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Characterization of Endothelin Receptors in Mesangial Cells: Evidence for Two Functionally Distinct Endothelin Binding Sites

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SUMMARY

Endothelin (ET) peptides are growth factors that bind to G protein-coupled receptors and serve as a useful model to study mitogenic signal transduction by vasoactive peptides. To begin to define the molecular mechanisms underlying mitogenic signaling by ET-1, we analyzed expression of ET receptor subtypes in glomerular mesangial cells and antagonism of ET-1-induced mitogenic responses by ET receptor antagonists. Competitive displacement analysis of ^{125}I-ET-1 binding revealed a shallow multicomponent curve consistent with the presence of two ET-1 binding sites (K_d values of 32 pM and 1.2 nM). Nonlinear regression analysis demonstrated that a two-site model fit the data better than a one-site model ($p = 0.0063$). Analysis of ^{125}I-ET-1 binding sites by affinity cross-linking revealed incorporated radioactivity in two distinct protein bands in mesangial cell membranes. Both ET_A-specific (BQ 123) and nonselective (PD 142893/PD 145065) receptor antagonists displaced ^{125}I-ET-1 from the low affinity site. The K_i values for BQ 123 and PD 145065 were similar to the IC_{50} values for inhibition of ET-1-induced increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by these antagonists. The ET_B-specific ligands S6c and [Ala^{1,3,11,15}]ET-1(6–21) were unable to displace ^{125}I-ET-1 from either low affinity or high affinity binding sites. Analysis of ET receptor mRNA by reverse transcription-polymerase chain reaction, using primers predicted from DNA sequences conserved through evolution in ET_A and ET_B genes, demonstrated that mesangial cells express a canonical ET_A receptor. Collectively, these data suggest that the low affinity, high capacity ^{125}I-ET-1 binding site is an ET_A receptor and that the high affinity, low capacity site is not accounted for by canonical ET receptors. We further demonstrated that BQ 123 and PD 142893/PD 145065 inhibited ET-1-stimulated [^{3}H]thymidine uptake but at higher concentrations than required for inhibition of $[Ca^{2+}]_i$ increases. Preincubation (as opposed to coincubation) with antagonists was required to inhibit ET_A-mediated increases in $[Ca^{2+}]_i$ produced by ET-1. These results suggest that the unique, nearly irreversible binding of ET-1 to ET_A receptors explains why high concentrations of ET receptor antagonists are required to block longer term actions such as mitogenesis.

ET-1 is a potent vasoconstrictor peptide secreted by the endothelium in diverse vascular beds (1). ET-1 belongs to a family of three 21-amino acid isopeptides (ET-1, ET-2, and ET-3), each encoded by separate genes at distinct chromosomal loci (see Refs. 2 and 3 for review). ET peptides are homologous to sarafotoxin peptides isolated from *Atractaspis engaddensis* (4), and both sequence homology and conserved bioactivity between ET and sarafotoxin peptides suggest that they form a supergene family with common evolutionary origins. In vertebrates, ET peptides are widely secreted at both vascular and nonvascular sites, in an isopeptide-specific fashion. Precise physiological roles for ET peptides remain to be elucidated, but most experiments suggest that ETs are multifunctional, act in a paracrine or autocrine mode to regulate vasomotor tone, Na^+ and water handling in the kidney, and release of pituitary hormones, renin, catecholamines, and atrial natriuretic factor, and might contribute to neurotransmission or neuromodulation. In addition to stimulating short term events, in many cells ET-1 also acts as a growth factor (5, 6). ET-1 is a mitogen for a variety of cell types, causes hypertrophy in cardiac myocytes, and therefore serves as a useful paradigm for mitogenic signaling by vasoactive peptides that bind to G protein-coupled receptors. Dysregulation of cell growth by ET-1 has been implicated in vascular remodeling in pathophysiological conditions including atherosclerosis, cardiac hypertrophy, and glomerulosclerosis (2, 3).

Transmembrane signaling by ET is initiated by binding to three subtypes of G protein-coupled receptors, (i) a selective ET_A receptor that binds only ET-1 and ET-2 with high affinity, (ii) a nonselective ET_B receptor that binds all three ET isopeptides with equal affinity, and (iii) an ET_C receptor that selectively binds ET-3 (7–9). Distinct ET_A- and ET_B-specific domains in the receptors have been mapped using ET_A/ET_B

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ABBREVIATIONS: ET, endothelin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DSS, disuccinimidyl suberate; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; bp, base pair(s).

chimeras transfected into COS-7 cells (10). In the vasculature, ET_A receptors mediate vasoconstriction by activating a phosphoinositide-based signaling cascade and by increasing the opening probability of receptor- and voltage-operated Ca^{2+} channels. Although in some vascular beds ET_B receptors also mediate vasoconstriction (11, 12), in most cases activation of ET_B receptors leads to vasorelaxation by increasing levels of nitric oxide/cGMP and prostaglandins. In contrast, the ET receptor subtypes regulating growth of vascular cells remain poorly characterized. ET receptors also have the unusual property among G protein-coupled receptors of binding ET ligands in a nearly irreversible fashion (see Ref. 13 for review). Thus, another unresolved issue concerning ET receptors and cell growth is whether conventional ET receptor antagonists can effectively block the longer term mitogenic actions of ET-1.

To further define the mechanisms through which ET-1 stimulates hypertrophy and hyperplasia of vascular cells, in the present study we analyzed expression of ET receptor subtypes in glomerular mesangial cells and asked whether conventional ET receptor antagonists can block cell growth stimulated by ET-1. Glomerular mesangial cells are microvascular pericytes from the renal glomerulus that serve as a useful model system for studying mitogenic signaling by ET-1 (3). In the glomerulus, the major source of ET-1 is thought to be secretion by glomerular endothelial cells (14). Moreover, the observation that an ET receptor antagonist blocks compensatory proliferation of mesangial cells in a model of progressive nephron loss in rats provides strong support for the notion that ET-1 is an important renal growth factor (15). We report here that mesangial cells express two ET-1 binding sites, i.e., a canonical ET_A receptor that mediates mitogenic signaling and another apparently distinct ET-1 receptor not linked to mitogenesis. In addition, we also present evidence that formation of nearly irreversible ET-1-ET_A complexes dictates that high concentrations of conventional ET receptor antagonists must be employed to block longer term actions such as [^{3}H]thymidine uptake.

Experimental Procedures

Materials. ET-1, ET-3, and arginine-vasopressin were from Peptide Institute (Tokyo, Japan). S6c was from Sigma Chemical Co. (St. Louis, MO). [$Ala^{1,3,11,15}$]ET-1(6–21) was from Peninsula Laboratories (Belmont, CA). [^{125}I-Tyr^{13}]ET-1 was from New England Nuclear. DSS was from Pierce (Rockford, IL). BQ 123 was a generous gift from Eliot Ohlstein, SmithKline Beecham Pharmaceuticals (King of Prussia, PA), and PD 142893 and PD 145065 disodium salts were kindly provided by Annette Doherty, Parke-Davis (Ann Arbor, MI). Fura-2/acetoxymethyl ester and ionomycin were purchased from Molecular Probes (Eugene, OR). Oligodeoxynucleotides were synthesized by National Biosciences (Plymouth, MN).

