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Mechanisms of vasorelaxation to 17β-oestradiol in rat arteries

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Abstract

We have investigated the involvement of the endothelium, K^+ channels, oestradiol receptors, and Ca^{2+} influx in 17β-oestradiol-induced vasorelaxation in rat mesenteric arterial beds and aortae. 17β-Oestradiol (10 pM–1 mM) caused acute vasorelaxations in mesenteric arterial beds and aortae from male and female rats. In male rat mesenteric vessels and aortae, the vasorelaxations were mostly independent of the endothelium and nitric oxide (NO). However, indomethacin (10 μM) enhanced the relaxant responses to 17β-oestradiol. In male rat mesenteric beds, 60 mM KCl, tetrabutylammonium chloride (300 μM), 4-aminopyridine (1 mM), and barium chloride (30 μM), charybdotoxin (100 nM), but not glibenclamide (10 μM) and tamoxifen (10 μM), inhibited vasorelaxation to 17β-oestradiol. In male rat aortae, 60 mM KCl did not affect vasorelaxation to 17β-oestradiol. However, in the presence of indomethacin, vasorelaxation to 17β-oestradiol was enhanced but this was sensitive to 60 mM KCl. Pre-treatment with 17β-oestradiol (100 μM) inhibited $CaCl_2$-induced contraction. The present findings indicate that, in rat mesenteric beds and aortae, 17β-oestradiol causes acute and potent vasorelaxation which may be enhanced in the presence of a cyclooxygenase inhibitor. In mesenteric arterial bed, 17β-oestradiol-induced vasorelaxation occurs primarily via activation of K^+ channels. In the aorta, vasorelaxations involved activation of K^+ efflux when the cyclooxygenase pathway was inhibited, and also inhibition of Ca^{2+} influx.

Keywords: 17β-Oestradiol; K^+ channel; Vasorelaxation; Ca^{2+} influx

1. Introduction

Epidemiological evidence indicates that pre-menopausal women have a reduced incidence of cardiovascular diseases compared to postmenopausal women, and men of a comparable age (Rosselli et al., 1995; Stampfer et al., 1991). This is supported by evidence that oestrogen replacement therapy reduces the risk of cardiovascular disease in postmenopausal women by approximately 50% (Stampfer et al., 1991). Vasorelaxant effects of oestrogen are thought to contribute towards a reduction in the incidence of cardiovascular disease (Gilligan et al., 1994; Guetta et al., 1997; Stampfer et al., 1991). However, the exact vascular mechanisms of action of oestrogens are controversial.

Previous studies have shown that the acute vesorelaxant effects of 17β-oestradiol are mediated via endothelium- and nitric oxide (NO)-dependent pathways (Andersen et al., 1999; Collins et al., 1994). For example, Collins et al. (1994) showed in coronary arterial rings from oestrogen-treated oophorectomized female rabbits that vasorelaxation to 17β-oestradiol was abolished by both removal of the endothelium and the presence of a nitric oxide synthase (NOS) inhibitor. In addition, in cultured arterial endothelial cells of human and animals, acute and long-term treatments with 17β-oestradiol stimulate endothelial NOS (eNOS) activity, leading to an increase in NO production (Caulin-Glaser et al., 1997; Hayashi et al., 1995; Lantin-Hermoso et al., 1997; Stefano et al., 2000). Moreover, several observations have suggested that NO production, synthesized and released from the endothelium, was dependent on gender (Hayashi et al., 1992; Kauser and Rubanyi, 1994; Knot et al., 1999). In this respect, findings carried out in rat and rabbit aortae showed that NO release was greater in females than males, suggesting that NO synthesis is regulated by oestrogens (Hayashi et al., 1992; Kauser and Rubanyi, 1994).

In contrast to the proposed involvement of NO, others have reported that vasorelaxation induced by 17β-oestradiol was unaffected by NOS inhibitors, and independent of the

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endothelium (Freay et al., 1997; Gonzales and Kanagy, 1999; Jiang et al., 1991, 1992; Nadarali et al., 1999; Shaw et al., 2000). One target may be K^+ channels, as previous findings have demonstrated, in rat and rabbit coronary arteries, that 17β-oestradiol acutely induces vasorelaxation via ATP-sensitive K^+ (K_{ATP}) channel activation, which is sensitive to glibenclamide (Hügel et al., 1999; Salom et al., 2002). Furthermore, vasorelaxation to 17β-oestradiol was also due to stimulation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in porcine coronary (White et al., 1995), ewe uterine (Rosenfeld et al., 2000), and rabbit coronary arteries (Salom et al., 2002). In addition, Valverde et al. (1999) have shown that 17β-oestradiol activates Maxi-K^+ channels in Xenopus leavis oocytes by binding to the Maxi-K^+ channels at the regulatory β subunit.

The ability of 17β-oestradiol to inhibit Ca^{2+} influx has been reported in several animal species (Gonzales and Kanagy, 1999; Han et al., 1995; Nadarali et al., 1999; Shan et al., 1994; Zhang et al., 1994). Previous studies in the porcine coronary artery and the rat mesenteric artery showed that 17β-oestradiol caused vasorelaxation by inhibition of extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores (Han et al., 1995; Nadarali et al., 1999). However, others have demonstrated, in rat tail and mesenteric arteries, that vasorelaxation to 17β-oestradiol is mediated by inhibition of Ca^{2+} influx from the extracellular space, but not Ca^{2+} release from intracellular stores (Gonzales and Kanagy, 1999).

In view of the controversy surrounding the precise mechanisms of action of 17β-oestradiol, the principal aim of the present investigation was to determine its mechanisms of vasorelaxation in isolated mesenteric arterial beds and aortae from male and female rats. The experiments were designed to examine the contribution of endothelium-derived relaxing factors in vasorelaxation to 17β-oestradiol. The involvement of K^+ channels was also investigated. It was also determined whether vasorelaxation to 17β-oestradiol was affected by gender. Finally, the effect of 17β-oestradiol on Ca^{2+} influx was examined. A preliminary account of these findings has already been communicated to the British Pharmacological Society (Tep-areenan et al., 2001, 2002).

2. Methods

2.1. Preparation of the rat isolated mesenteric arterial bed

Aged-matched male (250–350 g) and female (180–250 g) Wistar rats were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and killed by cervical dislocation, and this was followed by a midline laporatomy. The superior mesenteric artery was cannulated, and dissected away from guts and placed in a jacketed organ bath as previously described (McCulloch et al., 1997). The arterial vasculature was perfused with oxygenated ($95\%O_2/5\%CO_2$) Krebs-Henseleit solution (composition, mM: NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2; D-glucose, 10) at a constant flow rate 5 ml/min and maintained at 37 °C. In most experiments, indomethacin (10 μM) was added to the perfusion fluid, but indomethacin was omitted in some experiments to investigate the involvement of cyclooxygenase products in 17β-oestradiol-induced vasorelaxation.

