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Endothelin receptors and their cellular signal transduction mechanism in human cultured prostatic smooth muscle cells

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1 Endothelin (ET) receptors, and their cellular signal transduction mechanism, were characterized in a primary culture of human prostatic smooth muscle cells (HP cell).

2 [^{125}I]-ET-1 and [^{125}I]-ET-3 binding studies revealed that both ET_A and ET_B receptors were present in the HP cells, and the ratio of ET_A to ET_B receptors was 1.4:1.

3 Analysis of ET receptor mRNA by reverse transcription-polymerase chain reaction also demonstrated that HP cells express both ET_A and ET_B receptors.

4 ET-1 and ET-3 increased intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the HP cells in a concentration-dependent manner. Use of subtype selective antagonists BQ-123 and BQ-788, indicated that both ET_A and ET_B receptors were coupled to an increase in $[Ca^{2+}]_i$.

5 Pretreatment of the cells with pertussis toxin resulted in a significant but partial attenuation of the $[Ca^{2+}]_i$ increase mediated through the ET_A and ET_B receptors. However, sensitivity to pertussis toxin (PTX) was significantly different between them.

6 In conclusion, HP cells possess ET_A and ET_B receptors. Further, these two endothelin receptor subtypes evoke an increase in $[Ca^{2+}]_i$ possibly via the action of different GTP-binding proteins.

Keywords: Prostate; smooth muscle cell; endothelin receptor; intracellular Ca^{2+}; GTP-binding proteins; signal transduction

Introduction

Endothelin-1 (ET-1), isolated from culture medium of porcine vascular endothelial cells, was initially identified as a potent vasoconstrictor peptide (Yanagisawa *et al*., 1988). It is one of a family of three isopeptides (ET-1, ET-2, and ET-3) collectively termed endothelins (ETs). ETs have a variety of important biological activities in both vascular and nonvascular tissues, including cardiac, pulmonary and renal effects (Ishikawa *et al*., 1988; Advenir *et al*., 1990; Harris *et al*., 1991), modulation of neural function (Stojikovic *et al*., 1990; Samson *et al*., 1991) and cell mitogenesis (Imokawa *et al*., 1992; Simonson & Herman, 1993; Kanse *et al*., 1995). These activities are exerted through cell surface ET receptors. On the basis of the binding profile of ETs, three types of ET receptors (ET_A, ET_B and ET_C) have been identified, cloned and characterized (Sakurai *et al*., 1990; Arai *et al*., 1993; Karne *et al*., 1993). The ET_A receptor has much greater affinity for ET-1 and ET-2 than ET-3, the ET_C receptor for ET-3 than ET-1 and ET-2, whereas the ET_B receptor has no isopeptide selectivity. These receptors belong to a family of GTP-binding protein (G protein) coupled receptors.

Kobayashi *et al* showed that [^{125}I]-ET-1 binding sites were present in human prostate (1994a), and that both ET_A and ET_B receptors mediate potent contractions of prostate (1994b). Prostate is composed of stroma and epithelial components. Smooth muscle cells existing in the stroma represent 22% of total prostate area (Shapiro *et al*., 1992) and mediate prostate contraction. However, the endothelin receptor in the prostatic smooth muscle cell and its intracellular signal transduction mechanism have not yet been characterized.

We recently established a primary culture of smooth muscle cells from human prostate (HP; Yazawa *et al*., 1994). In the present study, we characterized the ET-receptor subtypes in the cells and investigated the intracellular signal transduction mechanism. We conclude that ET_A and ET_B receptors are expressed in HP cells, and that both subtypes evoke an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) possibly via the action of different G proteins.

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Methods

Cell culture

A primary culture of smooth muscle cell from human prostate (HP cell) was obtained by an explant method as described previously (Yazawa *et al*., 1994). Cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 2.5 μg ml^{-1} testosterone propionate, 1 μg ml^{-1} hydrocortisone, 5 μg ml^{-1} insulin, 5 μg ml^{-1} human transferrin, 5 ng ml^{-1} sodium selenite, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in a 5% CO_2 incubator. In this study, we used cells in passages 6 to 20. There was not any significant difference in results in passages below 20.

Membrane preparation

Cells were collected with a scraper into phosphate-buffered saline and centrifuged at $800 \times g$ for 4 min. The collected cells were homogenized with a ultrasonication homogenizer (TAITEC VP-30s; setting output 7 for 3 s × 6 times) in 250 mM sucrose, 50 mM Tris-HCl (pH = 7.4) and 10 mM $MgCl_2$ at 4°C, and centrifuged at $1000 \times g$ for 15 min at 4°C. The supernatant was again centrifuged at $40{,}000 \times g$ for 20 min at 4°C. The resulting pellets were suspended in 50 mM Tris-HCl (pH = 7.4), 10 mM $MgCl_2$ with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at $40{,}000 \times g$ for 20 min at 4°C. The final pellet was resuspended in 50 mM Tris-HCl (pH = 7.4), 10 mM $MgCl_2$ and stored at −80°C until use.

Binding assay

The binding assay was carried out at 25°C for 5 h in 0.5 ml of incubation buffer (50 mM HEPES (pH = 7.4), 10 mM $MgCl_2$,

