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Lack of Endothelin ET_B Receptor Binding and Function in the Rat with a Mutant ET_B Receptor Gene

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Congenital aganglionosis rat is a mutant with an autosomal recessive gene (*sl*). Recent studies have revealed that the endothelin ET_B receptor gene of *sl*/*sl* rat has a deletion of 301-bp region spanning exon 1 and intron 1 corresponding to the first and the second transmembrane domains of the receptor. In the present experiments, we examined the functions of ET_B receptors in the *sl*/*sl* rats. In the membranes of cerebellum, heart, and lung of control (+/+ and *sl*/+) rats, ET-1 induced a monophasic, competitive displacement of [^{125}I]ET-1 binding, whereas ET-3, IRL 1620, and BQ-123 showed biphasic displacement. In the membranes of *sl*/*sl* rats, in contrast, ET-1, BQ-123, ET-3, and IRL 1620 showed only monophasic displacement. Scatchard analysis revealed a single [^{125}I]ET-3 binding site in the membrane of control heart but not in the *sl*/*sl* rat heart, and the specific binding sites for [^{125}I]ET-1 in both control and *sl*/*sl* rat hearts. In the control rat aorta but not in the *sl*/*sl* rat aorta, ET-3 induced endothelium-dependent relaxation. These results suggest that *sl*/*sl* rats do not have functional ET_B receptors.

Endothelin (ET) is a potent vasoconstrictor peptide with various physiological functions (1,2). There are three distinct isoforms of ET, ET-1, ET-2 and ET-3. These isoforms are expressed in various tissues in various proportions (3). Diverse responses to ETs are mediated by two types of receptors, the ET_A and ET_B subtypes (4). Both of these receptors belong to the G protein-coupled receptor gene family (5,6). The major difference between the ET_A and the ET_B receptor is that the former is activated selectively by ET-1 and ET-2 whereas the latter is activated non-selectively by these ETs. Southern blot analysis of human DNA also revealed the existence of two ET receptor genes, probably corresponding to the ET_A and ET_B genes (4,7).

Congenital aganglionosis (AR) rat has an aganglionic bowel caused by an autosomal recessive gene (*sl*) (8). This rat is characterized by a megacolon and white coat-color with a small pigmented spot on the head. A 301-bp deletion was found in the endothelin ET_B receptor (*EDNRB*) gene of the *sl*/*sl* rat (9,10). The deletion resulted in transcripts of various forms arising from cryptic splicing donor sites. In spite of the absence of the frame shift in the reading frame of the major transcript, the product of the transcript has neither the first nor the second transmembrane domains of the G protein-coupled heptahelical receptor (9). Since the transcripts of the mutant *EDNRB* gene were detected in the brain, lung, kidney, and eye ball of the *sl*/*sl* rats, it is possible that the mutant ET_B receptors exist in the tissues of the *sl*/*sl* rats. In the present experiments, we measured the ligand binding and function of the ET_B receptor to know if the tissues of the *sl*/*sl* rats have the ET_B receptor that binds ETs.

MATERIALS AND METHODS

The AR rat strain are maintained in our laboratories by mating between heterozygous *sl*/+ male and *sl*/+ female. Three to four week-old rats were killed by a sharp blow to the neck and exsanguination. Cerebellum, heart and lung were removed

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for binding assay. Thoracic aorta was isolated and cut into ring strips (1 mm wide) to measure contractile force. Tissues isolated from +/+ and *sl*/+ rats were used as control. Normal physiological salt solution contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, ethylenediamine tetraacetic acid 0.01 and glucose 5.5. High KCl solution was made by substituting NaCl with equimolar KCl. These solutions were saturated with a 95% O_2 and 5% CO_2 at 37°C and pH 7.4. Contractile force was recorded isometrically under a resting force of 2 mN. High KCl solution was repeatedly applied until the peak force was reproducible before starting experiment. The relaxant effect of ET-3 was examined only once in one muscle strip because the second application of ET-3 was ineffective due to desensitization of the ET_B receptor (11).

For the receptor binding assays, cerebellum, heart and lung were homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) three times at top speed for 30 sec each in 9 volumes of ice-cold 0.25 M sucrose solution containing 20 mM Tris-HCl (pH 7.4), 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, 0.1 mM EDTA and 0.5 mM EGTA. After centrifugation of the homogenate at 1,000 × g for 10 min at 4°C, the supernatant was centrifuged again at 48,000 × g for 30 min. The resulting pellet was washed twice as described above and used as membrane sources. The membranes were stocked in aliquots at −80°C until use.

