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Allosteric Inhibition of Endothelin ETA Receptors by 3,5-Dibromosalicylic Acid

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ABSTRACT

Derivatives of salicylic acid (SA) and benzoic acid prevent endothelin-1 (ET-1) binding to ETA receptors. This study analyzed actions of 30 derivatives of benzoic acid and salicylic acid on ^{125}I-ET-1 binding to recombinant rat ETA receptors. The most active compounds were 3,5-dibromosalicylic acid (Br2SA, K_i = 0.5 mM) and 3,5-diiodosalicylic acid (K_i = 0.3 mM). They were about 50 times more potent than SA and aspirin. Br2SA inhibited equilibrium ^{125}I-ET-1 binding in an apparently competitive manner. It accelerated 8-fold the dissociation of ^{125}I-ET-1 receptor complexes and did not modify the second order rate constant of association of ^{125}I-ET-1 to its receptors. Br2SA also decreased the affinity of ETA receptors for receptor antagonists BQ-123 and bosentan. Br2SA accelerated dissociation of ^{125}I-ET-1-solubilized ETA receptor complexes and decreased the apparent molecular size of solubilized receptors. Br2SA and 3,5-diiodosalicylic acid inhibited two cellular actions of ET-1: the mobilization of intracellular Ca^{2+} stores in isolated cells and contractions of rat aortic rings. They accelerated the relaxing action of BQ-123 and bosentan in ET-1-treated aortic rings. The results suggest the existence of an allosteric modifier site on ETA receptors that recognizes selected derivatives of SA. SA derivatives might be of therapeutic interest to relieve tight ET-1 binding and to favor actions of receptor antagonists.

Endothelin-1 is a potent vasoconstrictor peptide that recognizes G protein-coupled receptors that stimulate the phospholipase C-signaling cascade (Van Renterghem et al., 1988; Yanagisawa et al., 1988). A unique property of ET-1 is its capacity to bind almost irreversibly to its receptors (Waggoner et al., 1992). Quasi-irreversible binding of ET-1 has many functional and pharmacological consequences that have only recently been appreciated. The affinity of ET-1 for its receptors is overestimated in many binding experiments because of time-limited second order kinetic conditions (Desmarets et al., 1996). Tight binding has been proposed to be responsible for the lack of action of guanine nucleotides on ET-1 binding (Nambi et al., 1996) and for the long-term refractoriness that follows actions of ET-1 (Leite et al., 1994; Hilal-Dandan et al., 1997). Tight binding imposes conditions in which short-range (autocrine) actions of ET-1 are favored over long-range (paracrine) actions. It provides an explanation for the observation that functional receptors serve as clearance receptors (Frelin and Guedin, 1994). It has also been suggested that, because of its irreversible binding, ET-1 is more likely to contribute to long-term physiological or physiopathological regulations than to short-term regulations (Hilal-Dandan et al., 1997). Another consequence of tight ET-1 binding is that circulating levels of ET-1 are not representative of the real amount of ET-1 present in tissues (Ferrari et al., 1998). Finally, tight binding may limit the access of competitive receptor antagonists to the receptors. Indeed, if ET-1 already sits on a receptor, receptor antagonists cannot act until ET-1 leaves the site and gives the antagonist a chance to compete with ET-1 for the occupancy of newly accessible sites (Talbodec et al., 2000). This simple fact may be a reason for the limited usefulness of ET receptor antagonists against endogenous ET-1.

Several G protein-coupled receptors are regulated by allosteric ligands. Well studied examples are D2 dopamine receptors (Hoare and Strange, 1998), muscarinic receptors (Tucek and Proska, 1995), adenosine A1 receptors (Bruns and Fergus, 1990), and α2-adrenergic receptors (Nunnari et al., 1987). We previously reported that aspirin and salicylic acid are allosteric inhibitors of ETA receptors (Talbodec et al., 2000). These actions of salicylates were observed at concentrations >10 mM, which were too large to carry out a detailed investigation of their mechanism of action. We therefore screened a number of derivatives of SA and benzoic acid to find compounds that would be more potent. This procedure led to the identification of dihalogenated derivatives of SA that are about 50 times more potent than aspirin.