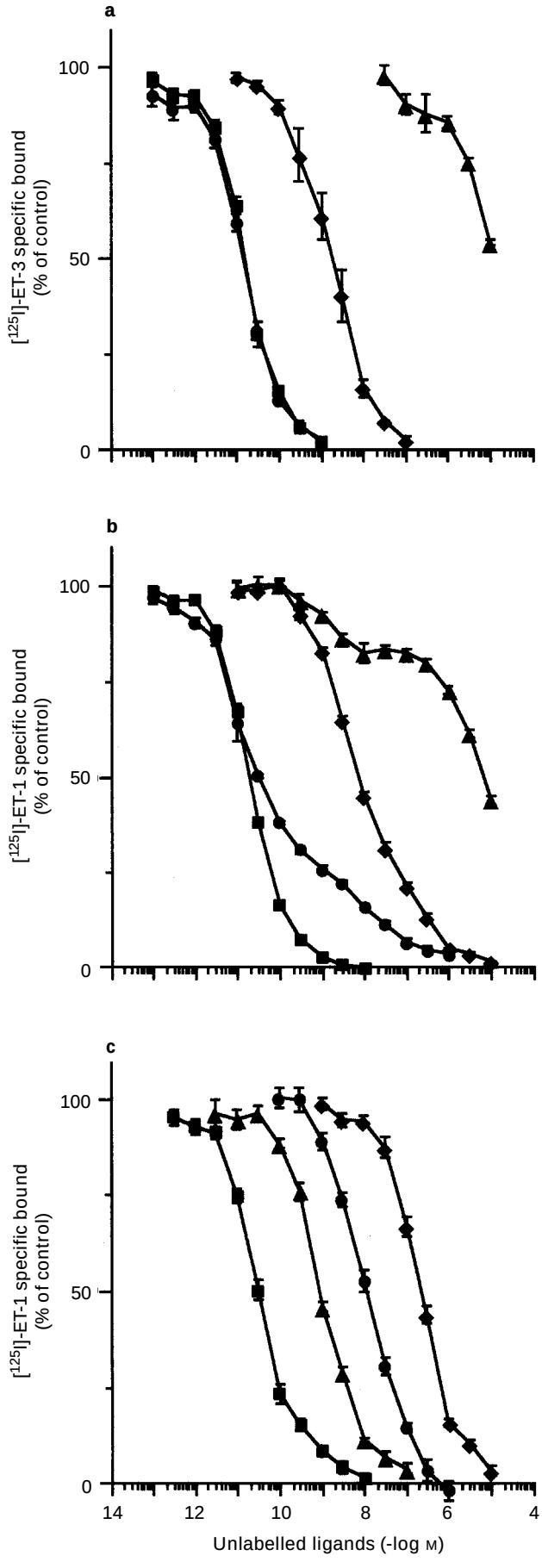

Figure 1 Concentration-dependent inhibition of binding of 10 pM [^{125}I]-ET-3 (a), 10 pM [^{125}I]-ET-1 (b) and 10 pM [^{125}I]-ET-1 in the presence of unlabelled 1 nM ET-3 (c) to membranes of a primary culture of smooth muscle cells from human prostate by unlabelled (■) ET-1, (●) ET-3, (◆) BQ-788 and (▲) BQ-123. Results are expressed as a percentage of control (specific binding in the absence of unlabelled ligand). Control c.p.m. values in (a), (b) and (c) were 3430 ± 340, 2580 ± 340 and 1070 ± 170 ($n = 7$), respectively. Each point represents mean of 3 separate experiments; vertical lines show s.e.

0.1% (w/v) BSA, 0.17 mg ml^{-1} bacitracin, 3 μM phenylmethylsulphonyl fluoride and 0.22 Tiu ml^{-1} aprotinin). The membranes (2 μg protein) were incubated with 10 pM [^{125}I]-ET-1 or [^{125}I]-ET-3 for competition experiments in the presence and absence of various concentrations of unlabelled ligands. Specific saturable binding was investigated by adding increasing concentrations of [^{125}I]-ETs to the membranes. Incubation was terminated by rapid filtration through Whatman GF/C filters (Maidstone, Kent, U.K.) by use of a Brandel cell harvester (Gaithersburg, MD, U.S.A.). The filter was then rinsed 6 times with 3 ml of ice-cold 50 mM Tris-HCl (pH = 7.4) and 0.01% BSA. Radioactivity retained on filters was counted with a γ-counter. Nonspecific binding was determined in the presence of 0.1 μM ET-1 or ET-3. Nonspecific binding was subtracted from total binding and the difference was defined as specific binding.

Analysis of endothelin receptor mRNA by polymerase chain reaction (PCR)

Total RNA from HP cells was isolated by the acid guanidium thiocyanate/phenol/$CHCl_3$ method (Chomczynski & Sacchi, 1987), with ISOGEN (Nippon Gene, Tokyo, Japan) and 1 μg of total RNA was reverse transcribed by use of random hexamer as a primer with Super-Script (Gibco, Grand Island, NY). Amplification of cDNA was performed by 35 cycles of polymerase chain reaction. The reaction cycles were 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The following primers were used: for ET_A receptor, upstream sense primer (nucleotides 35–54) and downstream antisense primer (nucleotides 642–661). For ET_B receptor upstream sense primer (nucleotides 123–142) and downstream antisense primer (nucleotides 600–619). No cross-amplification was observed under these conditions. ET_A receptor cDNA has restriction enzyme *Hinc*II cleavage site at nucleotide 545 and ET_B receptor cDNA has restriction enzyme *Sph*I cleavage site at nucleotide 398. Digestion of the ET_A receptor cDNA with *Hinc*II would produce 510 and 117 bp fragments, while digestion of the ET_B receptor cDNA with *Sph*I would produce 275 and 222 bp fragments. The PCR products and their digested fragments were electrophoresed to a 3% agarose gel and stained with ethidium bromide.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in HP cells was measured by fura-2 fluorometry as described previously (Saita *et al.*, 1994). Briefly, cells were grown to confluency on a cover glass and medium was changed to serum-free RPMI 1640 for 24 h before the experiments. The pertussis toxin (PTX) treatment of cells was carried out by incubating the cells with the indicated concentrations of PTX for 12 h at 37°C in a 5% CO_2 incubator before the addition of ET ligands. Cells were washed twice with HBSS (composition in mM: HEPES-NaOH (pH = 7.4) 2.0, NaCl 140, KCl 4, K_2HPO_4 1, $MgCl_2$ 1, $CaCl_2$ 1 glucose 10 and BSA 0.05%) and then loaded with 4 μM fura2-AM in HBSS for 30 min at 37°C. The cells were then subjected to the indicated concentrations of ET agonists or antagonists.

In vitro *ADP-ribosylation*

To estimate the effectiveness of PTX treatment under our experimental conditions, *in vitro* ADP-ribosylation was performed in the presence of [^{32}P]-nicotinamide adenine dinucleotide (NAD) in membrane either treated or untreated with PTX. The membranes (20 μg) were incubated with

10 μg ml^{-1} preactivated PTX for 40 min at 30°C in 50 mM Tris-HCl (pH = 7.5), 1 mM EDTA, 10 mM thymidine, 2.5 mM $MgCl_2$, 15 mM isonicotinohydrazide and 2.5 μM [^{32}P]-NAD in a reaction volume of 50 μl. Membrane was solubilized in the sodium dodecyl sulphate (SDS)-sample buffer and analysed by 10% SDS polyacrylamide gel electrophoresis followed by autoradiography.

