British Journal of Pharmacology (1999) 127, 1091–1098
http://www.stockton-press.co.uk/bjp

Pharmacological characterization of endothelin receptor subtypes in the guinea-pig prostate gland

[1]Winnie A.K. Lau, [1,3]Sandra L. Cox, *[,1]Jocelyn N. Pennefather & [2]Fred J. Mitchelson

[1]Department of Pharmacology, Monash University, Wellington Road, Clayton, Victoria, Australia 3168 and [2]Department of Pharmaceutical Biology & Pharmacology, Victorian College of Pharmacy, Monash University, Parkville, Victoria, Australia 3052

1 Experiments have been conducted to investigate the actions of endothelins on the guinea-pig prostate gland.

2 Saturation experiments with [^{125}I]-endothelin-1 (2–800 pM) in guinea-pig prostatic homogenates indicated the presence of high affinity binding sites with an equilibrium dissociation constant (K_D) of 230 ± 50 pM, a maximum number of binding sites (B_{max}) of 52 ± 16 fmol mg^{-1} protein or 269 ± 61 fmol g^{-1} tissue and a Hill coefficient (n_H) of 1.01 ± 0.03 ($n = 3$). Competition experiments revealed that binding of [^{125}I]-endothelin-1 (20 pM) was inhibited with the following order of potency: endothelin-1 $>>$ BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-Trp[1-CO_2CH_3-D-Nle-ONa]) $>$ BQ-123 (cyclo-D-Asp-L-Pro-D-Val-Leu-D-Trp) $\geqslant$ sarafotoxin S6c.

3 At concentrations with negligible influence on smooth muscle tone, endothelin-1, endothelin-2 and sarafotoxin S6b (1 nM – 0.1 μM) produced concentration-dependent potentiation of the contractions evoked by electrical field stimulation with trains of 20 pulses at 10 Hz every 50 s, 0.5 ms pulse width and a dial setting of 60 V. In contrast, the endothelin ET_B receptor-preferring agonist endothelin-3 (1 nM – 1 μM) was much less potent, and the endothelin ET_B receptor-selective agonists sarafotoxin S6c and BQ-3020 (Ac-[Ala^{11,15}]-endothelin-1 (6-21)), up to 1 μM, were without effect.

4 The endothelin ET_A receptor antagonist BQ-123 (1 μM) markedly inhibited the potentiation induced by endothelin-1, endothelin-2 and sarafotoxin S6b while the endothelin ET_B receptor antagonist BQ-788 (1 μM) was less effective.

5 While our binding data indicates the presence of ET_A and ET_B binding sites in the guinea-pig prostate, the endothelin-induced facilitation of neurotransmission to the prostatic smooth muscle is mediated largely *via* activation of endothelin receptors of the ET_A subtype.

Keywords: Endothelin ET_A and ET_B receptors; endothelin-1, -2 and -3; sarafotoxins S6b and S6c; BQ-123; BQ-788; BQ-3020; smooth muscle contraction; field stimulation; guinea-pig prostate

Abbreviations: BQ-123, cyclo-D-Asp-L-Pro-D-Val-Leu-D-Trp; BQ-788, *N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-Trp[1-CO_2CH_3-D-Nle-ONa; BQ-3020, Ac-[Ala^{11,15}]-endothelin-1 (6-21); ECE, endothelin converting enzyme

Introduction

Since the discovery of endothelin in the porcine vascular endothelial cell cultures in 1988 by Yanagisawa *et al.* (1988), the presence of this peptide and its receptors has been demonstrated in a variety of tissues and organs (see reviews by Huggins *et al.*, 1993; Rubanyi & Polokoff, 1994; Sokolovsky, 1995). The existence of three genes encoding a family of structurally similar peptides, endothelin-1, -2 and -3, has been described (Inoue *et al.*, 1989). Endothelins have been reported to have roles not only in smooth muscle contraction of vascular and non-vascular tissues, but also in promoting cell growth (see reviews by Battistini *et al.*, 1993; Hay, 1995) and in the modulation of neuroeffector transmission in the airways (Takimoto *et al.*, 1993; Henry & Goldie, 1995; Fernandes *et al.*, 1996) and in the urogenital tract (urinary bladder: Saenz de Tejada *et al.*, 1992; Donoso *et al.*, 1994; vas deferens: Wiklund *et al.*, 1989; Donoso *et al.*, 1992; Lau *et al.*, 1995; Maas *et al.*, 1995).

There is now a growing body of evidence that indicates the presence of endothelins in the prostate. Endothelin precursors and the endothelin converting enzyme (ECE-1) have been reported to be expressed in the human prostate (Rossi *et al.*, 1995; Prayer-Galetti *et al.*, 1997; Walden *et al.*, 1998). To date, two major endothelin receptor subtypes have been identified (Masaki *et al.*, 1994), and both endothelin ET_A and ET_B receptors are detected in human cultured prostatic smooth muscle cells (Saita *et al.*, 1997), but the ET_A receptors are predominantly expressed in prostatic membrane homogenates (Le Brun *et al.*, 1996) and slide-mounted prostatic sections (Kobayashi *et al.*, 1994a; Imajo *et al.*, 1997). Quantitative receptor autoradiographic studies by Kobayashi *et al.* (1994b), have, however, shown a preferential localization of endothelin ET_A and ET_B receptors in stroma and epithelium, respectively.

Endothelin-1 immunoreactivity is prominent in the glandular epithelium (Langenstroer *et al.*, 1993). It is possible that endothelins play a physiological role in modulation of secretion in the gland, or a physiological or pathophysiological role to attenuate apoptosis (Wu-Wong *et al.*, 1997a) or to exert mitogenic effects in human prostatic smooth muscle cells (Saita *et al.*, 1998). In addition, endothelin-1 potently contracts the human prostate *in vitro*. These contractions are shown to be mediated by both endothelin receptor subtypes (Kobayashi *et al.*, 1994a) although it has also been proposed that in prostatic stromal cells, contraction is mediated by ET_B receptors (Webb *et al.*, 1995). Endothelins may thus play an important role in the regulation of the contractile function of smooth muscle cells associated with prostatic tension and secretory processes.

* Author for correspondence.
[3]Current address: Pharmakologisches Institut, Freiburg Universitat, Hermann-Herderstr. 5, D-79104 Freiburg, Germany.

Recent reports of upregulated endothelin binding sites in the benign hyperplastic prostate (Kondo *et al*., 1995; Moriyama *et al*., 1996) and of increased levels of endothelin-1 with decreased expression of endothelin ET_B binding sites in prostate cancer (Nelson *et al*., 1996) suggest a possible involvement of endothelins in prostatic disorders. Prayer-Galetti *et al*. (1997) have proposed that zonal-specific distribution of endothelin receptors in the human prostate may be of relevance in the pathogenesis of benign prostatic hyperplasia and prostate cancer.

