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Effect of 5-hydroxytryptamine on the membrane potential of endothelial and smooth muscle cells in the pig coronary artery

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1 Many endothelium-dependent vasodilators hyperpolarize the endothelial cells in blood vessels. It is not known whether these hyperpolarizations are linked to nitric oxide synthesis or to an endothelium-derived hyperpolarizing phenomenon, since most of the vasodilators release both factors. In this context, we first verified that the endothelium-dependent relaxations induced by 5-hydroxytryptamine (5-HT) on pig coronary arteries are due only to the activation of the nitric oxide pathway. Then we studied the effects of 5-HT on membrane potential of endothelial and smooth muscle cells.

2 In the absence of endothelium, 5-HT caused a concentration-dependent contraction of coronary artery strips. No change of the smooth muscle cell membrane potential was observed during contraction to 1 μM 5-HT.

3 In the presence of 1 μM ketanserin to suppress the contractile effect of 5-HT, 5-HT induced concentration-dependent relaxation of endothelium-intact strips precontracted by 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). These relaxations were suppressed by 1 μM N^G-nitro-L-arginine, an inhibitor of nitric oxide synthesis, showing that they were produced predominantly by nitric oxide.

4 In the presence of 1 μM ketanserin, 1 μM 5-HT did not change the smooth muscle cell membrane potential of strips precontracted by either 10 μM $PGF_{2\alpha}$ or by 10 μM acetylcholine (ACh). In the same conditions, 1 μM 5-HT caused a weak 2.6 ± 0.4 mV hyperpolarization, of the endothelial cells.

5 In conclusion, the fact that 5-HT did not change the membrane potential of smooth muscle cells and only weakly hyperpolarized the endothelial cells during relaxations, suggests that in both cell types no electrical events accompany activation of the nitric oxide pathway. This is in contrast to the hyperpolarizations observed in endothelial and smooth muscle cells when the endothelium-derived hyperpolarization factor (EDHF) pathway is activated.

Keywords: Electrophysiology; endothelial cells; 5-HT; nitric oxide; pig coronary artery; membrane potential

Introduction

Many endothelium-dependent vasodilators relax vascular smooth muscles by inducing the release of endothelial agents such as nitric oxide, prostacyclin and the putative endothelium-dependent hyperpolarizing factor (EDHF) (Rubanyi & Vanhoutte, 1990). These agents are produced in different proportions by the endothelial cells, depending on the blood vessel and the vasodilator inducing their release.

In pig coronary arteries, the two kinins substance P (SP) and bradykinin (BK), relax the smooth muscle in an endothelium-dependent manner by releasing nitric oxide from the endothelium, and by triggering the phenomenon known as EDHF (Pacicca *et al.*, 1992). During these endothelium-dependent relaxations caused by kinins, the membrane potential of endothelial and of underlying smooth muscle cells simultaneously hyperpolarizes in the same manner, even when the synthesis of nitric oxide is inhibited (Bény *et al.*, 1986; 1987; Brunet & Bény, 1989; Bény, 1990a,b; Pacicca *et al.*, 1992). However, it is not known whether the two hyperpolarizations are cause-effect related. In addition, exogenous nitric oxide does not hyperpolarize the smooth muscle cells of pig coronary artery (Bény & Brunet, 1988), or of canine mesenteric artery (Komori *et al.*, 1988). Unlike the kinins, which stimulate nitric oxide synthesis and EDHF phenomenon, 5-hydroxytryptamine (5-HT) releases only nitric oxide from the pig coronary artery endothelium and probably no or very little EDHF (Vanhoutte, 1987; Richard *et al.*, 1990; Bruning *et al.*, 1993). Our purpose in the present study was to determine the role of membrane potential changes in endothelium and smooth muscle cells when nitric oxide, but not the EDHF pathway, is activated.

