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Expression of Endothelin Receptor Subtypes in Rabbit Saphenous Vein

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SUMMARY

Recent investigations have revealed the presence of vasoconstrictory endothelin (ET)-B receptors in several tissues, including the rabbit saphenous vein (RSV). To determine the nature of the ET binding sites in RSV, radioligand-receptor binding studies with selective ligands and Northern analyses with probes from the ET-A and ET-B receptor cDNAs were conducted. ET-1 inhibited ^{125}I-ET-1 binding to RSV in a monophasic manner, with an inhibition constant (K_i) of 0.08 ± 0.02 nM and a slope factor of 0.9 ± 0.1. ET-3 inhibition of ^{125}I-ET-1 binding was biphasic, with 68% of the ^{125}I-ET-1 binding sites being displaceable with a K_i value of 31 ± 4 nM. The remaining 32% of the sites displayed high affinity for ET-3 (K_i = 0.2 ± 0.1 nM). The ET-A-selective peptide BQ-123 inhibited ^{125}I-ET-1 binding in a biphasic manner, with K_i values of 10.4 ± 1.9 nM and 3.2 ± 0.9 μM. The high affinity BQ-123 site comprised 70% of the binding sites, whereas the low affinity site comprised 30%. The correspondence of high affinity binding sites for BQ-123 and low affinity binding sites for ET-3 is consistent with the suggestion that 70% of the ^{125}I-ET-1 binding sites in this tissue are ET-A receptors. To further investigate the nature of the ET-B binding sites in RSV, ^{125}I-ET-3 competition binding experiments were conducted. ET-1 and BQ-123 inhibited ^{125}I-ET-3 binding in RSV with K_i values of 40 ± 7 pM and 7.2 μM, respectively, whereas inhibition curves for ET-3 and the ET-B receptor-selective agonist sarafotoxin S6c (S6c) were best fit to two-site models. Resultant K_i values for ET-3 and S6c were 50 pM (71%)/4 pM (29%) and 0.7 nM (71%)/95 nM (29%), respectively. Binding in RSV differed from that in rat cerebellum, where ET-3 and S6c inhibition of ^{125}I-ET-3 binding was monophasic (K_i values of 70 pM and 1.1 nM for ET-3 and S6c, respectively). The presence of the nonhydrolyzable guanine nucleotide analog guanosine-5′-*O*-(3-thio)triphosphate (200 μM) did not affect ^{125}I-ET-3 binding. Low stringency Northern analysis of RSV RNA with [α-^{32}P]dCTP-labeled fragments from the ET-A or ET-B receptor cDNAs revealed similar hybridization patterns with both probes, with two resolved RNA species migrating at 4.7 and 1.8 kilobases. These data indicate that the ET receptor population in RSV is composed of approximately 70% ET-A receptors and 30% ET-B receptors, with a component of the ET-B population displaying very high affinity for ET-3 and moderate affinity for S6c.

The ET and sarafotoxin peptides comprise a family of structurally and pharmacologically related agents with potent biological activity (1). At present, there are as many as eight known members of the ET/sarafotoxin family, i.e., ET-1, ET-2, ET-3, vasoactive intestinal contractor, and sarafotoxins a, b, c, and d. The ET peptides arise from specific prepro-ET proteins, each of which is encoded by a separate gene. ET, originally isolated from porcine endothelial cells (2), has now been shown to be produced by numerous tissues, including lung, kidney, eye, gastrointestinal tract, and many nuclei in the central nervous system (3, 4). Among the diverse actions exerted by ETs are contraction (4, 5) and vasodilation (6) of smooth muscle, pressor (7) and depressor (8) effects in the renal vasculature, positive myocardial inotropy and chronotropy (9), aldosterone and prostaglandin release (10, 11), mitogenicity (12–14), inhibition of prolactin secretion (15), and neuromodulation (16). These diverse actions are widely attributed to the existence of multiple ET receptor subtypes with discrete and regulated cellular distributions and functions.

To date, two ET receptor types have been identified and cloned. ET-A receptors are distinguished by selectivity for ET-1 and ET-2 binding over that of ET-3 (17). In contrast, ET-B receptors bind ET-1, ET-2, and ET-3 with similar affinities (18). ET-A and ET-B receptors are generally thought to mediate opposing actions, i.e., ET-A receptors mediate vasoconstriction and are located on smooth muscle cells, whereas ET-B receptors mediate vasodilation and are found on endothelial cells (19). However, more recent evidence suggests that activation of ET-B receptors leads to pressor and contractile responses. Williams *et al.* (20) showed that the ET-B receptor-selective peptide S6c increased blood pressure in pithed rats, and Harrison *et al.* (21) found that ET-3 and S6c stimulated

ABBREVIATIONS: ET, endothelin; S6c, sarafotoxin S6c; RSV, rabbit saphenous vein; HPLC, high pressure liquid chromatography; TI, trypsin inhibitor; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; CHO, Chinese hamster ovary; kb, kilobase(s).

tension development in endothelium-denuded pig coronary arteries. The vasoconstrictory responses to ET-1 and S6c in RSV (22) and endothelium-denuded porcine coronary artery (23) were not totally reversed by high concentrations of the ET-A receptor antagonist BQ-123, consistent with the notion that constriction was mediated in part by a non-ET-A receptor. In guinea pig pulmonary artery, the vasomotor responses to ET-1 and ET-3 did not cross-sensitize; rather, the contractile responses to these agonists were additive (24). An understanding of the ET receptor subtypes is critical to the characterization and development of ET receptor-selective antagonists. The purpose of this investigation was to examine ET receptors in RSV, a tissue in which vasoconstrictory ET-B receptors have been identified.