The main aim of the present study was to examine the actions of endothelins and to characterize endothelin receptor subtype/s in the prostate, using the guinea-pig as an experimental model. The guinea-pig prostate has a substantial stromal component, which comprises predominantly smooth muscle cells (Ricciardelli *et al*., 1989). With age, the guinea-pig prostate develops a stromal hyperplasia which is histologically indistinguishable from that observed in ageing men (Horsfall *et al*., 1994). The influences of endothelins and related sarafotoxins on prostatic smooth muscle contractility and on neurotransmission in the guinea-pig isolated prostate were examined. Pharmacological tools including the endothelin ET_B receptor agonists sarafotoxin S6c (Williams *et al*., 1991) and BQ-3020 (Ihara *et al*., 1992b), and the endothelin ET_A and ET_B receptor-selective antagonists, BQ-123 (Ihara *et al*., 1992a) and BQ-788 (Ishikawa *et al*., 1994), respectively, have been employed in the characterization of endothelin receptor subtype/s in the guinea-pig prostate gland.

Preliminary accounts of some of the results of this study have been communicated to the Australian Neuroscience Society (Pennefather *et al*., 1998; Lau *et al*., 1999).

Methods

Animals

Adult male Dunkin-Hartley and Monash strain guinea-pigs (450–680 g) were housed in open runs at 22°C with a 12 h : 12 h light : dark cycle. Rodent chow, fruits, vegetables and water were provided *ad libitum*. Prior approval for animal experimentation was obtained from the Monash University Standing Committee on Ethics in Animal Experimentation (SCEAE Approval No. 96/069). Guinea-pigs were killed by cervical dislocation followed by exsanguination. The prostates were rapidly dissected out and trimmed of connective tissue and fat.

Radioligand binding studies

The [^{125}I]-endothelin-1 binding assay was conducted as described previously by Williams *et al*. (1991). Immediately after removal from the guinea-pig, the whole prostate was placed in ice-cold Tris buffer containing Tris HCl (50 mM), sucrose (250 mM) and the protease inhibitors pepstatin A (7 μg ml^{-1}) and leupeptin (0.5 μg ml^{-1}) at pH 7.4. The prostate was blotted dry, weighed (mean weight = 0.46 ± 0.02 g, $n = 18$), finely minced with scissors and homogenized twice for 10 s in 10 ml of ice-cold buffer with a 2 min interval on ice using a Polytron homogenizer (setting 5). The homogenates were centrifuged at 47,766 × *g* at 0–4°C for 10 min. The supernatant was discarded, the pellets homogenized and centrifuged as before for a further 30 min. The resultant pellets, containing membranes, were finally suspended in 7 ml of Tris buffer.

[^{125}I]-endothelin-1 binding was conducted in assay buffer containing potassium phosphate (KH_2PO_4 50 mM; pH 7.5) with 0.1% bovine serum albumin (BSA). For the binding assay, 100 μl of the membrane suspension was added to tubes containing a final volume of 250 μl (approximately 60 μg of protein as determined by the method of Lowry *et al*. (1951)). Saturation experiments with [^{125}I]-endothelin-1 were performed at eight concentrations in duplicate ranging from 2–800 pM. In competition experiments, at least twelve concentrations in triplicate of unlabelled endothelin-1 (10 pM–200 nM), sarafotoxin S6c (50 nM–5 μM), BQ-123 (1 nM–20 μM) and BQ-788 (0.5 nM–20 μM) were used with [^{125}I]-endothelin-1 (20 pM). Non-specific binding was determined in the presence of unlabelled endothelin-1 (200 nM). Following a 60 min incubation at 37°C, the binding reaction was terminated by rapid filtration through Whatman GF/B glass fibre filters using a Brandel M-48 cell harvester. The filters had been presoaked overnight in 2% BSA to reduce non-specific binding to filters. After washing twice with 5 ml of ice-cold buffer and partial drying under vacuum, tissue-bound radioactivity was determined by a United Technologies Packard gamma counter with an efficiency of 100%.

Functional studies

Preparations of the guinea-pig isolated prostate were mounted vertically in 5-ml siliconised (Coatasil, Searle) organ baths containing Krebs-Henseleit solution (pH 7.4, maintained at 37°C and bubbled with 5% CO_2 in O_2) of the following composition (mM): NaCl 118.1, KCl 4.87, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4.7H_2O$ 0.5, $CaCl_2.2H_2O$ 2.5. Tissues were allowed to equilibrate for 30 min under a resting force of 0.5 g, with bath medium changes every 5–10 min. Nerve terminals within the prostatic smooth muscle preparations of the guinea-pig were then electrically field stimulated with trains of 20 pulses at 10 Hz every 50 s with a dial setting of 60 V and 0.5 ms pulse width *via* two parallel electrodes incorporated into the tissue holder, connected to a Grass S88 stimulator. Isometric contractions of the prostatic smooth muscle were recorded with Grass FT03C force-displacement transducers connected to a MacLab data acquisition system (Chart 3.3) interfaced with a Macintosh LC575 computer.

After the field stimulated preparations of the guinea-pig prostate had been allowed to equilibrate for 30 min and when the stimulation-induced contractions were stable, the effects of endothelin receptor agonists including endothelin-1, endothelin-2 (1 nM–0.1 μM), endothelin-3 (1 nM–1 μM), sarafotoxin S6b (1 nM–0.1 μM) and sarafotoxin S6c and BQ-3020 (1 nM–1 μM) on prostatic smooth muscle tone and on field stimulation-induced contractions were assessed. Log concentration-response curves to the endothelin receptor agonists were constructed cumulatively using half log unit increments of agonist concentration. Increasing concentration of agonist was added when responses to the previous concentration reached a plateau; this usually occurred within 5 min for lower concentrations, or after agonist exposure time of 10 min.

To investigate the endothelin receptor subtype/s involved in mediating the agonist-induced influence on neurotransmission to the smooth muscle of the prostate from the guinea-pig, log concentration-response curves to the endothelin receptor agonists were constructed in the absence and presence of the subtype-selective endothelin receptor antagonists BQ-123 (ET_A: 1 μM) or BQ-788 (ET_B: 1 μM), with antagonist incubation period of 30 min. Control experiments were also conducted in parallel in order to determine whether agonist concentration-response curves, at 60–80 min intervals, were

reproducible over the experimental period and to correct for any tissue sensitivity changes due to time and/or vehicle. The vehicle was 0.01% dimethyl sulphoxide (DMSO). The effects of the endothelin receptor antagonists, alone and in combination, on the magnitude of field stimulation-induced contractions were examined after 15–30 min exposure of the prostatic preparations to BQ-123 (1 μM) or BQ-788 (1 μM).

In a separate series of experiments, the postjunctional action of endothelin-1 was determined by generating discrete concentration-contractile response curves to noradrenaline (0.1 μM–1 mM), noradrenaline exposure time of 60 s with a 10 min dose-cycle, before and 10 min after incubating prostatic preparations with endothelin-1 (10 nM).

Measurement and analysis of data

Binding data were analysed using software programs EBDA and LIGAND (McPherson, 1985).

In functional studies, the effects of endothelin receptor agonists on the magnitude of contractions evoked by trains of stimuli were expressed as percentage increases in field stimulation-induced responses. Mean peak force developed (in g) of four field stimulation-induced contractions just prior to and 5–10 min after exposure to each concentration of agonists were obtained in the absence and presence of antagonists. Mean log concentration-response curves to agonists were constructed by pooling data from individual curves. Agonist concentration required to produce 50% of the increase in stimulation-induced response induced by maximal concentration (0.1 μM) of agonist examined, $EC_{50\ (0.1\ \mu M)}$ with 95% confidence limits (C.L.) and degrees of freedom (d.f.), was derived from non-linear regression analyses using the GraphPad PRISM software program. The $EC_{50\ (0.1\ \mu M)}$ values were then converted to the negative logarithm and expressed as $pEC_{50\ (0.1\ \mu M)}$.