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ABBREVIATIONS: ET-1, endothelin-1; SA, salicylic acid; Br2SA, 3,5-dibromosalicylic acid; I2SA, 3,5-diiodosalicylic acid; BQ-123, cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate.

This paper defines the properties of interaction of Br2SA with recombinant rat ETA receptors and analyzes some of the pharmacological properties of Br2SA and I2SA.

Materials and Methods

Chemicals. BQ-123 was from Néosystem (Strasbourg, France). ET-1, indo-1/AM, CHAPS, digitonine, and protease inhibitors were from the Sigma Chemical Co. (St. Louis, MO). ^{125}I-ET-1 (2200 Ci/mmol) was prepared as previously described (Desmarets et al., 1996) and stored at −20°C. Salicylic acid and benzoic acid derivatives were purchased from Avocado (Heysham, UK), Acros Organics (Geel, Belgium), or Sigma. Sodium salts were used. Bosentan was obtained from Dr. M. Clozel (Actelion, Basel, Switzerland).

Cell Cultures. Stable transfectant CCl39 cells expressing functional rat ETA receptors were prepared as previously described (Gresser et al., 1996). Cell membranes were prepared as previously described (Desmarets et al., 1996). They were resuspended in a buffer of the following composition: 5 mM EGTA, 250 mM sucrose, 10 mM Tris-Cl, pH 7.4, supplemented with a cocktail of protease inhibitors (1 μM bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin). Membranes (4–8 mg of protein/ml) were stored at −20°C until use. Proteins were determined according to the Bradford (1976) method using bovine serum albumin as standard. Rat brain capillary endothelial cells of the B7 clone were grown as previously described (Vigne et al., 1990).

Binding Experiments. All experiments were performed at room temperature. Membranes (0.1–20 μg of protein/ml) were incubated in binding buffer supplemented with 1 to 120 pM ^{125}I-ET-1 and effectors. The binding buffer was an Earle's salt solution (140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 25 mM HEPES, pH 7.4) supplemented with 0.05% bovine serum albumin and protease inhibitors. After selected times of incubation, aliquots of the incubation solutions were filtered under reduced pressure onto Sartorius 0.2-μm filters and washed three times with 3 ml of 0.1 M $MgCl_2$. Filters were then counted. Nonspecific binding was measured in parallel experiments using 100 nM unlabeled ET-1. Triplicate experiments were performed.

A first type of binding experiment was competitive binding assays. In these experiments, the binding of a fixed concentration of ^{125}I-ET-1 was measured in the presence of a range of concentrations of putative inhibitors. Membranes (20 μg of protein/ml) were incubated for 4 h with 20 pM ^{125}I-ET-1 and different concentrations of inhibitors in 0.7 ml of assay buffer. Specific binding represented 5000 to 8000 cpm. Nonspecific binding was 10 to 15% of the total binding component.

Saturation analysis of ^{125}I-ET-1 binding was carried out using a large assay volume (4 ml) and a very low protein concentration (0.1 μg of protein/ml) in the assay. Under these conditions, the total concentration of receptors was about 1 pM. Membranes were incubated in the presence of a range of concentrations of freshly prepared ^{125}I-ET-1 (1–120 pM) in the absence or the presence of Br2SA (0.25 or 0.75 mM). After 16 h of incubation at room temperature, the whole incubation solution was filtered. The nonspecific binding component was determined in parallel incubations. Triplicate experiments were performed.

In dissociation experiments, membranes (20 μg of protein/ml) were incubated in the presence of 15 to 25 pM ^{125}I-ET-1. The total volume was 14 ml. After 4 h of incubation at room temperature, dissociation kinetics were initiated by the addition of 100 nM unlabeled ET-1. Duplicate, 200-μl aliquots were filtered after different times. In experiments using Br2SA or I2SA, SA derivatives were added at the start of the association process. Maximum binding was 10,000 to 12,000 cpm. We checked that, as previously described for SA (Talbodec et al., 2000), Br2SA did not induce a degradation of ET-1 either in the free form or in a receptor-bound form.