Experimental Procedures

Materials. ^{125}I-ET-1 (2200 Ci/mmol), ^{125}I-ET-3 (2200 Ci/mmol), and ^{125}I-IRL 1620 (2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA), ET-1, ET-3, and S6c from Peninsula Labs (Belmont, CA), cell culture reagents from GIBCO, Nuserum from Collaborative Research, and transfection reagents from Specialty Media. BQ-123 was prepared at Bristol-Myers Squibb Pharmaceutical Research Institute.

Preparation of RSV membranes. Male New Zealand White rabbits were sacrificed by pentobarbital injection and saphenous veins were excised and placed in ice-cold buffer. After the connective tissue was dissected away, veins were minced and homogenized in ice-cold 50 mM Tris·HCl, 0.24 TI units/ml aprotinin, 1 mM EDTA buffer (Brinkman Polytron, three bursts of 6 sec at setting 8). The homogenate was centrifuged at 700 × *g* for 10 min, and the resulting supernatant was further centrifuged at 100,000 × *g* for 60 min at 4°. The membrane pellet was resuspended in 50 mM Tris·HCl, 0.24 TI units/ml aprotinin, 1 mM EDTA. Aliquots were stored frozen at −80°. Before use in the binding assay, membranes were rehomogenized as described above.

Preparation of rat cerebellar membranes. Cerebellar tissue from Sprague-Dawley rats was homogenized for 30 sec (Brinkman Polytron, setting 8) in ice-cold 50 mM Tris·HCl, pH 7.4, with 0.24 TI units/ml aprotinin and 1 mM EDTA. The crude particulate matter was removed by centrifugation at 750 × *g* for 10 min at 4°. The membranes were sedimented from the supernatant fraction by centrifugation at 48,000 × *g* for 30 min. Membrane pellets were resuspended in the aforementioned buffer and stored in aliquots at −80° until use.

Radioligand binding assays. ET receptor binding assays were conducted as described previously (25). For competition binding, membranes (10–30 μg of RSV protein or 0.5–5 μg of rat cerebellar protein) were incubated with 50 pM ^{125}I-ET-1 or ^{125}I-ET-3, in the absence or presence of 100 nM ET-1 or ET-3, respectively, in a final volume of 0.25 ml of assay buffer (50 mM Tris·HCl, pH 7.4, 0.1% bovine serum albumin, 2 μM phosphoramidon), at 37° for 2 hr. For saturation binding, 0.01–1.0 nM ^{125}I-ET-1 or 0.01–0.4 nM ^{125}I-ET-3 was used in the binding reaction. Binding reactions were terminated by rapid filtration in a Tomtec cell harvester over a Filtermat B (Pharmacia LKB, Uppsala, Sweden) that had been presoaked for 1 hr in assay buffer. The filtermat was rinsed with 150 mM NaCl, 5 mM Tris·HCl, pH 7.4 at 4°, microwaved, and counted in a Betaplate liquid scintillation counter (Pharmacia LKB) in the presence of Meltilex solid scintillant wax (Pharmacia LKB). Counting efficiency was 65%. 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 (26). 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 (27).

HPLC analysis of peptides. ET-3 and S6c were chromatographed on a Waters HPLC.

RNA preparation and Northern analyses. Saphenous veins were removed from rabbits and dissected free from surrounding fat and connective tissue. The tissue was wrapped individually in sterile aluminum foil, quick-frozen in liquid N_2, and stored at −20° until use. The wrapped tissues were crushed into powder in a mortar and pestle, transferred into 5 ml of RNAzol solution (Biotecx Laboratories, Inc., Houston, TX), and homogenized at 4° (Brinkman Polytron, setting 8 for 30 sec). Chloroform was added to the homogenate, which was mixed vigorously for 15 sec and kept on ice for 15 min. The preparation was centrifuged at 12,000 × *g* for 15 min at 4°. The aqueous phase was recovered, mixed with an equal volume of isopropanol, and stored at −20° for 45 min. After centrifugation at 12,000 × *g* for 15 min at 4°, the RNA precipitate was washed twice with 75% alcohol, resuspended in 50 μl of formamide, heated at 65° for 15 min, and stored at −20° (28). Total RNA (10 μg) from RSV was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to Duralose-UV membranes (Stratagene, La Jolla, CA). Membranes were hybridized for 16–20 hr at 35° with either ET-A or ET-B receptor or actin DNA (labeled with [α-^{32}P]dCTP by random priming; Pharmacia, Uppsala, Sweden) in Hybrisol I containing 50% formamide (Oncor, Gaithersburg, MD). ET receptor probes were the 1600-base pair *Hind*III-*Asp*300 ET-A or 1700-base pair *Eco*RI ET-B DNA excised from the pCDM8/huET-A or pCDM8/huET-B vectors, respectively, and represent the entire coding sequences of the human ET-A (29) and ET-B[1] receptors. β-Actin DNA was a generous gift from Dr. Joe Fargnoli of BMSPRI. The blots were washed in 2× standard saline citrate (0.15 M NaCl, 0.015 M Na_3 citrate), 0.1% sodium dodecyl sulfate, twice for 15 min at room temperature and once for 1 hr at 52° and were autoradiographed with intensifying screens at −70° for at least 8 hr.