Data are presented as mean values with vertical bars representing standard error of the mean (s.e.mean); n represents the number of experimental animals. Statistical evaluation of data was performed with the GraphPad PRISM software program using Student's paired t-tests and one- or two-way analyses of variance (ANOVA), where appropriate. In all cases, values of $P<0.05$ were considered statistically significant.

Drugs

Endothelin-1, endothelin-2, endothelin-3, BQ-3020 (Ac-[$Ala^{11,15}$]-endothelin-1 (6-21)), sarafotoxin S6b, sarafotoxin S6c, BQ-123 (cyclo-D-Asp-L-Pro-D-Val-Leu-D-Trp and BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-Trp[1-CO_2CH_3-D-Nle-ONa] were obtained from the American Peptide Company. [^{125}I]-endothelin-1, at specific activity of 2000 Ci $mmol^{-1}$, was prepared at the Baker Institute (Australia). Pepstatin A and leupeptin were from Auspep. (−)-Arterenol (noradrenaline) bitartrate was from Sigma. Bovine serum albumin (BSA) was from Commonwealth Serum Laboratory Ltd. (Australia). Stock concentrations of endothelins (0.1 mM), BQ-3020 (0.1 mM) and BQ-788 (1 mM) were prepared in 5–10% dimethyl sulphoxide (DMSO). BQ-123, sarafotoxins and leupeptin were dissolved in distilled water and noradrenaline was dissolved and diluted in a catecholamine diluent (mM: NaCl 154.0, NaH_2PO_4 1.2 and ascorbic acid 0.2). Pepstatin A was first dissolved in equivalent volumes of glacial acetic acid and ethanol and then made up to a desired concentration of 1 mg ml^{-1} in distilled water. Subsequent dilutions of peptides were made in KH_2PO_4 buffer with 0.1% BSA and Krebs-Henseleit solution in binding and functional experiments, respectively.

Results

Radioligand binding studies

Saturation experiments Specific binding of [^{125}I]-endothelin-1 (2–800 pM) to the guinea-pig prostatic homogenates was saturable and of high affinity with a K_D of 230±50 pM and a B_{max} of 52±16 fmol mg^{-1} protein or 269±61 fmol g^{-1} tissue. The Hill coefficient (n_H) was 1.01±0.03 ($n=3$), indicating the presence of a homogeneous population of binding sites. Typical saturation and Scatchard plots are shown in Figure 1. Non-

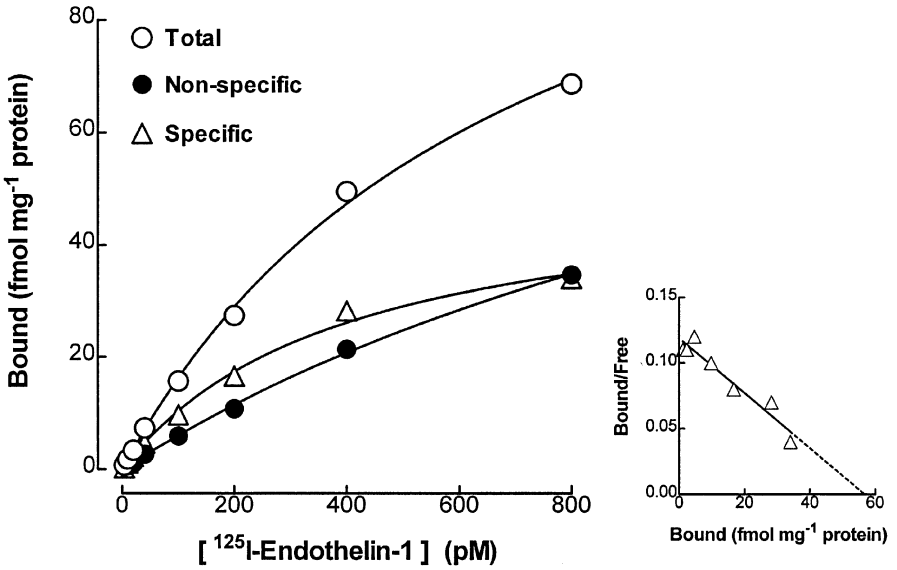

Figure 1 Saturation plot showing the specific binding of [^{125}I]-endothelin-1, determined as the difference between total and non-specific (in the presence of 200 nM endothelin-1) binding in the guinea-pig prostatic homogenates. Each data point represents the mean of duplicate samples in a single experiment. Corresponding Scatchard transformation of specific [^{125}I]-endothelin-1 is shown in the inset. These figures are representative of three similar experiments.

specific binding, determined in the presence of unlabelled endothelin-1 (200 nM), represented 20–50% of the total binding of [^{125}I]-endothelin-1, at the concentration range employed (2–800 pM) (Figure 1).

Competition experiments In competition studies the specific [^{125}I]-endothelin-1 (20 pM) binding to the guinea-pig prostatic homogenates was inhibited by endothelin-1, sarafotoxin S6c, BQ-123 and BQ-788 (Figure 2). Individual competition curves for endothelin-1 and BQ-788 were consistently better fitted by a one-site model whilst curves for BQ-123 were better fitted by a two-site model. Sarafotoxin S6c, at the highest concentration used (5 μM), caused approximately 70% inhibition of the specific [^{125}I]-endothelin-1 binding to the guinea-pig prostatic homogenates. The rank order of potency was endothelin-1 $>>$ BQ-788 $>$ BQ-123 $\geqslant$ sarafotoxin S6c with respective mean values of the negative logarithm of dissociation constants (pK_i) and slopes of the Hill plot (n_H) from estimates of individual experiments shown in Table 1.

Functional studies

Electrical field stimulation, delivered as trains of 20 pulses at 10 Hz every 50 s with 0.5 ms pulse width and a dial setting of 60 V, of nerve terminals within the prostate evoked regular and reproducible contractions (0.12 ± 0.2 g; $n=12$) of the prostatic smooth muscle preparations from the guinea-pig. It has previously been shown that neurotransmission to the guinea-pig prostatic smooth muscle is predominantly sympathetic and noradrenergic in nature (Ohkawa, 1983; Lau & Pennefather, 1995; Najbar-Kaszkiel *et al*., 1997; Lau *et al*., 1998).

Cumulative addition of endothelin-1, endothelin-2 and sarafotoxin S6b (1 nM–0.1 μM) to the guinea-pig prostatic smooth muscle preparations produced concentration-dependent potentiation of the field stimulation-induced contractions, but had negligible effect on smooth muscle tone. Endothelin-3 (1 nM–1 μM) was similarly without effect on smooth muscle tone and much less potent in producing facilitation. Figure 3 shows a representative trace illustrating the enhancing effects of endothelin-1 on field stimulation-induced contractions of the guinea-pig prostatic smooth muscle. Mean responses to endothelin-1, endothelin-2 and sarafotoxin S6b at the maximal concentration (0.1 μM) of agonist examined were not significantly ($P>0.05$, one-way ANOVA) different (Table 2 and Figure 4). The three agonists were equipotent in eliciting enhancement of stimulation-induced contractions (Table 2 and Figure 4). In sharp contrast, the endothelin ET_B receptor agonists sarafotoxin S6c and BQ-3020 (1 nM–1 μM) were without effect on the field stimulation-induced contractions of the guinea-pig prostatic preparations (Figure 4).

