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Identification and Characterization of a Novel Endothelin Receptor That Binds Both ET_A- and ET_B-Selective Ligands

PONNAL NAMBI, MARK PULLEN, JAMIE KINCAID, PARVATHI NUTHULAGANTI, NAMBI AIYAR, DAVID P. BROOKS, MIKLOS GELLAI, and CHANDRIKA KUMAR

Departments of Renal Pharmacology (P.N., M.P., J.K., D.P.B., M.G.), Cardiovascular Pharmacology (N.A.), and Molecular Genetics (C.K.), SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406-0939

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SUMMARY

This study demonstrates the presence of a novel endothelin (ET) receptor subtype that displays high affinity for both ET_A- and ET_B-selective ligands. This subtype has been identified in canine spleen membranes using ET_B-selective agonists ET-3, IRL-1620, sarafotoxin 6c (S6c) as well as ET_A-selective antagonists BQ123 and related cyclic pentapeptides. Binding of ^{125}I-ET-3 to canine spleen membranes was specific and saturable with an apparent dissociation constant of 130 pM and maximum binding (B_{max}) of 240.0 fmol/mg protein. Although the apparent affinities obtained with ^{125}I-ET-1 and ^{125}I-ET-3 were comparable (90 and 130 pM, respectively), the maximum binding obtained with ^{125}I-ET-3 was ~35% of that obtained with ^{125}I-ET-1, which indicates that canine spleen possesses both ET_A and ET_B receptors in the ratio 65:35. Competition binding experiments using ^{125}I-ET-3 and unlabeled ET-1, ET-3, S6c, and IRL-1620 suggested that although ET-1 and ET-3 displayed similar high affinity, S6c and IRL-1620 were 20–300-fold weaker than ET-1 and ET-3 in competing for ^{125}I-ET-3 binding to canine spleen membranes. In addition, BQ123, an ET_A-selective antagonist, displaced ^{125}I-ET-3 binding from canine spleen with an IC_{50} value of 30 nM. Similar profiles were obtained with related cyclic pentapeptides. Electrophysiological studies performed on *Xenopus laevis* oocytes injected with canine spleen poly(A)$^+$ RNA indicated that the ET_B receptor present in these tissues is functional and displays the same pharmacology as that observed in binding studies using these membranes. As a comparison, both binding and functional studies were performed in canine lung and the data indicate that the ET_B receptor present in this tissue is similar to that of the cloned human ET_B receptor but different from that present in canine spleen. These observations were further confirmed by performing cross-linking experiments on these membranes. Although canine lung and cloned human ET_B receptors displayed the same molecular weight bands with similar pharmacology, canine spleen ET_B receptors displayed different molecular weight bands and different pharmacology. In addition, the ET_B receptors present in canine spleen were also identified in canine bladder, monkey spleen and human spleen. Thus, the data presented in this manuscript provide evidence for the presence of a novel ET_B receptor in different tissues as well as different species including human.

ET-1, a 21-amino-acid peptide identified initially and isolated from endothelial cell culture medium, has attracted considerable attention because of its potent and long-lasting vasoconstrictor property (1). Soon after its discovery, two other isopeptides, ET-2 and ET-3, and a group of snake venom toxins (sarafotoxins) were identified (2, 3). The biological effects of these peptides were shown to be mediated through the binding of these peptides to specific cell surface receptors that were identified in a number of tissues and cell lines (4–7). ET receptors display subtype heterogeneity and, based on the binding properties of various ET-related peptides, they have been classified as ET_A and ET_B. ET_A receptors display high affinity for only ET-1 and ET-2, whereas ET_B receptors display high affinity for ET-1, ET-2, ET-3, and S6c (8). Both receptor subtypes have been cloned, sequenced, expressed and characterized from a number of species, including human (9–11). According to the initial classification, ET_A receptors were shown to mediate vasoconstriction and ET_B receptors were shown to mediate endothelium-dependent vasodilation (4–7). Development of subtype-selective antagonists played a critical role in demonstrating the involvement of ET_A and ET_B receptors in normal cellular functions as well as their role in various pathological conditions (12–15). BQ123, described originally by Ihara *et al.* (16) as an ET_A-selective antagonist, was used by many investigators in binding as well as functional studies because it displayed 1000-fold higher affinity for ET_A receptors than for ET_B receptors (17–21). However, conflicting data from whole animal studies, in which S6c caused potent vasoconstriction in addition to its originally described vasodilation, necessitated the search for additional subtypes of ET receptors, possibly ET_B receptor subtypes (22–25).

ABBREVIATIONS: ET, endothelin; S6c, sarafotoxin 6c; CHO, Chinese hamster ovary; BQ123, c(D-Trp-D-Asp-Pro-D-Val-Leu); BQ610, (*N*,*N*-hexamethylene)-carbamoyl-Leu-D-Trp(CHO)-D-; JKC 301, cyclic(D-Ile-Leu-D-Trp-D-Asp-Pro); JKC 302, cyclic(D-Val-Leu-D-Trp-D-Ser-Pro).

The present study was undertaken to identify and characterize subtypes of ET_B receptors by binding and functional studies. The data presented in this article demonstrate clearly for the first time that the ET_B receptors present in canine spleen, canine bladder, monkey spleen, and human spleen are novel and are different from those present in canine lung and cloned human ET_B receptors.

Materials and Methods

^{125}I-ET-1 (specific activity 2200 Ci/mmol) and ^{125}I-ET-3 (specific activity, 2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Unlabeled ET-1, ET-3, S6c, BQ123, BQ610, JKC 301, and JKC 302 were from American Peptides (Sunnyvale, CA). All other chemicals were of the highest grades available.