Mesangial cell culture and measurements of cell growth. Mesangial cell strains from male Sprague-Dawley rats were isolated and characterized as reported previously (5). Cells were maintained in RPMI 1640 medium supplemented with 17% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenite, at 37° in a 5% CO_2 incubator. Cells in passages 11–24 were used in this study. [^{3}H]Thymidine uptake in quiescent mesangial cells was measured as reported previously (16), with minor modifications. Mesangial cells at approximately 80% confluence in 24-well plates were incubated in DMEM/0.5% FBS for 24 hr to induce quiescence. Agonists were then added (time 0), and after a 16-hr incubation the cells were pulsed with 1.0 μCi/ml [*methyl*-^{3}H]thymidine for 2 hr. Radiolabeled DNA was extracted by washing the cells twice with Dulbecco's phosphate-buffered saline, fixing them in methanol/acetic acid/water (50:10:40) for 1 hr at 4°, and solubilizing them and counting the radioactivity in 1.0% SDS (5). In experiments with ET receptor antagonists, the cells were incubated with specific receptor blockers for 30 min at 37° before addition of ET-1. Viability of mesangial cells treated with ET receptor antagonists was confirmed by incubation with 5(6)-carboxyfluorescein diacetate and monitoring of the retention of the cleaved, fluorescent product carboxyfluorescein by epifluorescence microscopy, to assess membrane integrity, as described previously (16).

Determination of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ in quiescent mesangial cell monolayers grown on Aclar coverslips was determined by dual-wavelength spectrofluorometry using the Ca^{2+}-sensitive dye fura-2 (17), exactly as reported previously (5, 16). The fluorescent signal was calibrated with ionomycin followed by EGTA/Tris, and $[Ca^{2+}]_i$ was calculated by the formula of Grynkiewicz *et al.* (17), assuming the K_d of the fura-2-Ca^{2+} interaction to be 224 nM. All agonists were tested for autofluoresence. After long incubations (20–30 min), leakage of fura-2 was assessed by the addition of 100 μM Mn^{2+}, followed by rapid chelation with 4 mM diethylenetriaminepentaacetic acid.

ET receptor binding assays. Competitive displacement ^{125}I-ET-1 radioligand binding studies were performed as described by Martin *et al.* (18), with minor modifications. Briefly, mesangial cells in 24-well plates were made quiescent by incubation in DMEM/0.5% FBS for 48 hr, and the cells were washed twice at 4° with binding buffer consisting of Hanks' buffered salt solution (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1.25 mM $CaCl_2$, 4.0 mM $NaHCO_3$, 0.2 g/dl bovine serum albumin) buffered with 40 mM HEPES, pH 7.4. Binding reactions were then initiated by addition of ice-cold binding buffer (0.25 ml) containing 25 pM ^{125}I-ET-1 (specific activity, 2200 Ci/mmol), with or without increasing concentrations of unlabeled competitors as indicated. Equilibrium binding experiments were performed for 16 hr on an orbital shaker (100 rpm) at 4°. Measurements of carboxyfluorescein intensity, as described above, confirmed that cell viability was not significantly compromised after 16 hr at 4°. Binding reactions were terminated by rapid (<50 sec) washing of the wells four times with 1 ml of phosphate-buffered saline/0.2% bovine serum albumin, cells were solubilized in 0.2 N NaOH/1% SDS, and radioactivity was counted in a γ counter. Nonspecific binding was defined in the presence of 0.5 μM ET-1 and under these conditions was always <2.0% of total binding. Competition binding data were analyzed by the nonlinear regression curve-fitting program InPlot (GraphPAD Software, San Diego, CA). Data were fit to one- and two-component competition functions, and the statistical significance of the improvement of fit from a one-site to a two-site model was determined by an F test comparing the sum of squares of the residuals for each fit. K_i values were calculated by the method of Cheng and Prusoff (19).

Affinity cross-linking of ET receptors. Membranes were prepared from mesangial cells maintained for 24 hr in DMEM/0.5% FBS. Cells were scraped from Petri dishes, transferred to a glass homogenizer, and homogenized by 30 strokes with a Teflon pestle (4°) in 50 mM HEPES, pH 7.4, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 20 μg/ml aprotinin. Disrupted cells were centrifuged at 1000 × *g* for 10 min at 4°, and after the supernatant was reserved the pellet was again subjected to homogenization and centrifugation as described above. Combined postnuclear supernatants were centrifuged at 30,000 × *g* for 45 min at 4°, and the resultant pellet was suspended in HEPES buffer at a final concentration of 4 mg/ml. All labeling experiments were conducted at 4° to minimize proteolytic degradation of ligand or receptors. Membranes (400 μg/reacation) were incubated with 100 pM ^{125}I-ET-1 for 90 min in the absence or presence of 100 nM nonradiolabeled ET-1. After centrifugation for 20 min at 30,000 × *g*, the pellet was resuspended and incubated with 1 mM DSS for 15 min at 4°. The reaction was quenched by addition of 50 mM Tris, pH 8.0, 1 mM EDTA, 250 mM sucrose, the pellet was collected by centrifugation, and the proteins were resolved

by electrophoresis on 12% SDS-polyacrylamide gels and detected by autoradiography at −70°.

Analysis of ET receptor mRNA using PCR. Total mesangial cell RNA from quiescent mesangial cells was prepared as described previously (16), and 2 μg of RNA were used in a reverse transcription reaction containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, 1 mM levels of each deoxynucleoside triphosphate, 1 unit/μl RNasin, 1 μM downstream ET receptor primer, and 2.5 units/μl AMV reverse transcriptase, at 42° for 30 min, at 52° for 30 min, at 99° for 5 min, and at 5° for 5 min. A 20-μl aliquot of the reverse transcription reaction was then used for PCR in a reaction containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus). Samples were denatured for 2 min at 95°, and PCR was conducted for 40 cycles (2 min at 95°, 2 min at 50°, and 5 min at 72°), followed by a final extension for 7 min at 72°. PCR products were resolved on a 1.6% agarose gel stained with ethidium bromide. For sequence comparisons of ET receptor cDNAs using matrix methods, all sequences were downloaded from GenBank version 76.0. Oligodeoxynucleotide PCR primers were chosen with the help of Oligo (National Biosciences, Plymouth MN) and are given in Fig. 7B.

Results

Different classes of ET receptor antagonists effectively block increments in $[Ca^{2+}]_i$ induced by ET-1. To begin analyzing expression of ET receptor subtypes in mesangial cells, we asked whether either ET_A-specific ET receptor antagonists (i.e., BQ 123) (20) or antagonists of both ET_A and ET_B receptors (i.e., PD 142893 and PD 145065) (21, 22) would block ET-1-induced Ca^{2+} signaling. ET-1 (100 nM) induced a typical biphasic Ca^{2+} waveform (Fig. 1A) that was inhibited by BQ 123; Ca^{2+} signaling produced by a subsequent application of arginine-vasopressin was unaffected by BQ 123 (Fig. 1B). The ET-1-induced $[Ca^{2+}]_i$ waveform, but not the arginine-vasopressin-induced waveform, was similarly inhibited by the nonselective ET receptor antagonists PD 142893 and PD 145065 (Fig. 1, C and D). In agreement with our previously published data (16), the ET_B receptor-specific agonists [$Ala^{1,3,11,15}$]ET-1(6–21) and S6c did not increase $[Ca^{2+}]_i$ when added to mesangial cells and did not block the increase in $[Ca^{2+}]_i$ induced by subsequent addition of 100 nM ET-1 (Fig. 1, E and F).