In pig coronary arteries, at least two types of 5-HT receptors are present. The 5-HT_1, characterized as 5-HT_{1D}-subtype by Schoeffter & Hoyer (1990), is located on the endothelial cells. Its activation leads to vascular relaxation (Cocks & Angus, 1983; Cohen *et al.*, 1983) via the release of nitric oxide (Richard *et al.*, 1990). The 5-HT_2 receptor is located on smooth muscle cells and produces vasoconstriction when activated by 5-HT (for review see Vanhoutte *et al.*, 1984). This effect is inhibited by the selective 5-HT_2 receptor antagonist, ketanserin (R 41468) (Van Neuten *et al.*, 1981; Leysen *et al.*, 1981).

We first verified that, with our methodology, 5-HT relaxes pig coronary arteries in an endothelium-dependent manner only via the nitric oxide pathway. Then we observed the membrane potential changes in endothelial or smooth muscle cells during contraction or relaxation of coronary artery strips induced by 5-HT.

Methods

Preparation of tissues

Anterior descending branches of pig coronary arteries were obtained at the slaughterhouse. The coronary lumen were rinsed by injection of cold, oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.7, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 24.8, $MgSO_4$ 1.2, glucose 10.1; pH 7.3-7.4). Segments of the coronary artery were cleaned out of adherent tissue, and cut into rings of about 2 mm width. These rings were cut longitudinally to give strips about 5 mm in length. In some experiments, the endothelium was removed by rubbing the luminal face of the strip with a cotton-tip.

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The lack of response to SP or BK was taken as evidence for the complete removal of the endothelium (Bény *et al.*, 1986; 1987).

Electrophysiology of endothelial and smooth muscle cells

Mechanical isometric tension and transmembrane potential of the smooth muscle or the endothelial cells were simultaneously recorded as previously described (Bény *et al.*, 1986). The strip was incubated in a 200 µl Perspex bath continuously perfused with oxygenated Krebs (3.7 ml min^{-1}) at 36°C with a peristaltic pump. Peptides and drugs were delivered to the preparations by diluting them directly in the plastic beaker containing the perfusion solution. This avoided any perturbation in the perfusion flow rate.

One extremity of the strip was pinned on a silicon rubber surface with the intimal surface facing up. The other extremity was fixed horizontally to a force transducer. Changes in tension were measured isometrically with a transducer (Grass FT03C, USA), amplified (Lectromed) and recorded on a polygraph (W&W Electronics, USA). A force of about 10 mN was first applied to the strip by pulling the transducer with a micromanipulator. The tension stabilized to about 5 mN. In experiments studying relaxation to 5-HT, 10 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or 10 µM acetylcholine (ACh) were then added throughout the experiment to the perfusion fluid to produce a reproducible state of initial tension of the strip.

The membrane potential was measured with a conventional glass microelectrode (60–80 MΩ) filled with 3 M KCl. Cells were impaled near the fixed points of the tissue in order to reduce problems due to muscle movements. The criteria for accepting a record were a stable membrane potential and a sharp rise to 0 mV when the electrode was withdrawn from the recorded cell.

The technique for determining which cell type is impaled was the following. We slowly advanced the microelectrode to the intimal face of the strip. It first penetrated an endothelial cell, and then touched the internal elastic lamina which presented a mechanical resistance, visible as an erratic change in the potential measured by the electrode. After crossing the internal elastic lamina the electrode penetrated smooth muscle cells. The efficiency of this technique was proved by microiontophoretic injection of the lucifer yellow dye through the recording electrode, followed by examination with a fluorescence microscope.

Lucifer yellow injection

By injection of lucifer yellow we verified that the experimentally selected cells were actually endothelial cells. This identification was performed in approximately 50% of the cases. The method has already been described in detail (Bény & Gribi, 1989; Bény & Connat, 1991). Briefly, the cell membrane potential was measured with a glass microelectrode with its tip filled with a lucifer yellow solution (5% in water), back-filled with 150 mM LiCl. The microiontophoretic injection of the fluorescent dye was achieved by passing a direct current of 0.35 nA through the electrode for 0.5–5 min. To identify the injected cells, the tissue was fixed with 4% paraformaldehyde. The luminal face of the strip was examined with a fluorescence microscope (Nikon diaphot; excitation wavelength, 450–490 nm). The injected endothelial cells appeared as a cluster of fluorescent, ellipsoidal cells, whereas the smooth muscle cells appeared as a bundle of fluorescent, fusiform cells (Bény & Connat, 1991).