2.2. Experimental protocol of the rat isolated mesenteric arterial bed

The perfusion pressure in the superior mesenteric arterial bed was continuously monitored by a pressure transducer coupled to a MacLab 4e recording system (AD Instruments, New South Wales, Australia). Flow rate was maintained at 5 ml/min and so alterations in perfusion pressure represent changes in resistance of the mesenteric arterial bed.

Following a 30-min equilibration period, methoxamine (2–55 μM) was added to the perfusion fluid to increase tone by approximately 80–100 mm Hg (approximately 70% of maximal tone). In experiments investigating the effects of the oestrogen receptor antagonist, tamoxifen and the K_{ATP} channel inhibitor, glibenclamide on responses to 17β-oestradiol, the tone was induced by adding methoxamine plus 5-hydroxytryptamine to perfusion fluid as methoxamine alone was inadequate. Once stable tone was established, 17β-oestradiol was added cumulatively to the perfusion fluid (10 pM–10 μM). In preliminary experiments, vehicle controls with ethanol, used at the same concentrations present following addition of 17β-oestradiol, there was no relaxation of tone.

In order to investigate the role of the endothelium in the vasorelaxant responses to 17β-oestradiol, the endothelium was removed in some preparations by perfusion with 0.5% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-l-propanesulphonate, Okasaki et al., 1998). The preparation was deemed to be endothelium-denuded if vasorelaxation to 55 nmol carbachol was less than 15%.

The role of NO in vasorelaxation to 17β-oestradiol was assessed by using N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor (Randall and Griffith, 1991), at a concentration of 300 μM. In the presence of L-NAME, a lower concentration of methoxamine was used to achieve equivalent tone as methoxamine was more potent.

To investigate the involvement of K^+ channels in vasorelaxation to 17β-oestradiol, 60 mM KCl was used to induce tone by substituting an equimolar concentration of NaCl with KCl (McCulloch et al., 1997). This was used to assess the possible involvement of a hyperpolarizing mechanism. In addition, 300 μM tetrabutylammonium chloride, a nonselective K channel inhibitor (Randall and Kendall, 1997), 10 μM glibenclamide, a selective K_{ATP} channel inhibitor (Randall and Griffith, 1993), 1 mM 4-aminopyridine, a K_V channel inhibitor (Honda et al., 1999), 30 μM

barium chloride, a voltage-dependent inward rectifier K^+ (K_{IR}) channel inhibitor (Harris et al., 2000), were also independently used to examine the types of K^+ channels involved in vasorelaxations to 17β-oestradiol (10 pM–10 μM). To investigate the effects of an inhibitor of K_V and K_{Ca} channels, charybdotoxin at the concentration of 100 nM (Randall and Kendall, 1998), 1 μM 17β-oestradiol was chosen as a test concentration and the effects of the toxin were examined against its response.

The involvement of oestrogen receptors was investigated by using an oestrogen receptor antagonist, tamoxifen, at a concentration of 10 μM (Han et al., 1995).

In each case, the appropriate inhibitors were added to the perfusion fluid to establish the desired concentrations and allowed to equilibrate for 30 min. Then, 17β-oestradiol was added in a cumulative fashion with the concentration being increased every 20 min.

2.3. Preparation of the rat aorta

Following a thoracotomy, the thoracic aorta was dissected from the rat. The aorta was cleaned of fat and connective tissue and cut into 5 mm in length. Each ring was transferred to a jacketed organ bath filled with 50 ml of Krebs-Henseleit solution. The solution in the bath was maintained at a temperature of 37 °C and bubbled with 95% O_2 and 5% CO_2 mixture. The perfusion solution in the bath was exchanged every 15 min for 1 h. The rings were mounted between two triangle stainless steel hooks that were passed through the lumen and stretched to optimal passive tension about 1 g and maintained at this tension for 1 h. Tension was measured by an isometric force displacement transducer (LETICA 210), and recorded on a MacLab 4e recording system (AD Instruments).

2.4. Experimental protocol of the rat aorta

Following a 1-h equilibration period, a high concentration of methoxamine (60–150 μM) was used to increase tone by approximately 0.80–1.40 g. However, in the presence of L-NAME, low concentrations of methoxamine (10–20 μM) were required to induce equivalent tone. In some experiments, 60 mM KCl was also used to induce tone in order to investigate the possible involvement of K^+ channels in vasorelaxation to 17β-oestradiol in the presence and in the absence of indomethacin. Once stable tone was established, various concentrations of 17β-oestradiol (1 pM–1 mM) were added in a cumulative manner at 10-min intervals. In experiments involving indomethacin (10 μM), L-NAME (300 μM), Charybdotoxin (100 nM), and tamoxifen (10 μM), these inhibitors were added to the buffer to establish the desired concentration and allowed 30 min before cumulative concentrations of 17β-oestradiol was added.

In vehicle-control experiments, ethanol alone was added cumulatively in the same volumes as those used in the experiments with 17β-oestradiol until the maximum concentration in the organ bath was 1.24% (v/v).

To investigate the effects of 17β-oestradiol on Ca^{2+} influx, concentration–response curves to $CaCl_2$ (10 μM–30 mM) were obtained in the absence and in the presence of 17β-oestradiol at the concentration of 100 μM. After aortic rings were allowed to equilibrate for 30 min, the rings were washed three times at 10-min interval with Ca^{2+}-free Krebs solution (Salom et al., 2002). Then, the rings were bathed with Ca^{2+}-free, high KCl (100 mM) Krebs solution (Jiang et al., 1991) with or without 17β-oestradiol (100 μM). In vehicle-control experiments, absolute ethanol was added in the same volume as 17β-oestradiol. After the rings were incubated with 17β-oestradiol or ethanol for 30 min, the concentration–response curves to $CaCl_2$ (10 μM–30 mM) were constructed.

2.5. Data and statistical analysis

The concentration of vasorelaxant giving the half-maximal relaxation (EC_{50}) was obtained from the concentration–response curve. The data were best-fitted to one- or two-site models (i.e. with a high potency and a low potency); the most appropriate model was determined by regression analysis and the r^2 values are stated. The EC_{50} values of curves fitted to a one-site model were determined by the logistic equation:

$$R = R_{max} \times A^{n_H} / EC_{50}^{n_H} + A^{n_H}$$

where R is the reduction in tone, A is the concentration of the relaxant, R_{max} is the maximum relaxation of the established tone, n_H is the slope function and EC_{50} is the concentration of vasorelaxant giving half-maximal relaxation.