Membrane preparation. The tissues (canine lung, spleen, bladder, monkey spleen and human spleen) were washed in ice cold saline, dissected into small pieces and frozen in liquid N_2. The frozen tissues were homogenized in buffer (1 g of tissue/10 ml) containing 20 mM Tris, pH 7.5, 5 mM EDTA, 0.25 M sucrose, 100 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 1 mg/ml leupeptin) using a Tekmar tissuemizer (Cincinnati, OH) model TR-10 polytron at a setting of 80 for 4 × 15 sec on ice. Homogenates were centrifuged for 15 min at 1000 × *g* at 4°. The supernatants were filtered through two layers of cheesecloth and recentrifuged at 40,000 × *g* for 30 min at 4°. The pellets were resuspended in 50 mM Tris·HCl, pH 7.5 and 10 mM $MgCl_2$ and stored in small aliquots after freezing in liquid N_2.

Radioligand binding. ^{125}I-ET-1 and ^{125}I-ET-3 binding to membranes prepared from the above mentioned tissues were performed as described previously (26). Assay volumes were 50 μl and the concentrations of membrane proteins were 5–15, 20–40, 0.5–1.5, 2–4 and 3–6 μg/tube for canine lung, spleen, bladder, monkey spleen, and human spleen, respectively. The concentrations of the radioligands were 30–1000 pM for saturation binding and 100–300 pM for competition binding experiments. Nonspecific binding was measured in the presence of 1 μM unlabeled ET-1 or ET-3. Incubations were for 60 min at 30° and bound and free ligands were separated by filtration using GF/C filter paper and Brandel cell harvester (Brandel, Gaithersburg, MD). Each experiment was performed 2–4 times with multiple membrane preparations and the variation between experiments was less than 10%. The data shown are from one experiment which is representative of all the experiments performed under that condition.

Cross-linking of ET_B receptors. ET_B receptors were cross-linked following the procedure of Nambi *et al.* (27) with minor modifications. Briefly, 50 pM ^{125}I-ET-3 was added to membranes prepared from CHO cells expressing human ET_B receptors, canine lung and canine spleen (6–7 fmol of ET-3 binding activity) in the binding buffer and incubated for 60 min at 30° in the absence and presence of 0.3 μM unlabeled ET-3 (to measure nonspecific binding), 30 nM IRL-1620 or 300 nM BQ610. At the end of incubation, disuccinimidyl suberate dissolved in dimethyl sulfoxide was added at a concentration of 5 mM to each tube, and the incubations were continued for another 30 min at room temperature. The reaction was stopped by centrifuging the mixture and washing the pellets several times before resuspending in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing β-mercaptoethanol. These samples were run on a 10% polyacrylamide gel, and the gel was exposed to x-ray film after being dried.

***Xenopus laevis* oocyte electrophysiology.** Large *X. laevis* females were anesthetized by hypothermia and ovaries were removed surgically. Individual defolliculated oocytes were obtained by manual dissection and stage V oocytes were selected for microinjection (28). For each experimental group, 20–30 oocytes were injected (Drummond injection apparatus) with 50 nl of water containing 25 ng of canine spleen or lung poly(A)$^+$ RNA. Injected oocytes were maintained in Barth's medium at 18° for 48 hr to allow for ET receptor protein expression. Electrophysiology was performed using the voltage clamp technique, with an oocyte voltage clamp apparatus (Warner Instrument, CT). Oocyte membrane potentials were clamped at −60 mV and the Ca^{2+}-activated Cl^- channel activity was recorded in Barth's medium at room temperature as described (29).

Results and Discussion

Saturation binding experiments using ^{125}I-ET-1 (binds to both ET_A and ET_B receptors with the same affinity) and ^{125}I-ET-3 (binds to ET_B receptors with high affinity) were performed in membranes prepared from canine lung and spleen to quantify the proportion of ET_A and ET_B receptors. As shown in Figs. 1 and 2, both tissues displayed high affinity binding sites for ^{125}I-ET-1 and ^{125}I-ET-3, indicating the presence of both ET_A and ET_B receptors in these tissues. The apparent dissociation constants (K_ds) were 33 and 45 pM for