Analysis of data

Results are expressed as the mean ± s.e. Competition curves for the radioligand binding experiments were analysed by nonlinear least-squares regression analysis as provided in the software program ReceptorFit Competition (Lundon Software, OH, U.S.A.). When a statistical difference between two means was determined, an unpaired two-tailed Student's *t* test was performed; for multiple comparisons a one-way analysis of variance followed by Dunnet's multiple comparison test was used. Probabilities of less than 5% ($P<0.05$) were considered to be significant.

Materials

Plasmids harboring human ET receptor were the kind gift of Prof. Goto (Tsukuba University, Japan). The following drugs were used: [^{125}I]-ET-1 (81.4 TBq mmol^{-1}) and [^{32}P]-NAD (1.11 TBq mmol^{-1}) were purchased from New England Nuclear (Boston, MA, U.S.A) and [^{125}I]-ET-3 (74 TBq mmol^{-1}) was from Amersham (Bucks, U.K.). ET-1, ET-3, sarafotoxin S6c and IRL 1620 (Suc-[Glu^{9}, Ala^{11,15}]endothelin-1-(8-21)) were from Peptide Institute (Osaka, Japan); fura-2-acetoxymethylester (fura2-AM) from Dohjin Chemicals (Kumamoto, Japan). U-73122 ({1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione}) from Biomol (Plymouth Meeting, PA, U.S.A.); BQ-123 (cyclo(D-Trp-D-Asp (ONa)-Pro-D-Val-Leu) and BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γmetyl Leu-D-Trp-(COOMe)-D-Nle-ONa) were synthesized by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Other chemicals used were of analytical grade.

Results

ET_A and ET_B receptor binding studies

Binding sites for [^{125}I]-ET-1 and [^{125}I]-ET-3 were detected in the membranes of cultured smooth muscle cells from human prostate (HP cells). Binding was time- and temperature-dependent with submaximal binding obtained at 5 h at 25°C (data not shown). Nonspecific binding was always less than 15% of total binding. Competitive binding experiments are shown in Figure 1). The binding of 10 pM [^{125}I]-ET-3, which mainly binds to ET_B receptors, to cell membranes was inhibited by ET-1 and ET-3 in a dose-dependent manner with similar affinities, and the apparent K_d values for ET-1 and ET-3 were 6 and 5 pM, respectively (Figure 1a, and Table 1). These results demonstrate the presence of ET_B receptors in the cell membrane. The ET_A receptor-selective antagonist, BQ-123, and the ET_B receptor-selective antagonist, BQ-788, also showed monophasic competition. However, BQ-123 had approximately 1,000,000 fold weaker affinity than either ET-1 or ET-3, and approximately 5,000 fold weaker affinity than BQ-788 as judged by apparent K_d values.

[^{125}I]-ET-1 is a common ligand for both ET_A and ET_B receptors. ET-1 showed monophasic competition against [^{125}I]-ET-1 binding, but ET-3 showed biphasic competition (Figure 1b). ET-3 displaced [^{125}I]-ET-1 binding with the same affinity as ET-1 at concentrations from 0.1 pM to 10 pM, indicating that ET_B receptor was responsible for binding. ET-3 required about 100 fold higher concentrations than ET-1 to displace the remaining [^{125}I]-ET-1 binding, indicating that the ET_A receptor was responsible for binding. K_d values of ET-3 for the ET_B and ET_A receptor were 6 and 6,500 pM, respectively (Table 1). BQ-123 also showed biphasic inhibition of [^{125}I]-ET-1 binding. The curve for BQ-788 fit with a two-site rather than a one-site binding model in the analysis of a least squares curve-fitting programme (Figure 1b). K_d values of BQ-123 for the ET_A and ET_B receptor were 510 pM and 7.1 μM, while those of BQ-788 were 160 and 1.7 nM, respectively (Table 1).

To characterize the binding to the ET_A receptor only, 1 nM ET-3 was added to mask the ET_B receptor binding sites for [^{125}I]-ET-1 (Figure 1c). ET-3 1 nM almost completely inhibited the binding of [^{125}I]-ET-3 to the ET_B receptor (Figure 1a). Therefore, this condition primarily reflects [^{125}I]-ET-1 binding to the ET_A receptor only. Under this condition, [^{125}I]-ET-1 binding was inhibited monophasically by each ET ligand, with K_d values for ET-1, ET-3, BQ-123, and BQ-788 of 21, 10,000, 930, and 190,000 pM, respectively (Table 1).

Analyses of saturable binding for the ET_A and ET_B receptor are shown in Figure 2a and b, respectively. Scatchard plots of specific binding are shown in insets. Saturable binding of [^{125}I]-ET-1 in the presence of 1 nM ET-3 represented a single class of

Table 1. Dissociation constants and maximum binding capacities of endothelin receptors in human prostatic cell membranes

		Apparent K_d (pM)	
Ligand	*Competitor*	*ET_A receptor*	*ET_B receptor*
[^{125}I]-ET-1	ET-1	ND	ND
	ET-3	6,500 ± 2,200	6 ± 0
	BQ-123	510 ± 160	7,100,000 ± 2,100,000
	BQ-788	160,000 ± 18,000	1,700 ± 180
[^{125}I]-ET-3	ET-1		6 ± 2
	ET-3		5 ± 1
	BQ-123		5,800,000 ± 2,400,000
	BQ-788		1,100 ± 320
[^{125}I]-ET-1 + ET-3 (1 nM)	ET-1	21 ± 6	
	ET-3	10,000 ± 1,800	
	BQ-123	930 ± 42	
	BQ-788	190,000 ± 31,000	
	Ligand	K_d (pM)	B_{max} (fmol mg^{-1} protein)
ET_A receptor	[^{125}I]-ET-1 + ET-3 (1 nM)	70 ± 9	1400 ± 50
ET_B receptor	[^{125}I[-ET-3	10 ± 2	1000 ± 100**

Apparent K_d values were obtained by competition experiments. Competition of ET-3, BQ-123 and BQ-788 for [^{125}I]-ET-1 binding were fitted with a model having two rather than one binding site. Data are the mean ± s.e.mean of at least three separate experiments. ND, not determined because the data were not fitted by a two binding site model. K_d and B_{max} values were obtained by saturation experiments. **$P<0.01$ compared with the data obtained with B_{max} of ET_A receptor ($n=4$).

binding sites for the ET_A receptor with a K_d of 70 ± 9 pM and a B_{max} of 1400 ± 50 fmol mg^{-1} protein (Figure 2a and Table 1). Saturable binding of [^{125}I]-ET-3 represented a single class of binding sites for the ET_B receptor with a K_d of 10 ± 2 pM and a B_{max} of 1000 ± 100 fmol mg^{-1} protein (Figure 2b and Table 1).