Establishment of concentration-response curve

When only mechanical tension was measured to obtain concentration-response curves, ligatures were attached to both ends of the strips, which were mounted with a resting isometric tension of about 10 mN in a 85 µl tissue bath as previously described (Bény *et al.*, 1986; 1987). To establish concentration-response curves for 5-HT, strips, contracted by 10 µM $PGF_{2\alpha}$, were superfused with a Krebs solution containing each concentration of 5-HT in an ascending, non-cumulative manner, with sufficient time between each challenge to allow full relaxation (a 20–40 min wash-out period was performed between successive concentration). 5-HT was administered to the preparations by diluting it directly in the plastic beaker that contained the perfusion solution. Where indicated in the results section, different inhibitors were used: 10 µM indomethacin to block cyclo-oxygenase, 1 µM ketanserin to suppress the contracting effect of $5\text{-}HT_2$ receptors, and 1 µM N^G-nitro-L-arginine (L-NOARG) for inhibiting nitric oxide synthase. These inhibitors were administered to the strips in the perfusion fluid for at least 25 min before the first application of 5-HT.

Preparation of peptides and chemicals

The peptides, BK and SP, were prepared at a concentration of 1 mg ml^{-1} in 0.25% acetic acid. They were stored in 50 µl aliquots and kept frozen at −20°C until use. The $PGF_{2\alpha}$ was prepared at a concentration of 1 mg ml^{-1} in 75% ethanol. L-NOARG was prepared at a concentration of 10 mg ml^{-1} in 0.02% HCl, and indomethacin at a concentration of 2 mg ml^{-1} in >99.8% ethanol. Ketanserin was prepared at a concentration of 3.95 mg ml^{-1} in N-N dimethyl formamide (DMF) and aliquots of 200 µl were kept frozen at −20°C until use. 5-HT was prepared at a concentration of 0.4 mg ml^{-1} in water. $PGF_{2\alpha}$, peptides and inhibitors were diluted subsequently to the desired concentrations with Krebs solution.

Drugs

$PGF_{2\alpha}$, ACh, indomethacin and lucifier yellow were obtained from Sigma (St. Louis, MO, U.S.A.). SP and BK were obtained from Bachem Feinchemikalien (AG, Budendorf, Switzerland). L-NOARG was obtained from Aldrich (Steinheim, Germany). 5-HT and ketanserin were obtained from Fluka (Buchs, Switzerland).

Statistical analysis

For electrophysiological experiments, *n* corresponds to the number of impalements. The number of coronary arteries used to obtain this value of *n* is specified in the results section. For the other experiments, *n* corresponds to the number of coronary arteries, each of which originates from a different pig heart. Data were calculated as the mean ± standard error of the mean (s.e.mean). Student's test was used to compare results. A P value <0.05 was taken as significant. The effective concentrations of 5-HT that provoked 50% of maximal effect (inhibitory concentration 50; IC_{50} or excitatory concentration 50; EC_{50}) was calculated for each concentration-response curve by interpolation between two points on either side of the half maximal response and followed by reading the corresponding concentration on the logarithmic scale. The mean ± s.e.mean of these readings was calculated.

Results

Dual effects of 5-HT on coronary strip isometric tension

These experiments were performed with an intact endothelium. Strips were continuously, tonically contracted by 10 µM $PGF_{2\alpha}$ in the presence of 10 µM indomethacin to suppress prostacyclin synthesis. 5-HT relaxed strips from a concentration of 10 nM up to a maximal relaxation that occurred at 30 nM. At higher concentrations of 5-HT, the relaxation diminished. Starting at a concentration of 1 µM, 5-HT con-

tracted strips, thus producing a biphasic concentration-response curve. The contraction reached a maximum at 3 μM 5-HT. This maximum represents an additional contraction of about 45% above the tonic contraction caused by $PGF_{2\alpha}$ ($n = 4$, Figure 1).

