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Cloning and Expression of an Endothelin Receptor Subtype B from Human Prostate that Mediates Contraction

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SUMMARY

Recent evidence suggests a role for endothelin (ET) in contraction of human prostate [*J. Urol.* **149**:495–499 (1993)]. Although both ET_A and ET_B receptors have been shown to mediate contraction of smooth muscle, the molecular identity of the contractile ET_B receptor is controversial. The aim of this study was to examine the receptor subtype that mediates ET-induced contraction in prostate from patients with benign prostatic hyperplasia. Saturation binding with ^{125}I-ET-1 and ^{125}I-ET-3 in prostate stromal cells (PSC) indicated the presence of receptors with subnanomolar affinity for these radioligands, with equivalent receptor densities. Inhibition of specific ^{125}I-ET-1 or ^{125}I-ET-3 binding in PSC revealed a rank order of potency of ET-1 = ET-3 = sarafotoxin S6c ≫ BQ-123. These data are consistent with a predominance of ET_B receptors in PSC. The functional effects of ET stimulation of PSC were examined in a collagen gel contraction assay. ET-1 and ET-3 caused contraction of underlying collagen gel matrices with EC_{50} values of 0.4 ± 0.04 and 0.7 ± 0.2 nM, respectively. To determine the molecular nature of the contractile ET_B receptor in PSC, reverse transcription-polymerase chain reactions were conducted with oligonucleotide primers to the 5′ and 3′ ends of the coding sequence of the full length human ET_B receptor. DNA sequence analysis of the 1.3-kilobase DNA product showed 99% homology to other human ET_B receptor cDNAs. The encoded protein has a deduced amino acid sequence identical to that of other human ET_B receptors, with the exception of two conservative substitutions. Expression of the PSC ET_B cDNA in COS-7 cells resulted in a binding profile similar to that observed in parent cells. Polymerase chain reaction analysis revealed the presence of prepro-ET-1 mRNA in PSC. Collectively, these data indicate that PSC from patients with benign prostatic hyperplasia express ET_B receptors that mediate ET-induced contraction.

The ETs are a family of structurally and pharmacologically related peptides with potent effects on smooth muscle contractility (1). Yanagisawa *et al.* (2) demonstrated that ET produces a potent, slowly developing, and long-lasting vasoconstriction in pig, rat, cat, rabbit, dog, and human arteries. Subsequent studies (3, 4) demonstrated long-lasting vasoconstrictor and pressor effects of ET in humans. ETs are produced in many tissues, including lung, kidney, eye, gastrointestinal tract, many nuclei in the central nervous system (5, 6), and, as determined more recently, human prostate (7).

BPH is a neoplasm of the prostate that affects 70% of men by age 70, necessitating transurethral surgical resection in approximately 30% of these cases (8, 9). Immunohistochemical analysis revealed positive staining for ET-1 in the glandular epithelium of the human prostate (7), and radioligand binding studies demonstrated the presence of ET receptors in both the stroma and epithelium (10, 11). Moreover, ET-1 induced contraction of prostatic tissue strips in isometric force studies (7), suggesting that ET-1 may act as a paracrine or autocrine mediator in BPH.

The actions of ET in mammals are mediated through at least two related receptor subtypes, termed ET_A and ET_B. The cloning of the human ET_A and ET_B receptors revealed significant homology (60%) at the nucleic acid and protein levels (12, 13). ET receptors that are distinct from the mammalian subtypes have been identified in *Xenopus* but, thus far, mammalian homologues have not been identified (14, 15). Despite the degree of homology between human ET_A and ET_B receptor subtypes, these proteins are pharmacologically distinct. Whereas the ET_A receptor binds ET-3 and sarafotoxin S6c with low affinity and BQ-123 (D-Trp-D-Asp-Pro-D-Val-Leu) and BMS-182874 with high affinity, the ET_B subtype binds ET-3 and sarafotoxin S6c with high affinity and BQ-123 and BMS-182874 with low affinity (12, 13, 16, 17). In addition, although ET_A and ET_B receptors were originally thought to mediate vasocontraction and vasodilation, respectively, it is now recognized that activation of ET_B receptors also leads to contraction of vascular smooth muscle in select circulatory beds. Harrison *et al.* (18) found that ET-3 and sarafotoxin S6c, two ET_B receptor-selective agonists, stimu-

ABBREVIATIONS: ET, endothelin; BPH, benign prostatic hyperplasia; PSC, prostate stromal cell(s); PPET-1, prepro-endothelin-1; FCS, fetal calf serum; PCR, polymerase chain reaction; bp, base pair(s); RT, reverse transcription; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

lated force development in endothelium-denuded pig coronary arteries. The vasoconstrictory responses to sarafotoxin S6c in rabbit saphenous vein (19) and endothelium-denuded porcine coronary artery (16) were not completely reversed by high concentrations of the ET_A receptor antagonist BQ-123, consistent with the suggestion that contraction was mediated by non-ET_A receptors. Pharmacological studies demonstrated that the contractions mediated by non-ET_A receptors were not blocked by PD 142893 (acetyl-D-Phe-D-Phe-Leu-Asp-Ile-Ile-Trp), an antagonist of both ET_A and ET_B receptors, in the rabbit pulmonary artery but were blocked by the same compound in the rat mesentery (20). These data have led to the notion that, in addition to ET_A and ET_B subtypes, a third distinct, but ET_B-like, mammalian ET receptor subtype mediates some non-ET_A receptor contractions of isolated smooth muscle (20).