To determine the rank order of potency for antagonism of ET-1-induced Ca^{2+} signaling, we measured the peak value of $[Ca^{2+}]_i$ waveforms in the presence of increasing concentrations of ET receptor antagonists. All antagonists inhibited the peak $[Ca^{2+}]_i$ signal in a dose-dependent fashion, but with significantly different potencies (Fig. 2). IC_{50} values calculated from the semilogarithmic analysis were as follows: PD 145065, 9.8 nM; BQ 123, 10.4 nM; and PD 142893, 62.0 nM. These results confirm and extend our earlier contention that mesangial cells express an ET_A receptor subtype and that ET_A receptors mediate the increase in $[Ca^{2+}]_i$ produced by ET-1 (16).

^{125}I-ET-1 binds to two sites on glomerular mesangial cells. Previous experiments suggested the presence of two binding sites for ET-1 in mesangial cells, but radioligand binding studies to characterize these receptors have yielded conflicting results and the receptor subtypes have not been characterized (18, 23, 24). Thus, we performed ^{125}I-ET-1 binding studies utilizing competitive displacement for analysis of ET receptor subtypes and for estimation of ET-1-receptor equilibrium binding constants. Binding of ^{125}I-ET-1 to sites on mesangial cells at 4° reached steady state levels 7 hr after addition of radioligand and remained constant for up to 24 hr (Fig. 3). Competition displacement curves for displacement of ^{125}I-ET-1 binding

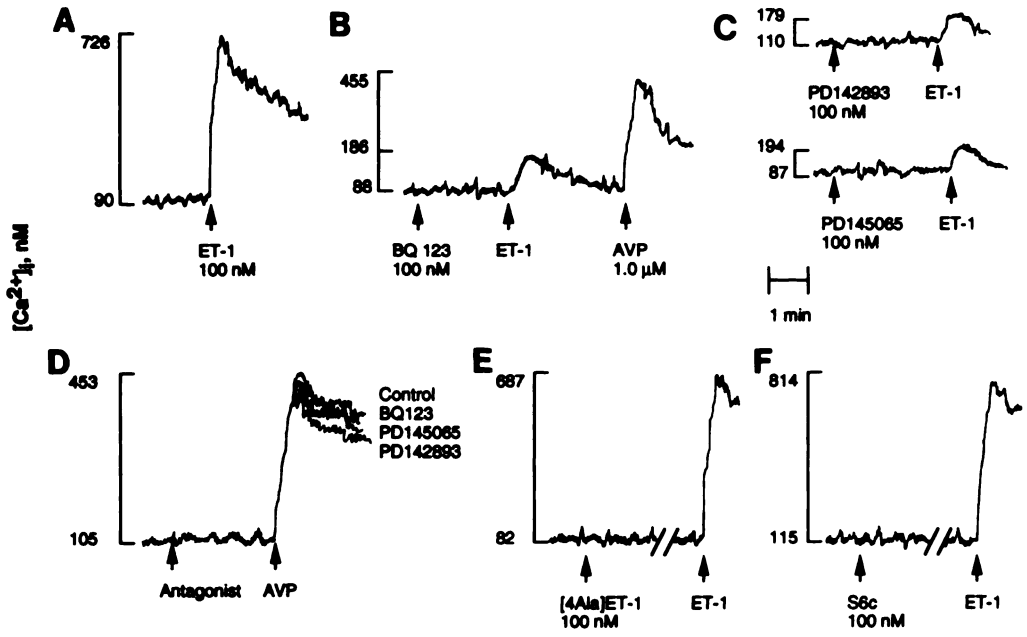

Fig. 1. Evidence for expression of an ET_A receptor subtype in glomerular mesangial cells. Quiescent mesangial cells on coverslips were loaded with the Ca^{2+}-sensitive dye fura-2, and changes in $[Ca^{2+}]_i$ were measured as described in Experimental Procedures. A-C, $[Ca^{2+}]_i$ waveforms stimulated by ET-1 were inhibited by an ET_A-specific antagonist (BQ 123) and by nonselective ET_A/ET_B-selective antagonists (PD 142893 and PD 145065). D, $[Ca^{2+}]_i$ waveforms induced by arginine-vasopressin (*AVP*) were unaffected by any of these antagonists (tracings are superimposed to facilitate comparison). E and F, Specific agonists of the ET_B receptor [S6c and [$Ala^{1,3,11,15}$]ET-1(6–21)] were unable to elevate $[Ca^{2+}]_i$ when added to mesangial cells. *Arrows*, time of addition for the indicated agonists or antagonists. Data are representative of three or four independent experiments for each condition.

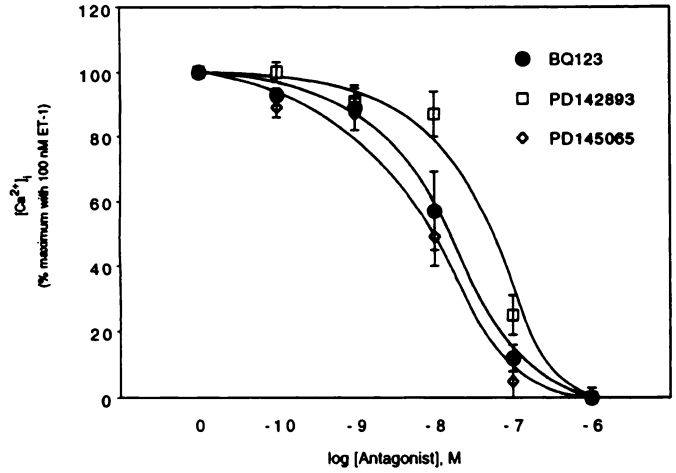

Fig. 2. Semilogarithmic plots for analysis of antagonism of ET-1-induced $[Ca^{2+}]_i$ waveforms by BQ 123, PD 142893, and PD 145065. ET-1 was added in the presence of increasing concentrations of the three ET receptor antagonists, and the peak increase in the ET-1-stimulated $[Ca^{2+}]_i$ waveform was measured. Nonlinear curve fits were calculated and used to estimate IC_{50} values. Data are mean ± standard error from three or four independent experiments.