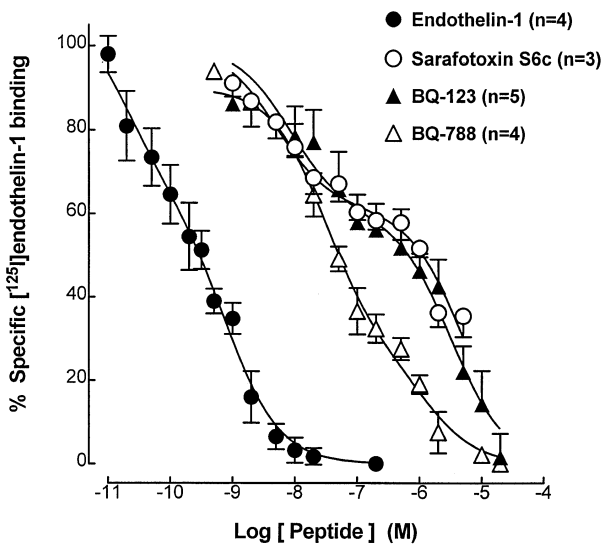

Figure 2 Competition of specific [^{125}I]-endothelin-1 binding to the guinea-pig prostatic homogenates by unlabelled endothelin-1 ($n=4$), sarafotoxin S6c ($n=3$), BQ-123 ($n=5$) and BQ-788 ($n=4$). Non-specific binding was determined in the presence of endothelin-1 (200 nM). Mean values $\pm$ s.e.mean of 3–5 separate experiments performed in triplicates are shown.

Table 1 pK_i and n_H values for peptide ligands in inhibiting specific [^{125}I]-endothelin-1 binding to the guinea-pig prostatic homogenates

Peptide	n	*Mean $pK_i \pm$ s.e.mean.*	$n_H \pm$ s.e.mean
Endothelin-1	4	9.60 ± 0.12	0.97 ± 0.10
BQ-123	5	6.42 ± 0.20	0.83 ± 0.24*
BQ-788	4	7.24 ± 0.22	0.91 ± 0.11
Sarafotoxin S6c	3	–	–

n = Number of animals. *$P<0.05$, Slope significantly less than one, analysis of data showed that competition of BQ-123 for [^{123}I]-endothelin-1 binding was fitted better with a two-site model than a one-site model, however, binding parameters of the predominant (80%) binding site are shown. –, Binding parameters for sarafotoxin S6c could not be computed as complete inhibition of binding was not achieved at the highest concentration examined (5 μM).

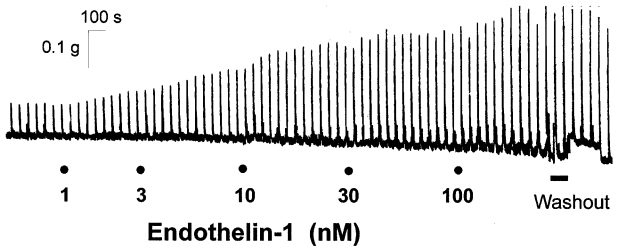

Figure 3 Representative trace showing the enhancing effect of endothelin-1 (1 nM–0.1 μM) on contractions evoked by trains of stimuli (0.5 ms pulse width, a dial setting of 60 V, 20 pulses at 10 Hz every 50 s) of the guinea-pig prostatic preparation.

Table 2 Potencies of agonists in facilitating neurotransmission to the guinea-pig prostatic smooth muscle

Agonist	n	$E_{max\ (0.1\ \mu M)}$ *(% increase)*	$pEC_{50\ (0.1\ \mu M)}$ *(95% confidence limits; d.f.)*
Endothelin-1	13	115.21 ± 13.26	7.89 (7.73, 8.05; 61)
Endothelin-2	15	123.54 ± 12.77	7.64 (7.52, 7.77; 69)
Sarafotoxin S6b	16	138.99 ± 23.46	7.69 (7.52, 7.86; 78)

n = Number of animals. $E_{max\ (0.1\ \mu M)}$ represents the magnitude of the effect as per cent increase in field stimulation-induced response produced by a concentration (0.1 μM) of agonist and $pEC_{50\ (0.1\ \mu M)}$ is the negative logarithm of agonist concentration producing 50% of the increase induced by a concentration (0.1 μM) of agonist.

In time and/or vehicle control experiments, neither the magnitude of field stimulation-induced contractions ($P>0.05$, Student's paired t-tests) (Table 3) nor the log concentration-response curves to the endothelin receptor agonists ($P>0.05$, two-way ANOVA) were affected in the presence of 0.01% DMSO. Exposure of the prostatic preparations to BQ-123 (1 μM) or BQ-788 (1 μM) alone and in combination had no significant ($P>0.05$, Student's paired t-tests) effect on the magnitude of field stimulation-induced contractions (Table 3). The agonist-induced enhancements were, however, markedly attenuated in the presence of BQ-123 (1 μM) (Figure 5A). BQ-788 (1 μM) caused a slight but significant ($P<0.05$, Student's paired t-tests) inhibition of the enhancing effects of endothelin-1 (30 and 100 nM) and sarafotoxin S6b (10 and 30 nM), but not those of endothelin-2, in the field stimulated preparations of the guinea-pig prostate (Figure 5B).

Application of exogenous noradrenaline (0.1 μM – 1 mM) produced concentration-dependent contractions of the guinea-

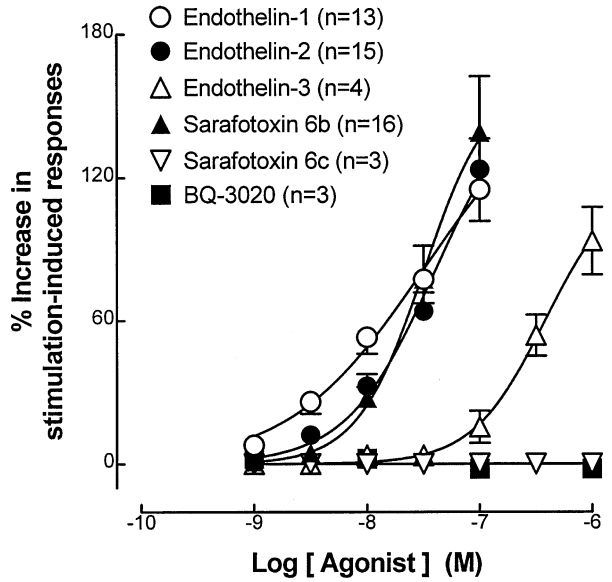

Figure 4 Mean log concentration-response curves for the facilitatory effects of endothelin-1 ($n=13$), endothelin-2 ($n=15$), endothelin-3 ($n=4$), sarafotoxin S6b ($n=15$), sarafotoxin S6c ($n=3$) and BQ-3020 ($n=3$) on the field stimulation-induced contractions in guinea-pig prostatic preparations.