Statistical analysis. Data are expressed as means ± standard errors. Statistical comparisons were made using Student's *t* test. The null hypothesis was rejected at $p < 0.05$.

Results

Saturation binding of ^{125}I-ET-1, ^{125}I-ET-3, and ^{125}I-IRL 1620 to RSV membranes was compared under equilibrium binding conditions, reached after 2 hr at 37° (data not shown). Because ET-1 binds to ET-A and ET-B receptors with equal affinity, ^{125}I-ET-1 (0.01–1.0 nM) saturation binding should reflect the sum of ET-A and ET-B receptors. In contrast, because ET-3 binds to ET-B receptors with high affinity (K_i = 0.1 nM) but to ET-A receptors with low affinity (K_i = 86 nM) (30), concentrations of ^{125}I-ET-3 up to 1 nM should occupy ≤1% of the ET-A receptors. ^{125}I-IRL 1620 has been reported to have 120,000-fold selectivity for ET-B receptors (31). For each radioligand, specific binding was defined in the presence of 100 nM unlabeled ligand. ^{125}I-ET-1, ^{125}I-ET-3, and ^{125}I-IRL 1620 binding to ET receptors in RSV was specific and saturable (Fig. 1), with high affinity binding sites detected for each radioligand (mean ± standard error K_d values of 50 ± 20, 20 ± 5, and 50 ± 5 pM, respectively, for three experiments). Specific binding typically ranged from 40 to 90% over radioligand concentration ranges of 0.01 to 0.5 nM. In all cases, total and nonspecific binding increased in a radioligand concentration-dependent fashion and resulted in specific binding isotherms suggestive of a single population of receptor binding sites. Scatchard transformation of these data (Fig. 2) indicated that the receptor densities for ^{125}I-ET-1 (399 ± 89 fmol/mg of protein) differed significantly ($p < 0.05$) from those for ^{125}I-ET-3 (116 ± 16 fmol/mg of protein) and ^{125}I-IRL 1620 (117 ± 8 fmol/mg of protein), which recognized the same quantity of receptors. These data indicate that binding sites for ^{125}I-ET-3 and ^{125}I-IRL 1620 comprise approximately 30% of the total ^{125}I-ET-1 receptor sites in RSV.

[1] P. Rose, unpublished observations.