Contractile effect of 5-HT on strips without endothelium

Strips without endothelium were concentration-dependently contracted by 5-HT. We observed this effect when 5-HT was applied to strips already contracted by 10 μM $PGF_{2\alpha}$ as well as on non-precontracted strips. The two concentration-response curves so obtained were superimposable. The contraction began in response to a concentration of about 20 nM 5-HT, and reached a maximum at about 20 μM. The excitatory concentration giving half maximal contraction was 680 ± 72 nM ($n = 4$) when strips were precontracted, and 795 ± 270 nM ($n = 4$) when not precontracted (Figure 1). In contrast, to the ACh provoked contractions, which begin with a phasic contraction followed by a weaker tonic contraction (Ito *et al.*, 1979), the 5-HT contractions are sustained as long as the agonist is present.

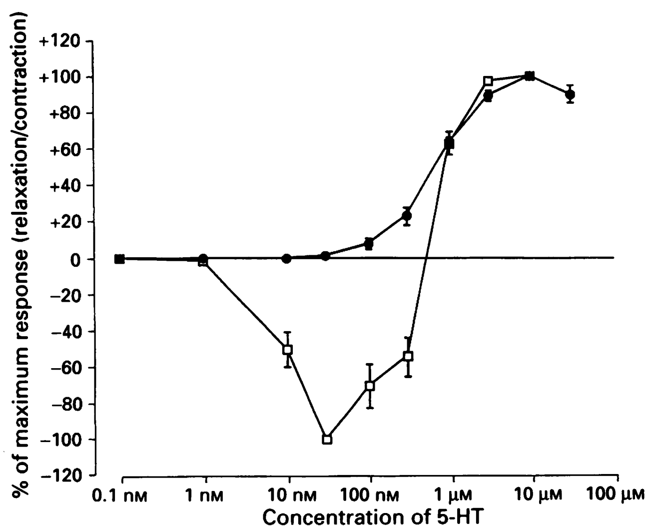

Figure 1 Concentration-response curves to 5-hydroxytryptamine (5-HT): (□) strip with an intact endothelium and tonically contracted by 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), in the presence of 10 μM indomethacin and without ketanserin ($n = 4$); (●) strip without endothelium and tonically contracted by 10 μM $PGF_{2\alpha}$ ($n = 4$). Values are mean ± s.e.mean.

Endothelium-dependent relaxant effect of 5-HT

In the presence of 10 μM indomethacin to inhibit cyclo-oxygenase activity, and 1 μM ketanserin to suppress the contractile effect mediated by $5\text{-}HT_2$ receptors, 5-HT relaxed precontracted strips (10 μM $PGF_{2\alpha}$) with an intact endothelium. The relaxation began at 3 nM 5-HT and reached a maximum at 0.3 μM (corresponding to an inhibition of about 70% of the contraction caused by $PGF_{2\alpha}$), with an IC_{50} of 21.3 ± 2.7 nM ($n = 5$, Figures 2a, b). Endothelium-dependent relaxation was suppressed by inhibition of nitric oxide synthase with 1 μM L-NOARG ($n = 6$, Figure 2b).

Effect of 5-HT on smooth muscle cell membrane potential in strips without endothelium

In the absence of endothelium and ketanserin, the smooth muscle cell membrane potential was -47 ± 2 mV ($n = 11$; 9 arteries). 5-HT (1 μM) contracted the strip without any effect on smooth muscle cell membrane potential, which remained at -46.4 ± 2.4 mV ($n = 11$; $P > 0.4$) during the onset of contraction (Figure 3). Because of the strong strip contraction, the microelectrode was generally dislodged after the contraction had reached approximately 20% of its maximal value. Consequently, membrane potential was also measured in cells