To reconcile the pharmacology and molecular biology of ET and to identify the nature of the receptor subtypes that mediate the effects of the ET isopeptides in human prostate, we derived a cell line from prostate stroma from patients with BPH. The aim of this study was to characterize the ET receptor subtypes in PSC and to determine the actions of the ETs in these cells.

Materials and Methods

Cell culture. Primary cultures of PSC were obtained using a modification of the technique of Wilding *et al.* (21). Prostate tissue obtained from three patients undergoing transurethral resection of the prostate was minced and digested overnight in dissociation medium [RPMI 1640 medium (Life Technologies, Grand Island, NY), 10% FCS (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 200 units/ml collagenase (type I; Worthington Enzymes, Freehold, NJ), 100 μg/ml DNase I (type II; Sigma Chemical Co., St. Louis, MO)]. After dissociation, the suspension was centrifuged and the pellet was washed and resuspended in Hanks' balanced salt solution and then poured through a 53-μm filter. The flow-through fraction consisted predominantly of PSC, which were collected and plated in RPMI 1640 medium containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. At confluence, the cells were split 1:4. Pure cultures of PSC were obtained after two passages. Stromal cells were verified by positive staining with anti-vimentin antibody and the absence of staining for low molecular weight cytokeratin (22). PSC were maintained in RPMI 1640 medium containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified environment of 95% air/5% CO_2. Experiments were conducted with cells between passages 2 and 12. PSC membranes were prepared by harvesting cells at 4°, followed by homogenization with a Polytron homogenizer (at setting 8) for 30 sec in ice-cold 50 mM Tris·HCl, pH 7.4, with 0.24 units/ml aprotinin and 1 mM EDTA. Crude particulate matter was removed by centrifugation at 750 × *g* for 10 min at 4°. Membranes were sedimented by centrifugation at 48,000 × *g* for 30 min at 4°. Membrane pellets were resuspended in the aforementioned buffer and stored in aliquots at −80° until use.

Radioligand binding. ET receptor binding assays were conducted as described previously (23). Data were analyzed by iterative curve fitting to a one- or two-binding site model, and K_i values were calculated from IC_{50} values (24). Analysis of saturation binding data was performed using nonlinear least-squares curve fitting to the nontransformed data. Linear transformation of data was conducted as described (25). Three separate primary cell line isolates from the prostate stroma were used in subsequent experiments.

Molecular cloning and expression. PSC mRNA and cDNA were purified by using a Micro-Fast Track mRNA kit and cDNA cycle kits (Invitrogen, San Diego, CA). PSC cDNA was amplified by two rounds of PCR using *Thermus aquaticus* polymerase (Boehringer Mannheim) and primers (Genosys, Woodlands, TX) to the full length human ET_B receptor. Under the conditions used, *T. aquaticus* polymerase is estimated to have a rate of base misincorporation of 1/10,000 to 1/50,000. Over the 1300 bases of the ETB cDNA, the overall error rate is ≈0.002%. PCR products were cloned into the pCRII vector (Invitrogen), and transformed colonies (PSC/pCRII) were screened and used for DNA sequencing according to the method of Sanger *et al.* (26). To express PSC/pCRII in mammalian cells, insert DNA was subcloned into the pCDM8 vector (Invitrogen) (yielding PSC/pCDM8). Expression of the PSC/pCDM8 cDNA was conducted by transfection of COS-7 cells using the polycationic lipid Lipofectamine (Life Technologies). Cells were harvested in buffer A (Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.4 at 37°, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor) 48–72 hr after transfection, homogenized, and centrifuged at 100,000 × *g* for 1 hr at 4°. The supernatant was discarded and the membrane pellet was resuspended in buffer A. Membranes were homogenized and stored in aliquots at −80° until used in radioligand binding experiments.

Collagen gel contraction assay. Collagen gel contraction was conducted essentially as described (27). PSC monolayers were prepared by seeding each well of 24-well plates with 2×10^4 cells. The next day, PSC were harvested in serum-free RPMI 1640 medium and mixed with the prepared collagen solution Vitrogen 100 (Celtrix Pharmaceuticals, Santa Clara, CA), at 1×10^6 cells/ml of collagen, and 0.5 ml of the collagen-PSC mixture was placed in each well over the established PSC monolayer. Plates were placed in a non-CO_2 incubator for 60 min for gelation to occur. The collagen gel was then gently separated from the sides of the well with a 22-gauge needle. Each well received 1 ml of serum-free RPMI 1640 medium containing different concentrations of ET-1 or ET-3. The positive control group received RPMI 1640 medium with 10% FCS and the negative control group received serum-free RPMI 1640 medium only. Quantitation of gel contraction was conducted 24 hr after culturing in a humidified environment of 5% CO_2/95% air at 37°, using Image Analyst software. Contraction of the total gel area was expressed as a percentage of the total area in the well.