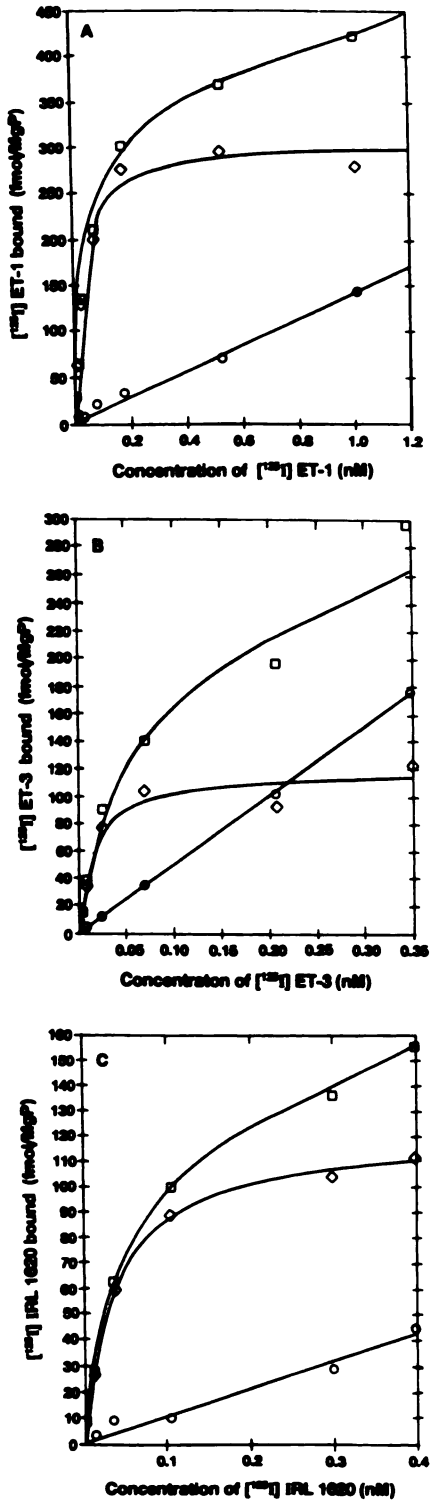

Fig. 1. Saturation binding isotherms for ^{125}I-ET-1 (A), ^{125}I-ET-3 (B), and ^{125}I-IRL 1620 (C). Saturation binding to RSV membranes was conducted at 37° for 2 hr, as described in Experimental Procedures. Nonspecific binding for each radioligand was defined in the presence of 100 nM unlabeled ligand. □, Total binding; ○, nonspecific binding; ◇, specific binding. The results are representative of at least three similar experiments.

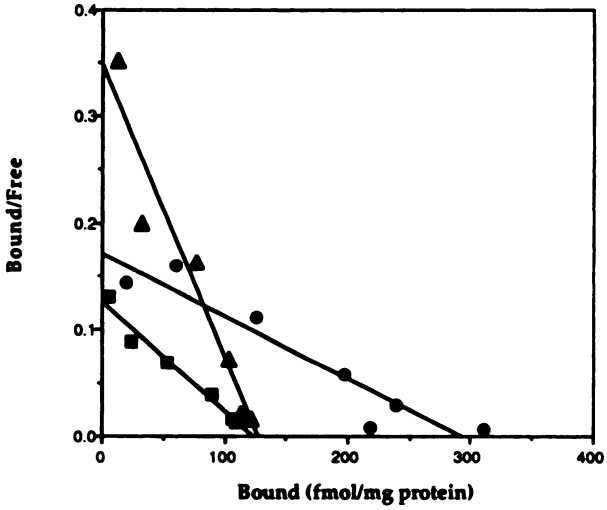

Fig. 2. Scatchard transformation of saturation binding data for ^{125}I-ET-1, ^{125}I-ET-3, and ^{125}I-IRL 1620. ●, ^{125}I-ET-1; ▲, ^{125}I-ET-3; ■, ^{125}I-IRL 1620. The results are representative of three similar experiments.

To further investigate the ET receptor subtypes in RSV, a series of inhibition binding studies were conducted with ^{125}I-ET-1 and ^{125}I-ET-3. ET-1 inhibited ^{125}I-ET-1 binding to RSV in a monophasic manner, with a K_i value and slope factor of 0.08 ± 0.02 nM and 0.9 ± 0.1, respectively (Fig. 3A). In contrast, ET-3 inhibition of ^{125}I-ET-1 binding was biphasic and the data were best fit to a two-site model (Fig. 3B). The high affinity site (K_i = 0.2 nM) represented 32% of the receptor population, whereas the low affinity site (K_i = 31 nM) represented 68% of the sites (Table 1). Similarly, the ET-A-selective peptide BQ-123 also inhibited ^{125}I-ET-1 binding in a biphasic manner (Fig. 3C). The high (K_i = 10 nM) and low (K_i = 3.2 μM) affinity BQ-123 sites comprised 70% and 30% of the binding sites, respectively (Table 1). These data are consistent with the presence of subtypes of ET receptors in RSV.

To further examine the ET receptor subtypes in RSV, competition binding experiments with ^{125}I-ET-3 (0.03 nM) were conducted. ET-1 and BQ-123 inhibition of ^{125}I-ET-3 binding was monophasic, whereas ET-3 and S6c inhibited ^{125}I-ET-3 binding to RSV membranes in a dose-dependent, biphasic fashion (Fig. 4). The ET-3 and S6c data were best fit to two-site binding models, with K_i values for ET-3 of 50 and 4 pM and for S6c of 0.7 and 95 nM (Table 1). The 4 pM affinity ET-3 site comprised only 29% of the total binding population. This proportion was consistent with that of the 95 nM affinity S6c site, which also represented 29% of the binding sites.

The possibility that the presence of a binding component with very high affinity for ET-3 and moderate affinity for S6c in RSV was due to an artifact of the binding conditions was excluded by performing similar radioligand-receptor binding studies in rat cerebellum. In comparison with the two-site ET-3 and S6c inhibition of ^{125}I-ET-3 binding seen in RSV, the slopes of the inhibition curves for rat cerebellar membranes did not differ significantly from unity (Table 2). Moreover, the ET-3 K_i value in rat cerebellum was 70 pM, compared with 50 and 4 pM in the RSV. This K_i value was consistent with the 50 ± 0.01 pM (three experiments) K_d value obtained from ^{125}I-ET-3 saturation binding experiments in rat cerebellum.

