and SB20646 (1 μM, right panel) on 5-HT-induced relaxation of the contraction induced by 1 μM $PGF_{2\alpha}$. 5-HT was cumulatively applied during the $PGF_{2\alpha}$ contraction in the presence and absence of each antagonist. Data are shown as mean ± SEM.

Figure 10A) but it did not modify the 5-HT-induced relaxation on that contraction ($n = 6$; $P = 0.84$; Figure 10B).

The PKA inhibitor Rp-cAMPS (0.1 mM) did not modify the contraction induced by 1 μM $PGF_{2\alpha}$ ($n = 4$; $P > 0.1$) but it significantly attenuated the 5-HT-induced relaxation of the $PGF_{2\alpha}$ contraction in endothelium-denuded strips (Figure 11A, $n = 6$; $P < 0.01$). Similarly, the AC inhibitor SQ22536 (0.3 mM) did not modify the contraction induced by 1 μM $PGF_{2\alpha}$ ($n = 4$; $P > 0.5$) but it significantly attenuated the relaxation induced by 10^{-8}–10^{-5} M 5-HT (Figure 11B, $n = 4$; $P < 0.01$).

Discussion and conclusions

In this study, we found that 5-HT induced a two-phase smooth muscle cell hyperpolarization in the endothelium-intact rabbit jugular vein: a transient hyperpolarization followed by a small, sustained hyperpolarization. The former response, but not the latter, required the presence of the endothelium. 5HT induced an endothelium-independent relaxation during the $PGF_{2\alpha}$ contraction, and this relaxation was blocked by the selective $5\text{-}HT_7$ receptor antagonist SB269970. In the presence of SB269970, 5-HT induced a contraction that was blocked by the $5\text{-}HT_{2A}$ receptor antagonist sarpogrelate. These results indicate that in the rabbit jugular vein, although the net response to 5-HT is relaxation, this agent activates both smooth muscle $5\text{-}HT_7$ receptors (relaxation) and smooth muscle $5\text{-}HT_{2A}$ receptors (contraction).

5-HT-induced electrical activities

In line with previous findings in the porcine coronary artery (Nagao *et al.*, 1995), 5-HT induced a transient smooth muscle cell hyperpolarization in endothelium-intact (but not endothelium-denuded) preparations of rabbit jugular vein. This transient hyperpolarization gradually declined in amplitude when 5-HT was repeatedly applied (tachyphylaxis), whereas the 5-HT-induced sustained hyperpolarization did not. The latter hyperpolarization, and the 5-HT-induced relaxation, were reversible responses. The 5-HT-induced transient hyperpolarization was blocked by charybdotoxin (a blocker of IK_{Ca} and BK_{Ca} channels). Moreover, it was attenuated by the $5\text{-}HT_{2A}$ receptor antagonists sarpogrelate and MDL 11939 but not by the $5\text{-}HT_{2B/2C}$ receptor antagonist SB200646 or by the $5\text{-}HT_7$ receptor antagonist SB269970. Furthermore, a faint response indicative of $5\text{-}HT_{2A}$ receptors was immunohistochemically identified in the endothelial layer of the vein. These results suggest that 5-HT acts on endothelial $5\text{-}HT_{2A}$ receptors to induce a transient smooth muscle cell hyperpolarization via activation of endothelial IK_{Ca} channels through an increase in the cellular concentration of Ca^{2+}. At present, however, we do not know the roles played by this response in the 5-HT-induced relaxation of the $PGF_{2\alpha}$ contraction as the relaxation was similar in the presence and absence of endothelium.

After the transient hyperpolarization response to 5-HT, there was a small sustained smooth muscle cell hyperpolarization in endothelium-intact preparations as well as in endothelium-denuded ones. The $5\text{-}HT_7$ receptor antagonist