Expression of PPET-1 in PSC. PSC cDNA, prepared as described above, was analyzed for PPET-1 (the human PPET-1 gene was generously provided by Dr. Thomas Quertermous, Vanderbilt University) by amplification in PCRs using primers designed to amplify a 559-bp fragment in the 3′ untranslated region of PPET-1 mRNA. PCR was conducted for 40 cycles, after which 10 μl of the reaction mixture were electrophoresed on a 2% agarose gel; DNA bands were visualized under UV light.

Statistical analysis. Data are expressed as mean ± standard error. Statistical significance was determined by Student's *t* test.

Results

To elucidate the ET receptor subtypes expressed in PSC, ^{125}I-ET-1 and ^{125}I-ET-3 saturation and competition binding experiments were conducted in primary cell cultures derived from three patients with BPH. Because ET-1 binds to ET_A and ET_B receptors with equal affinity, ^{125}I-ET-1 saturation binding reflects binding to both receptor subtypes. In contrast, ^{125}I-ET-3 binds to ET_B receptors with high affinity but to ET_A receptors with low affinity (K_i values of ≈0.1 and ≈100 nM, respectively) (23). Thus, concentrations of ^{125}I-ET-3 up to 1 nM can be expected to occupy ≤1% of the available ET_A receptors. Specific ^{125}I-ET-1 and ^{125}I-ET-3 binding varied from 40 to 90% over the radioligand concentration range. In all cases, total and nonspecific binding increased in a radioligand concentration-dependent fashion

and resulted in specific binding isotherms consistent with the presence of saturable, high affinity binding sites (Fig. 1). The equilibrium dissociation constant (K_d) was 60 ± 20 pM for ^{125}I-ET-1 and 70 ± 30 pM for ^{125}I-ET-3. Scatchard transformation (Fig. 1, *inset*) of the ^{125}I-ET-1 and ^{125}I-ET-3 saturation data indicated that the receptor densities (B_{max}) for ^{125}I-ET-1 (B_{max} = 320 ± 90 fmol/mg of protein, three experiments) and ^{125}I-ET-3 (B_{max} = 350 ± 85 fmol/mg of protein, three experiments) were not statistically different. Equivalent binding site maxima for ^{125}I-ET-1 and ^{125}I-ET-3 are consistent with the suggestion that PSC express the ET_B receptor subtype.

Further molecular pharmacological investigation of the ET receptor subtypes in PSC was conducted using competition binding experiments. Inhibition of specific ^{125}I-ET-1 (50 pM) binding, defined in the absence and presence of 100 nM ET-1, was evaluated with the nonselective peptide ET-1, two ET_B-selective peptides (ET-3 and sarafotoxin S6c), and the ET_A-selective cyclic pentapeptide BQ-123. The rank order of potency for inhibition of specific ^{125}I-ET-1 binding was ET-1 = ET-3 = sarafotoxin S6c ≫ BQ-123 (Table 1). Similar experiments to inhibit specific ^{125}I-ET-3 (50 pM) binding, defined in the absence and presence of 100 nM ET-3, also revealed a rank order of potency of ET-1 = ET-3 = sarafotoxin S6c ≫ BQ-123 (Table 1).

The functional effects of agonist binding to PSC ET_B receptors were assessed in collagen gel contraction assays. This assay has been previously used to study the *in vitro*, cell-based contraction of fibroblasts in response to ET (28), transforming growth factor-β and colchicine (27), serum (29), and cell culture conditions (30). Guidry and Hook (28) demonstrated that the half-maximally effective concentration (EC_{50}) of ET-1 for stimulation of collagen gel contraction was 30 pM, consistent with the affinity of ET-1 for ET receptors. In the present experiments, PSC in collagen gels were incubated for 24 hr in serum-free medium containing increasing concentrations of agonist peptide. Decreases in collagen gel area induced by ET-1 or ET-3 were compared with those induced by medium containing 10% FCS or serum-free medium. As shown in Fig. 2A, low concentrations (1 nM) of ET-3 consistently stimulated collagen gel contraction, as did medium containing 10% FCS, whereas serum-free medium failed to induce significant contraction of the gel matrix. Quantitation of these gels demonstrated that 1 nM ET-3 was approximately as efficacious as 10% serum in stimulating gel contraction (Fig. 2B). Both ET-1 and ET-3 induced contraction of collagen gels in a concentration-dependent fashion (Fig. 3). EC_{50} values were 0.4 ± 0.04 nM for ET-1 and 0.7 ±

TABLE 1

Inhibition of specific ^{125}I-ET-1 or ^{125}I-ET-3 binding in PSC membranes

Data are the means ± standard errors of the number of experiments given in parentheses.