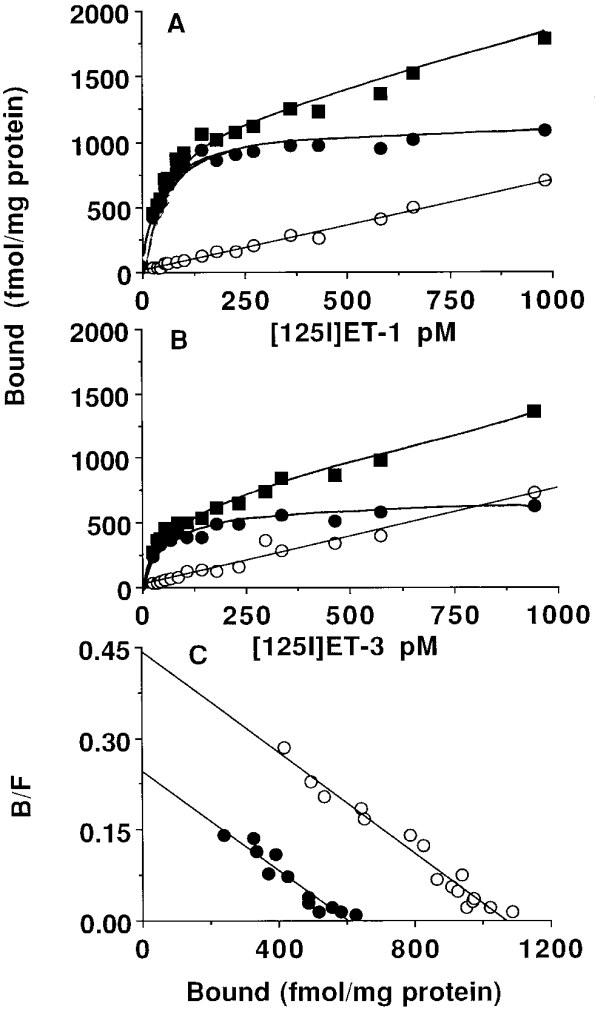

Fig. 1. Saturation binding of ^{125}I-ET-1 (A) and ^{125}I-ET–3 (B) to membranes prepared from canine lung. Increasing concentrations of ^{125}I-ET-1 or ^{125}I-ET-3 were added to membranes in the absence (total binding) or presence (nonspecific binding) of 1 μM unlabeled ET-1 or ET-3 and incubated for 60 min at 30°. The reactions were stopped by filtering the incubation mixture as explained in Materials and Methods. The data presented are from one experiment that is representative of two or three experiments. ■, Total binding; ○, nonspecific binding; ●, specific binding. C, Scatchard plots of the specific binding for ^{125}I-ET-1 (○) and ^{125}I-ET-3 (●) obtained from saturation binding experiment presented in A and B.

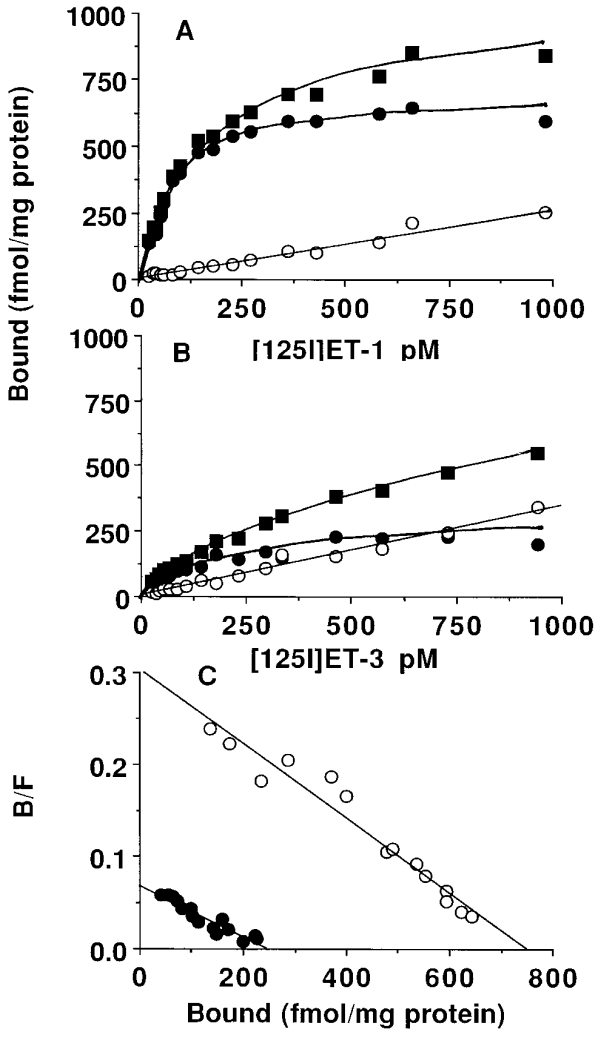

Fig. 2. Saturation binding of ^{125}I-ET-1 (A) and ^{125}I-ET-3 (B) to membranes prepared from canine spleen membranes. The experiment was performed as explained in Fig. 1 legend. ■, Total binding; ○, nonspecific binding; ●, specific binding. C, Scatchard plots of the specific binding for ^{125}I-ET-1 (○) and ^{125}I-ET-3 (●) obtained from the saturation binding experiments.

^{125}I-ET-1 and ^{125}I-ET-3, respectively, in canine lung membranes (Fig. 1C), whereas they were 90 and 130 pM for ^{125}I-ET-1 and ^{125}I-ET-3, respectively, in canine spleen membranes (Fig. 2C). The maximum binding was 1100 and 750 fmol/mg for ^{125}I-ET-1 and 600 and 240 fmol/mg for ^{125}I-ET-3 in canine lung and spleen, respectively (Fig. 1C and 2C). Thus, the proportion of ^{125}I-ET-3 binding was 55% and 35% of that obtained with ^{125}I-ET-1 in canine lung and spleen, respectively (Fig. 1C and 2C). The nonspecific binding for ^{125}I-ET-1 and ^{125}I-ET-3 was from 5–25% and 5–50%, respectively, in both tissues (Fig. 1, A and B, and Fig. 2, A and B).

Because the focus of this effort was to identify subtypes of ET_B receptors, ^{125}I-ET-3 was used as the radioligand for competition binding experiments because it will bind only to ET_B receptors at the concentrations used for the binding studies. For comparison, CHO cell membranes expressing recombinant human ET_A and ET_B receptors were used in the competition binding experiments and ^{125}I-ET-1 and ^{125}I-ET-3 were used as the radioligands for ET_A and ET_B receptors, respectively. Competition of ^{125}I-ET binding by unlabeled ET-1 and ET-3 from human ET_A, human ET_B and canine lung and spleen ET_B receptors shown in Fig. 3. Although ET-1 gave monophasic superimposable competition curves in all four membranes (Fig. 3, *top*), ET-3 displayed high affinity competition curves in CHO/ET_B, canine lung and spleen membranes, whereas it was 90–120-fold less potent in competing for CHO/ET_A receptors (Fig. 3, *bottom*; Table 1). This is not surprising because ET-3 displays very weak affinity for ET_A receptors. Fig. 4 presents the competition binding data obtained with the ET_B-selective agonists, S6c and IRL-1620. As shown in Fig. 4, *top*, S6c was as potent as ET-1 and ET-3 in competing for human ET_B receptors, whereas in canine lung and spleen, it displayed two affinities (Fig. 4, *top*). In canine lung, the predominant proportion (68%) of binding showed high affinity for S6c, whereas in canine spleen, the predominant proportion (74%) displayed low affinity for S6c (Fig. 4, *top*). As expected, S6c showed very weak affinity for human ET_A receptors (Fig. 4, *top*). Similar binding profiles were obtained with IRL-1620 in these four preparations. Although IRL-1620 showed extremely weak binding to ET_A receptors, it remained 6–10-fold less potent than ET-1, ET-3, and S6c for ET_B receptors (Fig. 4, *bottom*).