As appropriate, some data were fitted to a double hyperbola according to the following equation:

$$R = (R_{max-1} \times A^{n_H-1} / EC_{50-1}^{n_H-1} + A^{n_H-1}) + (R_{max-2} \times A^{n_H-2} / EC_{50-2}^{n_H-2} + A^{n_H-2})$$

where R is the reduction in tone, A is the concentration of the relaxant, R_{max-1} is the maximum relaxation of the established tone at the high potency site, $n_H - 1$ is the slope function at the high potency site and EC_{50-1} is the concentration of vasorelaxant giving half-maximal relaxation at the high potency site. R_{max-2} is the maximum relaxation of the established tone at the low potency site, $n_H - 2$ is the slope function at the low potency site and EC_{50-2} is the concentration of vasorelaxant giving half-maximal relaxation at the low potency site. The appropriateness of fitting the curves to one- or two-site model was determined by comparing their r^2 values.

Maximal responses are expressed as mean ± S.E.M., and pEC_{50} values ($-\log$ of EC_{50} values) are expressed as means with 95% confidence intervals (CI). The number of

animals in each group is represented by *n*. The data were compared, as appropriate, by the Student's unpaired *t*-test or analysis of variance (ANOVA) with statistically significant differences between groups being determined by Bonferroni's post-hoc test. The curve-fitting and graph plotting were carried out using the graphical package GraphPad Prism.

2.6. Drugs and chemicals

All drugs and chemicals were purchased from Sigma (UK), except charybdotoxin, which was from Latoxan. 17β-Oestradiol, indomethacin, and tamoxifen were dissolved in absolute ethanol. 17β-Oestradiol at a concentration of 10 mM was diluted to various concentrations in Krebs-Henseleit solution. Glibenclamide was dissolved in dimethyl sulphoxide. Barium chloride and 4-aminopyridine were dissolved in distilled water. The remaining drugs were dissolved in the perfusion fluid. All drugs were made up on the day of the experiment.

3. Results

3.1. The effects of indomethacin on vasorelaxation to 17β-oestradiol in the rat isolated mesenteric arterial bed

17β-Oestradiol (10 pM–1 mM) caused concentration-related relaxations of methoxamine-induced tone, described by a two-site model, with a high potency site $pEC_{50\text{-}1}$) and a low potency site ($pEC_{50\text{-}2}$) ($pEC_{50\text{-}1}$ = 8.15 (7.04–9.25, 95% CI), $pEC_{50\text{-}2}$ = 5.04 (4.69–5.39) with maximal relaxation (R_{max}) = 97.7 ± 6.2%, *n* = 9). The presence of indomethacin (10 μM) significantly ($P < 0.001$) enhanced the potency of 17β-oestradiol at both high and low potency sites, but had no effects on maximal relaxation ($pEC_{50\text{-}1}$ = 10.1 (9.7–10.6), $pEC_{50\text{-}2}$ = 6.14 (5.69–6.58) with R_{max} = 91.3 ± 3.5%, *n* = 16, Fig. 1).

3.2. The effects of endothelial denudation or L-NAME in the presence of indomethacin on 17β-oestradiol-induced vasorelaxation in the rat isolated mesenteric arterial bed

In the presence of indomethacin, removal of the endothelium significantly reduced the potency of 17β-oestradiol-induced vasorelaxation at its high potency site ($pEC_{50\text{-}1}$: indomethacin = 10.1 (9.7–10.6), *n* = 16; indomethacin and endothelium-denuded = 8.98 (8.35–9.60), *n* = 10, $P < 0.01$), but did not affect responses at the low potency site and maximal relaxation to 17β-oestradiol (indomethacin: $pEC_{50\text{-}2}$ = 6.14 (5.69–6.58) with R_{max} = 91.3 ± 3.5%, *n* = 16; indomethacin and endothelium-denuded: $pEC_{50\text{-}2}$ = 5.56 (5.13–6.00) with R_{max} = 89.9 ± 3.1%, *n* = 10, Fig. 1).

In the presence of indomethacin, addition of L-NAME (300 μM) partially reduced vasorelaxation to 17β-oestradiol at the high potency site ($pEC_{50\text{-}1}$: indomethacin = 10.1 (9.7–10.6), *n* = 16; indomethacin and L-NAME = 9.24 (8.97–9.50), *n* = 7, $P < 0.05$). However, the low potency site and maximal relaxation to 17β-oestradiol were not affected (indomethacin: $pEC_{50\text{-}2}$ = 6.14 (5.69–6.58) with R_{max} = 91.3 ± 3.5%, *n* = 16; indomethacin and L-NAME: $pEC_{50\text{-}2}$ = 5.54 (4.81–6.26) with R_{max} = 105 ± 10%, *n* = 7, Fig. 1).

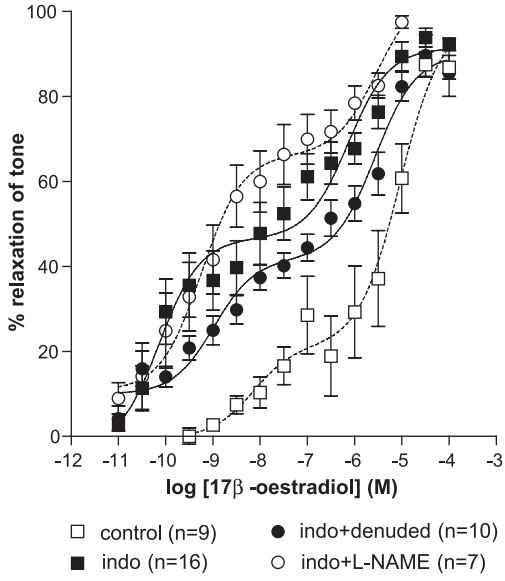

Fig. 1. The effects of indomethacin (indo, 10 μM), and N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) or removal of the endothelium plus indomethacin (10 μM) on 17β-oestradiol-induced vasorelaxation in mesenteric arterial beds from male rats precontracted with methoxamine. Data are shown as mean ± S.E.M.