Competitor	K_i	
	^{125}I-ET-1	^{125}I-ET-3
	nM	
ET-1	0.4 ± 0.1 (3)	0.1 ± 0.01 (4)
ET-3	0.1 ± 0.01 (3)	0.1 ± 0.01 (4)
Sarafotoxin S6c	0.5 ± 0.1 (3)	0.2 ± 0.1 (4)
BQ-123	>10,000 (3)	>10,000 (2)

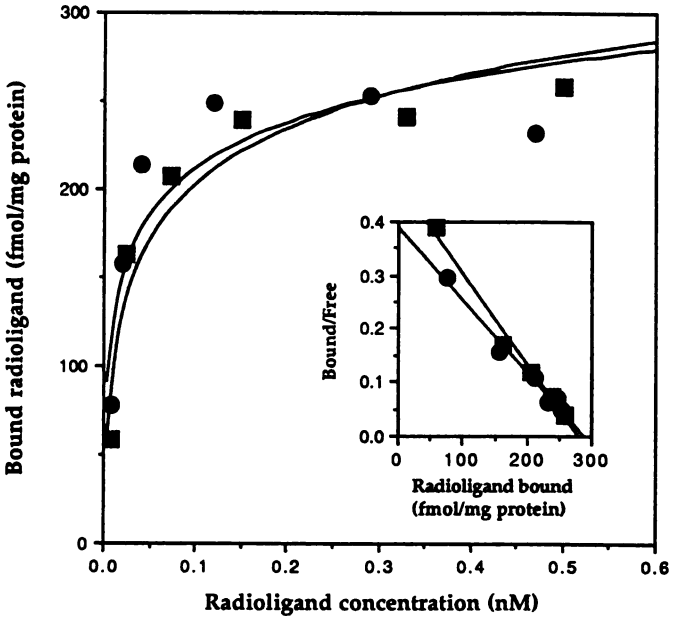

Fig. 1. Saturation binding isotherms and Scatchard transformation (*inset*) of ^{125}I-ET-1 (●) and ^{125}I-ET-3 (■) binding in PSC. Specific binding was defined in the presence of 100 nM ET-1 or 100 nM ET-3, respectively. Data points are the means of duplicate or triplicate samples, and the figure is representative of three similar experiments.

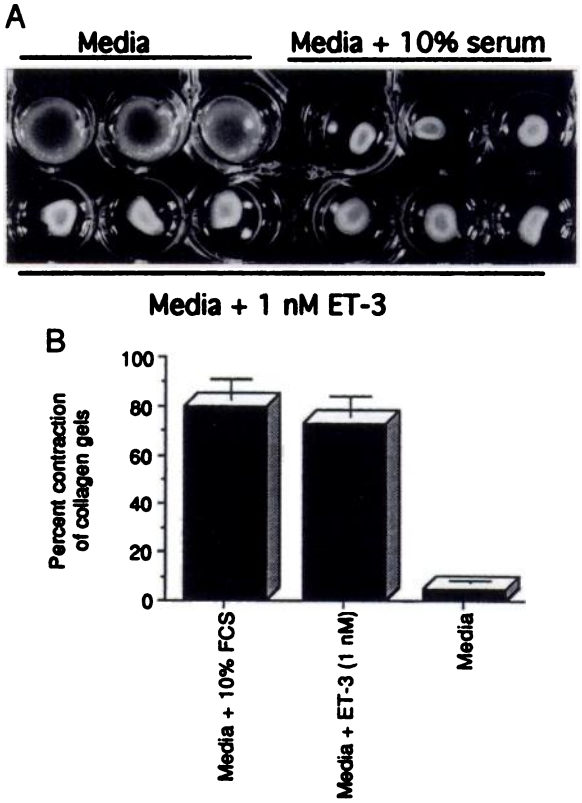

Fig. 2. PSC contraction of collagen gels induced by ET. Cells were seeded in collagen gels as described in Materials and Methods and were treated with ET-1 or ET-3 for 24 hr. Contraction of the gel area was expressed as a percentage of the total area in the well. A, Photograph of contraction of collagen gels (in a six-well plate) induced by 1 nM ET-3, compared with that induced by medium containing 10% FCS or serum-free medium. B, Quantitation of photograph in A. Data points are the means of three to six wells, and the figure is representative of three similar experiments.