The question arises as to whether the biphasic curves for ET-3 and S6c inhibition of ^{125}I-ET-3 binding represent a distinct subtype of ET-B receptor or an agonist-modulated G protein-coupled state of the ET-B receptor. If the latter were

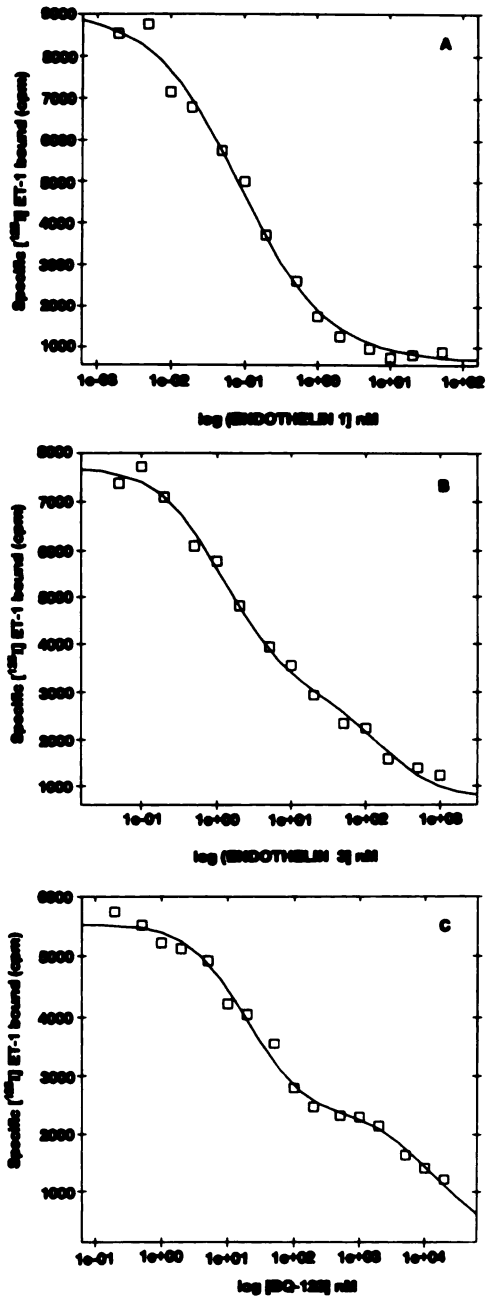

Fig. 3. ET-1 (A), ET-3 (B), and BQ-123 (C) inhibition of ^{125}I-ET-1 (0.05 nM) binding to RSV membranes. Binding was conducted at 37° for 2 hr, as described in Experimental Procedures. Nonspecific binding was defined in the presence of 100 nM ET-1. The results are representative of at least three similar experiments.

true, one would expect that the high affinity binding site would be shifted to a lower affinity form in the presence of guanine nucleotides. This seemed unlikely, because this binding component recognizes ET-3 with very high affinity and S6c with moderate affinity. Nevertheless, we examined the possibility that G protein coupling modulates the binding of ^{125}I-ET-3 to RSV membranes. Inhibition and dissociation binding experiments were conducted in the absence and presence of the nonhydrolyzable guanine nucleotide GTPγS. GTPγS (200 μM) caused a slight (10%) but consistent decrease in binding of the agonist to the receptor. Inhibition by S6c of ^{125}I-ET-3 binding

TABLE 1

Inhibition constants (K_i) and slope factors (SF) for ^{125}I-ET-1 (50 pM) and ^{125}-ET-3 (30 pM) binding in RSV

Values are means ± standard errors of three to seven experiments.

	^{125}I-ET-1	^{125}I-ET-3
ET-1		
K_i (nM)	0.08 ± 0.02	0.04 ± 0.007
SF	0.9 ± 0.1	0.9 ± 0.04
ET-3		
K_{i_1} (nM)	37.0 ± 10 (68%)[a]	0.05 ± 0.008 (71%)
K_{i_2} (nM)	0.2 ± 0.1 (32%)	0.004 ± 0.001 (29%)
SF	1.0[b]	1.0[b]
BQ-123		
K_{i_1} (nM)	10.4 ± 1.9 (70%)	7216 ± 939
K_{i_2} (nM)	3215 ± 918 (30%)	
SF	1.0[b]	0.7 ± 0.09
S6c		
K_{i_1} (nM)		0.7 ± 0.3 (71%)
K_{i_2} (nM)		95 ± 19 (29%)
SF		1.0[b]

[a] Percentages represent proportions of total binding.
[b] Slope constrained to 1.

was unchanged in the presence of 200 μM GTPγS (Table 3). In addition, the presence of 200 μM GTPγS did not affect the rate of dissociation of ^{125}I-ET-3 from RSV membranes (data not shown).

The possibility that proteolytic degradation of competing peptides was a factor in the biphasic inhibition by ET-3 and S6c of ^{125}I-ET-3 binding was examined. To determine whether prolonged incubation with RSV membranes may have led to cleavage of ET-3 or S6c peptides, resulting in altered inhibitory potency, HPLC of ET-3 and S6c was conducted before and after exposure of these peptides to RSV membranes under conditions identical to those used in radioligand binding assays (2 hr at 37° in the radioligand assay buffer). ET-3 and S6c eluted as distinct peaks at 20 min both before and after incubation with RSV membranes (data not shown). Thus, it appears that metabolism of ET-3 and S6c cannot account for the biphasic ^{125}I-ET-3 inhibition curves.