3.3. The effects of gender on vasorelaxation to 17β-oestradiol in the presence of indomethacin in the rat isolated mesenteric arterial bed

In mesenteric arterial beds from male rats, 17β-oestradiol (10 pM–10 μM) caused concentration-related vasorelaxations in the presence of indomethacin, and the data were best-fitted (r^2 = 0.997) to a two-site model ($pEC_{50\text{-}1}$ = 10.1 (9.7–10.6), $pEC_{50\text{-}2}$ = 6.14 (5.69–6.58) with R_{max} = 91.3 ± 3.5%, *n* = 16). In female rat mesenteric vessels, 17β-oestradiol (1 pM–100 μM) induced vasorelaxation in a concentration-dependent manner in the presence of indomethacin. The data were best-fitted (r^2 = 0.998) to a two-site model ($pEC_{50\text{-}1}$ = 8.56 (7.96–9.15), $pEC_{50\text{-}2}$ = 5.33 (4.97–5.68) with R_{max} = 95.2 ± 3.3%, *n* = 17, Fig. 2). The potency of 17β-oestradiol at both high and low potency sites was significantly ($P < 0.001$ and $P < 0.01$, respectively) greater in mesenteric vessels from male rats compared to females.

3.4. The effects of high KCl and K^+ channel inhibitors on 17β-oestradiol-induced vasorelaxation in the presence of indomethacin in the rat isolated mesenteric arterial bed

In the presence of indomethacin, increasing extracellular K^+ to 60 mM abolished vasorelaxation to 17β-

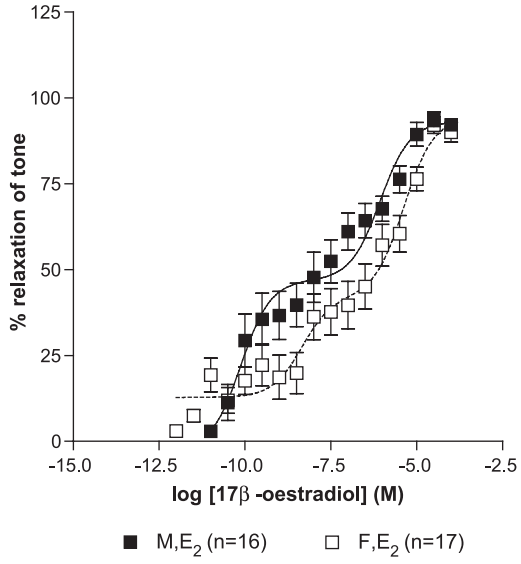

Fig. 2. Log concentration–response curves for vasorelaxation to 17β-oestradiol (E_2) in the presence of indomethacin (10 μM) in male (M) and female (F) rat mesenteric arterial beds precontracted with methoxamine. Data are shown as mean ± S.E.M.

oestradiol (10 pM–3 μM), except at a concentration of 10 μM 17β-oestradiol at which there was a vasorelaxation of 59.9 ± 14.5% ($n = 4$), but this was significantly ($P < 0.05$) less than the control value of 92.2 ± 4.4% ($n = 12$) in the presence of a normal extracellular K^+ concentration (Fig. 3A). Similarly, addition of tetrabutylammonium chloride (300 μM) in the presence of indomethacin abolished vasorelaxation induced by 17β-oestradiol at concentrations from 10 pM to 3 μM. At 10 μM 17β-oestradiol, vasorelaxation to 17β-oestradiol was significantly ($P < 0.01$) inhibited by tetrabutylammonium (indomethacin = 92.2 ± 4.4%, $n = 12$; indomethacin and tetrabutylammonium = 60.3 ± 5.6%, $n = 5$, Fig. 3A).

Pre-treatment with 4-aminopyridine (1 mM) in the presence of indomethacin reduced the potency of 17β-oestradiol-induced vasorelaxation. However, the maximal relaxation to 17β-oestradiol was not affected by 4-aminopyridine (R_{max}: indomethacin = 91.4 ± 3.5%, $n = 16$; indomethacin and 4-aminopyridine = 104 ± 7%, $n = 6$, Fig. 3B).

In the presence of indomethacin, addition of barium chloride ($BaCl_2$, 30 μM, $n = 6$) significantly reduced the potency of 17β-oestradiol-induced responses at both its high and low potency sites ($pEC_{50\text{-}1}$: indomethacin = 10.1(9.7–10.6), indomethacin and $BaCl_2$ = 8.33 (7.17–9.49), $P < 0.001$; $pEC_{50\text{-}2}$: indomethacin = 6.14 (5.69–6.58), indomethacin and $BaCl_2$ = 5.18 (4.87–5.48), $P < 0.05$), but did not affect maximal relaxation (indomethacin: R_{max} = 91.4 ± 3.5%; indomethacin and $BaCl_2$: R_{max} = 97.5 ± 4.6%, Fig. 3B).

Pre-treatment with charybdotoxin (100 nM) in the presence of indomethacin significantly inhibited vasorelaxations to 17β-oestradiol (1 μM) (indomethacin: 71.6 ± 4.2%, $n = 12$; indomethacin and charybdotoxin: 15.6 ± 7.5%, $n = 3$, $P < 0.001$, Fig. 4).

In the presence of indomethacin, pre-treatment with glibenclamide (10 μM) did not inhibit maximal relaxation to 17β-oestradiol (100 pM–3 μM) (indomethacin: pEC_{50} = 8.40 (6.84–9.96) with R_{max} = 53.3 ± 6.4%, $n = 5$; indomethacin and glibenclamide: pEC_{50} = 6.99 (6.28–7.70) with R_{max} = 64.9 ± 5.5%, $n = 6$).

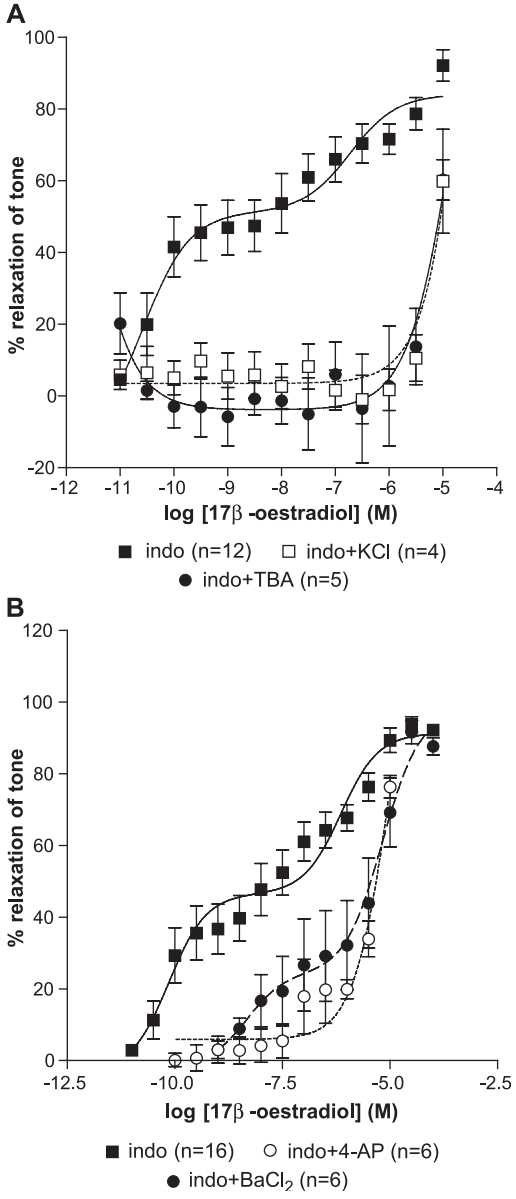

Fig. 3. (A) The effects of 60 mM KCl and tetrabutylammonium chloride (tetrabutylammonium, 300 μM). (B) The effects of 4-aminopyridine (4-aminopyridine, 1 mM) and barium chloride ($BaCl_2$, 30 μM) on 17β-oestradiol-induced vasorelaxation in the presence of indomethacin (indo, 10 μM) in mesenteric arterial beds from male rats precontracted with methoxamine. Data are shown as mean ± S.E.M.