Receptor binding assay was performed as reported previously (12). In brief, the membranes (0.5–25 μg protein) were incubated at 37°C for 1 h with [^{125}I]ET-1 or [^{125}I]ET-3 (74 TBq/mmol) (Amersham, Bucks, UK) in the presence or absence of various amounts of unlabeled ligand, IRL 1620, BQ-123 (synthesized at Ciba-Geigy Japan), ET-1 or ET-3 (Peptide Institute, Osaka, Japan), in a total volume of 1.0 ml of 20 mM Hepes (pH 7.4), 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 1.0 mM EGTA, 0.1% BSA and 0.02% bacitracin. Binding was terminated by centrifugation at 20,000 × g for 20 min at 4°C. The membrane-associated radioactivity was measured in an autogamma counter. Specific binding was defined as total binding minus non-specific binding which was measured in the presence of 100 nM unlabeled ET-1. Total binding was less than 10% of the total radioactivity added. Protein concentration was determined using a BCA assay kit (Pierce). Results of the experiments are expressed as mean ± S.E.M.

RESULTS AND DISCUSSION

Fig. 1 shows the results of competitive binding assays in the cerebellum membrane preparations. In the control membrane (Fig. 1A), the maximum binding of [^{125}I]ET-1 was 600 fmol/mg protein. ET-1 displaced [^{125}I]ET-1 in a monophasic manner with IC_{50} of 100 pM. In contrast, a relatively selective ET_B ligand, ET-3, and a selective ET_B ligand, IRL 1620 (11,12), showed biphasic displacement curve. Approximately 90% of the [^{125}I]ET-1 binding was displaced with IC_{50} of 100 pM whereas remaining 10% was displaced by higher concentrations. A selective ET_A ligand, BQ-123 (13), displaced 10% of the [^{125}I]ET-1 binding at IC50 $\leq$ 100 pM whereas it was almost ineffective on the remaining 90% binding (IC50 > 1 μM). These results indicate that 90% of the ET receptor in the rat cerebellum membrane is the ET_B subtype whereas 10% is the ET_A subtype.

In the *sl*/*sl* rat cerebellum membrane (Fig. 1B), maximum binding of [^{125}I]ET-1 was only 10%

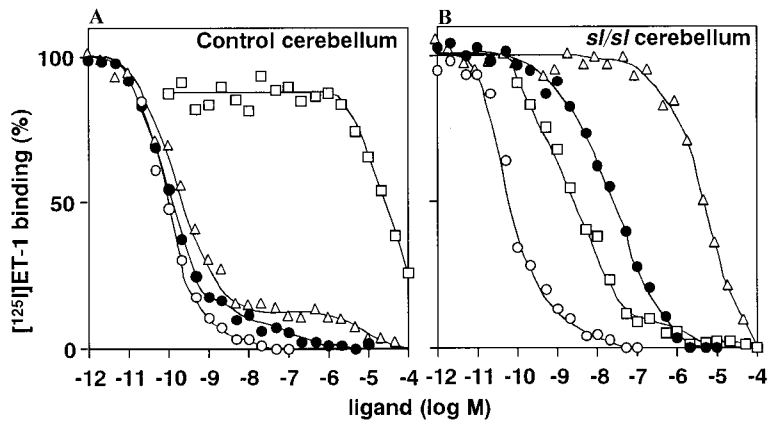

FIG. 1. Competitive binding of [^{125}I]ET-1 with unlabeled ligands in the cerebellum membranes of control (A) and *sl*/*sl* rats (B). The membrane preparations were incubated with 30 pM [^{125}I]ET-1 in the presence or absence of various concentrations of unlabeled ET-1 (○), ET-3 (●), IRL 1620 (△), and BQ-123 (□). 100% represent the specific binding (600 and 69 fmol/mg protein in control and *sl*/*sl*, respectively).

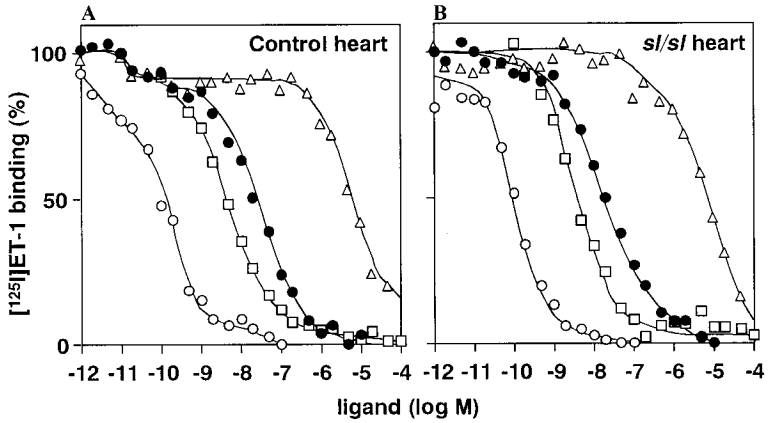

FIG. 2. Competitive binding of [^{125}I]ET-1 with unlabeled ligands in the heart membranes of control (A) and *sl*/*sl* rats (B). 100% represent the specific binding (450 fmol/mg protein in both control and the *sl*/*sl*). For further details see Fig. 1.