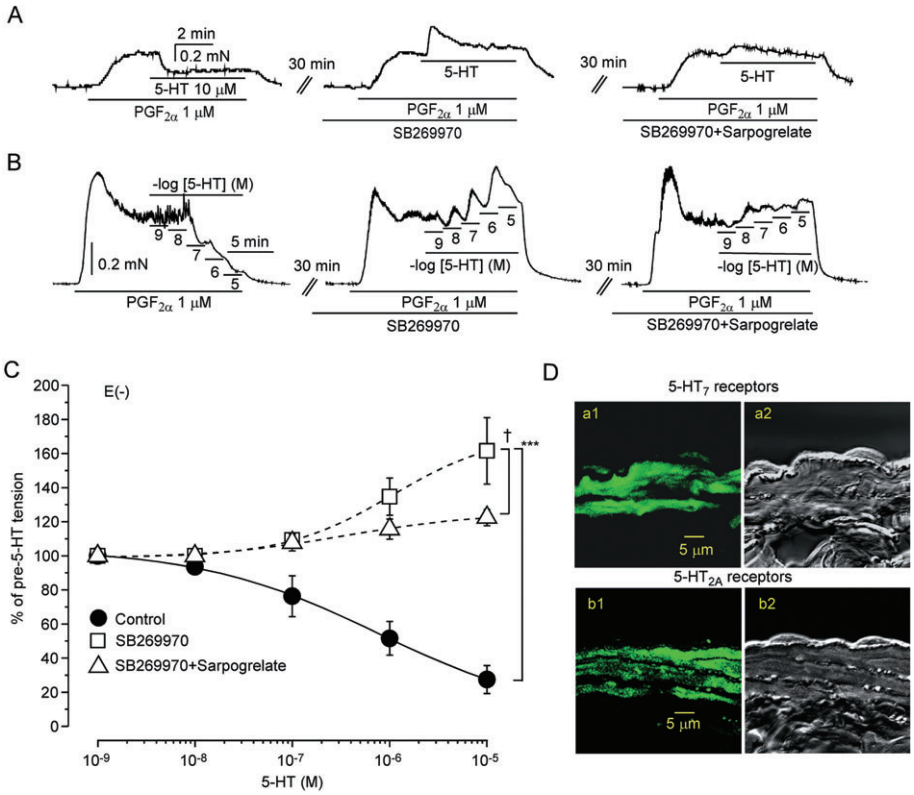

Figure 8

Effects of SB269970 with or without sarpogrelate on 5-HT-induced relaxation in endothelium-denuded strips. (A) 5-HT (1 μM) was applied for 5 min during the contraction induced by 1 μM $PGF_{2\alpha}$ in the absence (left record) and presence of 0.3 μM SB2699701 without (middle record) or with 1 μM sarpogrelate (right record). (B) Effects of cumulatively applied 5-HT on the $PGF_{2\alpha}$-induced contraction in the absence and presence of SB269970 (0.3 μM) with or without sarpogrelate (3 μM). After the control 5-HT response ('Control') had been recorded and a subsequent 30 min washout, the preparation was pretreated with SB269970 for 5 min and then 5-HT was applied during the $PGF_{2\alpha}$ contraction in the presence of SB269970 ('SB269970'). This was followed by a 30 min washout. SB269970 with sarpogrelate was then applied as a pretreatment for 5 min and the effect of 5-HT was examined in the presence of these antagonists ('SB269970+Sarpogrelate'). (C) Summary of the results of cumulatively applied 5-HT ($n = 5$). For each condition, tension is expressed as a percentage of the value obtained just before application of the first concentration of 5-HT. Data are shown as mean ± SEM. ***$P < 0.001$ versus 'Control'. †$P < 0.05$ versus 'SB269979'. (D) Fluorescence images showing localization of antibodies against either the 5-HT_7 (a1) or 5-HT_{2A} (b1) receptor in cross-sections of jugular vein, together with the corresponding bright-field images (a2 and b2). Similar observations were made in three other preparations.

SB269970 blocked this hyperpolarization and the selective 5-HT_7 receptor agonist AS 19 induced a smooth muscle cell hyperpolarization. Furthermore, neither a 5-HT_{2A} receptor antagonist (sarpogrelate or MDL 11939) nor a $5\text{-HT}_{2B/2C}$ receptor antagonist (SB200646) modified the 5-HT-induced sustained hyperpolarization. Moreover, our immunohistochemical analysis revealed positive responses against 5-HT_7 receptors within the smooth muscle layer of the rabbit jugular vein. These results suggest that smooth muscle 5-HT_7 receptors may be responsible for the 5-HT-induced sustained hyperpolarization. Interestingly, we found that 5-HT induced a depolarization via activation of 5-HT_{2A} receptors when the 5-HT_7 receptor was being blocked. In addition, we found that the selective 5-HT_{2A} agonist TCB-2 induced a transient hyperpolarization followed by a sustained small depolarization, both phases being blocked by the 5-HT_{2A} receptor antagonist sarpogrelate. These results suggest that in rabbit jugular vein, although activation of smooth muscle 5-HT_{2A} receptors induces a membrane depolarization, this is masked by a 5-HT_7-mediated hyperpolarization when these two receptor-subtypes are activated simultaneously.