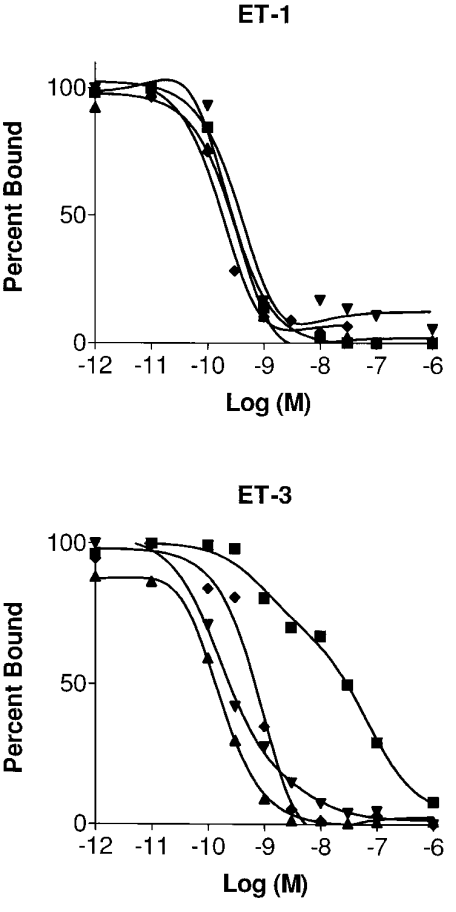

Fig. 3. Competition of ^{125}I-ET-1 binding to human ET_A or ^{125}I-ET-3 binding to human ET_B, canine lung and canine spleen membranes by unlabeled ET-1 (*top*) or ET-3 (*bottom*). Increasing concentrations of unlabeled ET-1 or ET-3 were added to human ET_A (■), ET_B (▲), canine lung ET_B (♦), and canine spleen ET_B (▼) receptors to compete with 0.3 nM ^{125}I-ET-1 or ^{125}I-ET-3. The incubations were for 60 min at 30° and the bound and free ligands were separated as explained in Materials and Methods. The data presented are from one experiment that is representative of three or four similar experiments.

TABLE 1

Analysis of competition binding data obtained from recombinant human ET_A and ET_B receptors and canine lung and spleen ET_B receptors

Competition binding experiments were done as explained in Materials and Methods and legends to Figs. 3–7. The radioligands used were ^{125}I-ET-1 for human ET_A and ^{125}I-ET-3 for ET_B canine lung and spleen. %, percentage of receptor; H, high affinity; L, low affinity. The data were analyzed using in Plot (Graph Pad) program. The data presented are from one experiment that is representative of two or three experiments.

Ligands	ET_A		ET_B		Canine lung		Canine spleen	
	K_i	%	K_i	%	K_i	%	K_i	%
	nM		*nM*		*nM*		*nM*	
ET-1	0.3	100	0.24	100	0.15	100	0.32	100
ET-3	20.7	100	0.17	100	0.17	100	0.23	100
S6c	1,220	100	0.53	100	0.23	68 (H)	0.34	26 (H)
					125	32 (L)	100	74 (L)
IRL-1620	8,860	100	3.9	100	0.87	63 (H)	0.58	27 (H)
					872	37 (L)	2,530	73 (L)
BQ123	41.4	100					12.3	76 (H)
							63,000	24 (L)
BQ610	13.3	100					4.06	69 (H)
							100,000	31 (L)
JKC 301	115	100	31,500	100	15	34 (H)	7.3	72 (H)
					46,000	66 (L)	302	28 (L)
JKC 302	9,000	100					8,000	

In canine spleen and lung, IRL-1620 competed with two affinities. The percentages of high affinity IRL-1620 binding sites in canine lung and spleen were 63 and 27, respectively (Fig. 4, *bottom*).

As a negative control for ET_B receptors, we also tested BQ123, the putative ET_A-selective antagonist, in these preparations. The unexpected results of these experiments are shown in Fig. 5, *top*. BQ123 was very potent in competing for ^{125}I-ET-3 binding to canine spleen membranes (Fig. 5, *top*). In addition, canine spleen ET_B receptors displayed two affin-

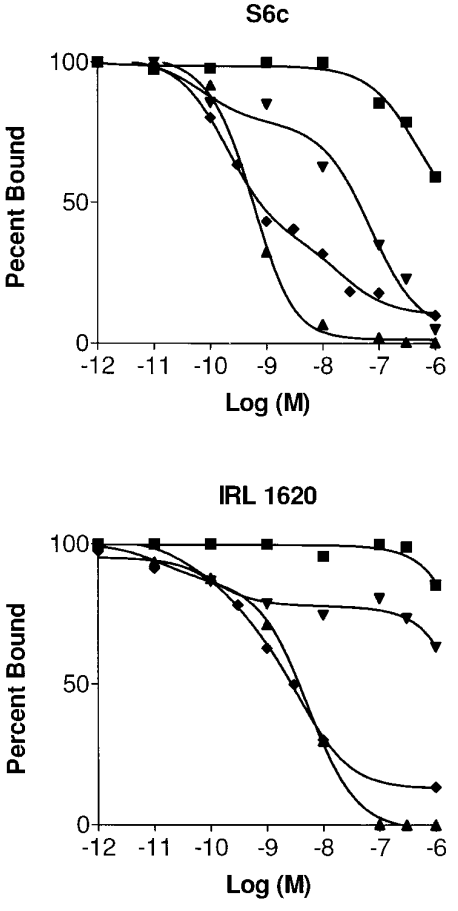

Fig. 4. Competition of ^{125}I-ET-1 binding to human ET_A (■) and ^{125}I-ET-3 binding to human ET_B (▲), canine lung ET_B (♦), and canine spleen ET_B (▼) receptors by unlabeled S6c (*top*) and IRL-1620 (*bottom*). The experiment was performed as explained in legend to Fig. 3.