Association experiments were performed as described previously (Talbodec et al., 2000). Control experiments were performed at 0.5 μg of membrane protein/ml. This concentration was raised in experiments using Br2SA to compensate for the decrease in the specific binding. They were 5 and 10 μg/ml in experiments using 2 and 7 mM Br2SA, respectively. The concentration of ^{125}I-ET-1 used was 9 to 20 pM. The total volume was 14 ml.

Reversibility of Actions of Br2SA. Three samples of membranes (40 μg of protein/ml in 14 ml of binding buffer) were processed in parallel. Sample A was treated with 7 mM Br2SA. After 30 min of incubation at room temperature, all samples were centrifuged (10 min, 16,000 rpm) and resuspended into 14 ml of binding buffer. After 40 min at room temperature, all samples were centrifuged. Pellets were resuspended into 14 ml of binding buffer supplemented with 20 pM ^{125}I-ET-1. Sample B was supplemented with 7 mM Br2SA. Association was allowed to proceed for 3 h, and the dissociation kinetics were initiated by the addition of 100 nM unlabeled ET-1.

Solubilization and Gel Filtration of ETA Receptors. All experiments were performed at 4°C. Membranes were diluted in the same volume of solubilization buffer (300 mM NaCl, 10 mM EDTA, 4 mM EGTA, 1% digitonin, 0.76% CHAPS, 2 μM bacitracin, 0.2 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 40 mM Tris-Cl, pH 7.4). After 1 h under agitation, the mixture was centrifuged at 100,000*g* for 30 min. The supernatant was harvested and loaded onto an Ultrogel ACA34 gel filtration column (Sigma Chemical Co.). The column (100 × 1.5 cm) was equilibrated in solubilization buffer containing 0.025% digitonine and 0.1% CHAPS and eluted with the same buffer. The column was calibrated with the MW-GF200 kit (Sigma Chemical Co.). Two-milliliter fractions were collected. ^{125}I-ET-1 binding was assessed on 700-μl aliquots using 100 pM ^{125}I-ET-1. After 90 min of incubation at room temperature, three aliquots (200 μl) of the incubation mixture were filtered onto polyethyleneimine-treated (0.3%) Sartorius filters. Nonspecific binding was assessed for each fraction in parallel incubations using 100 nM ET-1.

In a second series of experiments, solubilized receptors were incubated for 1 h with 1 to 3 nM ^{125}I-ET-1. Bound and free radioactivities were separated on a Sephadex G50 column. The bound radioactivity was loaded onto the ACA34 column. Two-milliliter fractions were collected and counted.

In experiments using Br2SA, Br2SA (2 mM) was added to the elution buffer.

Intracellular Ca^{2+} Measurements. B7 cells express ETA receptors. Their activation leads to large increases in the intracellular Ca^{2+} concentration that have previously been documented (Vigne et al., 1990, 1993). Changes in indo-1 fluorescence ratio are conveniently monitored by flow cytometry analysis of indo-1-loaded cells (Vigne et al., 1990, 1993). Suspended cells were incubated for 30 min in the presence of 5 μM indo-1/AM, centrifuged at 1000*g*, and resuspended into an Earle's salt solution at a density of 10^6 cells/ml. ET-1 was added to the cell suspension. After mild vortexing, tubes were inserted into a FACS Vantage SE cytometer (Becton Dickinson). Mean fluorescence ratios were determined for 1000 cells after different times of exposure to ET-1. Acquisition time was <2 s. Concentration-response curves were defined from indo-1 fluorescence ratios sampled between 8 and 10 s after the addition of ET-1. This time corresponded to the peak of the intracellular Ca^{2+} transients. Fluorescence ratios were calculated in arbitrary units set to a value of 100 for unstimulated cells.

Contraction Experiments. Thoracic aorta from 200-g Wistar rats were cleaned of adherent fat and cut into rings, and the endothelium was removed. Rings were mounted under 2 g of resting tension in organ baths (3 ml, 37°C, bubbled with a 5% CO_2 and 95% O_2 gas mixture) containing a Krebs' bicarbonate solution. The composition of the solution was 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, and 5.8 mM glucose, pH 7.4. After a 60-min equilibration during which the buffer was changed at 15-min intervals, three contraction/relaxation cycles were performed using 40 mM KCl. Rings were then exposed to 100 nM ET-1, and contractions were allowed to develop for 30 min.