Existence of ET_A and ET_B receptor mRNAs in HP cells

To confirm the expression of the ET_A and ET_B receptors in HP cells, we tried to detect their mRNAs. Total RNA were isolated from HP cells and reverse transcribed to obtain HP cell cDNA as a PCR template. PCR amplification of the HP cell cDNA with ET_A and ET_B receptor-specific primer pairs yielded a single cDNA band of 627 and 497 bp, respectively, which was consistent with PCR products of the cloned human ET_A and ET_B receptor cDNA (Figure 3, lanes 1–4). PCR products of HP cell cDNA amplified with the ET_A and ET_B receptor-specific primer pairs were digested with *Hinc*II or *Sph*I, respectively. The digestion with *Hinc*II and *Sph*I produced theoretical 510 and 117 bp and theoretical 275 and 222 bp, respectively (Figure 3, lanes 6 and 7). Restriction mapping of the cDNA product was consistent with the ET_A and ET_B receptor subtype (Figure 3, lanes 5–8). These results also indicate that both ET_A and ET_B receptors are expressed in HP cells.

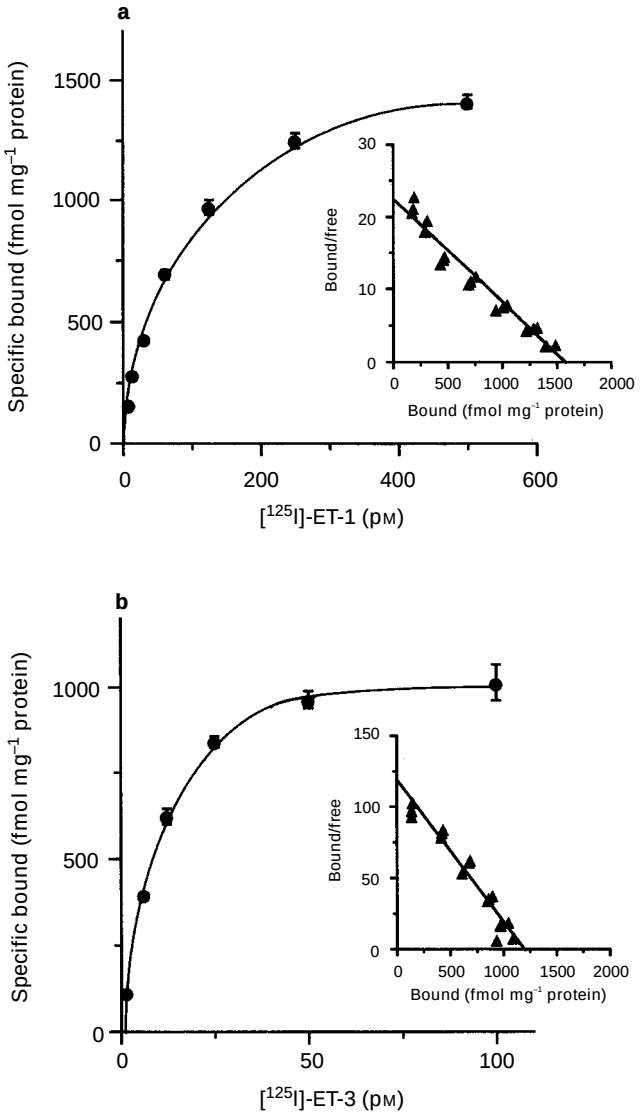

Figure 2 Saturable binding of [^{125}I]-ET-1 in the presence of unlabelled 1 nM ET-3 (a), and [^{125}I]-ET-3 (b) to membranes of primary culture of smooth muscle cells from human prostate. Scatchard plots of specific binding are shown in insets. Values are means of triplicate determinations. Similar results were obtained from at least 4 separate experiments.

$[Ca^{2+}]_i$ increase mediated through ET_A and ET_B receptors

We investigated the effect of ETs on $[Ca^{2+}]_i$ in HP cells. ET-1 and ET-3 elicited an increase in $[Ca^{2+}]_i$ in the cells (Figure 4a and b). This increase was composed of two phases, a transient increase in $[Ca^{2+}]_i$ that finished within 2 min, and a sustained increase that lasted over 20 min. Removal of extracellular Ca^{2+} had little effect on the transient phase, but abolished the sustained phase. These results suggest that the source of Ca^{2+} in the transient phase was from intracellular Ca^{2+} stores, while that of the sustained phase was from an influx of extracellular Ca^{2+}. Furthermore, the $[Ca^{2+}]_i$ response to ET-1 was inhibited by the phospholipase C (PLC) inhibitor U-73122 (data not shown). We therefore suggest that ET receptors in this cell evoke a $[Ca^{2+}]_i$ increase via PLC.