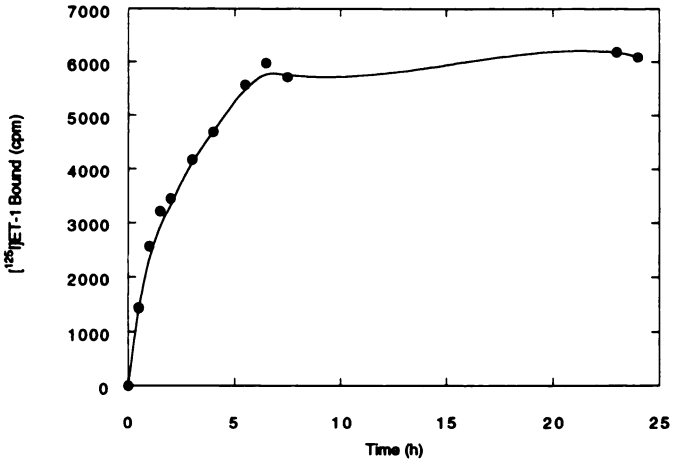

Fig. 3. Kinetics of ^{125}I-ET-1 binding to cultured glomerular mesangial cells at 4°. Quiescent mesangial cells in 24-well plates were incubated with 25 pM ^{125}I-ET-1 for the times indicated before separation of bound and free ligand and determination of nonspecific binding, as described in Experimental Procedures. Data are the mean of two independent experiments in triplicate.

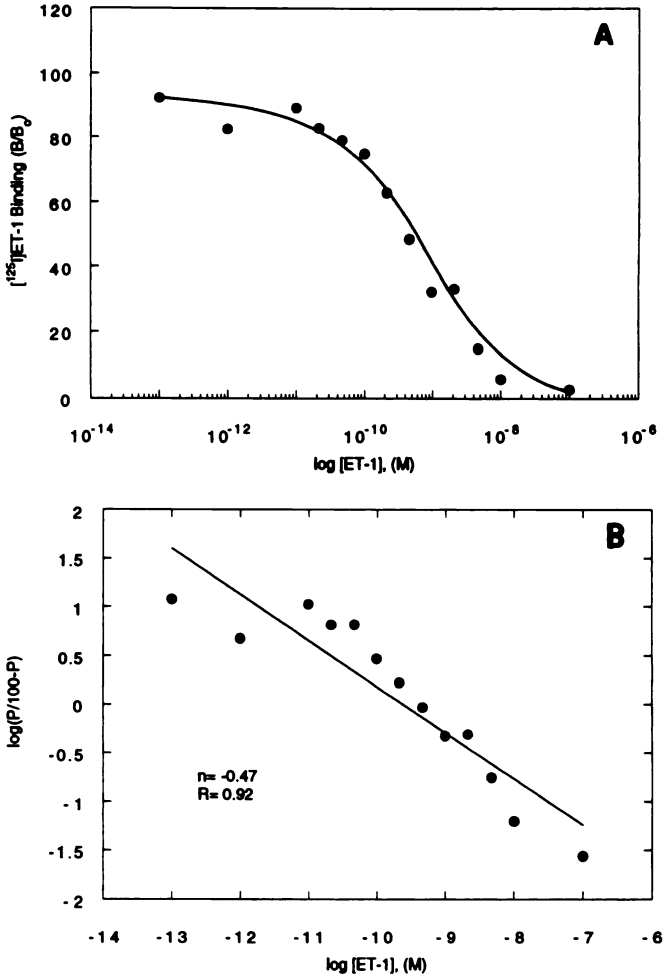

Fig. 4. Competition displacement analysis of ^{125}I-ET-1 binding, demonstrating a shallow multicomponent curve consistent with the presence of two binding sites for ET-1. A, ^{125}I-ET-1 was incubated in the presence of increasing concentrations of nonradiolabeled ET-1. Data were plotted as percentage of maximal binding versus concentration of nonradiolabeled ET-1. Nonlinear curve fitting was used to estimate values for K_d and B_{max} for a two-site model. Each *point* represents the mean of three or four independent determinations, in triplicate. B, Logit-log analysis of ^{125}I-ET-1 binding isotherms confirms that the competition displacement binding data are not fit by a single-site model.

by unlabeled ET-1 were shallow and extended over >2 log units, suggesting the presence of two ET-1 binding sites (Fig. 4A). Log-logit transformation of the competition binding data gave a "pseudo-Hill" coefficient of −0.47, which is also consistent with a two-site model (Fig. 4B). Nonlinear curve fitting of the binding data was used to generate the following estimates of K_d and B_{max} values in a two-site model: higher affinity site, K_d = 32 pM, B_{max} = 105 fmol/mg of protein; lower affinity site, K_d = 1.2 nM, B_{max} = 467 fmol/mg of protein. A two-site model fit the data significantly better than a one-site model (p = 0.0063). Affinity cross-linking techniques were used to confirm that ET-1 binds to two distinct proteins in mesangial cell membranes. Radioactivity was specifically incorporated into two protein bands, at 34 and 45 kDa (Fig. 5). Taken together, these data indicate that mesangial cells express both a high affinity, low capacity site and a lower affinity, high capacity site for ET binding.

Mesangial cells express two distinct ET receptor subtypes. Both BQ 123 and PD 145065 competed effectively with ^{125}I-ET-1 for occupancy of the lower affinity, high capacity site (Fig. 6A), indicative of binding to an ET_A receptor subtype (K_i for BQ 123 = 1.71 nM; K_i for PD 145065 = 10.2 nM). K_i values were similar to the IC_{50} values for inhibition of ET-1-induced Ca^{2+} signaling reported in Fig. 2. We predicted that, if mesangial cells express an ET_B receptor subtype, nonradiolabeled ET-1 and ET-3 should compete with equal potency for ^{125}I-ET-1 binding. However, ET-3 only minimally displaced ^{125}I-ET-1 binding to the high capacity binding site and failed to displace ^{125}I-ET-1 from the high affinity site (Fig. 6B). The ET_B-specific agonists S6c and [Ala^{1,3,11,15}]ET-1(6–21) were also unable to displace ^{125}I-ET-1 binding to either the high affinity or lower affinity sites (Fig. 6B). Collectively, these data suggest that the lower affinity, high capacity ^{125}I-ET-1 binding site is an ET_A receptor and that the high affinity, low capacity site is not accounted for by canonical ET receptors.

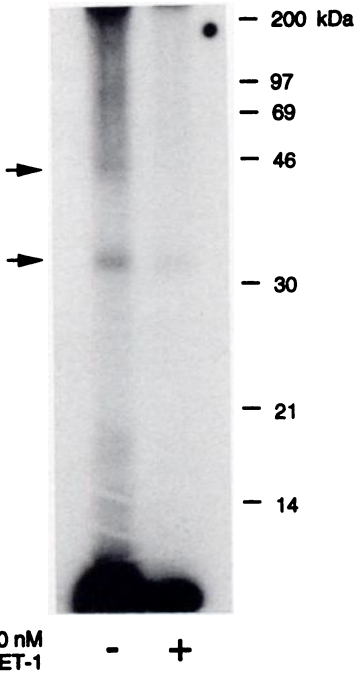

Fig. 5. Affinity cross-linking of ^{125}I-ET-1, demonstrating two different ET-1 binding sites in mesangial cell membrane preparations. Mesangial cell membranes (400 μg/lane) were incubated with ^{125}I-ET-1 in the presence or absence of 100 nM unlabeled ET-1. After addition of 1.0 mM DSS, membrane proteins were resolved on 12% SDS-polyacrylamide gels, and labeled bands were detected by autoradiography for 6 days at −70°. *Arrows on left*, two bands (34 and 45 kDa) specifically incorporating radioactivity. Similar results were observed in two independent experiments.