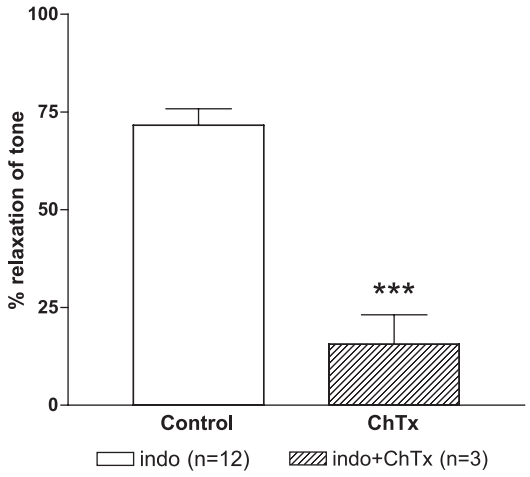

Fig. 4. The effects of charybdotoxin (100 nM) on vasorelaxation to 17β-oestradiol (1 μM) in the presence of indomethacin (indo, 10 μM) in mesenteric arterial beds from male rats precontracted with methoxamine. Data are shown as mean ± S.E.M. ***$P<0.05$ versus control.

3.5. The effects of tamoxifen on 17β-oestradiol-induced vasorelaxation in the presence of indomethacin in the rat isolated mesenteric arterial bed

In the presence of indomethacin, tamoxifen itself (100 pM–10 μM) caused potent concentration-dependent vasorelaxation, and the data were best-fitted ($r^2=0.999$) to a two-site model ($pEC_{50\text{-}1}=9.29$ (9.05–9.55), $pEC_{50\text{-}2}=6.02$ (5.31–6.72) with $R_{max}=94.1\pm3.2\%$, $n=5$).

Neither the potency nor maximal response to 17β-oestradiol (100 pM–10 μM) were affected by 10 μM tamoxifen in the presence of indomethacin (indomethacin: $pEC_{50}=8.40$ (6.84–9.96) with $R_{max}=53.3\pm6.4\%$, $n=5$; indomethacin and tamoxifen: $pEC_{50}=8.96$ (8.25–9.68) with $R_{max}=51.1\pm3.4\%$, $n=7$).

3.6. Vasorelaxation to 17β-oestradiol in the rat aorta

In the absence of indomethacin, 17β-oestradiol (30 pM–1 mM) induced vasorelaxation of aortic rings from male rats in a concentration-related manner and the data were best-fitted ($r^2=0.999$) to a two-site model ($pEC_{50\text{-}1}=8.87$ (8.17–9.57), $pEC_{50\text{-}2}=4.99$ (4.75–5.23) with $R_{max}=80.2\pm1.7\%$, $n=15$, Fig. 5). Moreover, in the absence of indomethacin, 17β-oestradiol (30 pM–1 mM) also caused concentration-dependent vasorelaxation of aortic rings from female rats, and the data were best-fitted ($r^2=0.999$) to a one-site model ($pEC_{50}=4.55$ (4.41–4.69), $R_{max}=80.4\pm1.7\%$, $n=15$), and the pEC_{50} was significantly ($P<0.01$) less than the pEC_{50} at the low potency site in males (Fig. 5). However, the maximal relaxation to 17β-oestradiol in aortic rings from female rats was not significantly different from those in males.

In vehicle-control experiments carried out in aortic rings from female rats, ethanol caused a relaxation of 10.5 ± 1.0% ($n=5$) at the maximal concentration used (1.24% (v/v), Fig. 5).

3.7. The effects of L-NAME on 17β-oestradiol-induced vasorelaxation in the male rat aorta

In the absence of indomethacin, in aortic rings from male rats, the presence of 300 μM L-NAME abolished the actions of 17β-oestradiol at its high potency site and the data were then best-fitted to a one-site model (control: $pEC_{50\text{-}1}=8.87$ (8.17–9.57), $pEC_{50\text{-}2}=4.99$ (4.75–5.23), $R_{max}=80.2\pm1.7\%$, $n=15$; L-NAME: $pEC_{50\text{-}2}=4.91$ (4.64–5.19), $R_{max}=82.9\pm4.2\%$, $n=6$, Fig. 5). The potency and maximal response to 17β-oestradiol of aortic rings from females were not affected by the addition of L-NAME in the absence of indomethacin (control: $pEC_{50}=4.55$ (4.41–4.69), $R_{max}=80.4\pm1.7\%$, $n=15$; L-NAME: $pEC_{50}=4.63$ (4.45–4.81), $R_{max}=74.6\pm2.8\%$, $n=12$, Fig. 5).

3.8. The effects of endothelial denudation and indomethacin on 17β-oestradiol-induced vasorelaxation in the male rat aorta

Removal of the endothelium significantly ($P<0.01$) inhibited the maximal relaxation to 17β-oestradiol, but had no effects on its potency (control: $pEC_{50\text{-}1}=8.87$ (8.17–9.57), $pEC_{50\text{-}2}=4.99$ (4.75–5.23) with $R_{max}=80.2\pm1.7\%$, $n=15$; rubbed: $pEC_{50\text{-}1}=8.31$ (5.26–11.4), $pEC_{50\text{-}2}=4.73$ (4.38–5.09) with $R_{max}=66.5\pm3.6\%$, $n=6$, Fig. 6).