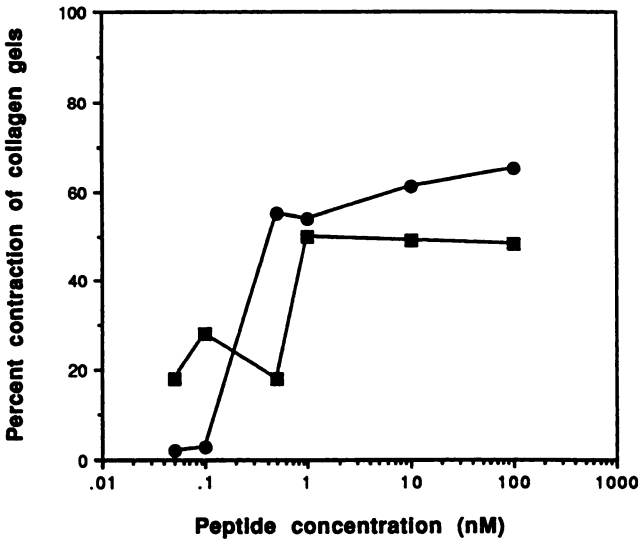

Fig. 3. Effect of increasing concentrations of ET-1 (●) and ET-3 (■) on contraction of collagen gels. Data points are the means of triplicate wells, and the figure is representative of three similar experiments.

0.2 nM for ET-3 (three experiments). In contrast, treatment of rat mesangial cells, which express the ET_A receptor subtype,[1] with 1 nM ET-1 also caused a reduction in collagen gel area, whereas 1 nM ET-3 was without effect (data not shown). Moreover, collagen gels treated with ET in the absence of cells did not contract (data not shown).

Collectively, these data indicated that PSC contain ET_B or ET_B-like receptors that mediate contraction. However, despite this pharmacological receptor characterization, it remained possible that the primary sequence of the receptor was altered in a way that was not distinguishable by differential ligand affinities. Indeed, one interpretation of functional evidence from isolated tissue preparations is that a putative third ET receptor, which has binding properties indistinguishable from those of the ET_B subtype, exists in mammals. Therefore, to discern the molecular identity of the ET_B-like receptor in PSC that mediated collagen gel contraction, RT-PCR was conducted with oligonucleotide primers to the 5′ and 3′ ends of the coding sequence for the full length, 1.3-kilobase, human ET_B receptor cDNA. We reasoned that, because the pharmacology and molecular pharmacology of the contractile ET_B-like receptor were similar to those of the ET_B subtype, the molecular structure of a putative third ET receptor subtype may also resemble this subtype. Agarose gel electrophoresis of the PCR products resolved a single discrete band of approximately 1.3 kilobases. Restriction analysis of DNA from ≈10 individual bacterial colonies with several 4-base cutters revealed similar restriction maps. A single colony, PSC/C3, was grown for further analysis. Nucleotide sequencing of PSC/C3 revealed a DNA sequence that was 99% identical to that of the ET_B receptor cDNA cloned from human liver (31), placenta (32), and jejunum (33). In the 1326-bp coding region, two nucleotides deviated from previously reported ET_B sequences, resulting in two conservative substitutions in the predicted 442-amino acid protein. Comparison of the deduced amino acid sequences of the human liver, placenta, jejunum, and prostate ET_B receptors shows that the prostate ET_B receptor has a lysine replacement for an arginine in the amino terminus and a valine replacement for isoleucine in the second transmembrane region (Fig. 4). As with other human ET_B receptor cDNAs, the nucleotide sequence of the PSC cDNA predicts that the encoded protein contains an amino-terminal, 26-amino acid, signal sequence (34), a site for *N*-linked glycosylation at asparagine 59 (35), seven hydrophobic regions presumed to be transmembrane regions, six cysteines in the carboxyl-terminal tail that may be sites for palmitoylation (36), and several serine residues in the intracellular loop regions and carboxyl-terminal tail that may serve as substrates for serine/threonine protein kinases and may be involved in receptor desensitization (37) (Fig. 4).

Transient expression of the PSC/C3 cDNA-encoded protein in COS-7 cells conferred specific ^{125}I-ET-1 binding to otherwise null cells. Inhibition of specific ^{125}I-ET-1 binding to membranes from transfected COS-7 cells demonstrated that the encoded protein had high affinity for the ET isopeptides ET-1, ET-3, and sarafotoxin S6c, but not for BQ-123 (Table 2). As in the parent cell line, the expressed protein had a rank order of potency of ET-1 = ET-3 = sarafotoxin S6c ≫ BQ-123.

The presence of ET receptors in PSC raised the question of whether ET could be an autocrine effector. To address this question, RT-PCR was conducted using oligonucleotide primers to the human PPET-1 gene. These primers were designed to amplify a 560-bp region of PPET-1 DNA. As shown in Fig. 5, a discrete product of ≈560 bp was resolved from template cDNA reverse transcribed from cells from two patients with BPH or from the control PPET-1 DNA. This result is in agreement with preliminary radioimmunoassay assay data that indicate the presence of 1- 6 pg/ml ET-1 in the culture medium from prostate cells (data not shown).

Discussion

The finding that ET_B receptor-selective agonists generated force as well as endothelium-dependent relaxation of smooth muscle (18–20, 38) led to the suggestion that a molecularly distinct, but ET_B-like, receptor subtype mediates non-ET_A receptor contractions in select isolated smooth muscle preparations. ET receptors have been shown to mediate contraction of isolated human prostate tissue strips (7, 10). Contractions induced by ET-1, but not sarafotoxin S6c, were inhibited by BQ-123, consistent with both ET_A and non-ET_A receptor-mediated actions of these agonists. In light of these findings and the paracrine activity of ET (39), the molecular nature and cellular distribution of ET receptor subtypes become critical for the elucidation of ET effects in tissues. In the present study, we provide evidence that PSC derived from three patients with BPH express an apparently homogeneous population of ET_B receptors that mediate ET-induced contraction of these cells.