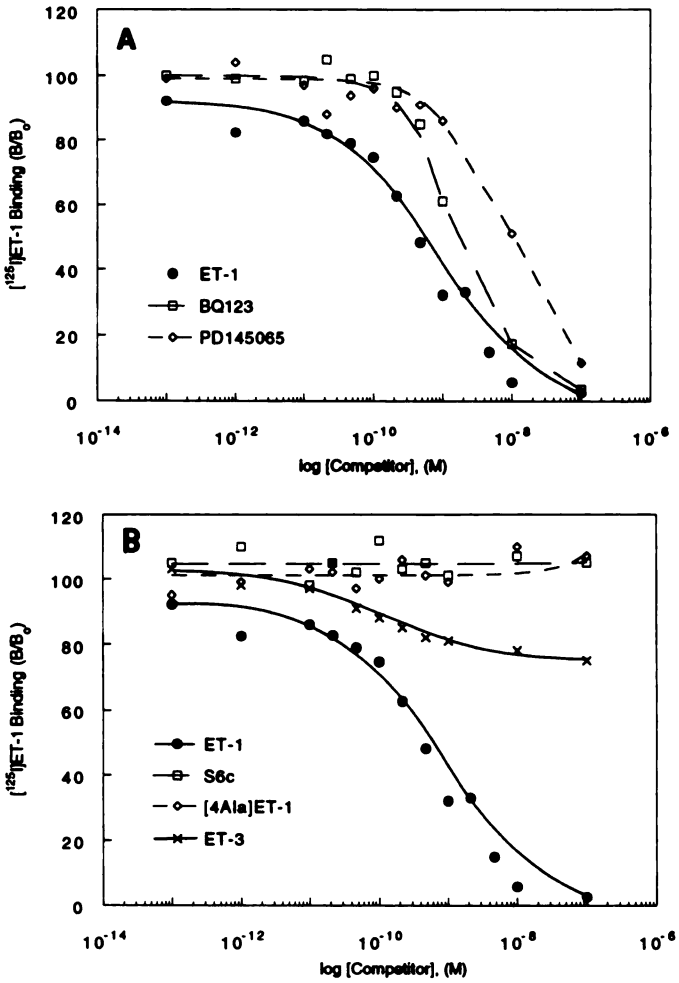

Fig. 6. Competitive displacement of ^{125}I-ET-1 binding by ET_A/ET_B receptor antagonists but not by ET_B-specific agonists. A, Binding studies were conducted as described in Fig. 4, in the presence of increasing concentrations of BQ 123 and PD 145065. Both antagonists competed with ^{125}I-ET-1 binding at the high capacity, low affinity site but not at the low capacity, high affinity site. B, ET_B-selective ligands S6c and [Ala^{1,3,11,15}] ET-1(6–21) failed to compete with ^{125}I-ET-1 binding to either site, and ET-3 competed minimally with ^{125}I-ET-1 binding to the low affinity site. Data are the mean of two independent experiments, in triplicate.

Analysis of ET_A receptor mRNA demonstrates that mesangial cells express a canonical ET_A receptor. Due to potential pitfalls in assigning receptor subtypes on the basis of pharmacological data alone, we used PCR to analyze ET receptor mRNA expression in mesangial cells. To design a set of optimal PCR primers for reverse transcription and amplification of ET receptor mRNAs, sequences of rat ET_A (8) and ET_B cDNA (7) were aligned to maximize homology (data not shown). Sequences from two segments (segment 1, residues 322–360 of the rat ET_A receptor and residues 382–420 of the rat ET_B receptor; segment 2, residues 910–948 of the rat ET_A receptor and residues 958–996 of the rat ET_B receptor) were found to be highly conserved through evolution and might therefore encode important domains for ET receptor function (Fig. 7A). Sequence similarity between any two receptor subtype sequences in this region ranged from 77% to 92%.

Optimal PCR primers complementary to sequences within these conserved regions were chosen, and the approximate locations of amino acid sequences represented by the PCR primers are shown in Fig. 7B. Surprisingly, these conserved amino acid sequences were located toward the cytoplasmic face of the receptor and partially included predicted transmembrane domains II and VI. Amplification of cDNAs for ET_A and ET_B mRNAs would yield double-stranded PCR products of 611 and 600 bp, respectively. Reverse transcription and PCR amplification of four different preparations of mesangial cell RNA yielded a single truncated cDNA of 611 bp (Fig. 7B). Restriction mapping of the truncated cDNA product with *Ava*II and *Sau*I was consistent with an ET_A receptor subtype (Fig. 7C). Further restriction mapping with *Hpa*II, *Eco*RV, and *Bgl*II confirmed the identity of this clone as ET_A (data not shown). In an attempt to isolate other $ET_{A/B}$-related transcripts, the annealing temperature of the PCR was lowered by 2° increments; however, additional ET receptor-related cDNAs could not be amplified with these primers. These results demonstrate that a canonical ET_A receptor is expressed in mesangial cells and that additional ET receptor-related transcripts could not be amplified using PCR primers from a highly conserved sequence of ET_A and ET_B mRNA.

Reversible ET receptor antagonists block [^{3}H]thymidine uptake stimulated by ET-1 but at higher concentrations than those required for inhibition of Ca^{2+} signaling. We next turned to the question of whether conventional ET receptor antagonists can block long term biological actions of ET-1 such as [^{3}H]thymidine uptake. As shown in Fig. 8, preincubation with BQ 123 dose-dependently inhibited [^{3}H] thymidine uptake stimulated by ET-1, but with a much higher IC_{50} value (225 nM) than that for inhibition of Ca^{2+} signaling (e.g., 10.4 nM in Fig. 2). Similarly, preincubation with the