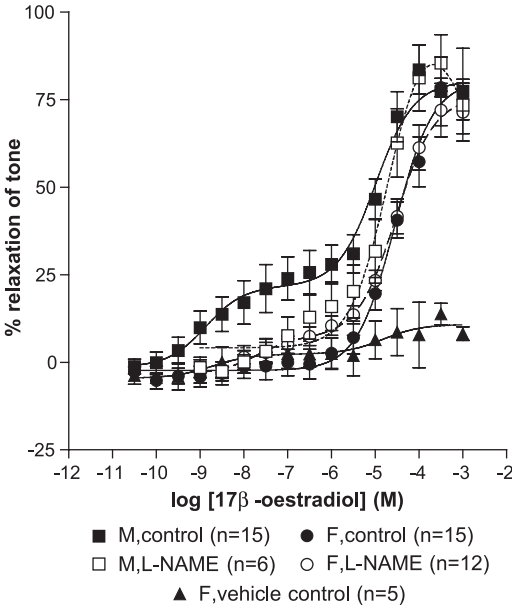

Fig. 5. Vasorelaxant responses to 17β-oestradiol in male (M) and female (F) aortae precontracted with methoxamine under control conditions and with ethanol vehicle-control data, and following the addition of N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM). Data are shown as mean ± S.E.M.

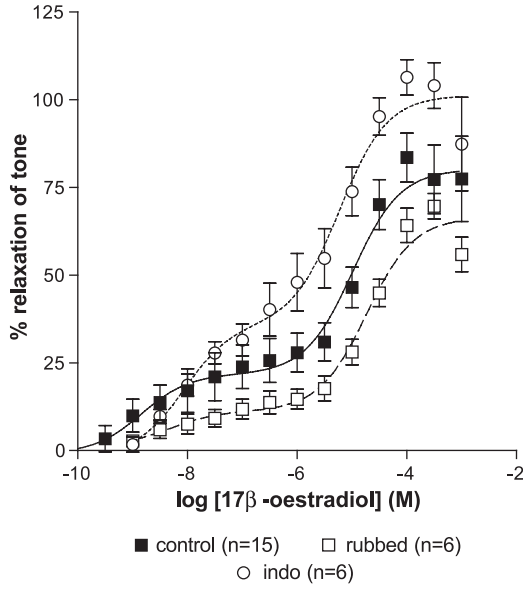

Fig. 6. The effects of indomethacin (indo, 10 μM) on 17β-oestradiol-induced vasorelaxation in male rat aortae precontracted with methoxamine. Data are shown as mean ± S.E.M.

Pre-treatment with indomethacin significantly ($P < 0.001$) enhanced the maximal relaxation to 17β-oestradiol but had no effects on the potency of 17β-oestradiol-induced relaxation (control: $pEC_{50\text{-}1} = 8.87$ (8.17–9.57), $pEC_{50\text{-}2} = 4.99$ (4.75–5.23) with $R_{max} = 80.2 \pm 1.7\%$, $n = 15$; indomethacin: $pEC_{50\text{-}1} = 8.06$ (7.05–9.07), $pEC_{50\text{-}2} = 5.22$ (4.79–5.65) with $R_{max} = 101 \pm 4\%$, $n = 6$, Fig. 6).

3.9. The effects of high extracellular K^+ and charybdotoxin on 17β-oestradiol-induced vasorelaxation in the male rat aorta

In the absence of indomethacin, high extracellular K^+ (60 mM KCl) inhibited the high potency portion of vasorelaxation to 17β-oestradiol (control: $pEC_{50\text{-}1} = 8.87$ (8.17–9.57), $pEC_{50\text{-}2} = 4.99$ (4.75–5.23), $R_{max} = 80.2 \pm 1.7\%$, $n = 15$; 60 mM KCl: $pEC_{50} = 4.76$ (4.60–4.92), $R_{max} = 143 + 5\%$, $n = 4$). However, high KCl significantly ($P < 0.01$) augmented vasorelaxations to 100 and 300 μM 17β-oestradiol, compared to control (Fig. 7A). Similarly, in the absence of indomethacin, charybdotoxin (100 nM) abolished the high potency site of vasorelaxation to 17β-oestradiol, and then the data were best-fitted to a one-site model (control: $pEC_{50\text{-}1} = 8.87$ (8.17–9.57), $pEC_{50\text{-}2} = 4.99$ (4.75–5.23), $R_{max} = 80.2 \pm 1.7\%$, $n = 15$; charybdotoxin: $pEC_{50} = 4.98$ (4.74–5.22), $R_{max} = 108 \pm 5\%$, $n = 4$, Fig. 7A).

In the presence of indomethacin, 60 mM KCl reduced the potency of 17β-oestradiol-induced vasorelaxation and the data were best-fitted to a one-site model (indomethacin: $pEC_{50\text{-}1} = 8.06$ (7.05–9.07), $pEC_{50\text{-}2} = 5.22$ (4.79–5.65), $n = 6$; indomethacin and 60 mM KCl: $pEC_{50} = 4.81$ (4.59–5.03), $n = 5$). However, there were no significant differences in vasorelaxations to 17β-oestradiol at concentrations from 30 μM to 1 mM, compared to indomethacin alone (Fig. 7B).

3.10. The effects of tamoxifen on 17β-oestradiol-induced vasorelaxation in the male rat aorta

In the presence of indomethacin, tamoxifen itself (1 nM–300 μM) caused concentration-dependent vasorelaxation, and the data were best-fitted ($r^2 = 0.999$) to a one-site model (Chen and Randall, unpublished observations).

In the absence of indomethacin, addition of tamoxifen (10 μM) did not inhibit relaxation and the potency of 17β-oestradiol-induced responses (control: $R_{max} = 80.2 \pm 1.7\%$,