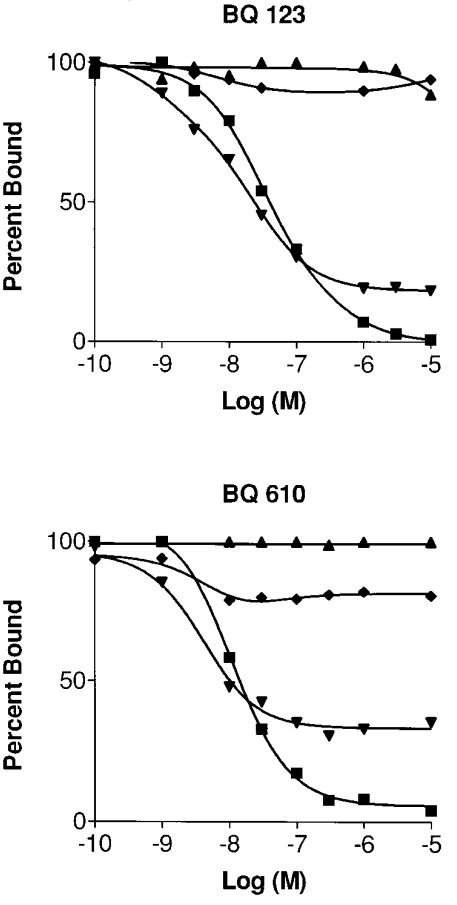

Fig. 5. Competition of ^{125}I-ET-1 binding to human ET_A (■) and ^{125}I-ET-3 binding to human ET_B (▲), canine lung ET_B (♦), and canine spleen ET_B (▼) receptors by unlabeled BQ123 (*top*) and BQ610 (*bottom*). The experiment was performed as explained in legend Fig. 3. The data are from one experiment that is representative of three or four similar experiments.

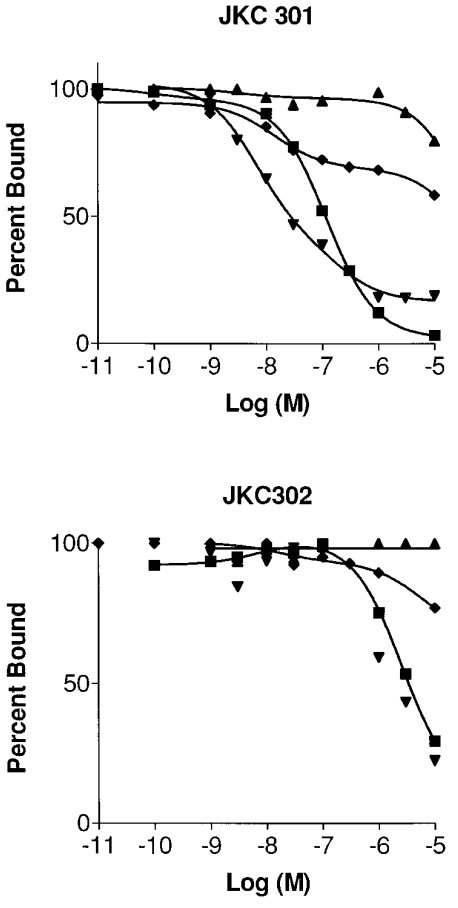

Fig. 6. Competition of ^{125}I-ET-1 binding to human ET_A (■) and ^{125}I-ET-3 binding to human ET_B (▲), canine lung ET_B (♦), and canine spleen ET_B (▼) receptors by unlabeled JKC 301 (*top*) and JKC 302 (*bottom*). The experiment was performed as explained in legend to Fig. 3.

ities for BQ123. The predominant (76%) receptor displayed high affinity (K_i = 12.3 nM) for BQ123, whereas 24% of the receptors displayed low affinity for BQ123 (K_i = 63 μM). This low affinity K_i value compared well with the published K_i value obtained for BQ123 with cloned human ET_B receptors (30). In addition, the proportion of canine spleen ET receptors that showed low affinity for BQ123 (K_i = 63 μM) had high affinity for S6c and IRL-1620 (K_i = 0.34 and 0.58 nM, respectively) and the proportion of canine spleen ET receptors that demonstrated high affinity (K_i = 12.3 nM) for BQ123 showed low affinities for S6c and IRL-1620 (K_i = 100 and 2530 nM, respectively) (Table 1). These data indicate clearly that BQ123 was binding to ET_B receptors present in canine spleen and that this ET_B receptor was different from the one present in canine lung and the cloned human ET_B receptors. Indeed, BQ123 displayed very similar binding profiles in canine lung and cloned human ET_B receptors (Fig. 5a).