Several lines of evidence demonstrate that PSC express a homogeneous population of ET_B receptors. Saturation binding with ^{125}I-ET-1 and ^{125}I-ET-3 indicates that these radioligands label equivalent numbers of ET receptors. Because ^{125}I-ET-3 occupies <1% of the available ET_A receptors over the concentration range used in this study, the ^{125}I-ET-3 binding site maximum reflects the quantity of ET_B sites. Moreover, because ^{125}I-ET-1 binds with high affinity to both ET receptor subtypes, equivalent receptor densities for these two radioligands indicate that the ET_B population accounts for all of the ^{125}I-ET-1 binding sites. The saturation binding

[1] M. L. Webb and E. C. K. Liu, unpublished observations.

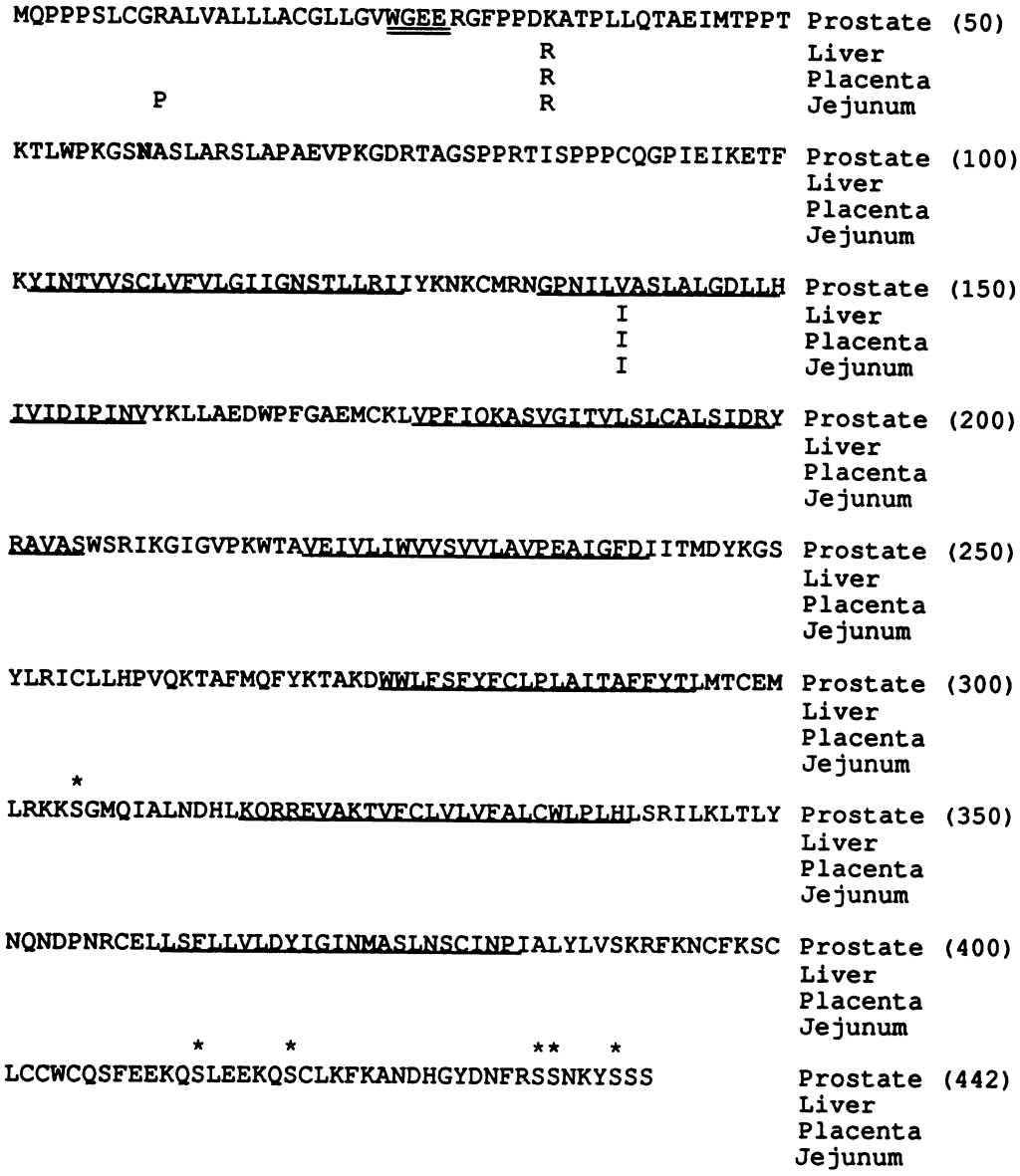

Fig. 4. Homology of the deduced amino acid sequences of human ET_B receptor cDNAs cloned from prostate, liver, placenta, and jejunum. Amino acids that differ from the prostate sequence are shown according to the single-letter code. The putative transmembrane domains of the prostate ET_B receptor are *underlined*. The putative signal sequence cleavage site, according to von Heijne's algorithm, is shown by *double underlining*. Asparagine 59, the predicted site for *N*-linked glycosylation, is shown in *bold*. *, Sites of potential phosphorylation.