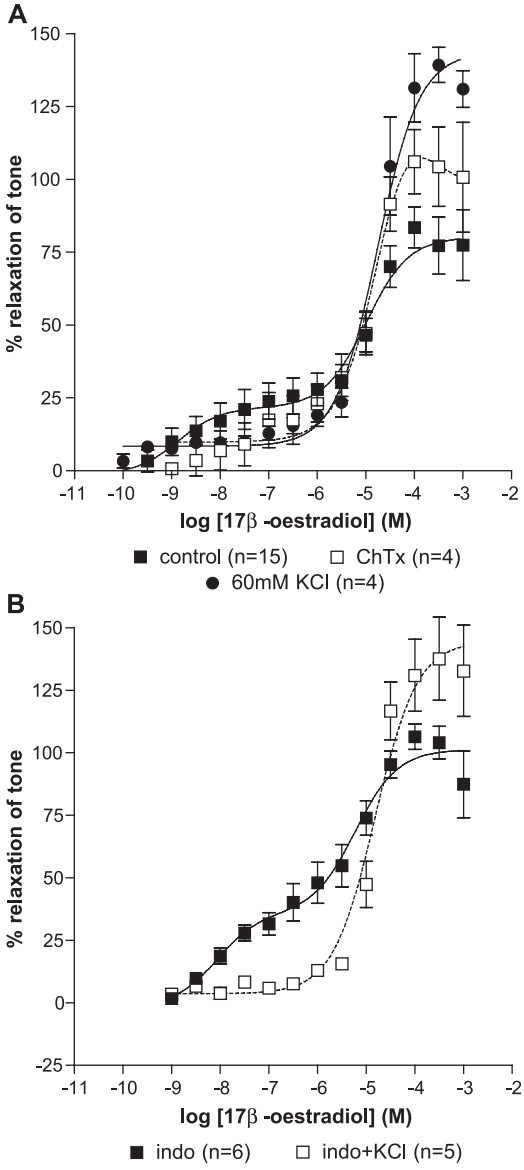

Fig. 7. (A) The effects of 60 mM KCl and charybdotoxin (100 nM) in the absence of indomethacin. (B) The effects of 60 mM KCl in the presence of indomethacin on 17β-oestradiol-induced vasorelaxation in male rat aortae. Data are shown as mean ± S.E.M.

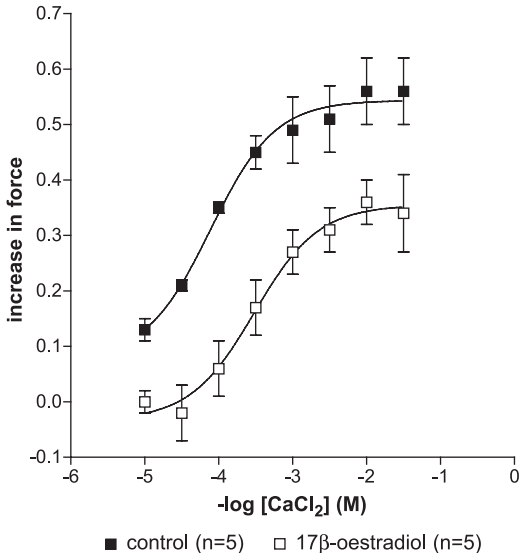

Fig. 8. The effects of 17β-oestradiol (100 μM) on $CaCl_2$-induced contraction in male rat aortae depolarized by 100 mM KCl. Data are shown as mean ± S.E.M.

$pEC_{50\text{-}1}$ = 8.87 (8.17–9.57), $pEC_{50\text{-}2}$ = 4.99 (4.75–5.23), n = 15; tamoxifen: R_{max} = 74.1 ± 2.8%, $pEC_{50\text{-}1}$ = 8.67 (8.25–9.07), $pEC_{50\text{-}2}$ = 4.47 (4.08–4.86), n = 6).

3.11. The effects of 17β-oestradiol on Ca^{2+}-induced contractions in the male rat aorta

In Ca^{2+}-free buffer, 100 mM KCl increased tension by 0.09 ± 0.04 g (n = 5) above basal tension. Pre-treatment with 17β-oestradiol reduced tension by 0.11 ± 0.08 g (n = 5) below basal tension.

$CaCl_2$ (10 μM–30 mM) caused contraction in a concentration-dependent manner in the rat aorta depolarized by 100 mM KCl. Pre-incubation with 100 μM 17β-oestradiol significantly inhibited $CaCl_2$-induced contraction at all concentrations such that the maximal contractions were 0.54 ± 0.01 g (control, n = 5) and 0.35 ± 0.01 g (in the presence of 17β-oestradiol, n = 5) (Fig. 8).

4. Discussion

In the present investigation, we have clearly shown that 17β-oestradiol caused acute and potent vasorelaxations in both mesenteric arterial vessels and aortae. Our findings have, therefore, confirmed several other studies which have shown that 17β-oestradiol causes vasorelaxant effects which are rapid in onset and are unlikely to be mediated via genomic effects (Nadarali et al., 1999; Shaw et al., 2000; Teoh et al., 2000; Salom et al., 2002). Consistent with this, others have shown that these responses are insensitive to inhibition of protein synthesis and gene transcription (Freay et al., 1997; Browne et al., 1999; Nadarali et al., 1999; Shaw et al., 2000; Teoh et al., 2000; Salom et al., 2002). One key finding of our study was that the responses to 17β-oestradiol occurred at low concentrations, which may suggest that physiological concentrations of 17β-oestradiol (0.02–1 nM in rat, Brown-Grant et al., 1970) may in fact influence vascular tone.

In both mesenteric arterial beds and aortae, it was apparent that 17β-oestradiol was more potent as a vasorelaxant in preparations from male rats compared to females. Similar observations have been recently reported by Nadarali et al. (2001) that 17β-oestradiol-induced vasorelaxation is more potent in mesenteric arteries form male guinea pigs than in those from females. From these results, it is indicated that vasorelaxant responses to 17β-oestradiol are gender-dependent. These differences have been suggested to result from excessive concentrations of testosterone or lower levels of 17β-oestradiol level in male animals (Nadarali et al., 2001). However, others have shown that vasorelaxations induced by 17β-oestradiol were greater in female rat aortae and coronary arteries from women than in male rats and men, respectively (Mügge et al., 1993; Le Tran et al., 1997).

The principal aim of the present study was to characterise the mechanisms of 17β-oestradiol-induced vasorelaxation. In both mesenteric arterial beds and aortae, it was observed that the vasorelaxation to 17β-oestradiol was largely endothelium-independent, as removing the endothelium only modestly affected the relaxant responses. This observation is compatible with the previous studies in rat aorta and mesenteric arteries, and in coronary arteries from bovine, rabbit and human sources showing little or no endothelium dependence (Jiang et al., 1991, 1992; Mügge et al., 1993; Freay et al., 1997; Le Tran et al., 1997; Kalanic et al., 2000; Chan et al., 2001). Coupled to this observation, it was also found that inhibition of NO synthesis similarly had only a modest effect on the relaxant responses to 17β-oestradiol in both mesenteric arterial vessels and aortae, confirming that endothelium-derived NO does not mediate these relaxation. Similar findings were also reported in the previous studies using rat mesenteric arteries (Nadarali et al., 1999; Shaw et al., 2000).

Although our analysis of responses at each individual concentration indicated that L-NAME did not significantly affect vasorelaxation to 17β-oestradiol, it is possible that NO may have played a modest role at the lower concentrations. Specifically, control response curves could readily be fitted to a two-site model, however, action at the high potency site was abolished in the presence of L-NAME. This might suggest that NO played some role in the vasorelaxation to 17β-oestradiol at low concentrations, but does not support a major role for NO at the higher concentrations used.