of control (69 fmol/mg protein). ET-1 displaced [^{125}I]ET-1 binding most strongly (IC_{50} = 100 pM) followed by BQ-123 > ET-3. In contrast, IRL 1620 was almost ineffective (IC_{50} > 1 μM). This result indicates that the *sl*/*sl* rat cerebellum membrane has only a small amount of a single population of the ET receptor, the ET_A subtype.

Fig. 2 and 3 show the results of competitive binding assay in the heart and lung membrane preparations, respectively. In the control preparations, the maximum binding of [^{125}I]ET-1 was 450 fmol/mg protein in the heart and 2,900 fmol/mg protein in the lung. ET-1 showed a monophasic displacement with an IC_{50} of 100 pM whereas ET-3, IRL 1620 and BQ-123 showed biphasic displacement (Figs. 2A and 3A). A part of the [^{125}I]ET-1 binding (10% in heart and 40% in lung) was displaced by lower concentrations of ET-3 (<1 nM) and IRL 1620 (<10 nM). The rest (90% in heart and 60% in lung) was displaced by higher concentrations of ET-3 (>1 nM) and IRL 1620 (>1 μM). In contrast, BQ-123 displaced larger part (90% in heart and 60% in lung) at lower concentrations (<100 nM). These results suggest that relative amount of the ET_A and the ET_B receptors are 90% and 10%, respectively, in the heart and 60% and 40%, respectively, in the lung.

In the *sl*/*sl* rat membranes, the maximum binding of [^{125}I]ET-1 was 66% of control (1,900 fmol/mg protein) in the heart and 100% of control (450 fmol/mg protein) in the lung. ET-1

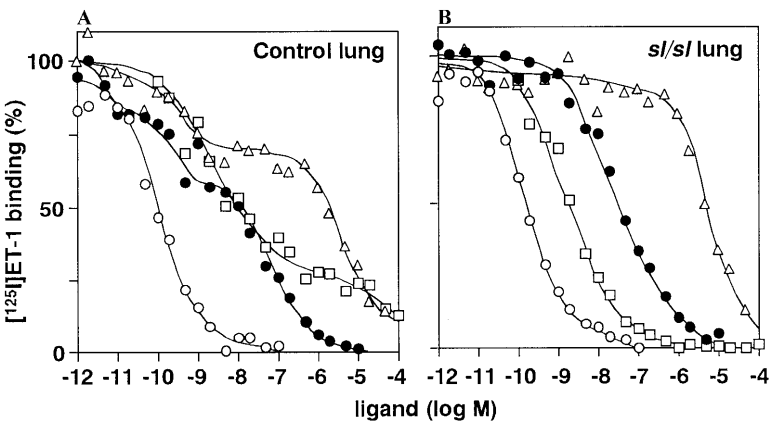

FIG. 3. Competitive binding of [^{125}I]ET-1 with unlabeled ligands in the lung membranes of control (A) and *sl*/*sl* rats (B). 100% represent the specific binding (2,900 and 1,900 fmol/mg protein in control and *sl*/*sl*, respectively). For further details see Fig. 1.

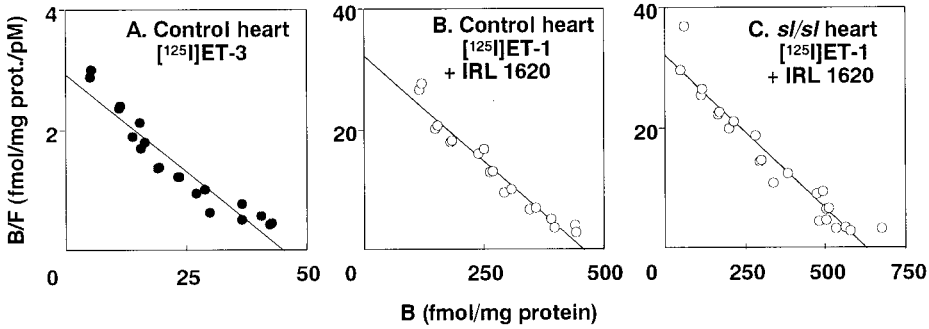

FIG. 4. Scatchard analysis of the specific binding of ET-3 to the control heart membranes (A) or ET-1 in the presence of 100 nM unlabeled IRL 1620 to the heart membranes of control (B) and *sl*/*sl* rats (C).

displaced [^{125}I]ET-1 binding most strongly (IC_{50} = 100 pM) followed by BQ-123 > ET-3 (Fig. 2B and 3B). In contrast, IRL 1620 was almost ineffective (IC_{50} > 1 μM). These results suggest that the *sl*/*sl* rat heart and lung membranes have only the ET_A receptor.