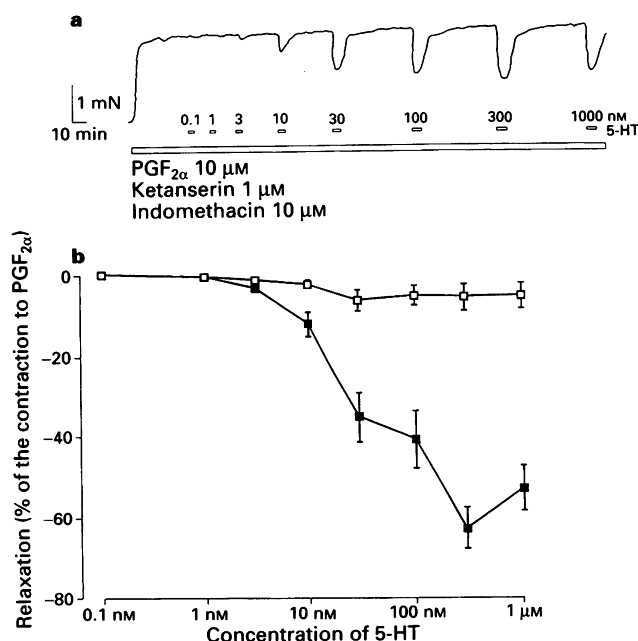

Figure 2 (a) Original recording of mechanical activity of a strip tonically contracted by 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the presence of 1 μM ketanserin and 10 μM indomethacin. Relaxations produced by graded concentrations of 5-hydroxytryptamine (5-HT) are shown. (b) Concentration-response curve for 5-HT applied to strips tonically contracted by 10 μM $PGF_{2\alpha}$ in the presence of 1 μM ketanserin and 10 μM indomethacin; with (□; $n = 6$) and without (■; $n = 5$) N^G-nitro-L-arginine (L-NOARG), an inhibitor of nitric oxide synthase. Values are mean ± s.e.mean.

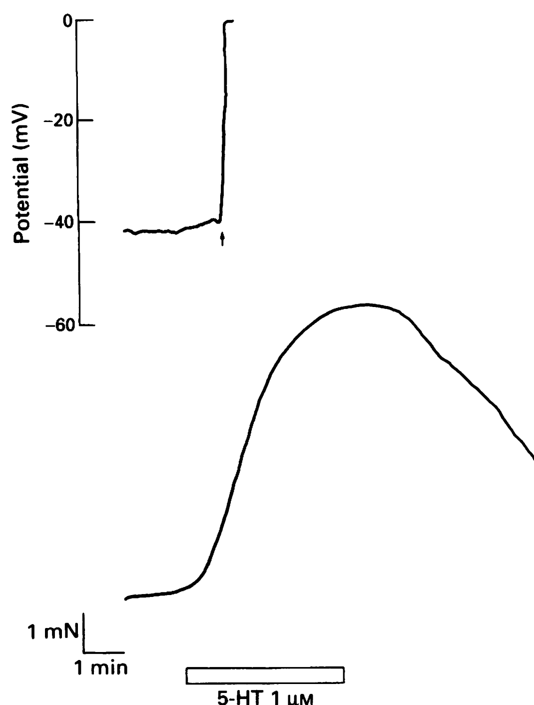

Figure 3 Simultaneous recording of smooth muscle cell membrane potential (upper trace) and isometric tension of the strip (lower trace) without endothelium. The strip was not precontracted. 5-Hydroxytryptamine (5-HT) 1 μM, did not change the membrane potential during the onset of the contraction. The arrow indicates when the microelectrode was withdrawn from the cell.