Table 3 The effects of vehicle (0.01% DMSO), BQ-123 and BQ-788 on the field stimulation-induced contractions in guinea-pig prostatic preparations

Treatment	*Change in force (g)**
Control	0.13±0.02
0.01% DMSO	0.14±0.03
BQ-123 alone	0.14±0.03
BQ-788 alone	0.12±0.03
BQ-123 and BQ-788 in combination	0.11±0.02

*Force developed in response to field stimulation. $n=4-8$.

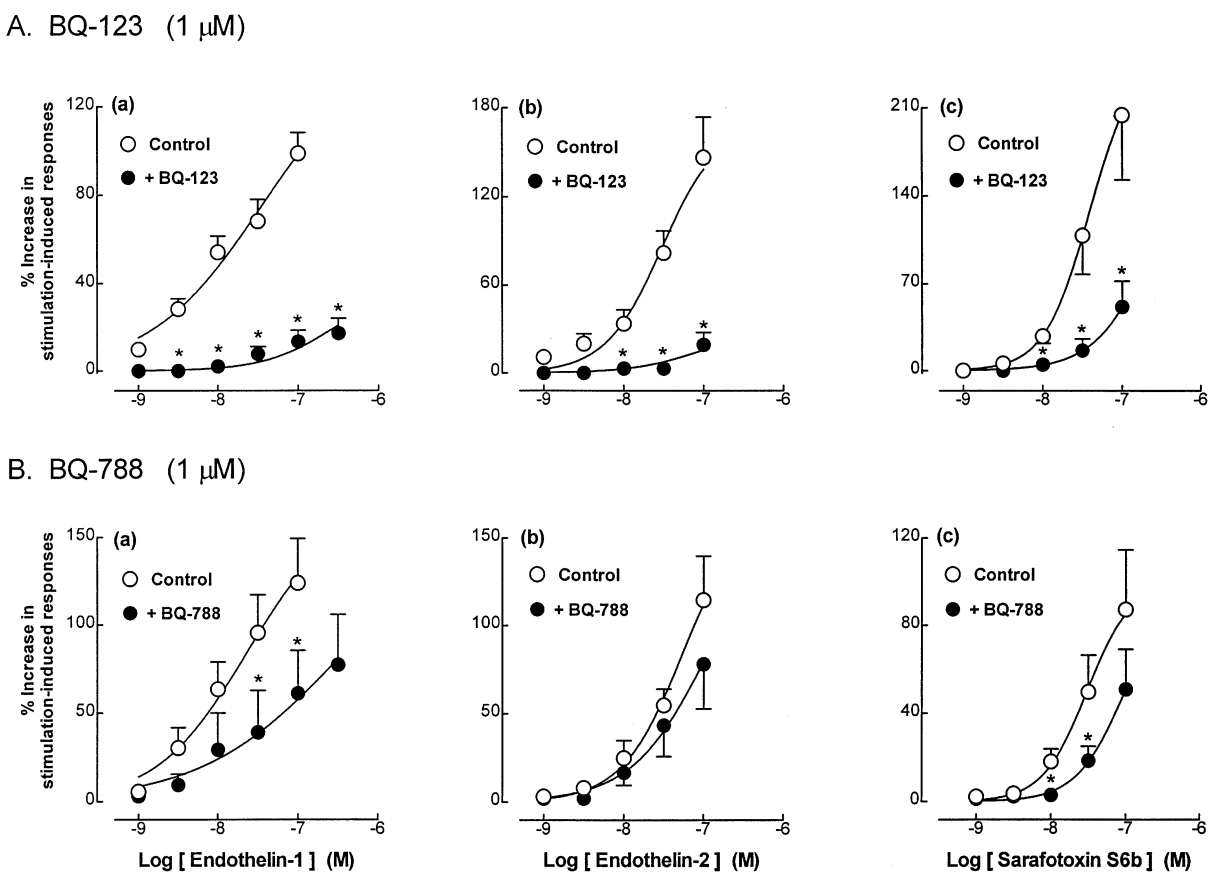

Figure 5 The effects of (A) BQ-123 (1 μM) and (B) BQ-788 (1 μM) on the mean log concentration-response curves to (a) endothelin-1 ($n=4-5$), (b) endothelin-2 ($n=4-5$) and (c) sarafotoxin S6b ($n=5-8$) in field stimulated preparations of the guinea-pig prostate. *$P<0.05$, 2-way ANOVA and Student's paired t-tests.

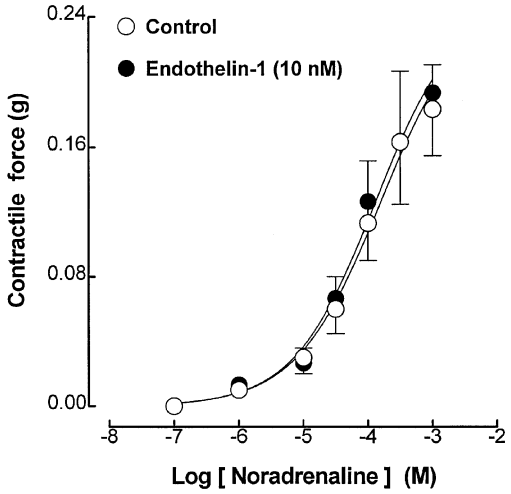

Figure 6 The effects of endothelin-1 (10 nM) on the mean log concentration-contractile response curves to noradrenaline in guinea-pig prostatic preparations ($n=3$).

pig prostatic smooth muscle preparations; these were not modified by the presence of endothelin-1 (10 nM) (Figure 6).

Discussion

Radioligand binding and functional experiments have been conducted to characterize the endothelin receptor subtype/s present and their role in smooth muscle contraction in the guinea-pig prostate gland. The major findings from this study are (1) that guinea-pig prostatic homogenates contain both endothelin ET_A and ET_B binding sites and (2) that endothelin-induced facilitation of neuromuscular transmission to the smooth muscle of the prostate from the guinea-pig is mediated predominantly through activation of the endothelin ET_A receptors.

Saturation binding experiments in the guinea-pig prostatic homogenates revealed that [^{125}I]-endothelin-1 binding involved an apparently single class of saturable and high affinity binding sites. The mean K_D value of 230 ± 50 pM obtained is well within the range of K_D values previously reported in the human prostate (70 pM: Saita *et al*., 1997; 550 pM: Imajo *et al*., 1997; 720 pM: Kobayashi *et al*., 1994a).

In competition binding studies, the rank order of affinity of endothelin-1 > > BQ-788 > BQ-123 $\geqslant$ sarafotoxin S6c in inhibiting [^{125}I]-endothelin-1 binding to the guinea-pig prostatic homogenates indicated the presence of both endothelin ET_A and ET_B binding sites. Endothelin-1 is known to bind to ET_A and ET_B receptors with equally high affinity. Reported pK_i values ranged from 9.24–10.64 at cloned human ET_A receptors transfected into Chinese hamster ovary cells, and from 9.92–10.97 at ET_B receptors in similar cells (Williams *et al*., 1993; Latifpour *et al*., 1995). The pK_i value (9.60) obtained for endothelin-1 in the present study approximates these ranges. Additionally, similar affinity constants were obtained for the radiolabelled (230 pM) and unlabelled (250 pM) endothelin-1 in saturation and competition experiments, respectively.