Stimulation of the 5-HT_7 receptor activates AC coupled to Gs protein, and thereby increases the concentration of cAMP within the smooth muscle cells of various arteries and veins (Martin and Wilson, 1995; Cushing *et al.*, 1996; Leung *et al.*, 1996; Morecroft and MacLean, 1998; Terrón and Falcón-Neri, 1999). In many arteries, it has been found that an increase in the cellular concentration of cAMP leads to an activation of K_{ATP} channels in the smooth muscle cells, thereby causing a hyperpolarization (Kuriyama *et al.*, 1998; Purves *et al.*, 2009). We found that both the PKA inhibitor Rp-cAMPS and the K_{ATP}-channel blocker glibenclamide inhibited the sustained, but not the transient, hyperpolarization induced by 5-HT. We also found that Rp-cAMPS inhibited the hyperpolarization induced by the selective 5-HT_7 receptor agonist AS 19. These results indicate that in the rabbit jugular vein, 5-HT induces a sustained hyperpolarization in part through activation of the 5-HT_7 receptor/cAMP/K_{ATP}-channel pathway.

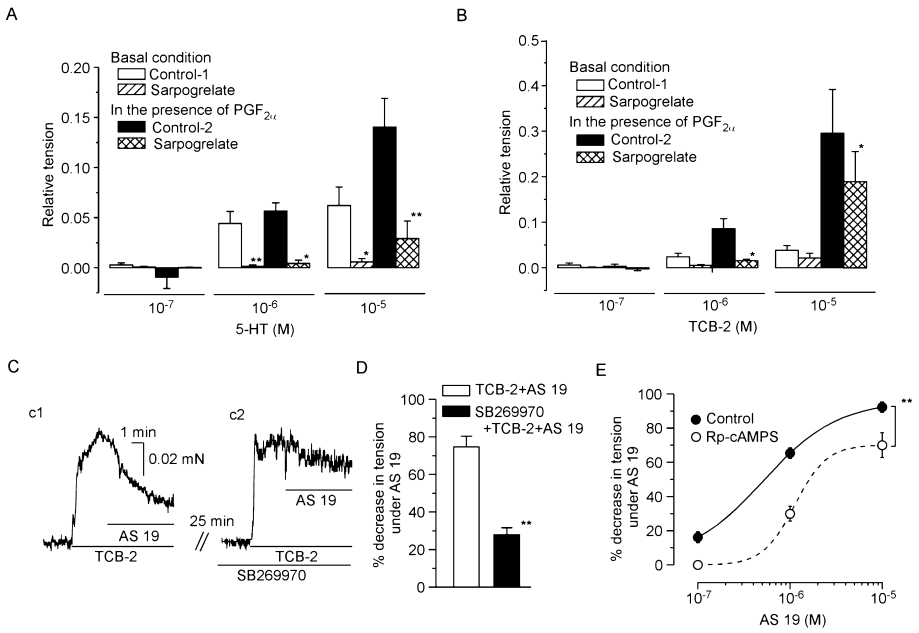

Figure 9

Mechanical responses induced by TCB-2 alone and 5-HT plus SB269970, each in the presence and absence of $PGF_{2\alpha}$, together with the effects of SB269970 and Rp-cAMPS on AS 19-induced relaxation, all in endothelium-denuded strips. (A) In the presence of SB269970, 5-HT (10^{-7}–10^{-5} M) was cumulatively applied without ('Control') or with sarpogrelate (1 μM). This was carried out in the absence ('Basal') and in the presence of 1 μM $PGF_{2\alpha}$. (B) Likewise, TCB-2 (10^{-7}–10^{-5} M) was cumulatively applied, without or with sarpogrelate (1 μM), in the absence and in the presence of 1 μM $PGF_{2\alpha}$. Data are shown as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ versus the relevant 'Control' (by paired *t*-test). The maximum tension induced by 128 mM K^+ in each preparation was normalized as 1.0. (C) Effects of AS 19 (10 μM) on the contraction induced by TCB-2 (10 μM) in the absence and presence of SB269970. (D) Summary of this blocking effect of SB269970 on the relaxation induced by AS 19. Ordinate shows the percentage decrease in the 'TCP-2-pre-contracted' tension that occurred under AS 19. $^{**}P < 0.01$ versus 'TCB-2+AS 19' (by paired *t*-test). (E) Effects of Rp-cAMPS (0.1 mM) on the relaxation induced by AS 19 in the presence of 10 μM TCB-2. $^{**}P < 0.01$ versus 'Control' (by two-way repeated-measures ANOVA).