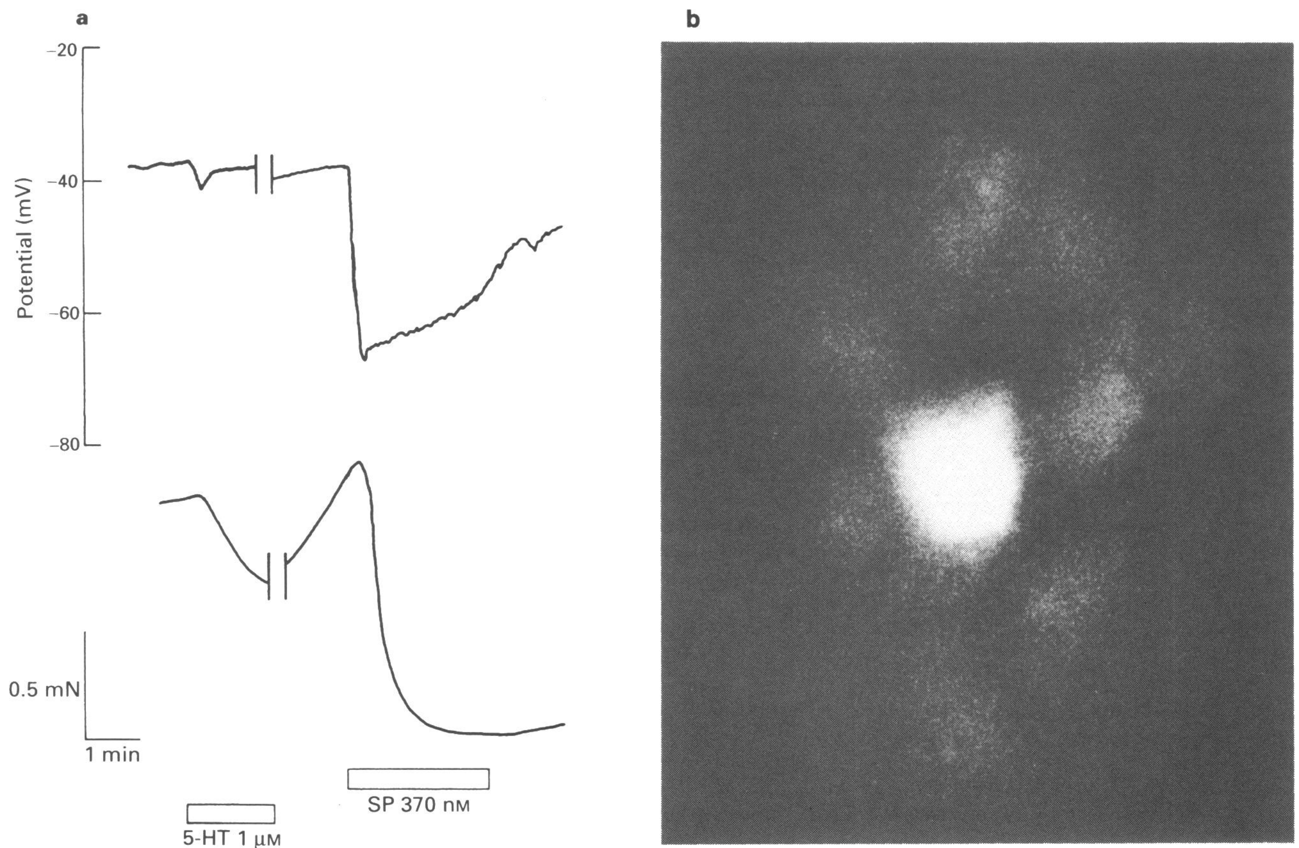

Figure 4 (a) Simultaneous recording of endothelial cell membrane potential (upper trace) and isometric tension of the strip (lower trace). The strip was tonically contracted by 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the presence of 10 μM indomethacin and 1 μM ketanserin. 5-Hydroxytryptamine (5-HT) 1 μM, had no significant effect on the membrane potential; by comparison, the effect of substance P (SP) 370 nM proved that the recorded cell and the strip were functional. For technical reasons, recording was interrupted during the lucifer yellow injection. (b) Fluorescent microscope image of the intimal face of the pig coronary artery strip. The endothelial cell in the middle of the photograph was injected with the fluorescent dye lucifer yellow during the membrane potential recording (a). The photograph shows clearly that the endothelial cells were dye coupled.

impaled after contraction reached a stable maximal value. In this condition, membrane potential was -43.9 ± 1.6 ($n = 12$; 4 arteries), which was not significantly different from the membrane potential ($P > 0.15$) measured before the application of 5-HT.

Effect of 5-HT on the membrane potential of endothelial cells

These experiments were achieved in strips contracted by 10 μM $PGF_{2\alpha}$, in the presence of 10 μM indomethacin and 1 μM ketanserin. The endothelial cell membrane potential was -42.4 ± 2 mV ($n = 10$; 7 arteries). 5-HT (1 μM) caused a weak change in the membrane potential of endothelial cells to -45 ± 2 mV, which corresponds to a hyperpolarization of 2.6 ± 0.4 mV. In comparison, SP (370 nM) hyperpolarized these cells by 28.8 ± 2.1 mV ($n = 5$, Figure 4a). The impaled cells were identified as endothelial cells by injection of lucifer yellow after application of 5-HT ($n = 4$, Figure 4b).