Effects of ET-1 and ET-3 on the initial transient increase in $[Ca^{2+}]_i$ are shown in Figure 5. ET-1 and ET-3 elicited an increase in $[Ca^{2+}]_i$ in a dose-dependent manner, with ED_{50} values of 3.4 ± 0.1 nM and 2.8 ± 0.4 nM, respectively. Submaximal effects were obtained at 10 nM ET-1 and ET-3. However, peak $[Ca^{2+}]_i$ response to 10 nM ET-1 was approximately 1.5 times higher than that to 10 nM ET-3. The ET_B receptor-selective agonist sarafotoxin S6c gave a similar magnitude of response as ET-3 (data not shown), but another ET_B receptor-selective agonist, IRL1620, was less potent than ET-3 (Table 2). Preincubation with 1 μM BQ-123 inhibited this 10 nM ET-1-induced $[Ca^{2+}]_i$ increase to a similar level to that obtained with 10 nM ET-3 alone (Figure 6a,b,d, and Table 2). Further, 1 μM BQ-123 had little effect on the $[Ca^{2+}]_i$ response to 10 nM ET-3 (Figure 6e and Table 2). Therefore, the $[Ca^{2+}]_i$ response to 10 nM ET-3 was considered to be mediated mainly through the ET_B receptor. BQ-788 at 0.1 μM was enough to inhibit the $[Ca^{2+}]_i$ increase mediated through the ET_B receptor, because preincubation with 0.1 μM BQ-788 completely inhibited the $[Ca^{2+}]_i$ response to 10 nM ET-3 (Figure 6f and Table 2). Therefore, the 10 nM ET-1-induced $[Ca^{2+}]_i$ increase on pretreatment with 0.1 μM BQ-788 reflects an increase mediated mainly through the ET_A receptor (Figure 6c and Table 2). Furthermore, application of both 1 μM BQ-123 and 0.1 μM BQ-788 completely abolished the 10 nM ET-1-induced increase (Table 2). Therefore 1 μM BQ-123 and 0.1 μM BQ-788 were able to inhibit the $[Ca^{2+}]_i$ increase through the ET_A and ET_B receptor, respectively. These results indicate that both ET_A and ET_B receptors couple to an increase in $[Ca^{2+}]_i$.

Distinct G proteins responsible for coupling of ET receptor subtypes to $[Ca^{2+}]_i$ mobilization

PTX catalyses the ADP-ribosylation of some G proteins and uncouples them from their linked receptors (Gilman, 1987). We investigated the effects of PTX on $[Ca^{2+}]_i$ increase to address whether the coupling of the ET_A and ET_B receptor to the $[Ca^{2+}]_i$ increase is mediated through different G proteins. Figure 7 shows the effects of various concentrations of PTX on peak $[Ca^{2+}]_i$ increase through activation of the ET_A and ET_B receptors. ET-1-induced $[Ca^{2+}]_i$ increase on pretreatment with BQ-788, which reflects $[Ca^{2+}]_i$ response through the ET_A receptor, was partially inhibited by PTX and the inhibition was significant at more than 10 ng ml^{-1} PTX. ET-3-induced $[Ca^{2+}]_i$ increase, which reflects $[Ca^{2+}]_i$ response through the ET_B receptor, was also inhibited by PTX in a dose-dependent manner and the inhibition was significant at more than 0.1 ng ml^{-1} PTX. PTX at 10 ng ml^{-1} reduced the $[Ca^{2+}]_i$ response through ET_A and ET_B receptors by $23.0 \pm 5.5\%$ and $54.1\% \pm 2.3\%$, respectively. Inhibition by PTX 10 ng ml^{-1} was significantly different between them. To confirm the efficiency of PTX treatment, we assessed ADP-ribosylation in membranes of HP cells treated with PTX (Figure 8). In PTX-untreated cell membranes, 41 kDa and 36 kDa proteins were

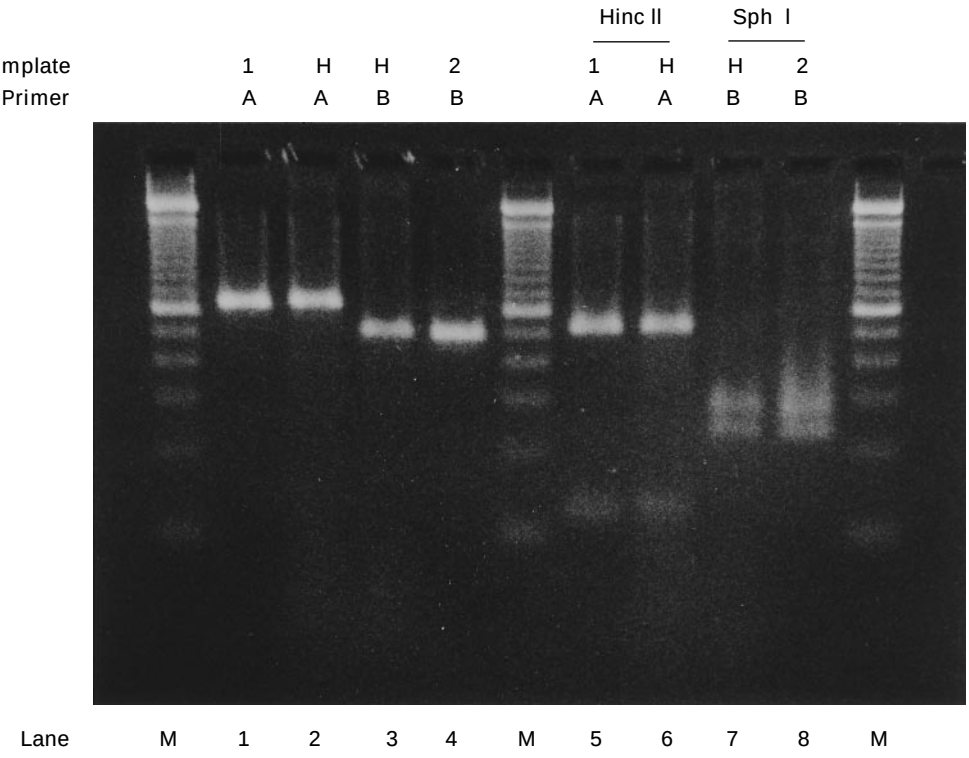

Figure 3 Existence of ET_A and ET_B receptor mRNA. Total RNA of human prostatic (HP) cells (1 μg) was applied to reverse transcription reaction, and the products (H) were subjected to polymerase chain reaction with ET_A (A) and ET_B (B) receptor-specific primer pairs as described under Methods. Human ET_A (1) and ET_B (2) cDNAs were used as PCR control. The products were electrophoresed on a 3% agarose gel (lanes 1–4). Lane M shows molecular size marker. Restriction mapping of the PCR product confirmed the expression of both ET_A and ET_B receptor mRNAs in HP cells (lanes 5–8).