To further confirm this novel observation, we performed additional experiments using other ET_A-selective antagonists (BQ610, JKC301, and JKC302) (Fig. 5, *bottom*, and 6). BQ610 displaced ^{125}I-ET-1 binding from human ET_A receptors with a K_i value of 13.3 nM and was very weak in displacing ^{125}I-ET-3 binding from human ET_B receptors (Fig. 5, *bottom*; Table 1). Similar to BQ123, it displayed two affinities for displacing ^{125}I-ET-3 from canine spleen. The K_i values were 4.06 nM and 100 μM for the high and low affinity sites, respectively; the proportion of high and low affinity sites was 69 and 31%, respectively (Fig. 5, *bottom*; Table 1). Similar binding curves were obtained with JKC301 (Fig. 6, *top*) and JKC302 (Fig. 6, *bottom*), two other ET_A selective antagonists. JKC302 was weaker than JKC301 in ET_A and canine spleen preparations (Fig. 6, *bottom*); however, the binding profiles were very similar. Because spleen tissue is highly proteolytic, it was important to find out whether the binding profiles obtained with BQ123 and related compounds were caused by proteolysis of these peptides. To address this possibility, competition experiments were performed in the presence (data not shown) and absence of a protease inhibitor cocktail; the data indicated that there was no difference in the binding profiles whether the protease inhibitors were present or absent.

Thus, the data presented thus far indicate that ET_A-selective antagonists displayed very similar affinities for cloned human ET_A receptors and canine spleen ET_B receptors. At the concentrations (100–300 pM) used in these competition binding studies, ^{125}I-ET-3 has been shown to bind only to ET_B receptors; thus, in competition binding studies, ET-3 did

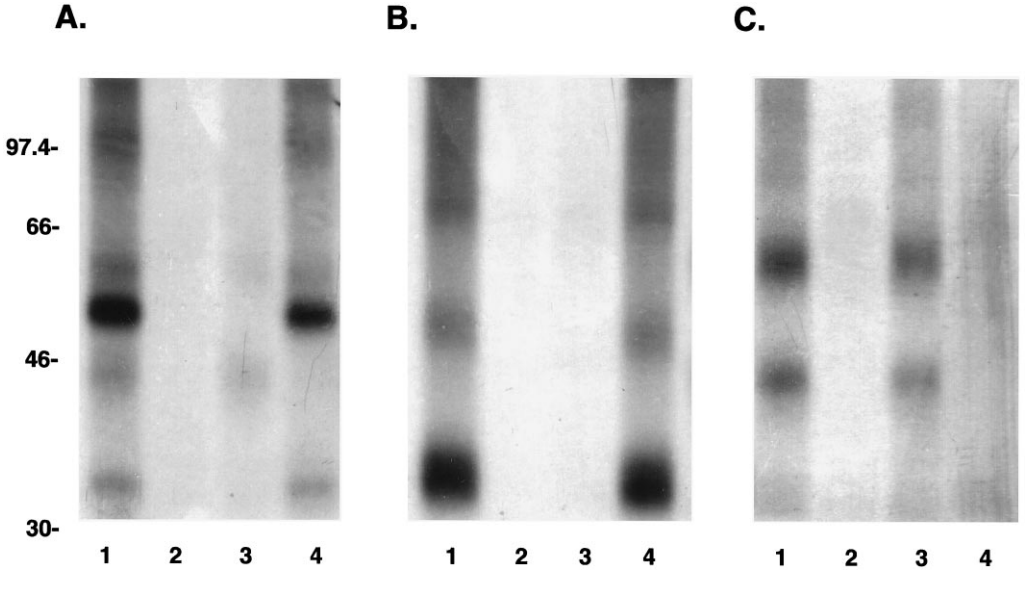

Fig. 7. Cross-linking of ^{125}I ET-3 to membranes prepared from CHO cells stably transfected with recombinant human ET_B receptors (A), canine lung (B) and canine spleen (C). Membranes were cross-linked with ^{125}I ET-3 in the absence (*lane 1*) or presence of 0.3 μM unlabeled ET-3 (*lane 2*), 30 nM IRL-1620 (*lane 3*), and 300 nM BQ610 (*lane 4*) as explained in Materials and Methods.