TABLE 2

Inhibition of specific ^{125}I-ET-1 binding in membranes from COS-7 cells transiently transfected with PSC/C3 cDNA

Inhibition constants are shown as means ± standard errors of the number of experiments given in parentheses.

Competitor	K_i	Slope factor
	nM	
ET-1	0.8 ± 0.07 (4)	1.2 ± 0.1 (4)
ET-3	0.1 ± 0.06 (3)	0.9 ± 0.2 (3)
Sarafotoxin S6c	0.8 ± 0.5 (4)	1.1 ± 0.2 (4)
BQ-123	≥50,000 (4)	1.0 ± 0.5 (3)

data are in agreement with the competition binding data, where the ET_B receptor-selective ligands ET-3 and sarafotoxin S6c were potent inhibitors of ^{125}I-ET-1 binding and the ET_A receptor-selective pentapeptide BQ-123 was a weak inhibitor. Numerous studies have reported that ET_A receptors have a rank order of potency of ET-1 ≫ ET-3 ≫ sarafotoxin S6c, whereas the ET_B receptor rank order of potency is ET-1 = ET-3 = sarafotoxin S6c (12, 13, 40). Consistent with the data reported here for PSC, we previously found that the K_i values for ET-1, ET-3, and sarafotoxin S6c at recombinant human placental ET_B receptors were 0.1, 0.2, and 0.1 nM, respectively (41). Kobayashi *et al.* (11) reported that approximately 5 times more ET_B than ET_A receptors were found in the prostate epithelium and, conversely, 2.5 times more ET_A than ET_B receptors were in the stroma. The prostate stroma is a heterogeneous tissue composed of smooth muscle cells, fibroblasts, and connective tissue elements. Immunohistochemical studies demonstrated that the stromal cell cultures used for the present studies were a pure population of mesenchymal cells (data not shown). The proportion of smooth muscle cells and fibroblasts was not quantified, and it may be that the cells used in this study are predominantly fibroblasts.

Another line of evidence that PSC express ET_B receptors was provided by assessment of the functional role of ET receptors in these cells in collagen gel contractility experiments. Several studies have utilized the contraction of collagen gels with epithelial cells or fibroblasts to assess cell function (27–30). In addition to ET-1, transforming growth

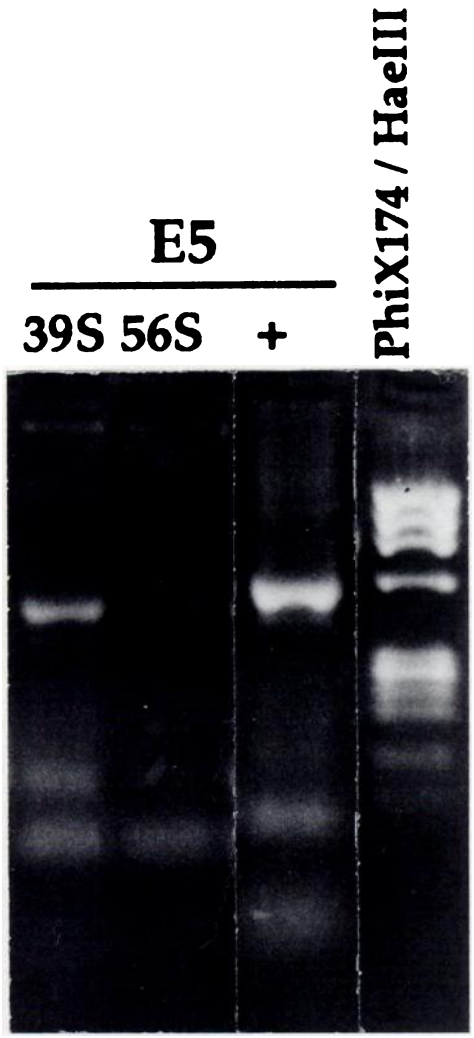

Fig. 5. Expression of PPET-1 mRNA in PSC from patients with BPH. Cells were analyzed for the presence of PPET-1 mRNA by RT-PCR, as described in Materials and Methods. Primers (*E5*) designed to amplify a 560-bp region of human PPET-1 DNA were used for PCR amplification of cDNA reverse transcribed from RNA prepared from cells from patients 39S and 56S. As a positive control (+), these primers were used to amplify sequences from the cloned human PPET-1 gene. DNA size markers are HaeIII restricted PhiX174.