Endothelium-dependent effect of 5-HT on smooth muscle cell membrane potential

The membrane potential was recorded in strips with an intact endothelium, tonically contracted by 10 μM $PGF_{2\alpha}$, in the presence of 10 μM indomethacin and 1 μM ketanserin. The smooth muscle cell membrane potential was -45.2 ± 2.9 mV ($n = 9$; 8 arteries). 5-HT (1 μM) had no significant effect ($P > 0.4$) on this potential, -46.1 ± 3.2 mV ($n = 9$), (for up to 90 s following the application of 5-HT). These results were obtained by continuous recording of the membrane potential

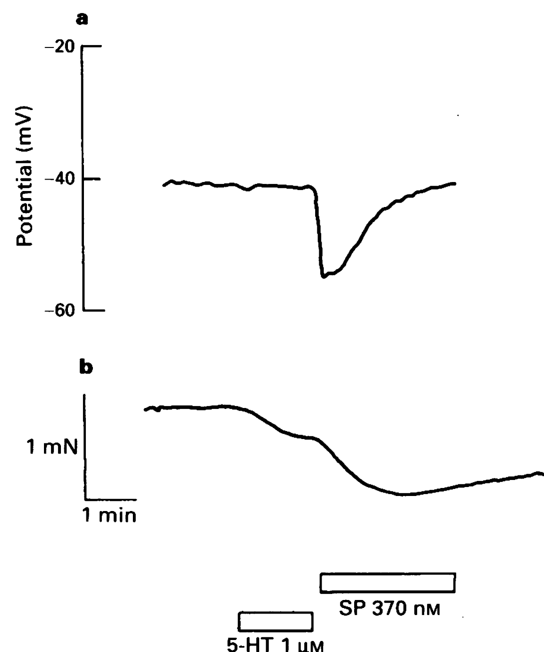

Figure 5 Simultaneous recording of smooth muscle cell membrane potential (a) and isometric tension of the strip (b). The strip was continuously contracted by acetylcholine (10 μM) in presence of 1 μM ketanserin and 10 μM indomethacin. 5-Hydroxytryptamine (5-HT) 1 μM, had no effect on the membrane potential; by comparison, the effect of substance P (SP) 370 nM proved that the recorded cell and the strip were functional.

before and during the application of 5-HT. We verified the responsiveness of these cells by applying 370 nM SP, which hyperpolarized the cells by 15.3 ± 1 mV ($n = 7$) in an endothelium-dependent manner. The relaxation caused by the application of 5-HT, recorded simultaneously with the membrane potential, proved that the strip was physiologically functional.

In our past publications, ACh was used as the contracting agent. To be able to compare the present results with those already obtained on this tissue, we also studied the effect of 5-HT on a strip continuously contracted by 10 μM ACh instead of $PGF_{2\alpha}$. The smooth muscle cell membrane potential was then -44.8 ± 1 mV ($n = 6$; 3 arteries). 5-HT (1 μM) did not change the membrane potential, which remained at -45.1 ± 0.9 mV ($n = 6$; $P > 0.4$). SP (370 nM) induced a transient hyperpolarization of 13.4 ± 1.8 mV ($n = 5$, Figure 5).

Discussion

The biphasic aspect of the concentration-response curve for 5-HT reflects the existence of two receptor types in this tissue (Cocks & Angus, 1983; Cohen *et al.*, 1983; Vanhoutte *et al.*, 1984; Houston & Vanhoutte, 1986; Angus, 1989). The biphasic curve results from the algebraic sum of the contractile effect of 5-HT (as displayed by the concentration-response curve obtained without endothelium), and the endothelium-dependent relaxant effect (in the presence of ketanserin that inhibits the $5\text{-}HT_2$ receptors). The IC_{50} (about 20 nM) and EC_{50} (about 700 nM) of dual 5-HT effects are compatible with those already determined for pig coronary arteries (Molderings *et al.*, 1989; Richard *et al.*, 1990; Schoeffter & Hoyer, 1990), and for human or bovine coronary arteries (Ratz & Flaim, 1985; Bax *et al.*, 1993).