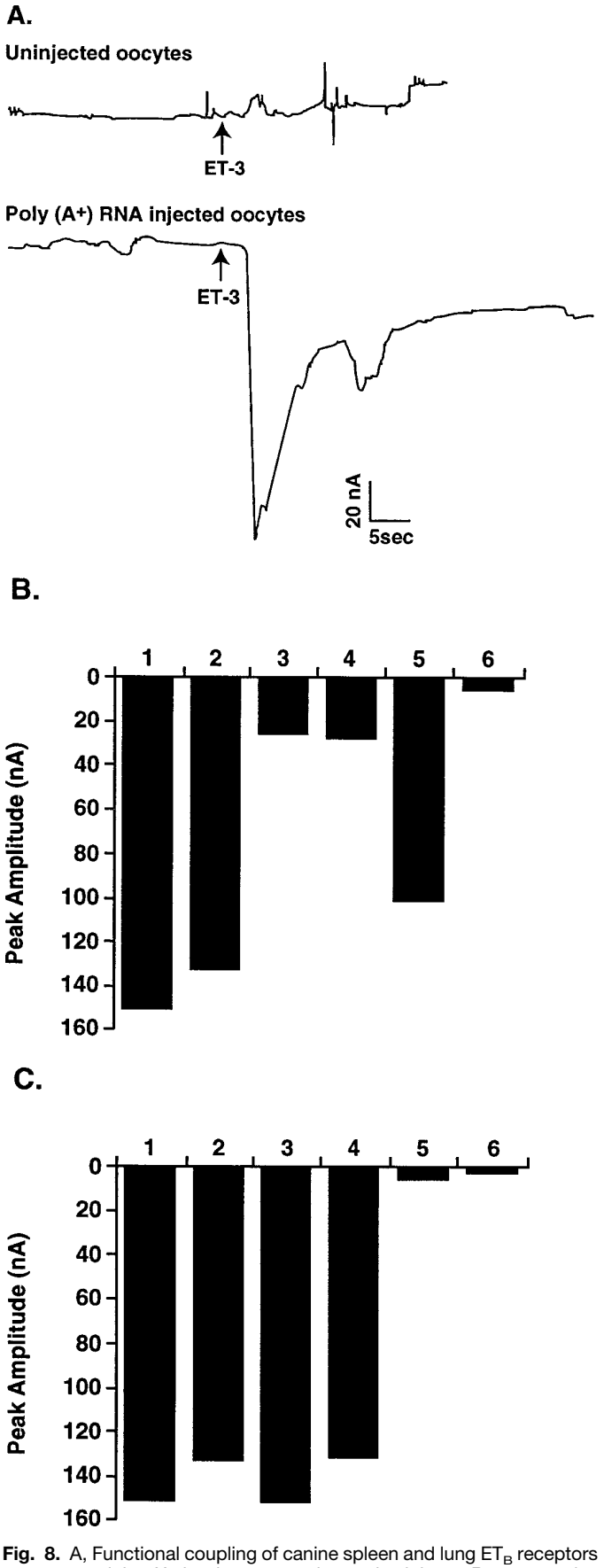

Fig. 8. A, Functional coupling of canine spleen and lung ET_B receptors as assessed by *X. laevis* oocyte electrophysiology. Representative tracings of ET-3- (1 nM) induced current (in nanoAmperes) induced 2 days after oocyte injection with water (*top*) or 25 ng of poly(A)$^+$ RNA isolated from canine lung or spleen. *Arrow,* addition of ET-3. The mean ± standard error peak current response to ET-3 is 160 ± 20 (n = 30). B, Histogram showing the chloride current induced in *X. laevis* oocytes injected with poly(A)$^+$ RNA isolated from canine spleen. The experiment was performed as explained in Materials and Methods. *Bars, 1*, ET-3 alone; *2*, S6c alone; *3*, ET-3 + BQ123; *4*, S6c + BQ123; *5*, ET-3 + RES701; *6*, ET-3 + SB 209670. The concentrations of ligands are 1 nM and 10 nM for ET-3 and S6c, respectively, and 100 nM for BQ123, RES701, and SB 209670. C, Histogram showing the chloride current induced in *X. laevis* oocytes injected with poly(A)$^+$ RNA isolated from canine lung. The experiment was performed as explained in Materials and Methods. *Bars, 1*, ET-3 alone; *2*, S6c alone; *3*, ET-3 + BQ123; *4*, S6c + BQ123; *5*, ET-3 + RES701; *6*, ET-3 + SB 209670. The concentrations of ligands are 1 nM and 10 nM for ET-3 and S6c, respectively, and 100 nM for BQ123, RES701, and SB 209670.

not have any effect on ^{125}I-ET-1 binding to ET_A receptors up to 1 nM (Fig. 3, *bottom*). Furthermore, ^{125}I-ET-1 was purposely not used for competition binding experiments in canine spleen and lung because it will bind to both ET_A and ET_B receptors and complicate the interpretation of the data. A summary of the K_i values and the ratio of different affinity sites are presented in Table 1. These interesting observations were further confirmed by performing cross-linking studies on these membranes; the data are presented in Fig. 7. Canine lung and cloned human ET_B receptors displayed two specific bands at approximate molecular masses of 56 and 34 kDa (Fig. 7, A and B, *lane 1*). The labeling of these two bands was blocked by 30 nM IRL-1620 (Fig. 7, A and B, *lane 3*), but not by 300 nM BQ610 (Fig. 7, A and B, *lane 4*). On the other hand, cross-linking of canine spleen membranes with ^{125}I ET-3 resulted in specific labeling of two bands at 65 and 46 kDa (Fig. 7C, *lane 1*), and the labeling of these two bands was blocked by 300 nM BQ610 (Fig. 7C, *lane 4*) and not 30 nM IRL-1620 (Fig. 7C, *lane 3*). Nonspecific labeling was identified in the presence of 300 nM unlabeled ET-3 (Fig. 7, *lanes 2*). The differences observed in the molecular masses of ^{125}I ET-3 labeled bands between canine spleen, canine lung, and recombinant human ET_B receptor may be caused by differences in post-translational modifications such as glycosylation of these receptors. Thus, these data further confirm the observations made in the binding studies.

Studies were also performed to examine whether these receptors are functional. Heterologous expression of this ET_B receptor in *X. laevis* oocytes resulted in increased Ca^{2+}-activated chloride current in response to ET-3. Actual tracings of the responses obtained with *X. laevis* oocytes injected with water or poly(A)$^+$ RNA isolated from canine spleen or lung are shown in Fig. 8A. In addition, S6c (10 nM) also increased Ca^{2+}-activated chloride current in oocytes injected with canine spleen poly(A)$^+$ RNA (Fig. 8B). BQ123 (100 nM), ET_A-selective antagonist, inhibited ET-3-mediated (1 nM) as well as S6c-mediated (10 nM) responses by 85%, whereas RES701 (100 nM), an ET_B-selective antagonist (31), inhibited ET-3-mediated response by only 30% (Fig. 8B). The reason why BQ123 did not inhibit ET-3- or S6c-mediated responses completely may be attributable to the presence of a small proportion (~20%) of the BQ123-insensitive ET_B receptor in these membranes (Table 1). RES701, which is a potent antagonist for cloned human ET_B receptors (IC_{50} = 100 nM) and a weak antagonist for canine spleen ET_B receptors (IC_{50} > 10 μM), inhibited only 30% of ET-3-mediated response in canine spleen, again suggesting the presence of two ET_B receptors in

this tissue (BQ123-sensitive and RES701-sensitive). These functional data agree with the binding data obtained with BQ123 and related compounds that show two site-competition binding curves (Figs. 5 and 6; Table 1). SB 209670 (nonselective ET receptor antagonist) inhibited ET-3-mediated response almost completely (Fig. 8B). In comparison, similar experiments were performed with oocytes injected with poly(A)$^+$ RNA isolated from canine lung; the data are presented in Fig. 8C. As observed in canine spleen, both ET-3 and S6c stimulated a chloride current, indicating the presence of ET_B receptors in canine lung; however, their responses were not inhibited by BQ123 (Fig. 8C), which suggests that these ET_B receptors are different from those present in canine spleen. On the other hand, RES701 completely abolished the ET-3-mediated response, further confirming the difference between canine spleen and lung ET_B receptors. As in canine spleen, SB 209670 also abolished ET-3-mediated response in canine lung, suggesting that SB 209670 did not distinguish between the ET_B receptors present in these two tissues. These functional data agree well with the binding data obtained with canine spleen and lung membranes.