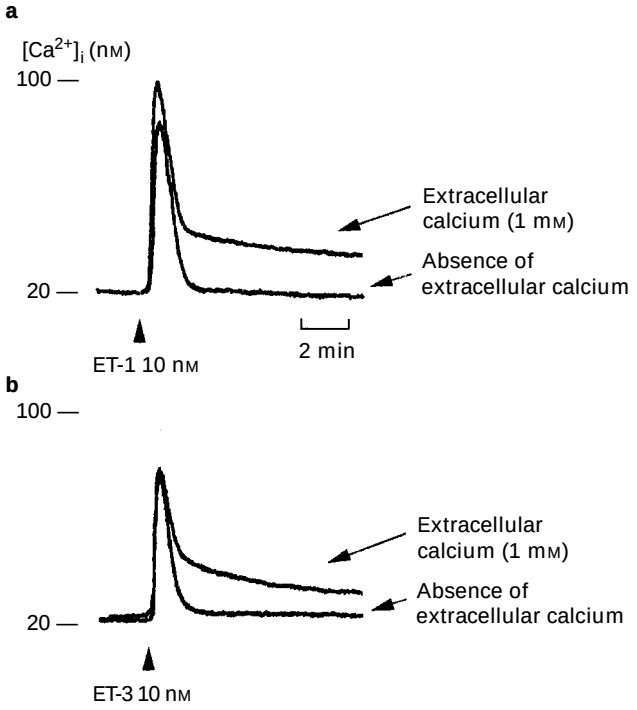

Figure 4 ET-1 (a) and ET-3 (b) -induced $[Ca^{2+}]_i$ responses in the presence and absence of extracellular calcium (1 mM). Cells were monitored in HBSS or Ca^{2+}-free HBSS containing 0.3 mM ethylene glycol *bis*(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) for 2 min before the addition of 10 nM ET-1 or 10 M ET-3. Data show typical charts of at least 3 separate experiments.

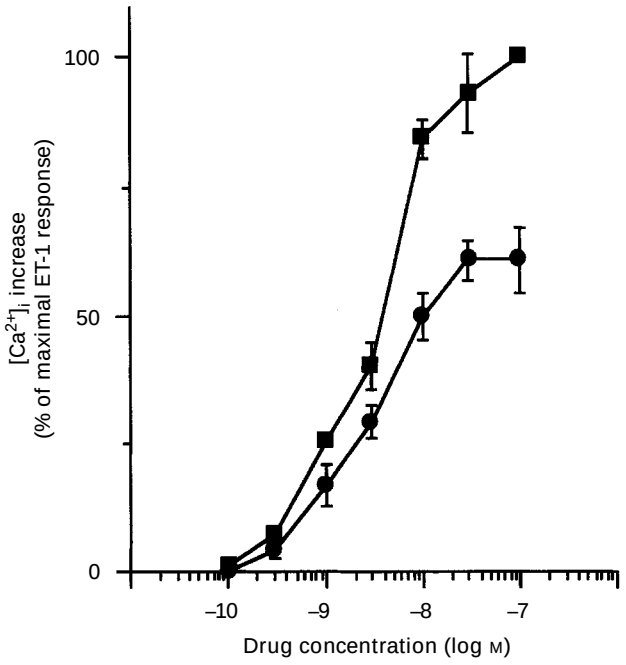

Figure 5 Concentration-dependence of initial transient increase in $[Ca^{2+}]_i$ induced by (■) ET-1 and (●) -ET-3. Values are expressed as percentage of the increase in $[Ca^{2+}]_i$ induced by 100 nM ET-1. Basal $[Ca^{2+}]_i$ was 22.7 ± 0.7 nM and peak $[Ca^{2+}]_i$ values induced by 10 nM ET-1 and ET-3 were 126.6 ± 6.1 and 80.9 ± 8.3 nM, respectively ($n = 5$). Each point shows the mean of at least 3 separate experiments; vertical lines indicate s.e.

Table 2 Agonist-induced $[Ca^{2+}]_i$ increase in human prostatic cells

Treatment	$[Ca^{2+}]_i$ increase from basal level (nM) ET-1	ET-3	IRL1620
None	103.0±13.0	65.9±7.7	6.9±2.2
BQ-123	64.5±6.9	59.6±5.6	NT
BQ-788	55.7±2.4	0±0	NT
BQ-123+BQ-788	0±0	NT	NT

Cells were pretreated with 1 μM BQ-123, 0.1 μM BQ-788 or 1 μM BQ-123 plus 0.1 μM BQ-788 for 2 min. Then, 10 nM ET-1, ET-3 or IRL1620 were applied to the cells, and peak $[Ca^{2+}]_i$ increase from basal level was measured. Data are the mean±s.e. of at least three separate experiments. NT, not tested.

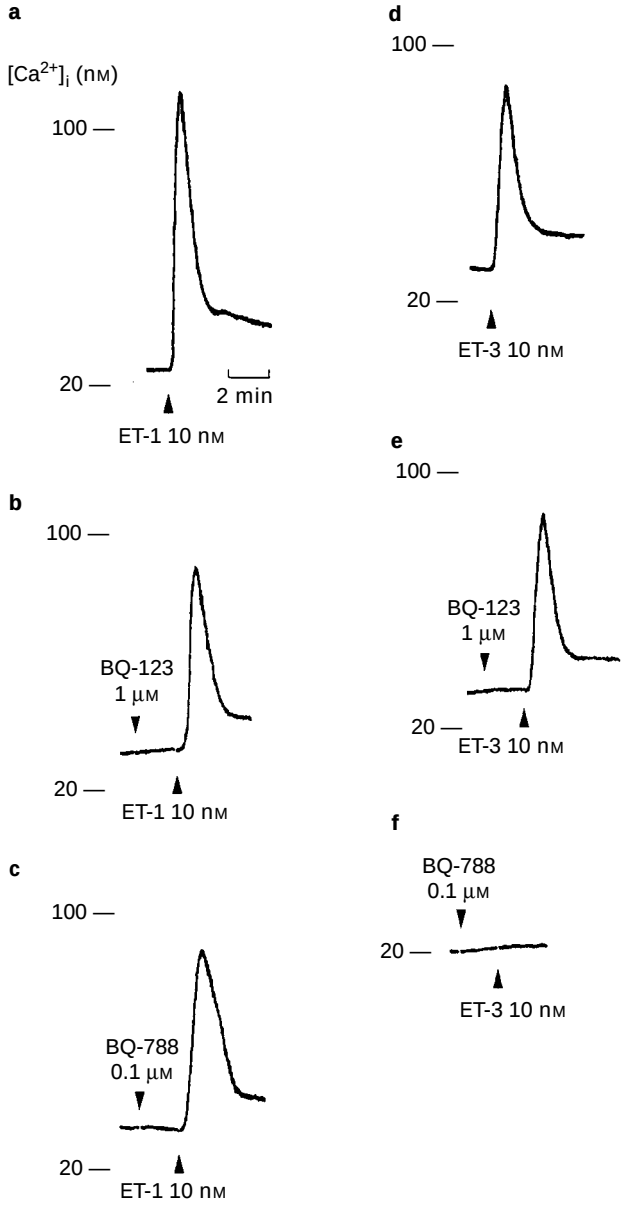

Figure 6 Effects of BQ-123 and BQ-788 on $[Ca^{2+}]_i$ increases induced by ET-1 and ET-3. Indicated concentrations of ET-agonists or antagonists were applied at the arrows. Representative charts are shown; similar results were obtained from at least three separate experiments.