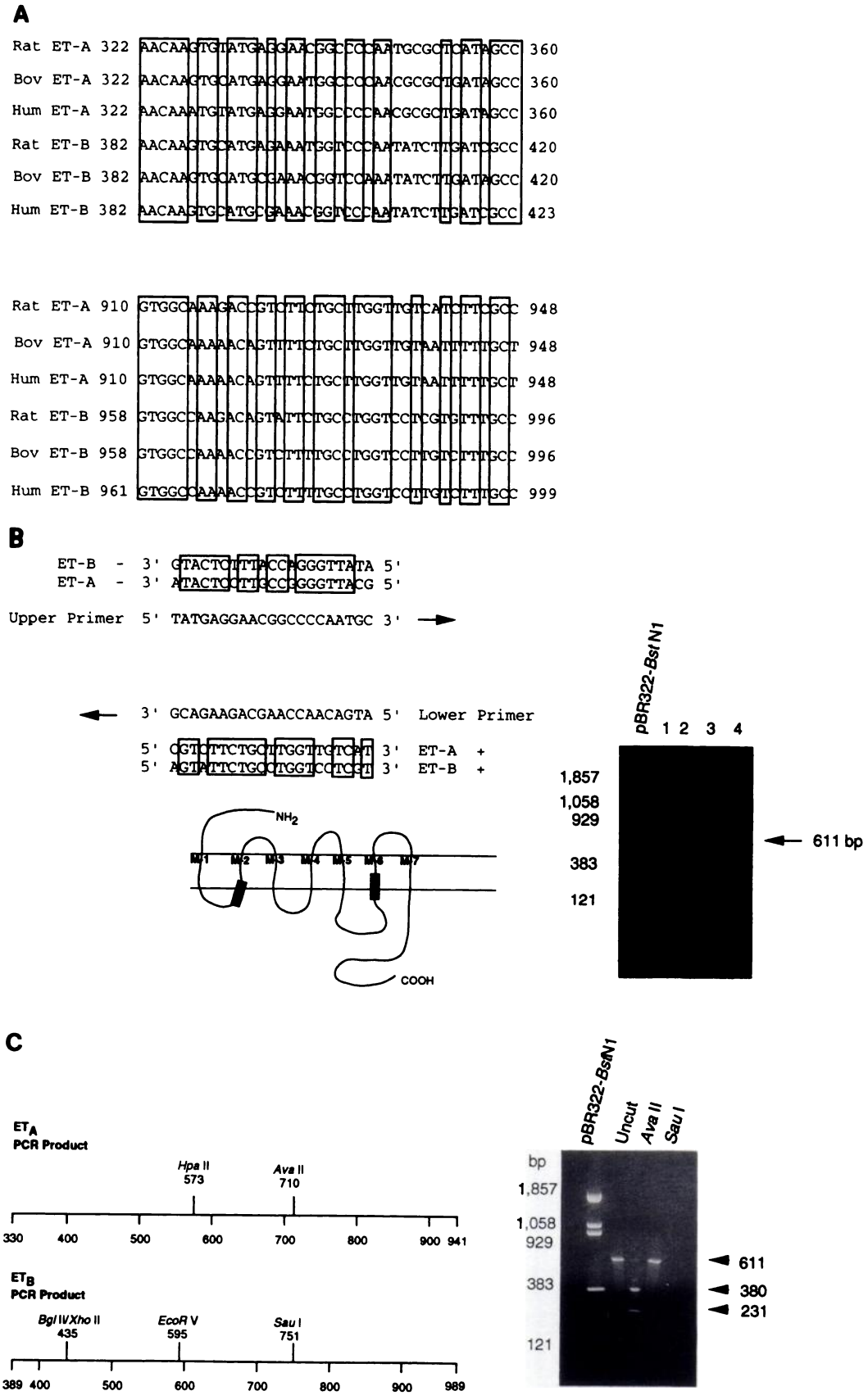

Fig. 7. Analysis of ET receptor mRNA, revealing that mesangial cells express a canonical ET_A receptor. A, Sequence similarities in cDNAs for rat ET_A and ET_B receptors were compared by matrix methods, and evolutionary conservation in matching regions was analyzed for bovine (*Bov*) and human (*Hum*) ET_A/ET_B sequences. DNA sequences within two of these segments were found to be highly conserved in evolution, suggesting that they encode amino acids required for ET receptor activity. *Boxed regions*, exact matches. B, Optimal PCR primers were derived from the highly conserved regions from rat ET_A cDNA (8) presented in A (*left*). Approximate domains of the ET receptor represented by the PCR primers are shown schematically. Total RNA (2 μg) from four different mesangial cell preparations was used in a reverse transcription-PCR to amplify a truncated cDNA. After 40 cycles of PCR the amplified product was resolved on a 1.6% agarose gel stained with ethidium bromide. A single band corresponding to the predicted size (611 bp) for the ET_A receptor was observed in *lanes 1-4* (*right*). C, Restriction mapping of the PCR product (*left*) confirmed its identity as a truncated cDNA encoding the ET_A receptor (*right*).

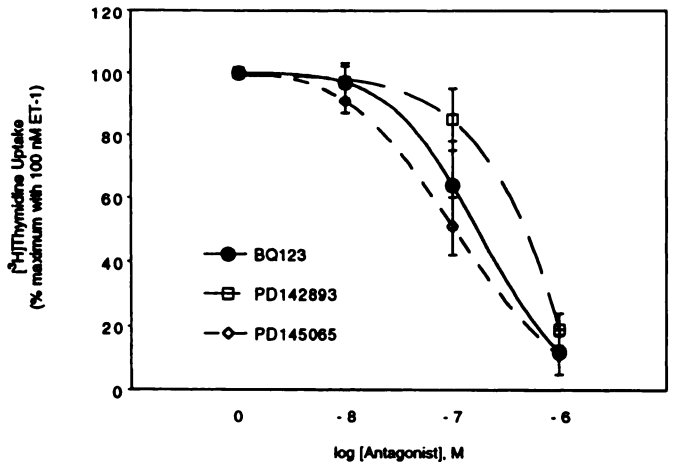

Fig. 8. Semilogarithmic plots for analysis of antagonism of ET-1-induced [^{3}H]thymidine uptake by ET receptor antagonists. Quiescent mesangial cells in 24-well plates were incubated with ET-1 in the presence of increasing concentrations of BQ 123, PD 142893, and PD 145065. After 16 hr the cells were pulsed with [^{3}H]thymidine and extracted as described in Experimental Procedures. Nonlinear curve fitting was used to fit the data and calculate IC_{50} values. Data are mean ± standard error for three independent experiments, in duplicate.

nonselective ET receptor antagonists PD 142893 and PD 145065 blocked ET-1-stimulated [^{3}H]thymidine uptake with higher IC_{50} values than those for blockade of Ca^{2+} signaling (Fig. 8). IC_{50} values for inhibition of [^{3}H]thymidine uptake by PD 142893 and PD 145065 were 740 nM and 105 nM, respectively (versus 62.0 nM for PD 142893 and 9.8 nM for PD 154065 in Fig. 2). To determine whether proteolytic degradation of ET receptor antagonists by mesangial cells might explain the relative inability of reversible ET blockers to inhibit [^{3}H]thymidine uptake, we performed additional experiments in which fresh aliquots of BQ 123 and PD 145065 (10 nM each) were added to the wells every hour for the first 7 hr of the [^{3}H]thymidine uptake assay. Replenishment of ET blockers did not alter the dose-inhibition curves for blockade of ET-1-induced [^{3}H]thymidine uptake (data not shown), which suggests that degradation of ET receptor antagonists by mesangial cells cannot explain the reduced capacity of the antagonists to inhibit ET-1-stimulated [^{3}H]thymidine uptake. These results demonstrate that two distinct classes of reversible ET receptor antagonists are less effective for blockade of the long term biological actions of ET-1 (i.e., [^{3}H]thymidine uptake) than for inhibition of short term events (i.e., Ca^{2+} signaling).