Salicylates or BQ-123 were then added without washing the preparation. Changes in tension were recorded on a TA4000 recorder (Gould, Cleveland, OH).

Data Presentation and Statistical Analysis. Data are given as means ± S.E. and the number of independent experiments performed. Equilibrium binding data were analyzed using the Ligand software (Jandel Scientific, Corte Madera, CA). Dissociation kinetics were linearized to yield k_d, the first order rate constant of dissociation of ^{125}I-ET-1 receptor complexes. Association kinetics were linearized according to a pseudo first order process to yield k'_a, the pseudo first order rate constant of association of ^{125}I-ET-1 to its receptors. The second order rate constant of association (k_a) was then calculated from the following relationship: $k'_a = k_a \times [\text{ET-1}] - k_d$. Curve fitting was performed using a logistic equation and the Sigma Plot software (Jandel Scientific).

Results

Derivatives of Benzoic Acid and SA Inhibit ^{125}I-ET-1 Binding to ETA Receptors. We first used competitive binding assays to define actions of 30 different derivatives of SA and benzoic acid. Figure 1 shows the structures of the major compounds used in this study. Membranes, isolated from ETA receptor-expressing fibroblasts, were incubated in the presence of ^{125}I-ET-1 and of different concentrations of SA or benzoic acid derivatives, and ^{125}I-ET-1 binding was measured after 4 h of incubation. The concentrations of derivatives that produced a 50% inhibition of the specific ^{125}I-ET-1 binding (IC_{50}) were calculated and are listed in Table 1. Table 1 shows that the potency of benzoic or SA derivatives was markedly increased by substitutions of the aromatic ring with halogen atoms. Monohalogenated compounds were less potent than dihalogenated compounds. Introduction of a third heteroatom did not improve activity of the compounds. The rank order of potency of different halogens was Br > Cl > F in the benzoic acid series. 3,5-Dichlorosalicylic acid (IC_{50} = 0.6 mM) and Br2SA (IC_{50} = 0.5 mM) were equally potent. I2SA (IC_{50} = 0.3 mM) was slightly more potent. 3,5-Diisopropylsalicylic acid (IC_{50} = 0.8 mM) was as potent as dihalogenated derivatives of SA. These compounds were 25 to 60 times more potent than SA (IC_{50} = 15 mM) and aspirin (IC_{50} = 20 mM). Figure 2A shows typical concentration-response curves for the inhibition of ^{125}I-ET-1 binding by Br2SA, I2SA, and SA. Actions of the two most potent compounds, Br2SA and I2SA, were analyzed in more detail.

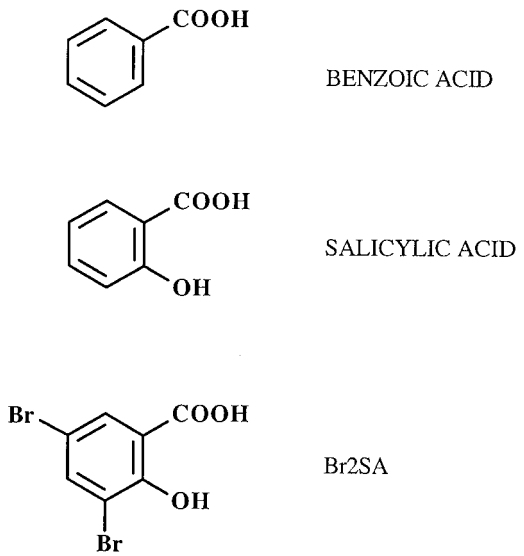

Fig. 1. Structures of the major compounds used in this study.