To ascertain the molecular nature of the ET receptor species in RSV, Northern analyses with ET-A and ET-B receptor cDNA probes were conducted under low stringency hybridization conditions. We reasoned that, if a distinct ET binding component were present, low stringency hybridization with the ET-A and ET-B probes might yield additional hybridizing species. Conditions for the hybridizations were determined by using RNA from CHO cells stably transfected with human placental ET-A (CHO/ET-A) and ET-B (CHO/ET-B) receptor cDNA.[2] The ET-A and ET-B probes were the entire coding sequences of these receptor cDNAs excised from pCDM8/huETR-A or pCDM8/huETR-B, respectively, agarose gel purified, and labeled with [α-^{32}P]dCTP. As shown in Fig. 5, the ET-A and ET-B probes clearly hybridized with two RNA species from RSV, migrating at approximately 4.2 and 1.8 kb. In some gels, a faint third band migrating at approximately 1.0 kb was apparent when probed with [α-^{32}P]dCTP-labeled ET-B DNA. This infrequent 1-kb species was not apparent under higher stringency conditions (data not shown). The presence of two hybridizing RNA species ranging from 1.7 to 5.0 kb has been observed previously in numerous tissues (17, 32–34) and

[2] P. Rose, unpublished observations.

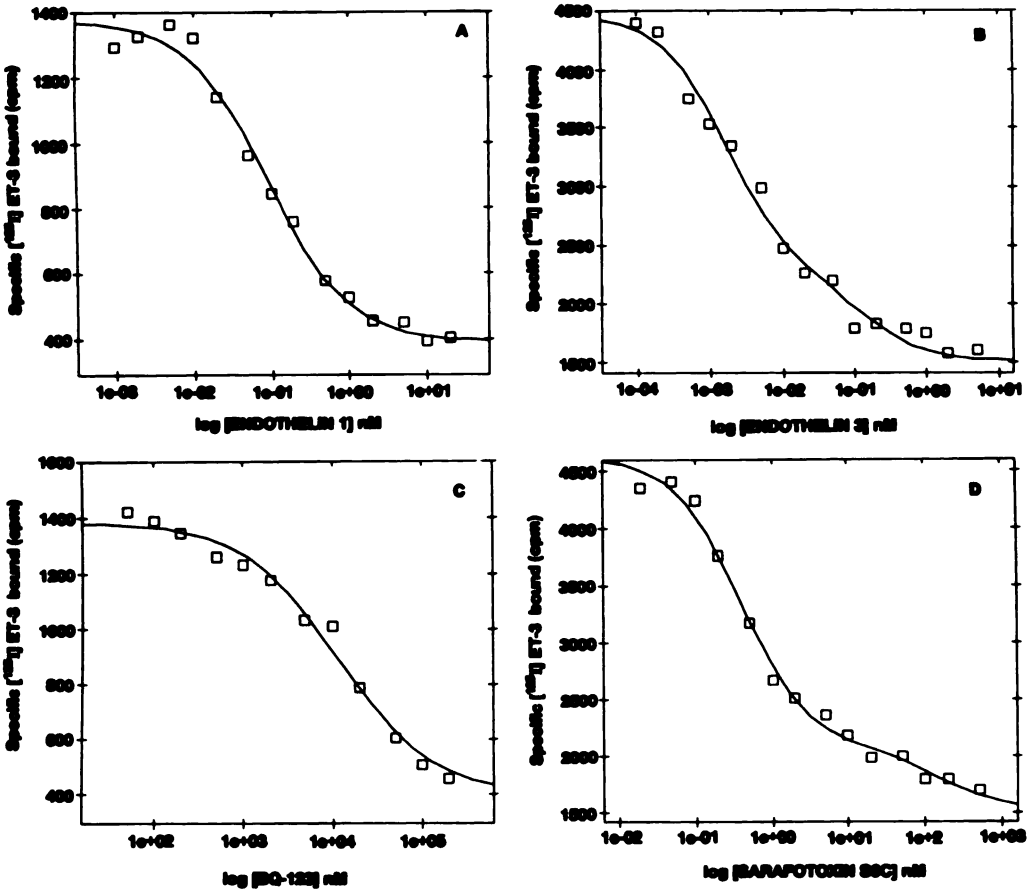

Fig. 4. ET-1 (A), ET-3 (B), BQ-123 (C), and sarafotoxin S6c (D) inhibition of ^{125}I-ET-3 (30 pM) binding to RSV membranes. Binding was conducted at 37° for 2 hr, as described in Experimental Procedures. Nonspecific binding was defined in the presence of 100 nM ET-3. The results are representative of at least three similar experiments.

TABLE 2

Inhibition constants (K_i) and slope factors for ^{125}I-ET-3 (30 pM) binding in rat cerebellum

Values are means ± standard errors of four to eight experiments.