Preincubation with ET receptor antagonists is required to block Ca^{2+} responses produced by subsequent addition of ET-1. We next asked whether the nearly irreversible binding of ET-1 to its receptor would contribute to the relatively high IC_{50} values for inhibition of [^{3}H]thymidine uptake. As expected, a 10-fold molar excess of BQ 123 blocked Ca^{2+} signaling when cells were preincubated with the drug before addition of ET-1 (Fig. 9A), whereas coincubation of cells with BQ123 and ET-1 resulted in only minimal reductions in the ET-1-stimulated Ca^{2+} waveform (Fig. 9B). Postincubations with BQ 123 were completely ineffective in reversing the sustained phase of Ca^{2+} waveforms induced by ET-1 (Fig. 9C). Similar results were also observed with the nonselective ET receptor antagonist PD 145065 (data not shown). Thus, we suggest that the relatively slow rate of dissociation of ET-1 from an ET_A receptor might explain why higher concentrations of antagonists were required to inhibit ET-1-induced [^{3}H]thymidine uptake, compared with Ca^{2+} signaling.

Discussion

Relatively little is known about how vasoactive peptides that bind to G protein-coupled receptors control signaling mechanisms linked to vascular cell growth. Mitogenic signaling by ET-1 in mesangial cells is a useful model to investigate molecular mechanisms of growth regulation by vasoactive peptides. To further define mechanisms by which ET-1 stimulates cell growth, we analyzed expression of ET receptor subtypes in mesangial cells. We conclude that mesangial cells express a canonical ET_A receptor and an uncharacterized ET binding site with pharmacological characteristics unlike those of ET_A, ET_B, or ET_C receptors. Mitogenic signaling, however, is mediated solely by the ET_A receptor subtype. Using two distinct classes of ET receptor antagonists, we showed that higher concentrations of antagonists are required to block ET_A-mediated [^{3}H]thymidine uptake, as opposed to Ca^{2+} signaling. These results suggest that the unique, nearly irreversible, nature of the ET-1 ligand-receptor interaction has important consequences for the design and potential utility of ET receptor antagonists.

Mesangial cells express an ET_A receptor subtype that mediates mitogenic signaling. Several lines of evidence support the contention that ET_A receptors mediate mitogenic signaling in mesangial cells. Here we demonstrate that mesangial cells express canonical ET_A receptors and that occupation of the ET_A receptor by ^{125}I-ET-1 occurs over the same concentration range (see Fig. 4A) as stimulation of [^{3}H]thymidine uptake by ET-1 (16). BQ 123 blocked ET-1-stimulated [^{3}H]thymidine uptake and effectively competed for occupation of the ET_A receptor by ^{125}I-ET-1. Nonselective ET_A/ET_B antagonists also blocked ET-1-stimulated [^{3}H]thymidine uptake and occupation of the ET_A receptor by ^{125}I-ET-1. However, previously published results showing that ET_B-specific agonists fail to stimulate [^{3}H]thymidine uptake (16), and the present results showing that S6c and [$Ala^{1,3,11,15}$]ET-1(6–21) also fail to compete with ^{125}I-ET-1 binding to mesangial cell ET receptors, suggest that mesangial cells do not express functional ET_B receptors. Previous studies using reverse transcription-PCR or Northern RNA blotting demonstrated that mesangial cells express mRNA transcripts for ET_B receptors, although pharmacological evidence of ET_B receptor expression was not presented (25, 26). We were unable to detect ET_B mRNA transcripts in the present study, but it seems possible that some mesangial cell strains express low levels of ET_B mRNA that do not result in functional expression of ET_B receptors. Further evidence for the lack of ET_B receptor expression in mesangial cells is the rank order of potency for [^{3}H]thymidine uptake stimulation by ET isopeptides (i.e., ET-1 > ET-3) (16). Moreover, our present data demonstrate that high concentrations of ET-3 displace only small amounts of ^{125}I-ET-1 from the ET_A receptor in mesangial cells. It is also noteworthy that BQ 123 blocks ET-1-stimulated tyrosine phosphorylation of cellular proteins (16) and activation of $pp60^{c\text{-}src}$,[1] which are both implicated in mitogenic signaling by ET-1. Additional evidence for involvement of ET_A receptors in vascular cell growth comes from studies by Ohlstein *et al.* (27), where BQ 123 was shown

[1] M. S. Simonson and W. H. Herman, unpublished observations.

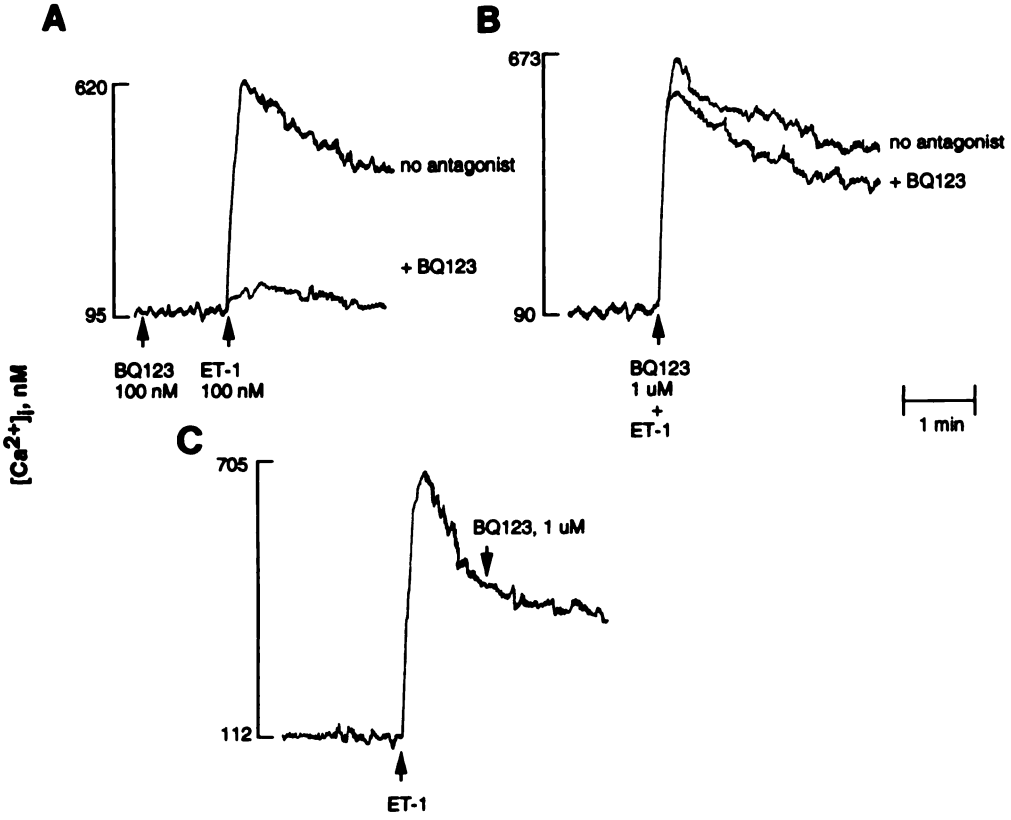

Fig. 9. Requirement for preincubation with reversible ET receptor antagonists to effectively block increments in $[Ca^{2+}]_i$ stimulated by ET-1. Quiescent mesangial cells loaded with fura-2 were either preincubated, coincubated, or postincubated with BQ-123 and then treated with ET-1. *Arrows*, agonist and antagonist additions. A, Preincubation with BQ 123 effectively blocked ET-1-stimulated increments in $[Ca^{2+}]_i$, whereas (B) coincubation with BQ 123 was only minimally effective. C, Postincubation with BQ 123 failed to reduce the magnitude or duration of the sustained phase of Ca^{2+} signaling. $[Ca^{2+}]_i$ tracings with and without antagonist were superimposed to facilitate comparison. Identical results were observed in three or four independent experiments.