Br2SA Decreases the Apparent Affinity of ETA Receptors for ET-1. Equilibrium binding experiments were used to define the mechanism of action of Br2SA. Figure 2B shows typical Scatchard plots for the specific ^{125}I-ET-1 binding to ETA receptors. ^{125}I-ET-1 recognized a single family of binding sites with a K_d value of 16 pM and a maximum binding capacity of 11 pmol/mg of proteins in the absence of Br2SA. In four independent experiments using the same membrane preparation, the mean K_d value of ^{125}I-ET-1 receptor complexes was 8.9 ± 2.4 pM. The maximum binding density was 11.2 ± 0.5 pmol/mg of protein. Figure 2B further shows that Br2SA (0.25 mM) decreased the apparent affinity of ETA receptors for ^{125}I-ET-1 and did not change the maximum number of binding sites. In two experiments, Br2SA (0.25 mM) increased the K_d value of ^{125}I-ET-1 receptor complexes 2.6- and 4.2-fold compared with the respective controls. In two other experiments, Br2SA (0.75 mM) increased the K_d value for ^{125}I-ET-1 receptor complexes 7.1- and 10.0-fold. Thus, Br2SA behaves as an apparent competitive antagonist of ET-1 binding.

Br2SA Accelerated Dissociation of ^{125}I-ET-1 Receptor Complexes. Figure 3A shows typical dissociation kinetics of ^{125}I-ET-1 receptor complexes. Complexes were first allowed to form for 4 h, and the dissociation kinetics were initiated by the addition of a large excess of unlabeled ET-1 (100 nM). Figure 3A shows that ^{125}I-ET-1 receptor complexes dissociated very slowly. In 10 independent experiments, the

TABLE 1

Inhibition of ^{125}I-ET-1 binding by derivatives of benzoic acid and SA

Concentration-response curves were established for each compound using competitive binding assays. Each concentration-response curve was performed using triplicates and repeated several times. Means ± S.E. are indicated wherever n was ≥3. Otherwise means of two determinations are indicated.

Compound	IC_{50}
	mM
5-Aminosalicylic acid	>30
Benzoic acid	25 ± 5 (n = 3)
3,5-Dinitrobenzoic acid	>20
Salicylamide	>20
Aspirin	20.6 ± 4.4 (n = 6)
4-Hydroxybenzoic acid	20
3-Hydroxybenzoic acid	20
Salicylic acid	15.4 ± 4.4 (n = 6)
3,5-Difluorobenzoic acid	>10
5-Bromo-2,4-dihydroxybenzoic acid	>10
2-Hydroxybenzyl alcohol	>10
Nicotinic acid	>10
Picolinic acid	>10
Salicylsalicylic acid	>10
3,5-Dinitrosalicylic acid	>10
Methylsalicylic acid	10
3-Methylsalicylic acid	10
3,5-Dibromo-4-hydroxybenzoic acid	7.6 ± 0.3 (n = 3)
5-Sulfosalicylic acid	7
3,5-Dichlorobenzoic acid	5.6 ± 1.3 (n = 3)
5-Chlorosalicylic acid	5.5 ± 2.0 (n = 3)
5-Bromosalicylic acid	2.5 ± 0.5 (n = 4)
3,5,6-Trichlorosalicylic acid	2
3,5-Dibromobenzoic acid	2.0 ± 0.6 (n = 4)
2,3,5-Triiodobenzoic acid	1.2 ± 0.4 (n = 4)
2 Amino-3,5-diiodobenzoic acid	1.0 ± 0.4 (n = 4)
3,5-Diisopropylsalicylic acid	0.8 ± 0.1 (n = 3)
3,5-Dichlorosalicylic acid	0.6 ± 0.3 (n = 3)
3,5-Dibromosalicylic acid	0.5 ± 0.2 (n = 5)
3,5-Diiodosalicylic acid	0.3 ± 0.05 (n = 3)