5-HT-induced mechanical responses

In the rabbit jugular vein, 5-HT at concentrations over 10^{-8} M induced a concentration-dependent relaxation of the $PGF_{2\alpha}$ contraction. The amplitude of the relaxation induced by 5-HT was similar between endothelium-denuded strips and endothelium-intact ones, and the relaxation in the endothelium-intact strips was not modified by the NO-synthase inhibitor L-NNA, indicating that neither endothelium-dependent hyperpolarization nor endothelial NO release play a significant role in the 5-HT-induced relaxation. In addition, we found that the 5-HT-induced relaxation was not modified by the 5-HT_{2A} receptor antagonists sarpogrelate or MDL 11939 or by the $5\text{-HT}_{2B/2C}$ receptor antagonist SB200646. However, the 5-HT_7 receptor antagonist SB269970 changed this relaxation to a contraction (i.e. 5-HT actually enhanced the $PGF_{2\alpha}$ contraction in the presence of SB269970). Moreover, we found that the AC inhibitor SQ22536 and the PKA inhibitor Rp-cAMPS both attenuated the 5-HT-induced relaxation of the $PGF_{2\alpha}$ contraction in endothelium-denuded strips. Taken together, these results suggest that in the rabbit jugular vein, 5-HT induces endothelium-independent relaxation during the $PGF_{2\alpha}$ contraction through activation of the smooth muscle 5-HT_7 receptor/AC/cAMP-PKA signal cascade.

We found that (i) the level of the sustained hyperpolarization induced by 5-HT (1 and 10 μM) was minimal (less than 2 mV); yet, 5-HT (10^{-8}–10^{-5} M) induced a concentration-dependent relaxation; (ii) Rp-cAMPS inhibited both the sustained hyperpolarization and relaxation induced by 5-HT; and (iii) glibenclamide blocked the 5-HT-induced sustained hyperpolarization but did not modify the 5-HT-induced relaxation. These results indicate that in the rabbit jugular vein, The 5-HT_7 receptor-mediated, cAMP and glibenclamide-sensitive hyperpolarization does not underlie the 5-HT-induced relaxation.

In the rabbit jugular vein, 5-HT did not increase basal tension but it induced a smooth muscle contraction when the 5-HT_7 receptor was blocked. The selective 5-HT_{2A} agonist TCB-2 by itself induced a smooth muscle contraction under basal conditions. These contractions were inhibited by the 5-HT_{2A} receptor antagonist sarpogrelate. Furthermore, the TCB-2-induced contraction was attenuated by the selective 5-HT_7 receptor agonist AS 19, and this effect of AS 19 was inhibited by SB269970 and also by Rp-cAMPS. Thus, our data indicate that in rabbit jugular vein, although 5-HT is able to activate both smooth muscle 5-HT_7 receptors (to induce relaxation) and smooth muscle 5-HT_{2A} receptors (to induce contraction), the net effect is that 5-HT induces a relaxation.