ADP-ribosylated. ADP-ribosylation of 36 kDa protein seemed to be a nonspecific event under our experimental conditions, because there is no evidence to suggest the presence of 36 kDa G protein subjected to ADP-ribosylation by PTX. Pretreatment of the cells with 10 ng ml^{-1} PTX resulted in complete inhibition of subsequent *in vitro* ADP-ribosylation of only the 41 kDa protein, possibly α subunit of Gi (Ui, 1984), indicating that this 41 kDa protein was the principal target of PTX in whole cells and that pretreatment with PTX effectively ADP-ribosylated 41 kDa protein in the cells. These results suggest the presence of two pathways linking the ET receptor to an increase in $[Ca^{2+}]_i$, one a PTX-sensitive and the other a PTX-insensitive G protein.

Discussion

The aim of this study was to characterize the ET receptor subtypes and cellular signal transduction mechanism in smooth muscle cells of human prostate. Our results indicate that both ET_A and ET_B receptor subtypes are localized in the cells. The presence of both subtypes in prostatic smooth muscle cells was identified by several experiments. Binding of [^{125}I]-ET-1 was inhibited by ET-1 in a monophasic manner. However, the subtype-selective ligands ET-3, BQ-123 and BQ-788 inhibited the binding of [^{125}I]-ET-1 over a broad range of concentrations. When competition curves for ET-3, BQ-123 and BQ-788 were analysed by a least–squares curve-fitting programme, the data fit a two-site rather than a one-site binding model. Saturation binding assays indicated the K_d value of the ET_A and ET_B receptor were 70±9 and 10±2 pM, respectively (Figure 2a and b). These values are similar to those obtained in binding studies on cloned human ET_A and ET_B

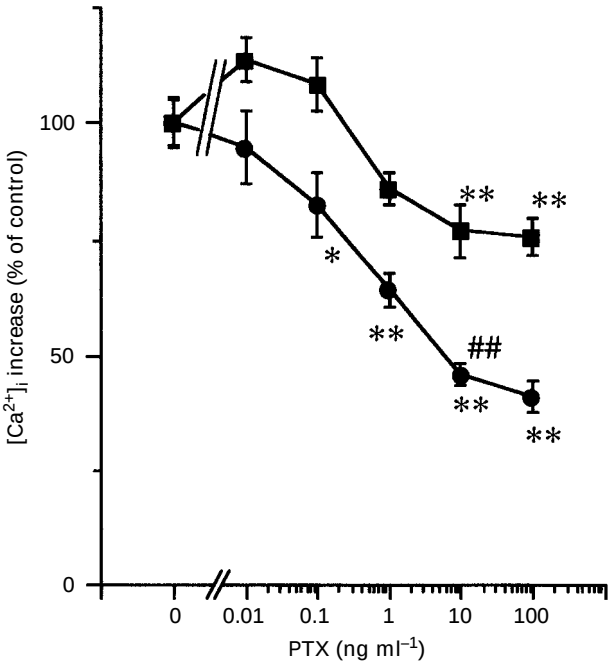

Figure 7 Effects of pertussis toxin (PTX) on $[Ca^{2+}]_i$ increases mediated through (■) ET_A and (●) ET_B receptors. Cells were pretreated with various concentrations of PTX for 12 h. Increases in $[Ca^{2+}]_i$ through ET_A and ET_B receptor are expressed as percentage of that in the absence of PTX. The $[Ca^{2+}]_i$ increases from basal level mediated through the ET_A and ET_B receptors were 58.6±3.2 and 80.7±4.4 nM ($n=12$), respectively. Each point shows the mean of at least 6 separate experiments; vertical lines indicate s.e. $^*P<0.05$, and $^{**}P<0.01$, compared with the data in the absence of PTX. $^{\#\#}P<0.01$ compared with the data for the ET_A receptor-mediated $[Ca^{2+}]_i$ increase.

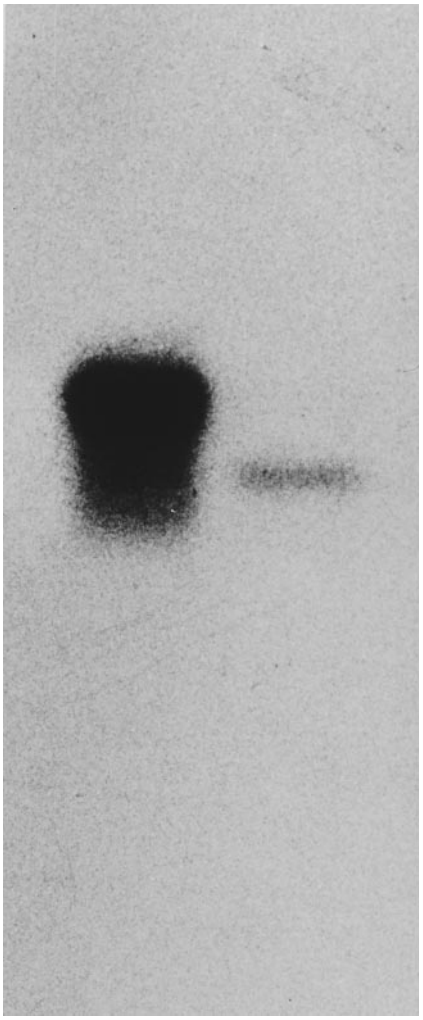

Figure 8 Pertussis toxin (PTX)-catalysed ADP-ribosylation of human prostatic (HP) cell membranes. HP cells were incubated in the absence (control) and presence of 10 ng ml^{-1} PTX for 12 h and membrane fractions were prepared. ADP-ribosylation was carried out in presence of activated PTX and [^{32}P]-NAD for 40 min at 30°C.

receptors (Kevin *et al*., 1994) and in guinea-pig tracheal smooth muscle cells (Inui *et al*., 1994).