factor-β and platelet-derived growth factor have been found to stimulate contraction of collagen gels by fibroblasts (27, 28). In the present study, ET-1 and ET-3 treatment of PSC stimulated collagen gel contraction with potencies consistent with ET_B receptor-mediated effects (EC_{50} for ET-1, 0.4 nM; EC_{50} for ET-3, 0.7 nM). These data are consistent with the suggestion that agonist binding to PSC ET_B receptors leads to collagen gel contraction. Preliminary experiments to study the effects of selective and nonselective ET receptor antagonists support the suggestion that contraction is ET_B subtype mediated.[2]

Lastly, the molecular nature of the ET receptor subtype in these cells was assessed by RT-PCR. The nucleotide sequence of the PSC/C3 cDNA is 99% homologous to those of human ET_B receptor cDNAs previously cloned from liver (31), jejunum (33), and placenta (32). Expression of PSC/C3 cDNA in COS-7 cells revealed that the encoded protein conferred high affinity binding for ET-1, ET-3, and sarafotoxin S6c, but not BQ-123, to COS-7 cells. Thus, the binding profile of COS-7 cells transfected with PSC/C3 cDNA is similar to that of the parent primary cell line. Taken together, the pharmacological and molecular data indicate that ET_B receptors are the predominant ET receptor subtype expressed in PSC and that the ET_B receptor mediates collagen gel contraction.

This is the first molecular characterization of the ET receptor subtype that mediates ET-3- or sarafotoxin S6c-induced contraction. Our data show that, of the 442 amino acids in the human ET_B receptors cloned from liver, placenta, and jejunum, two conservative substitutions, i.e., lysine for arginine in the amino termimus and valine for isoleucine in the second transmembrane region, appear in the PSC/C3 ET receptor cDNA characterized here. This is similar to work reported by Sakamoto *et al.* (33), in which the sequence of the human jejunum ET_B receptor has a single amino acid divergence, arginine to proline, in the amino terminus. Previously, Beinborn *et al.* (42) showed that substitution of valine in the sixth transmembrane region of the cholecystokinin-B/gastrin receptor with leucine altered benzodiazepine-based antagonist binding. Although this result demonstrates that variability in the aliphatic side chain of an amino acid residue can indeed alter receptor molecular pharmacology, no alterations in the binding of agonists to the PSC/C3 cDNA-encoded protein were observed. Given that only two ET receptor genes have been found in the human genome (43, 44), it seems unlikely that the PSC/C3 cDNA encodes a third ET receptor subtype that is related to the A and B subtypes. It should also be noted that, although a panoply of receptor subtypes for aminergic ligands exist, the size of receptor families for peptide hormones has thus far remained considerably smaller (45). In light of these data, and because the differences in PSC/C3 cDNA could not be explained by sequencing errors, a second interpretation, that the divergences noted in the PSC/C3 cDNA sequence represent an error in the PCR or allelic variance, seems more likely. Thus, the observed divergences probably do not represent significant alterations to previously cloned ET_B receptors, consistent with the suggestion that ET_B receptors mediate contraction in PSC. However, these data do not exclude the possibility that a molecularly distinct ET receptor subtype mediates vasoconstriction in other tissues where ET_B-selective agonists have been shown to stimulate contraction through non-ET_A receptors. As an example, sarafotoxin S6c-induced contractions of the rabbit pulmonary artery are not blocked by BQ-123 or PD 142893, whereas the sarafotoxin S6c-induced relaxation of rat mesentery is blocked by PD 142893 but not BQ-123 (20). These data are consistent with the suggestion that non-ET_A receptors mediate these contractions, but the molecular nature of the ET_B-like receptor is unknown.

The paracrine and autocrine nature of ETs is well known. Evidence that ET-1 immunoreactivity was prominent in the glandular epithelium of human prostate (7) raised the question of whether PSC responded to ET-1 released by epithelial cells or whether PSC had the capacity to produce ET-1. Our finding that the PPET-1 gene is expressed in PSC from patients with BPH confirms and extends previous results that demonstrated immunoreactive ET in the glandular epithelium of human prostate (7) and suggests that ET may play a paracrine or autocrine role in prostate cells.

[2] M. L. Webb and C-c. Chao, unpublished observations.

In conjunction with earlier reports (7, 10, 11), the present data indicate that ET mRNA and protein are made in the prostate and that ET receptors are active in prostate. To date, the data available have shown that ET stimulates contraction of the prostate, but it is well documented that ET is mitogenic for smooth muscle cells and fibroblasts (46, 47). Thus, it will be important to determine whether ET acts as a mitogen in the prostate. Although it remains to be determined whether ET is involved in the etiology or symptomatology of BPH, one can speculate that the encapsulated nature of the prostate leads to elevated local concentrations of ET, resulting in proliferation of prostate cells as well as enhanced prostate contractility. In summary, the evidence provided here is consistent with the expression of ET_B receptors that mediate contraction in PSC derived from patients with BPH, as well as with a role for ET in prostate function and possibly in prostate disease.

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