Analysis of binding data indicated that specific [^{125}I]-endothelin-1 binding to the guinea-pig prostatic homogenates was inhibited by the endothelin ET_A receptor-selective antagonist BQ-123 in a biphasic manner; the predominant proportion (80%) of binding yielding a pK_i value of 6.42. The endothelin ET_B receptor-selective antagonist BQ-788 competed for [^{125}I]-endothelin-1 binding with a pK_i value of 7.24. These estimates are somewhat lower than those reported at corresponding cloned ET_A and ET_B receptor systems. Values ranging from 7.1–7.9 have been reported for BQ-123, and from 8.7–9.2 for BQ-788 at cloned ET_A and ET_B receptors, respectively (Williams *et al*., 1993; Buchan *et al*., 1994; Ishikawa *et al*., 1994; Latifpour *et al*., 1995). In the human prostate pK_i values of 8.26 (Kobayashi *et al*., 1994a), 8.89 (Imajo *et al*., 1997) and 9.29 (Saita *et al*., 1997) were reported for BQ-123 and values of 8.77 (Saita *et al*., 1997) and 9.15 (Walden *et al*., 1998) for BQ-788. The reasons for the lower values we have obtained remain uncertain. It may be that these antagonists are binding to more than one receptor subtype in the guinea-pig prostate gland, but, as outlined by De Lean *et al*. (1982) further resolution of the binding profile would require 15–18 data points to validate this. Furthermore, differences in binding profiles may be influenced by the use of different preparations (intact cells vs cell membranes, Hara *et al*., 1998) and incubation conditions (temperature and time). The present study employed conditions similar to those described by Williams *et al*. (1991). Bovine serum albumin is included in the binding buffer in this study and that by Williams *et al*. (1991); serum albumin has been proposed to decrease the potency of antagonists in inhibiting endothelin receptor binding (Wu-Wong *et al*., 1997b).

The endothelin ET_B receptor agonist sarafotoxin S6c, at the highest concentration tested, caused approximately 70% inhibition. Taken together with the data for the endothelin antagonists, its low potency suggests that either low affinity ET_B as well as ET_A sites are present in the guinea-pig prostate. Additionally, atypical ET_B receptors or subtypes of endothelin ET_B receptors may be present in this tissue as proposed for other tissues including the human bronchus (Hay *et al*., 1998; see Bax & Saxena, 1994 for a review).

The results from our binding experiments indicated that the prostate gland of guinea-pig, as that in man, contains both endothelin ET_A and ET_B receptors. The guinea-pig prostate, like the human prostate, is composed primarily of glandular and stromal components. Although differential distribution and proportion of endothelin receptor subtypes between smooth muscle cells and other components in the guinea-pig prostatic homogenates may exist, the prostatic stroma from the guinea-pig has been shown to comprise predominantly (95%) smooth muscle cells (Ricciardelli *et al*., 1989; Horsfall *et al*., 1994). In the human prostate, ET_A receptors were originally shown to be mainly localized to the stromal compartment (Kobayashi *et al*., 1994a), although more recent studies by Webb *et al*. (1995) and Walden *et al*. (1998) indicate that ET_B receptors are the predominant subtype present in the stromal cells.

High levels of immunoreactive endothelin-1 have been shown to be present in human prostatic secretory epithelium (Langenstroer *et al*., 1993), suggesting that the prostate may contribute to the bulk of immunoreactive endothelin-1 found in the human seminal fluid (Casey *et al*., 1992). Exogenous application of endothelin-1 has been shown to potently contract human isolated prostate (Langenstroer *et al*., 1993; Kobayashi *et al*., 1994a; Webb *et al*., 1995). In our hands, the endothelin receptor agonists endothelins-1, -2 and -3, sarafotoxins S6b and S6c and BQ 3020 were without direct effect on the contractility of guinea-pig prostatic smooth muscle. This, together with the lack of effect of BQ-123 and BQ-788 on the magnitude of field stimulation-induced contractions of the guinea-pig prostatic smooth muscle suggests that endogenous endothelins are normally not

involved in neurotransmission to the smooth muscle of this tissue. However, endothelin-1, endothelin-2 and sarafotoxin S6b produced concentration-dependent potentiation of the field stimulation-induced contractions of these preparations. These three endothelin agonists were equally efficacious and potent in facilitating neurotransmission. In contrast the endothelin ET_B receptor-preferring agonist endothelin-3 was much less effective, and the endothelin ET_B receptor-selective agonists sarafotoxin S6c and BQ-3020 were completely inactive up to 1 μM. This series of experiments using agonists indicates that endothelins modulate neuromuscular transmission to the smooth muscle of the prostate from the guinea-pig through activation of ET_A receptors.

The involvement of ET_A receptors in facilitating neurotransmission to the prostate is further substantiated by findings that the endothelin ET_A receptor-selective antagonist BQ-123 markedly attenuated the endothelin-induced facilitation. The finding that the endothelin ET_B receptor-selective antagonist BQ-788, at 1 μM, reduced responses to endothelin-1 and to sarafotoxin S6b, might be attributable to an action at ET_A receptors since it has been reported to have an IC_{50} within the micromolar range at this subtype, while its K_D value at ET_B receptors is in the low nanomolar range (see review by Opgenorth *et al*., 1995).

Neurotransmission to the guinea-pig prostatic smooth muscle is predominantly sympathetic and noradrenergic (Ohkawa, 1983; Lau & Pennefather, 1995; Najbar-Kaszkiel *et al*., 1997; Lau *et al*., 1998). Since endothelin-1 was without appreciable influence on noradrenaline-induced contractions of the guinea-pig prostatic smooth muscle, it seems unlikely that endothelin-induced facilitation is due to a postjunctional interaction with noradrenaline. It is, however, possible that endothelins may interact postjunctionally with excitatory transmitters other than noradrenaline. Indeed, endothelin-1 has been shown to facilitate stimulation-induced contractions of the rat vas deferens (Donoso *et al*., 1992; Lau *et al*., 1995) and bladder (Donoso *et al*., 1994) by modulating the purinergic component of neurotransmission. In the guinea-pig prostate, acetylcholine does not contract stromal smooth muscle (Lau, 1998) and neither ATP nor its stable analogue α, β-methylene ATP, produce consistent effects (Lau, unpublished observations). Therefore postjunctional interactions of endothelins with these substances were not investigated. Nevertheless, the mechanism/s involved in the facilitation by endothelins on neurotransmission to the guinea-pig prostatic smooth muscle requires further investigation.

In conclusion, the present study has described the first pharmacological characterization of endothelin action on neurotransmission to the guinea-pig prostate gland. Both endothelin ET_A and ET_B binding sites have been shown to be present in this tissue. While the absence of an effect of endothelin antagonists on neurotransmission to the prostate indicates that endogenous endothelins are not normally involved in neurotransmission to the smooth muscle within this tissue, their ability to facilitate neurotransmission mainly *via* activation of ET_A receptors may be of potential physiological and pathophysiological significance.