Capacities of binding sites for the ET_A and ET_B receptor were 1400 ± 50 and 1000 ± 100 fmol mg^{-1} protein, respectively. Kobayashi *et al*. (1994b) demonstrated the presence of 2.5 times more ET_A than ET_B receptors in human prostate stroma. Prostate stroma is primarily composed of smooth muscle cells, fibroblasts and connective tissue elements. Our results may therefore suggest that ET receptor distribution and proportion are different between smooth muscle cells and other components in prostate stroma.

We also examined the intracellular signal transduction mechanism of each ET receptor subtype in HP cells. Stimulation of ET_A and ET_B receptors mediates an increase of phosphatidylinositol hydrolysis and $[Ca^{2+}]_i$ in various tissues and cultured cells (Catherine *et al*., 1992; Inui *et al*., 1994). Measurement of $[Ca^{2+}]_i$ in HP cells by fura-2 fluorometry demonstrated that both ET-1 and ET-3 elicited an initial transient and sustained increase in $[Ca^{2+}]_i$ (Figures 4 and 5). It is notable that the ED_{50} values for the $[Ca^{2+}]_i$ response were 40–300 fold higher than the K_d values obtained from saturation experiments. The mechanism behind such a discrepancy remains uncertain, but the differences in the binding and functional values may be attributable to the use of different preparations (cell membranes and cultured cells) and incubation conditions (temperature and time), the loss of ET peptides due to metabolism and internalization or may be complicated by the slow dissociation of the ET peptides from the receptor. Indeed, several laboratories have demonstrated that ED_{50} values for $[Ca^{2+}]_i$ response or phosphoinositide turnover induced by ETs are much greater than K_d values obtained in binding studies (Catherine *et al*., 1992; Inui *et al*., 1994).

Peak $[Ca^{2+}]_i$ response induced by 10 nM ET-1 was approximately 1.5 times higher than the value obtained for 10 nM ET-3. This result indicates that the ET-1 induced $[Ca^{2+}]_i$ increase was mediated through cooperation of both ET_A and ET_B receptors. Use of subtype-selective antagonists BQ-123 and BQ-788, indicated that both subtypes were coupled to an increase in $[Ca^{2+}]_i$ (Figure 6 and Table 2).

Recent studies have proposed that there are two types of ET_B receptors: an ET_B receptor subtype that is sensitive to IRL1620, RES-701-1 and IRL1038, termed ET_{B1}, and another subtype that is insensitive to these agents, termed ET_{B2} (Sudjarwo *et al*., 1994). Therefore, the ET_B receptor subtype in this cell may be the ET_{B2} because the $[Ca^{2+}]_i$ increase elicited by IRL1620 was very low compared to that induced by ET-3 (Table 2). However, the functional implications of the receptor on the HP cells being of the ET_{B2} subtype are uncertain.

ETs mediate smooth muscle contraction of various tissues and organs, such as uterus (Maggi *et al*., 1991), airway (Goldie *et al*., 1995), trachea (Yoneyama *et al*., 1995) and vascular beds (Yanagisawa *et al*., 1988; Shetty *et al*., 1993). Contraction of human prostate smooth muscle cells was not measured in this study, but our data indicate that stimulation of both subtypes evokes an increase in $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ will subsequently induce phosphorylation of myosin light chain and cause contraction of the cells (Kodama *et al*., 1994). These results are consistent with the findings that both ET_A and ET_B receptors mediate contraction of human prostate (Kobayashi *et al*., 1994b). However, functional differences between these receptors are not clear.

It has been shown that the ET_A receptor is coupled to phosphoinositide hydrolysis via a PTX-insensitive G protein in rat aortic vascular smooth muscle cells (Takuwa *et al*., 1990), human brain endothelial cells (Stanimirovic *et al*., 1994), and rat cardiac myocytes (Hilal-Dandan *et al*., 1994) but that the ET_B receptor acts via a PTX-sensitive G protein in bovine endothelial cells (Emori *et al*., 1991). Activation of PLC by $\beta\gamma$-subunits released from Gi protein may represent the mechanism by which PTX-sensitive G proteins stimulate PLC (Campus *et al*., 1992; Katz *et al*., 1992). We looked at the effects of PTX on the $[Ca^{2+}]_i$ increase, because an effect seen here would be suggestive of the presence of PTX-sensitive phosphoinositide hydrolysis. The difference between the $[Ca^{2+}]_i$ increase mediated through the ET_A and ET_B receptors was seen on pretreatment of cells with PTX (Figure 7). Sensitivity to PTX was significantly different between them. Furthermore, treatment of cells with 10 ng ml^{-1} PTX completely ADP-ribosylated 41 kDa protein, possibly α subunit of Gi, but resulted in only partial inhibition of the $[Ca^{2+}]_i$ increase. These results suggest the presence of multiple G proteins linking the ET receptor to $[Ca^{2+}]_i$ increase. The present study may be the first to demonstrate that a single cell possess two different ET receptor subtypes which evoke an increase in $[Ca^{2+}]_i$ via the action of different G proteins. Functional differences between the ET_A and ET_B receptor in human prostate may be attributable to these different signal transduction cascades.

Benign prostatic hyperplasia (BPH) results from hyperplasia of the cellular elements of the prostate gland. ET has been shown to be a mitogen for various cell types, including human vascular smooth muscle cells (Kanse *et al*., 1995) and rat mesangial cells (Simonson & Herman, 1993). It is possible that ETs may modulate the pathophysiology of BPH. It is of interest to investigate whether ET exerts a mitogenic effect on HP cells. In preliminary experiments, we have found that ET stimulates DNA synthesis and serves as a growth factor for HP cells.

In summary, we have shown that human prostatic smooth muscle cells possess ET_A and ET_B receptors and that both

subtypes evoke intracellular calcium mobilization. Furthermore, an increase in $[Ca^{2+}]_i$ through the ET_A and ET_B receptors is probably mediated via distinct G proteins. Data from this study will be helpful in investigations of the pathophysiology of BPH.

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