In the course of our study, we found, in both mesenteric vessels and aortae, that inhibition of the cyclooxygenase pathway enhanced the potency of 17β-oestradiol. This is a novel finding and may suggest that vasorelaxation to 17β-oestradiol is modulated by the endogenous release of vasoconstrictor prostanoids. Indeed, we have made a comparable observation with the relaxant responses to testoster-

one in the aorta (Tep-areenan et al., 2003). The present finding has practical implications in that the addition of cyclooxygenase inhibitors may amplify responses to 17β-oestradiol under experimental conditions, and this may explain some of the differences between the various publications. In addition, if prostanoid-dependent modulation occurs in man, then the use of cyclooxygenase inhibitors may also amplify the influence of endogenous oestrogens.

It has been proposed that 17β-oestradiol is a K^+ channel activator (White et al., 1995; Valverde et al., 1999; Rosenfeld et al., 2000) and the involvement of K^+ channels in vasorelaxation to 17β-oestradiol was examined. It was found in the mesenteric vessels that raising extracellular K^+ abolished the relaxant responses, except at the highest concentration used, and this suggests that a hyperpolarizing or repolarizing mechanism largely underlies the vasorelaxation to 17β-oestradiol. The involvement of K^+ channels was implicated by the ability of tetrabutylammonium chloride, a nonselective K^+ channel inhibitor, to inhibit the relaxant responses to 17β-oestradiol. In order to more fully characterise the K^+ channels involved, we used a range of channel inhibitors. It was found that charybdotoxin, which inhibits both K_{Ca} and K_V channels, blocked the relaxant responses to 17β-oestradiol. Blockade of K_V channels with 4-aminopyridine also inhibited these responses, as did blockade of K_{IR} channels with barium chloride. However, the K_{ATP} channel inhibitor, glibenclamide, did not affect responses to 17β-oestradiol. Taken together, these findings suggest that in the mesenteric vessels, a range of K^+ channels, which may include K_{Ca}, K_V, K_{IR} channels but not K_{ATP} channels, may mediate these responses. The present findings are in agreement with the observation by White et al. (1995), who showed, in porcine coronary arteries, that vasorelaxation to 17β-oestradiol was inhibited by charybdotoxin. Recently, Rosenfeld et al. (2000) demonstrated in ewe uterine arteries that tetraethylammonium chloride, an inhibitor of BK_{Ca} channels attenuated 17β-oestradiol-induced vasorelaxation. Furthermore, previous findings from patch-clamp studies showed that 17β-oestradiol acutely activated BK_{Ca} channels in smooth muscle cells of porcine coronary artery (White et al., 1995) and ewe uterine artery (Rosenfeld et al., 2000). In addition, Valverde et al. (1999) also demonstrated that Maxi-K^+ channels were activated by 17β-oestradiol, which directly bound to the β-subunit of Maxi-K^+ channels in Xenopus laevis oocytes. In the present study, only at the highest concentrations of 17β-oestradiol was there any vasorelaxation in the presence of high KCl or the K^+ channel blockers and this may reflect action at additional sites or incomplete blockade of K^+ channels.

The relaxant responses to 17β-oestradiol in the aorta were also modulated by prostanoids, as responses were enhanced in the presence of indomethacin. Once again this may be due to the concomitant release of vasoconstrictor prostanoids opposing the relaxant responses to 17β-oestradiol. Subsequent experiments investigating the mechanisms of action in the aorta in the absence of indomethacin found that both high KCl and charybdotoxin opposed the relaxant responses to 17β-oestradiol at low concentrations but at high concentrations KCl enhanced them. This may suggest that K^+ channels play a modest role in mediating these responses at low, but not high, concentrations of 17β-oestradiol. However, the apparent responses uncovered by indomethacin were entirely sensitive to high KCl. This may suggest that the action at this high potency site, which is normally modulated by the production of vasoconstrictor prostanoids, may involve a hyperpolarizing or repolarizing mechanism, possibly via K^+ channel activation.

The relaxant responses to 17β-oestradiol were not affected by the oestrogen receptor antagonist, tamoxifen and this would suggest that currently recognised nuclear oestrogen receptors do not mediate the relaxant responses. This insensitivity to tamoxifen has also been reported by previous studies in porcine coronary arteries (Han et al., 1995; Teoh et al., 1999, 2000). Furthermore, Shaw et al. (2000) similarly found in rat mesenteric arteries that the oestrogen receptor antagonist, ICI 182,780, did not affect responses to 17β-oestradiol. In the course of the experiments with tamoxifen, it was found that this agent also caused a relaxant response, which has also been reported in porcine coronary vessels (Hutchison et al., 2001). The mechanisms underlying relaxation to tamoxifen were not investigated in the present study; whether they share a common site of action or mechanism with 17β-oestradiol remains to be determined.

In the present study, the effect of 17β-oestradiol on Ca^{2+}-induced contractions was also investigated in the rat aorta depolarized by 100 mM KCl. We found that 17β-oestradiol inhibited $CaCl_2$-induced contractions in Ca^{2+}-free Krebs solution. These results indicated that 17β-oestradiol inhibited contractile responses of the aorta to $CaCl_2$ by inhibiting extracellular Ca^{2+} influx. This finding is in agreement with the previous observations in rabbit carotid and coronary arteries, rat tail and mesenteric arteries, and rat aorta (Jiang et al., 1991; Gonzales and Kanagy, 1999; Nadarali et al., 1999; Salom et al., 2002). It is suggested that 17β-oestradiol predominantly inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels in porcine coronary artery strips (Crews and Khalil, 1999), single coronary artery smooth muscle cells from pigs (Murphy and Khalil, 1999), and rat tail artery smooth muscle cells (Shan et al., 1994). This is supported by patch-clamp studies in vascular smooth muscle cells showing that 17β-oestradiol inhibits voltage-dependent Ca^{2+} channels through T- and L-type Ca^{2+} channels (Shan et al., 1994; Zhang et al., 1994; Nakajima et al., 1995; Kitazawa et al., 1997). We have therefore confirmed that inhibition of Ca^{2+} influx may contribute towards the relaxant effects of 17β-oestradiol in the aorta.

In summary, the present study in the rat mesenteric arterial bed and aorta has shown that acute vasorelaxation to 17β-oestradiol is largely mediated via endothelium- and

NO-independent pathways. In addition, the relaxant responses to 17β-oestradiol uncovered by cyclooxygenase inhibition. In the male rat mesenteric arterial bed, 17β-oestradiol-induced vasorelaxation occurs predominantly through K^+ channel activation, which may include K_v, K_{IR}, and K_{Ca} channels. In the aorta, vasorelaxations to 17β-oestradiol involve inhibition of Ca^{2+} influx and activation of K^+ efflux, but only when the cyclooxygenase pathway is inhibited.

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