Fig. 4 shows the Scatchard analysis of the ET binding sites in the rat heart. Fig. 4A shows that control heart membrane has a single component of specific binding sites for [^{125}I]ET-3 with a Kd of 15.4 pM and a Bmax of 45 fmol/mg protein. In contrast, we could not detect any specific binding of [^{125}I]ET-3 to the *sl*/*sl* rat heart membrane. Fig. 4B shows the specific [^{125}I]ET-1 binding to the control heart membrane. This experiment was done in the presence of 100 nM IRL 1620 to inhibit the binding to the ET_B receptors and to measure specific binding to the ET_A receptor. Results indicated that [^{125}I]ET-1 bound to a single binding site with a Kd of 14.2 pM and a Bmax of 460 fmol/mg protein. Fig. 4C shows that, in the presence of 100 nM IRL 1620, [^{125}I]ET-1 bound to a single component in the *sl*/*sl* rat heart membrane with a Kd of 19.6 pM and a Bmax of 630 fmol/mg protein. These results support the suggestion that control rat heart has both the ET_A and the ET_B receptors whereas the *sl*/*sl* rat heart has only the ET_A receptor.

Since vascular endothelium has the ET_B receptors that mediate nitric oxide release and relaxation of smooth muscle (11,14), we measured the endothelium-dependent relaxant effect of ET-3 in the rat thoracic aorta. As shown in Fig. 5, 100 nM ET-3 transiently inhibited the contraction induced by 1 μM arginine vasopressin by 34.1 ± 67.6% (n = 9). Sequential addition of 1 μM carbachol again inhibited the contraction by 89.6 ± 5.5% (n = 9). In contrast, neither ET-3 nor carbachol induced relaxation in the control aorta denuded of vascular endothelium (data not shown).

Fig. 5 also shows that, in the aorta isolated from *sl*/*sl* rat, 100 nM ET-3 did not change the contraction induced by arginine vasopressin. Sequential addition of 1 μM carbachol inhibited the contraction by 85.1 ± 5.1% (n = 9). In vascular endothelium, it has been reported that stimulation of the ET_B receptor or the muscarinic receptor increases cytosolic Ca^{2+}, activates nitric oxide synthase and produces nitric oxide (11,14). These results indicate that, although the vascular

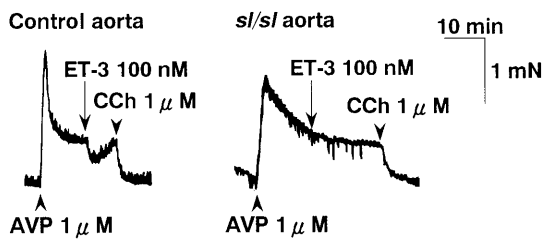

FIG. 5. The effects of 100 nM ET-3 and 1 μM carbachol (CCh) on the aorta of the *sl*/*sl* rats and control rats stimulated by 1 μM arginine vasopressin (AVP).

endothelium of the *sl*/*sl* rat has the muscarinic receptor and nitric oxide synthase, it does not respond to ET-3 because of lack of functionally active ET_B receptor.

Based on the selectivity to antagonists, it has been suggested that the ET_B receptor is further classified into ET_{B1} and ET_{B2} subtypes (15,16). In the present results, it was shown that mutation of the *EDNRB* gene totally abolished the ET_B receptors. Subtypes of the ET_B receptors may be generated by alternative splicing from a single *EDNRB* gene (17).

The loss of the ET_B receptor function has also been reported in the knock out mice of the *EDNRB* gene (*ednrb*/*ednrb*) and the s^l/s^l mutant mice (18). In all of these animals, aganglionic megacolon and coat color spotting are found. These results support the suggestion that mutation in the *EDNRB* gene is responsible for the myenteric aganglionosis and the coat color spotting phenotypes.

In summary, we found that the *sl*/*sl* rats do not have functional ET_B receptor. This rats is an excellent model for investigating the functions of the ET_B receptor.

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