Is this novel ET_B receptor present only in canine spleen or do other tissues and species display this subtype of ET_B receptor? To address this question, binding studies were conducted using membranes prepared from different tissues and different species, including human. The data presented in Fig. 9 demonstrate clearly that this novel ET_B receptor subtype is also present in canine bladder, monkey spleen, and human spleen. BQ123 (Fig. 9, *top*) displaced ^{125}I-ET-3 binding from canine bladder and monkey spleen with apparent K_i values of 7 and 16 nM, respectively. In human spleen, the K_i values were 8.16 nM and 25.7 μM, respectively, and the distribution of the receptors in high and low affinity states were 30% and 70%, respectively. Similar results were obtained with BQ610 (Fig. 9, *bottom*), which displayed K_i values of 2.2 and 4.5 nM for canine bladder and monkey spleen, respectively. The K_i values for human spleen were 0.4 nM and 10.3 μM, respectively; the distribution of receptors in high and low affinity states were 29% and 71%, respectively. These data indicate clearly that the presence of this novel ET_B receptor is not restricted to one tissue or species. It is present in different tissues and different species, including humans.

In summary, the data presented in this manuscript demonstrate clearly that there are subtypes of ET_B receptors and demonstrate for the first time the nonselective nature of BQ123 and related putative ET_A-selective antagonists. We and many other investigators have used BQ123 to characterize and quantify the subtypes of ET receptors and to study their involvement in different physiological and pathological conditions. Further studies are needed to revisit the old conclusions that were drawn based on these antagonists alone, especially in spleen and bladder. In addition, future experiments will be directed toward the identification of the expression of this receptor subtype using a wide range of tissues and species. At present, we do not know the physiological function of this newly identified ET receptor, although it is tempting to speculate that this may be the subtype of ET_B receptor that mediates vasoconstriction. Molecular cloning of this newly identified ET_B receptor subtype and its expression will enable further characterization of this receptor by binding and functional studies as well as its tissue distribution and regulation in normal and pathological conditions.

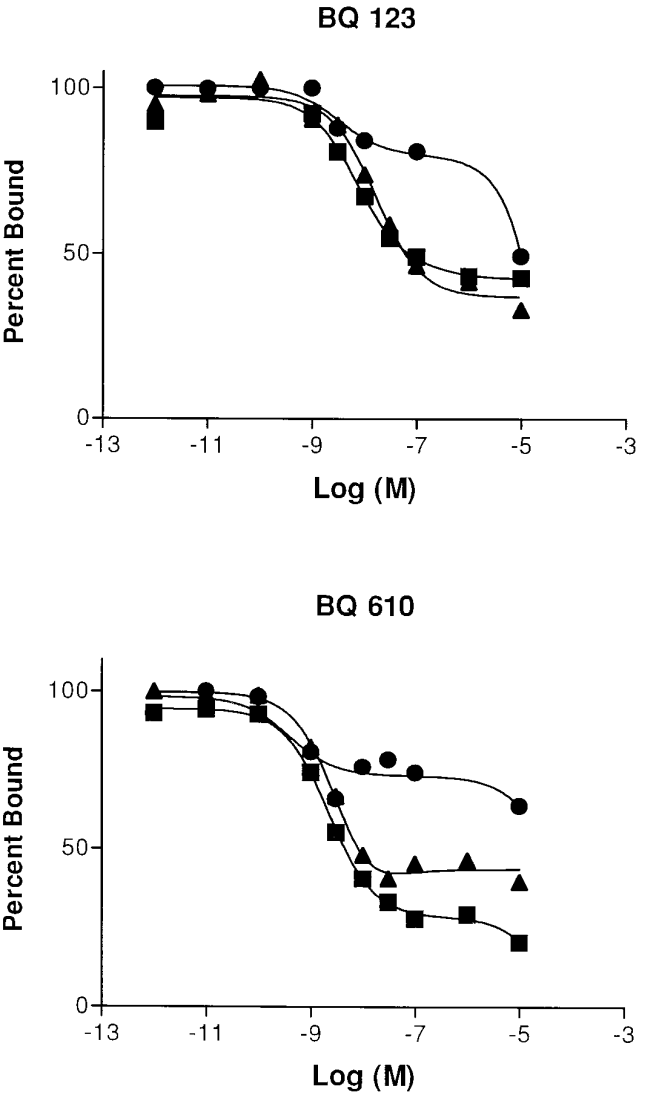

Fig. 9. Competition of ^{125}I-ET-3 binding to membranes prepared from human spleen (●), monkey spleen (▲), and canine bladder (■) by BQ123 (*top*) and BQ610 (*bottom*). The experiment was performed as explained in legend to Fig. 3.

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Send reprint requests to: Ponnal Nambi, Ph.D., SmithKline Beecham, Dept. of Renal Pharmacology, UW2521, P.O. Box 1539, King of Prussia, PA 19406-0939. E-mail: ponnal_nambi@sbphrd.com