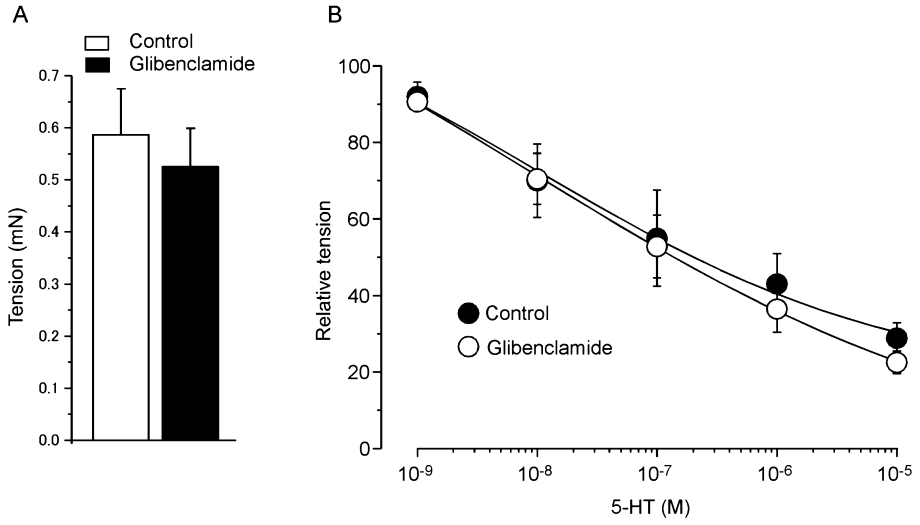

Figure 10

Effects of glibenclamide on 5-HT-induced relaxation of the contraction induced by phenylephrine in endothelium-denuded strips. (A) Effects of glibenclamide (10 μM) on the contraction induced by phenylephrine (3 μM). Glibenclamide tended, non-significantly, to inhibit this contraction. (B) Summary of the effect of glibenclamide on 5-HT-induced relaxation. 5-HT (10^{-9}–10^{-5} M) was cumulatively applied during the contraction induced by 3 μM phenylephrine. After a 25 min interval, glibenclamide (10 μM) was applied as a pretreatment for 5 min and 5-HT was then cumulatively applied during the phenylephrine contraction. The 'precontracted' tension (i.e. the tension just before the first application of 5-HT) was normalized to a relative tension of 100. Data are shown as mean ± SEM.

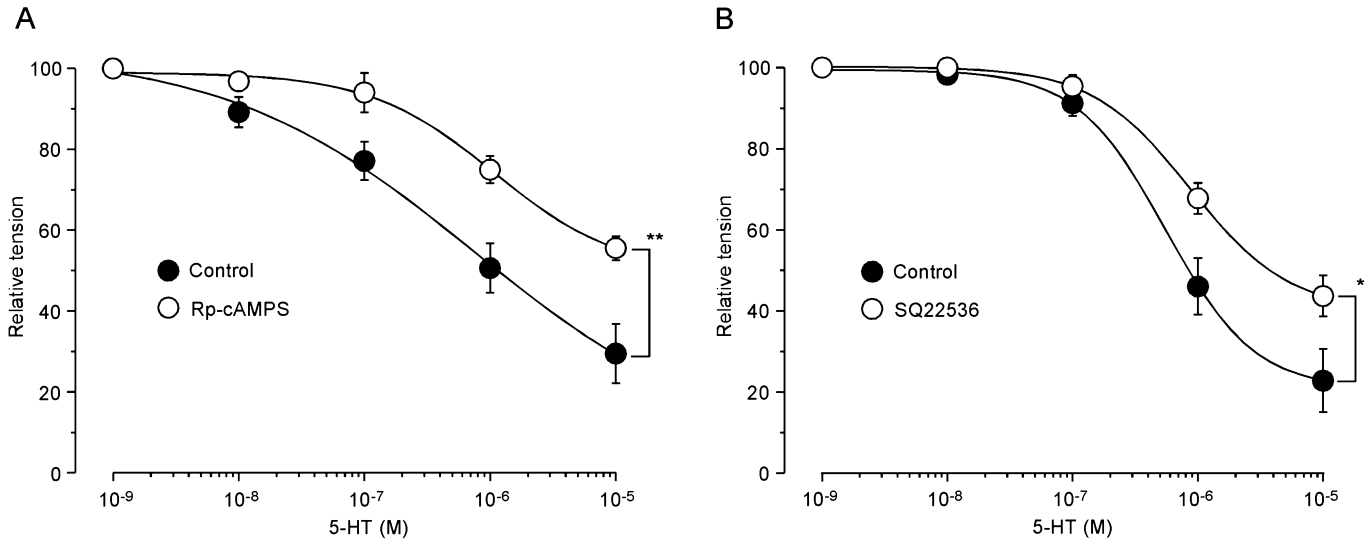

Figure 11

Effects of Rp-cAMPS and SQ22536 on 5-HT-induced relaxation in endothelium-denuded strips. 5-HT (10^{-9}–10^{-5} M) was cumulatively applied during the contraction induced by 1 μM $PGF_{2\alpha}$ in the absence and presence of either 0.1 mM Rp-cAMPS (A) or 0.3 mM SQ22536 (B). After the control 5-HT response had been recorded, the preparation was pretreated with Rp-cAMPS or SQ22536 for 30 min, which were present thereafter. The 'precontracted' tension (i.e. the tension just before the first application of 5-HT) was normalized to a relative tension of 100. Data are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus 'Control' (by two-way repeated-measures ANOVA).

We suggest that under physiological conditions, the smooth muscle 5-HT_{2A} receptor-mediated depolarization and contraction may be masked by the 5-HT_7 receptor-mediated hyperpolarization and relaxation, possibly through actions mediated by cAMP in this particular vein.

Acknowledgements

We thank Dr R. J. Timms for a critical reading of the manuscript. We also thank T Kanie and Y Ohta, medical students at Nagoya City University, for their technical assistance with the

present experiments. This work was partly supported by a Grant-In-Aid for Scientific Research from the Japan Society for the Promotion of Science.

Conflict of interest

None.

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