to block ET-1-stimulated [^{3}H]thymidine uptake in vascular smooth muscle cells. Benigni *et al.* (15) also showed that daily administration of FR139317, an ET_A-specific antagonist, completely blocked mesangial cell proliferation in an animal model of progressive renal injury. Although indirect effects of FR139317 (i.e., vasorelaxation leading to reduced intraglomerular pressures) could not be ruled out in that study, the finding that mesangial cell proliferation is blocked by an ET_A-specific receptor antagonist provides strong support for the idea that ET_A receptors mediate mitogenic signaling *in vivo*. Our present results do not rule out the possibility that ET_B receptors might also regulate cell growth. For example, ET-1 is a mitogen for some but not all endothelial cells, which express ET_B receptors (6). Thus, the question of whether ET_B receptors mediate cell growth in other cell types remains to be resolved.

Two major strategies have been developed for antagonism of ET receptors, (i) use of subtype-specific antagonists that exclusively block either ET_A (BQ 123 and FR139317) or ET_B (IRL 1038) receptors or (ii) development of nonselective antagonists (PD 145065) that inhibit binding of ET-1 to both ET_A and ET_B receptor subtypes. Our finding that ET_A receptors are responsible for mitogenic signaling raises the question of whether nonselective ET_A/ET_B antagonists would have any advantage over ET_A-specific antagonists. Although our data suggest that ET_A-specific antagonists would be sufficient to inhibit mesangial cell proliferation, the kidney expresses abundant levels of ET_B receptors linked to vasoconstriction (12, 28, 29). Thus, the use of nonselective ET_A/ET_B blockers might be beneficial, in that both vasoconstriction and proliferation produced by dysregulation of ET-1 secretion would be attenuated.

Higher concentrations of ET receptor antagonists are required to inhibit [^{3}H]thymidine uptake, compared with Ca^{2+} signaling, providing evidence for complex mechanisms of ET receptor antagonism. One explanation for the relative inefficiency of ET receptor antagonists in blocking [^{3}H]thymidine uptake stimulated by ET-1 lies in the nearly irreversible binding of ET-1 and the formation of nonequilibrium conditions under which receptor antagonists might not effectively compete with ET-1 for occupancy of the ET receptor in long term incubations. Experiments by Vigne *et al.* (30) showed that BQ 123 forms stable complexes with the ET_A receptor that are, however, less stable than ET-1-ET_A complexes. This concern is reinforced by the relatively high concentrations of currently available, reversible, ET receptor antagonists required to completely inhibit ET-1-induced contraction of isolated blood vessels. If the reverse rate constant (k_{-1}) for the ET-receptor interaction is smaller than the corresponding rate constant for the antagonist-receptor interaction (i.e, $k_{-1} < k_{-2}$), then time-dependent formation of stable ET-1-ET_A complexes would reduce occupancy of ET receptors by antagonists in long term incubations. If this explanation was valid, we reasoned that preincubation with ET receptor antagonists

might also be required to effectively block Ca^{2+} signaling in fura-2-loaded mesangial cells. In fact, formation of stable ET-1-ET_A complexes is consistent with our Ca^{2+} experiments, in which preincubation (not coincubation or postincubation) with ET receptor antagonists was required to attenuate Ca^{2+} signaling. The inability of conventional ET receptor antagonists to effectively compete for ET receptor occupancy in longer term incubations might also explain why these antagonists must be used at high concentrations and/or as continuous infusions to block ET-1 actions both *in vitro* and *in vivo* (see Refs. 3 and 31 for review). At present little is known about the mechanisms underlying the nearly irreversible ET-1-receptor interaction. In mesangial cells antagonism was not purely noncompetitive but might be better characterized as mixed antagonism.

Glomerular mesangial cells express two receptors for ET-1. In mesangial cells, the lower affinity, high capacity binding site has pharmacological properties consistent with those of an ET_A receptor. Analysis of an ET receptor cDNA product demonstrated that mesangial cells express a canonical ET_A receptor. Although three ET receptor subtypes have been unambiguously identified by molecular cloning, a few reports provide indirect evidence for the existence of novel ET receptor subtypes (32–34). The binding properties of these putative novel ET receptors appear to be different from those of the high affinity, low capacity site in mesangial cells. The high affinity site in mesangial cells does not bind ET-3, S6c, or [$Ala^{1,3,11,15}$]ET-1(6–21), suggesting that these sites are not ET_B receptors. Using a set of PCR primers for the ET_A and ET_B receptors, we were unable to amplify a novel ET receptor cDNA from mesangial cell RNA, which suggests that if mesangial cells express a second ET receptor the sequence for this receptor must be divergent from those of canonical ET_A and ET_B receptors.

Analysis of the competition displacement binding curves suggested a complex binding phenomenon that might be explained by independent populations of binding sites, negative cooperativity, or interconvertible affinity states of the ET receptor. However, several independent lines of pharmacological and biochemical evidence suggest that these data are best explained by proposing a two-site model for mesangial cells. First, our present experiments using competition binding assays, and previously published experiments in mesangial cells using saturation equilibrium techniques (23, 24), produced ET binding isotherms that could not be interpreted by a one-site model. One study also demonstrated that the lower affinity site (presumably the ET_A receptor) was approximately 4 times more abundant than the high affinity site (24), similar to the ratio of low affinity to high affinity sites in the present study. Second, affinity labeling of ET receptors in mesangial cell membrane preparations demonstrated the presence of two distinct proteins with different molecular weights (18, 35). The results presented here support the conclusion that ET-1 binds to two different proteins in mesangial cell membranes, although all three studies derived slightly different estimates of the molecular masses of these receptors. Third, occupation of these two binding sites by ET-1 evokes different mechanisms of signal transduction. ET-1, at concentrations that occupy only the high affinity receptor, stimulates a receptor-operated Ca^{2+} current, without release of Ca^{2+} from intracellular stores (5), and induction of c-*fos* but not c-*jun*, without a concomitant increase in AP-1 *cis*-element activity (36). Occupation of the high affinity receptor by ET-1 also fails to increase [^{3}H]thymidine uptake (5, 16). In contrast, addition of ET-1 at concentrations that occupy the lower affinity ET_A receptor stimulates phospholipase C and inositol phosphate turnover, protein kinase C, release of Ca^{2+} from intracellular stores (5), and induction of both c-*fos* and c-*jun* with concomitant activation of AP-1 (16, 36). Occupation of the lower affinity ET_A receptor also stimulates tyrosine phosphorylation of cellular proteins, $pp60^{c\text{-}src}$ activity, and mitogenesis (5, 16). Taken together, these data suggest that glomerular mesangial cells express two receptors for ET-1. Additional experiments are necessary before a more complete analysis of this second receptor is possible.

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