Peptide	K_i	Slope factor
	nM	
ET-1	0.1 ± 0.02	1.1 ± 0.06
ET-3	0.07 ± 0.01	1.0 ± 0.06
BQ-123	>20,000	
S6c	1.1 ± 0.2	0.9 ± 0.5

TABLE 3

Effect of GTP-γS (200 μM) on inhibition constants (K_i) for S6c inhibition of ^{125}I-ET-3 (30 pM) binding to RSV membranes

Values are means ± standard errors of six to ten experiments.

K_i	
Control	+GTPγS
nM	
0.6 ± 0.2 (75%)	0.1 ± 0.0 (79%)
100 ± 27 (25%)	88 ± 57 (29%)

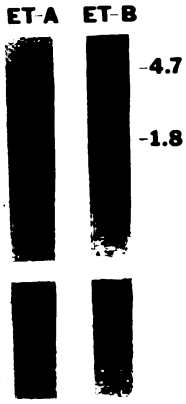

Fig. 5. Northern hybridization analysis of total RNA extracted from RSV. *Right margin*, positions and sizes (in kb) of the RNA markers. Hybridization conditions were as described in Experimental Procedures. *Upper*, hybridization with [α-^{32}P]dCTP-labeled ET-A DNA or [α-^{32}P]dCTP-labeled ET-B DNA. *Lower*, hybridization with [α-^{32}P]dCTP-labeled actin DNA as an internal control. The results are representative of several similar experiments.

has been attributed to differential processing of the transcript. The apparently greater amount of ET-B RNA is probably due to cross-hybridization of the ET-B probe with ET-A RNA under the low stringency conditions used here. Consistent with the binding data, high stringency Northern analysis indicated a 3–4-fold greater quantity of ET-A RNA than ET-B RNA (data not shown).

Discussion

The existence of ET-A and ET-B receptors is now well established (17, 18), and it has become clear that both types of ET receptors play a role in vasoconstriction of pulmonary artery, coronary artery, and saphenous vein (21–23). However, detailed molecular pharmacological examination of vasoconstrictory ET-A and ET-B receptors has proceeded slowly. In this study, selective ET receptor ligands were utilized to dem-

onstrate for the first time the presence of ET receptor subtypes in RSV.

Saturation binding data demonstrate that specific ^{125}I-ET-3 and ^{125}I-IRL 1620 sites comprise only 30% of the ^{125}I-ET-1 binding site maxima. Takai *et al.* (31) have recently shown that ^{125}I-IRL 1620 is 120,000-fold selective for ET-B receptors. Because ^{125}I-ET-3 and ^{125}I-IRL 1620 recognize the same receptor density, these data suggest that both radioligands are binding to ET-B receptors. Moreover, the proportion of ET-A and ET-B receptors identified by saturation binding is consistent with the ^{125}I-ET-1 competition binding data, which show that approximately 70% of the ^{125}I-ET-1-labeled sites display high affinity for the ET-A receptor-selective antagonist BQ-123. Thus, using ET receptor-selective ligands, the data presented here indicate that RSV contains approximately 70% ET-A receptors and 30% ET-B receptors.

The K_d values for binding of all three radioligands to RSV reported here (20–50 pM) are indicative of high affinity receptor-ligand interactions and are consistent with previous K_d values reported for ^{125}I-ET-1 binding to rat lung (29 pM) (35), porcine cerebellum (74 pM) (36), or human kidney (91 pM) (37) or for ^{125}I-ET-3 binding to rat lung (13 pM) (35), human kidney (117 pM) (37), or porcine cerebellum (120 pM) (38). Scatchard transformations of the present data were consistent with a single binding site for each radioligand, over the concentration range of radioligand. Sokolovsky *et al.* (39) recently demonstrated that two high affinity binding sites for ^{125}I-ET-3 in rat cerebellum, with K_d values of 50 and 600 pM, could be identified if the specific activity of the radioligand was reduced so that the concentration range for saturation binding could be expanded to 1 nM. In the present studies, concentrations of ^{125}I-ET-3 up to 0.4 nM did not reveal a second high affinity site in either the RSV or rat cerebellum. Whereas the absence of a 600 pM affinity site in the present studies may be due to the concentration range of ^{125}I-ET-3, Takayanagi *et al.* (38) used ^{125}I-ET-3 concentrations up to 4 nM and also failed to detect a second high affinity ET-3 site in porcine cerebellum. Although the reason for the variance in these results is unclear, it is possibly related to the use of different species.

Similar to findings in saturation binding experiments, competition with ^{125}I-ET-1 binding to RSV membranes was indicative of subtypes of ET receptors. The nonselective ET receptor ligand ET-1 produced a monophasic inhibition curve, but both the ET-A receptor-selective ligand BQ-123 and the ET-B receptor-selective ligand ET-3 inhibited ^{125}I-ET-1 binding in a biphasic manner. The proportion of sites with high affinity for BQ-123 corresponds to that of sites with low affinity for ET-3, characteristics of ET-A receptors. Conversely, the proportion of sites with high affinity for ET-3 corresponds to that of sites with low affinity for BQ-123, binding properties characteristic of ET-B receptors. Thus, these data are consistent with those from saturation binding analyses and indicate that 70% of the ET receptor population detected in RSV is ET-A and 30% is ET-B.