This study was supported by a grant from the Monash Research Fund. We are grateful to Dr I McCance for advice on the statistical analysis of the functional data.

References

BATTISTINI, B., CHAILLER, P., D'ORLEANS-JUSTE, P., BRIERE, N. & SIROIS, P. (1993). Growth regulatory properties of endothelins. *Peptides,* **14,** 395–399.

BAX, W.A. & SAXENA, P.R. (1994). The current endothelin receptor classification: time for reconsideration? *Trends Pharmacol. Sci.,* **15,** 379–386.

BUCHAN, K.W., ALLDUS, C., CHRISTODOULOU, C., CLARK, K.L., DYKES, C.W., SUMNER, M.J., WALLACE, D.M., WHITE, D.G. & WATTS, I.S. (1994). Characterization of three non-peptide endothelin receptor ligands using human cloned ET_A and ET_B receptors. *Br. J. Pharmacol.,* **112,** 1251–1257.

CASEY, M.L., BYRD, W. & MACDONALD, P.C. (1992). Massive amounts of immunoreactive endothelin in human seminal fluid. *J. Clin. Endocrinol. Metabolism,* **74,** 223–225.

DE LEAN, A., HANCOCK, A.A. & LEFKOWITZ, R.J. (1982). Validation and statistical analysis of a computer modelling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.,* **21,** 5–16.

DONOSO, M.V., MONTES, C.G., LEWIN, J., FOURNIER, A., CALIXTO, J.B. & HUIDOBRO-TORO, J.P. (1992). Endothelin-1 (ET)-induced mobilization of intracellular Ca^{2+} stores from the smooth muscle facilitates sympathetic cotransmission by potentiation of adenosine 5′-triphosphate (ATP) motor activity: studies in the rat vas deferens. *Peptides,* **13,** 831–840.

DONOSO, M.V., SALAS, C., SEPULVEDA, G., LEWIN, J., FOURNIER, A. & HUIDOBRO-TORO, J.P. (1994). Involvement of ET_A receptors in the facilitation by endothelin-1 of non-adrenergic non-cholinergic transmission in the rat urinary bladder. *Br. J. Pharmacol.,* **111,** 473–482.

FERNANDES, L.B., HENRY, P.J., RIGBY, P.J. & GOLDIE, R.G. (1996). Endothelin$_B$ (ET_B) receptor-activated potentiation of cholinergic nerve-mediated contraction in human bronchus. *Br. J. Pharmacol.,* **118,** 1873–1874.

HARA, M., TOZAWA, F., ITAZAKI, K., MIHARA, S-I & FUJIMOTO, M. (1998). Endothelin ET_B receptors show different binding profiles in intact cells and cell membrane preparations. *Eur. J. Pharmacol.,* **345,** 339–342.

HAY, D.W.P. (1995). Chapter 1: Endothelins. In *Airways smooth muscle: peptide receptors, ion channels and signal transduction*. eds Raeburn, D & Giembycz, M.A. pp. 1–50. Basel/Switzerland: Birkhäuser Verlag.

HAY, D.W.P., LUTTMANN, M.A., PULLEN, M.A. & NAMBI, P. (1998). Functional and binding characterization of endothelin receptors in human bronchus: evidence for a novel endothelin B receptor subtype? *J. Pharmacol. Exp. Ther.,* **284,** 669–677.

HENRY, P.J. & GOLDIE, R.G. (1995). Potentiation of endothelin-1 of cholinergic nerve-mediated contractions in mouse trachea via activation of ET_B receptors. *Br. J. Pharmacol.,* **114,** 563–569.

HORSFALL, D.J., MAYNE, K., RICCIARDELLI, C., RAO, M., SKINNER, J.M., HENDERSON, D.W., MARSHALL, V.R. & TILLEY, W.D. (1994). Age-related changes in guinea-pig prostatic stroma. *Lab. Invest.,* **70,** 753–763.

HUGGINS, J.P., PELTON, J.T. & MILLER, R.C. (1993). The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmac. Ther.,* **59,** 55–123.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1992a). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.,* **50,** 247–255.

IHARA, M., SAEKI, T., FUKURODA, T., KIMURA, S., FUKAMI, T., OZAKI, S., PATEL, A.C. & YANO, M. (1992b). A novel radioligand [^{125}I]BQ-3020 selective for endothelin (ET_B) receptors. *Life Sci.,* **51,** 47–52.

IMAJO, C., WALDEN, P.D., SHAPIRO, E., DOHERTY, A.M. & LEPOR, H. (1997). Evaluation of the effect of endothelin-1 and characterization of the selective endothelin A receptor antagonist PD155080 in the prostate. *J. Urol.,* **158,** 253–257.

INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 2863–2867.

ISHIKAWA, K., IHARA, M., NOGUCHI, K., MASE, T., MINO, N., SAEKI, T., FUKURODA, T., FUKAMI, T., OZAKI, S., NAGASE, T., NISHIKIBE, M. & YANO, M. (1994). Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 4892–4896.

KOBAYASHI, S., TANG, R., WANG, B., OPGENORTH, T., LANGENSTROER, P., SHAPIRO, E. & LEPOR, H. (1994a). Binding and functional properties of endothelin receptor subtypes in the human prostate. *Mol. Pharmacol.,* **45,** 306–311.

KOBAYASHI, S., TANG, R., WANG, B., OPGENORTH, T., STEIN, E., SHAPIRO, E. & LEPOR, H. (1994b). Localization of endothelin receptors in the human prostate. *J. Urol.,* **151,** 763–766.

KONDO, S., MORITA, T. & TASHIMA, Y. (1995). Benign prostatic hypertrophy affects the endothelin receptor density in the human urinary bladder and prostate. *Urol. Int.,* **54,** 198–203.

LANGENSTROER, P., TANG, R., SHAPIRO, E., DIVISH, B., OPGENORTH, T.J. & LEPOR, H. (1993). Endothelin-1 in the human prostate: tissue levels, source of production and isometric tension studies. *J. Urol.,* **149,** 495–499.

LATIFPOUR, J., FUKUMOTO, Y. & WEISS, R.M. (1995). Regional differences in the density and subtype specificity of endothelin receptors in rabbit urinary tract. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **352,** 459–468.

LAU, W.A.K. (1998). Neurotransmission to the prostate: role of acetylcholine. Masters of Science thesis, Monash University, Australia.

LAU, W.A.K., COX, S.L., PENNEFATHER, J.N. & MITCHELSON, F.J. (1999). Endothelin receptor subtypes in the guinea-pig prostate gland. *Proc. Aust. Neuroscience Soc.,* **10,** 163.

LAU, W.A.K. & PENNEFATHER, J.N. (1995). Neurotransmission to the smooth muscle of the prostate gland from the guinea-pig. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.,* **2,** 89.

LAU, W.A.K., VENTURA, S., JIANG, Q. & PENNEFATHER, J.N. (1995). Endothelin-induced facilitation of sympathetic neurotransmission to the rat vas deferens: effects of suramin. *Eur. J. Pharmacol.,* **272,** 31–38.