The fact that L-NOARG suppressed the relaxation indicates that the only endothelium-dependent relaxant factor released by 5-HT is nitric oxide. The effect of L-NOARG on the endothelium-dependent relaxation induced by SP or BK is rather different, since L-NOARG inhibits relaxation by only 30% (Pacicca *et al.*, 1992).

All the smooth muscle cell membrane potentials measured were of the same order of magnitude (range -38 to -62 mV) as those already published for the pig coronary arteries (Ito *et al.*, 1979; Bény *et al.*, 1986; Bény & Pacicca, 1994). This relatively depolarized state set the membrane potential far from the K^+ equilibrium potential, and thus would favour hyperpolarization and then vasodilatation.

In the basilar artery, Garland (1987) showed that contraction of smooth muscle cells caused by 5-HT is accompanied by a synchronized depolarization, even if contraction seems mainly due to other mechanisms. In our model, we did not observe any depolarization during the onset of contraction nor during the plateau of the tonic contraction. The fact that 5-HT can contract the smooth muscle cells without changing their membrane potential is not so surprising in this tissue. These cells are silent and usually never exibit action potentials. They fire only when their potassium channels are inhibited by a blocker such as tetrabutylammonium (Von der Weid & Bény, 1993). Moreover Ito *et al.* (1979) demonstrated that ACh causes a phasic contraction followed by a smaller tonic plateau without any change in the membrane potential of the smooth muscle cells. The phasic contraction is caused by the release of sarcoplasmic reticulum into the cytosol, whereas the entry of extracellular calcium into the cells is responsible for the weak tonic phase that follows (for review see Itoh, 1991). Interestingly, the 5-HT induced contraction is not biphasic and the force developed by the muscle lasts as long as 5-HT is applied. The mechanisms responsible for these distinct contractions remain to be established.

BK causes a hyperpolarization in the endothelial cells. It has been proposed that this hyperpolarization is necessary to maintain the electrochemical gradient that ensures the entry of extracellular calcium (Schilling, 1989; Lückhoff & Busse, 1990). This imported calcium allows the calcium-calmodulin-dependent synthesis of nitric oxide. But BK not only releases nitric oxide, it also triggers the phenomenon described by the acronym EDHF. Consequently, the exact role played by the hyperpolarization is difficult to assess. Our results using a pure nitric oxide releaser do not confirm the above-mentioned theory, at least in our model, since 5-HT induces nitric oxide production without hyperpolarizing the endothelial cells. In addition, use of a high potassium solution to separate nitric oxide-induced relaxations from EDHF-induced relaxations already indirectly showed that hyperpolarization is not necessary for nitric oxide synthesis (Kilpatrick & Cocks, 1994). Indeed, in a high potassium solution, endothelial cells cannot hyperpolarize, but are nevertheless able to release nitric oxide that relaxes smooth muscle cells (Kilpatrick & Cocks, 1994). This could indicate that an endothelial cell hyperpolarization induced by an agonist is the marker for the EDHF phenomenon and not for nitric oxide synthesis. However, this remains to be confirmed by other studies involving EDHF versus nitric oxide release in distinct tissues. In conclusion, the present study constitutes first observations of the effect on membrane potential of a pure nitric oxide releaser compared to substances like BK or SP that release both nitric oxide and EDHF. The difference we observed is that 5-HT, which releases only nitric oxide, does not markedly hyperpolarize the endothelial cells, whereas releasers of EDHF do. The observation that the endothelium-dependent relaxation of the smooth muscle cell is not accompanied by a hyperpolarization confirms previous findings showing that nitric oxide released by endothelial cells (in pig coronary and canine mesenteric arteries) is not responsible for endothelium-dependent smooth muscle cells hyperpolarization observed in these tissues during relaxation caused by SP or BK (Bény & Brunet, 1988; Komori *et al.*, 1988).

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