An interesting finding from further study of the ET-B receptor population was the biphasic ^{125}I-ET-3 competition curves. Competition curves from experiments using the ET-B receptor-selective ligands S6c and ET-3 to inhibit ^{125}I-ET-3 binding were best resolved by two-site models. The 50 pM ET-3 binding site corresponds to the "super-high" affinity site described by Sokolovsky *et al.* (39) and seen in the present saturation binding analyses. However, the other ET-3 site observed demonstrated 4 pM affinity for ET-3. The proportion of sites with 4 pM affinity for ET-3 corresponded to that of a site with only moderate affinity for S6c (K_i = 95 nM). Our observation of a 4 pM affinity ET-3 binding component is reminiscent of ^{125}I-ET-3 competition binding by ET-3 in bovine endothelial cells, where K_i values of 6 and 200 pM were obtained (40). High (16 pM) and low (4 μM) affinity S6c binding, corresponding to ET-B and ET-A receptors, respectively, has been reported in several tissues (20, 35). However, this is the first report of a moderate affinity S6c binding site. Given the proportions of the ET-3 and S6c binding components, it stands to reason that the 4 pM affinity ET-3 site and 95 nM affinity S6c site are characteristics of the same binding component. Cioffi *et al.* (35) detected two S6c sites for inhibition of ^{125}I-ET-3 binding in rat lung (20 pM and 4.6 nM). Consistent with data reported here, neither S6c site was altered by 1 mM GTPγS.

ET receptors are members of the superfamily of proteins with seven membrane-spanning domains and have been shown to be coupled to G proteins (17, 18, 30). Guanine nucleotides decrease the affinity of agonists for G protein-coupled receptors and increase the slope factor of the inhibition curve (41). A slight (10%) but consistent decrease in binding of the ^{125}I-ET-3 agonist was observed in these experiments, consistent with a decrease in agonist affinity in the presence of GTPγS. The lack of a GTPγS-induced shift in affinity or slope of the S6c competition curves for ^{125}I-ET-3 binding suggests that the two binding components are not due to different G protein-coupled states of the same receptor. It can also be argued that, because the same component shows very high affinity for ET-3 but only moderate affinity for S6c, it is unlikely to be due to G protein modulation. In addition, proteolytic degradation of the competing peptides appears not to have played a role in the experiments reported here, suggesting that ET-3 and S6c did not give rise to products that could account for a very high affinity ET-3 or moderate affinity S6c binding component. However, interpretation of agonist binding data is complicated by cation effects and possible interactions with other membrane components (42), distinct from G protein modulation and potential receptor heterogeneity. Moreover, determination of K_i values is predicated on reversible bimolecular interactions; the effect of slow reversibility of ET receptor-ligand interactions[3] on the Cheng and Prusoff (26) correction is unclear. Further experimentation with a high affinity, radiolabeled antagonist or purified receptors is warranted to determine whether the additional ET binding component represents a novel receptor subtype or is associated with agonist binding.

Northern hybridization experiments demonstrated the presence of transcripts in the RSV that hybridize with ET-A and ET-B receptor DNA probes. The presence of similar RNA hybridization patterns detectable with ET-A and ET-B DNA probes, coupled with the lack of other clearly detectable hybridizing RNAs under low stringency conditions, suggests that additional RNA species related to ET-A or ET-B are absent or are present as low-copy transcripts. Consistent with these findings is the recent report that only a single gene encoding each of the ET-A and ET-B receptors was found in the human (43) and bovine genomes (44), respectively. Thus, the ET receptor subtypes are products of distinct genes. These data

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

argue that, if additional ET receptor subtypes exist, such candidates may differ substantially in nucleic acid sequence from previously cloned ET receptors. Recently, a novel ET-3-preferring receptor cDNA was cloned from *Xenopus* that is 50% homologous to both the ET-A and ET-B receptors (45). Additional studies are necessary to determine whether the receptor encoded by the *Xenopus* cDNA is present in the mammalian genome.

In summary, this study demonstrates for the first time the presence of ET receptor subtypes in RSV. Although the precise role of ET-A and ET-B receptors in saphenous vein is unclear, previous work demonstrated that S6c-induced contractions of endothelium-denuded saphenous vein tissue strips were not inhibited by BQ-123 (22), indicating that contractile activity in saphenous vein smooth muscle is not mediated by ET-A receptors. Because the present work was conducted in membranes from saphenous veins that were not systematically denuded of endothelium, it is unclear which of the binding components represents the vasoconstrictory ET receptor. *In situ* hybridization experiments are in progress to determine the precise cellular location of ET receptor subtypes in the saphenous vein and to examine the potential pathological role of ET in vascular disease.

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