LAU, W.A.K., VENTURA, S. & PENNEFATHER, J.N. (1998). Pharmacology of neurotransmission to the smooth muscle of the rat and the guinea-pig prostate glands. *J. Auton. Pharmacol.,* **18,** 349–356.

LE BRUN, G., MOLDOVAN, F., AUBIN, P., ROPIQUET, F., CUSSENOT, O. & FIET, J. (1996). Identification of endothelin receptors in normal and hyperplasic human prostate tissues. *The Prostate,* **28,** 379–384.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MAAS, J., D'ORLEANS-JUSTE, P., YANO, M. & RAE, G.A. (1995). Evidence for atypical endothelin receptors and for presence of endothelin-converting enzyme activity in the mouse isolated vas deferens. *Eur. J. Pharmacol.,* **276,** 113–121.

MASAKI, T., VANE, J.R. & VANHOUTTE, P.M. (1994). V. International Union of Pharmacology Nomenclature of endothelin receptors. *Pharmacol. Rev.,* **46,** 137–142.

MACPHERSON, G.A. (1985). Analysis of radioligand binding experiment. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods,* **14,** 213–228.

MORIYAMA, N., KURIMOTO, S., MIYATA, N., YAMAURA, H., YAMAZAKI, R., SUDOH, K., INAGAKI, O., TAKENAKA, T. & KAWABE, K. (1996). Decreased contractile effect of endothelin-1 on hyperplastic prostate. *Gen. Pharmacol.,* **27,** 1061–1065.

NAJBAR-KASZKIEL, A.T., DI IULIO, J.L., LI, C.G. & RAND, M.J. (1997). Characterisation of excitatory and inhibitory transmitter systems in prostate glands of rats, guinea-pigs, rabbits and pigs. *Eur. J. Pharmacol.,* **337,** 251–258.

NELSON, J.B., CHAN-TACK, K., HEDICAN, S.P., MAGNUSON, S.R., OPGENORTH, T.J., BOVA, G.S. & SIMONS, J.W. (1996). Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res.,* **56,** 663–668.

OHKAWA, H. (1983). Sympathetic neuromuscular transmission in the smooth muscle of guinea-pig prostate gland. *Int. J. Fertil.,* **28,** 68–77.

OPGENORTH, T.J. (1995). Endothelin receptor antagonism. *Advances Pharmacol.,* **33,** 1–65.

PENNEFATHER, J.N., COX, S., LAU, W.A.K., MCCANCE, I. & MITCHELSON, F. (1998). Endothelin-induced facilitation of neurotransmission to the smooth muscle of the guinea-pig prostate. *Proc. Aust. Neuroscience Soc.,* **9,** 85.

PRAYER-GALETTI, T., ROSSI, G.P., BELLONI, A.S., ALBERTIN, G., BATTANELLO, W., PIOVAN, V., GARDIMAN, M. & PAGANO, F. (1997). Gene expression and autoradiographic localization of endothelin-1 and its receptors A and B in the different zones of the normal human prostate. *J. Urol.,* **157,** 2334–2339.

RICCIARDELLI, C., HORSFALL, D.J., SKINNER, J.M., HENDERSON, D.W., MARSHALL, V.R. & TILLEY, W.D. (1989). Development and characterization of primary cultures of smooth muscle cells from the fibromuscular stroma of the guinea-pig prostate. *In Vitro Cell Dev. Biol.,* **25,** 1016–1024.

ROSSI, G.P., ALBERTIN, G., FRANCHIN, E., SACCHETTO, A., CESARI, M., PALU, G. & PESSINA, A.C. (1995). Expression of the endothelin-converting enzyme gene in human tissues. *Biochem. Biophys. Res. Commun.,* **211,** 249–253.

RUBANYI, G.M. & POLOKOFF, M.A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathophysiology. *Pharmacol. Rev.,* **46,** 325–415.

SAENZ DE TEJADA, I., MUELLER, J.D., DE LAS MORENAS, A., MACHADO, M., MORELAND, R.B., KRANE, R.J., WOLFE, H.J. & TRAISH, A.M. (1992). Endothelin in the urinary bladder. I. Synthesis of endothelin-1 by epithelia, smooth muscle and fibroblasts suggests autocrine and paracrine cellular regulation. *J. Urol.,* **148,** 1290–1298.

SAITA, Y., KOIZUMI, T., YAZAWA, H., MORITA, T., TAKENAKA, T. & HONDA, K. (1997). Endothelin receptors and their cellular signal transduction mechanism in human cultured prostatic smooth muscle cells. *Br. J. Pharmacol.,* **121,** 687–694.

SAITA, Y., YAZAWA, H., KOIZUMI, T., MORITA, T., TAMURA, T., TAKENAKA, T. & HONDA, K. (1998). Mitogenic activity of endothelin on human cultured prostatic smooth muscle cells. *Eur. J. Pharmacol.,* **349,** 123–128.

SOKOLOVSKY, M. (1995). Endothelin receptor subtypes and their role in transmembrane signaling mechanisms. *Pharmac. Ther.,* **68,** 435–471.

TAKIMOTO, M., INUI, T., OKADA, T. & URADE, Y. (1993). Contraction of smooth muscle by activation of endothelin receptors on autonomic neurons. *F.E.B.S. Lett.,* **324,** 277–282.

WALDEN, P.D., ITTMANN, M., MONACO, M.E. & LEPOR, H. (1998). Endothelin-1 production and agonist activities in cultured prostate-derived cells: implications for regulation of endothelin bioactivity and bioavailability in prostatic hyperplasia. *The Prostate,* **34,** 241–250.

WEBB, M.L., CHAO, C.-C., RIZZO, M., SHAPIRO, R.A., NEUBAUER, M., LIU, E.C.K., AVERSA, C.R., BRITTAIN, R.J. & TREIGER, B. (1995). Cloning and expression of an endothelin receptor subtype B from human prostate that mediates contraction. *Mol. Pharmacol.,* **47,** 730–737.

WIKLUND, N.P., OHLEN, A., WIKLUND, C.U., CEDERQVIST, B., HEDQVIST, P. & GUSTAFSSON, L.E. (1989). Neuromuscular actions of endothelin on smooth, cardiac and skeletal muscle from guinea-pig, rat and rabbit. *Acta Physiol. Scand.,* **137,** 399–407.

WILLIAMS, D.L., JONES, K.L., ALVES, K., CHAN, C.P., HOLLIS, G.F. & TUNG, J.-S. (1993). Characterization of cloned human endothelin receptors. *Life Sci.,* **53,** 407–414.

WILLIAMS, D.L., JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINESCHMIDT, B.V. (1991). Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.,* **175,** 556–561.

WU-WONG, J.R., CHIOU, W.J., DICKINSON, R. & OPGENORTH, T.J. (1997a). Endothelin attenuates apoptosis in human smooth muscle cells. *Biochem. J.,* **328,** 733–737.

WU-WONG, J.R., DIXON, D.B., CHIOU, W.J. & OPGENORTH, T.J. (1997b). Endothelin receptor antagonists: effects of serum albumin on potency and comparison of pharmacological characteristics. *J. Pharmacol. Exp. Ther.,* **281,** 791–798.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature,* **332,** 411–415.

(Received September 2, 1998
Revised March 8, 1999
Accepted April 9, 1999)