half-life of the complexes was 5.9 ± 0.1 h. It corresponded to a k_d of 1.95 × 10^{-3} min^{-1}. Figure 3A further shows that Br2SA accelerated the dissociation of ^{125}I-ET-1 receptor complexes. In all cases, dissociation kinetics could be fitted by monoexponentials. Figure 3B shows the influence of different concentrations of Br2SA on the half-life of ^{125}I-ET-1 receptor complexes. It shows that the action of Br2SA was saturable. The half-maximum action of Br2SA was observed at 0.4 mM, similar to the value obtained in competitive binding studies (0.5 mM, Table 1). The mean value of the rate constant of dissociation of ^{125}I-ET-1 receptor complexes obtained at near saturating concentrations of Br2SA (3, 5, and 7 mM) was 15.3 ± 1.4 × 10^{-3} min^{-1}. It is important to note that about 30% of ^{125}I-ET-1 receptor complexes dissociated during a 3-h experiment under control conditions. More than 90% of the complexes dissociated during the same time period in the presence of 3 to 7 mM Br2SA. Finally, Fig. 4 shows that actions of Br2SA were fully reversible.

Four additional experiments were performed using I2SA. In this series of experiments, the half-lives of ^{125}I-ET-1 receptor complexes were 7.3 ± 0.8 h (n = 4) in the absence of I2SA and 2.0 ± 0.7 h (n = 4) in the presence of 0.5 mM I2SA. The same 3.6-fold increase in dissociation rate was obtained with 1 to 2 mM Br2SA (Fig. 3B). A 2.9-fold increase in the dissociation rate of ^{125}I-ET-1 receptor complexes is produced by 20 mM SA (Talbodec et al., 2000). Therefore, the rank order of potency for the action of SA derivatives is I2SA ≥ Br2SA ≫ SA.

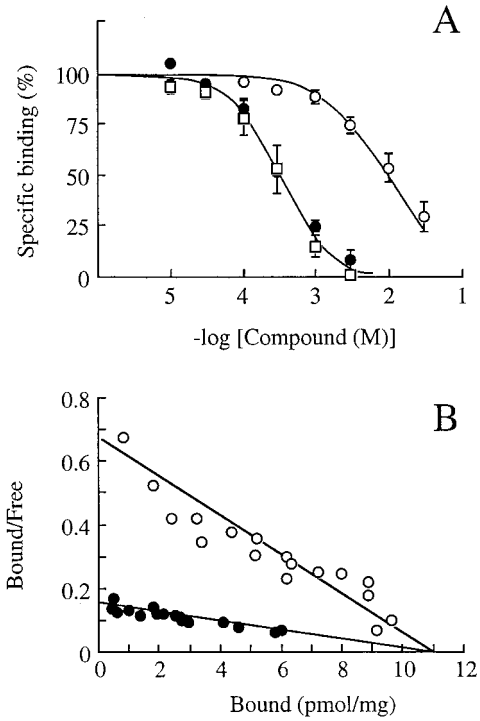

Fig. 2. Salicylates inhibit ^{125}I-ET-1 binding to ETA receptors. A, competitive binding assays were performed in the presence of 20 pM ^{125}I-ET-1 and the indicated concentrations of SA (○), I2SA (□), and Br2SA (●). Means ± S.E. from three determinations in representative experiments are shown. B, Scatchard plots for the specific ^{125}I-ET-1 binding to ETA receptors. Membranes (0.1 μg of protein/ml) were incubated in the presence of different concentrations of ^{125}I-ET-1 in the absence (○) or the presence (●) of 0.25 mM Br2SA for 16 h at room temperature, and the specific binding component was determined. Means of triplicates are shown. Bound-to-free ratios are expressed in pmol/mg/pM.

Association experiments were performed in the absence or the presence of Br2SA. The second order rate constant of association of ^{125}I-ET-1 to its receptors (k_a) was calculated and found to be unaffected by Br2SA at concentrations between 0.1 and 7 mM. The pooled k_a value was 11.2 × 10^8 ± 1.7 M^{-1} min^{-1} (n = 11). The equilibrium dissociation constant of ^{125}I-ET-1 receptor complexes was estimated from the k_a and k_d values given above (K_d = k_d/k_a). It was 1.7 pM in the absence of Br2SA and 13.6 pM in the presence of a near saturating concentration of Br2SA. The observation that Br2SA accelerated dissociation of ^{125}I-ET-1 receptor complexes and had no action on the association of ^{125}I-ET-1 to its

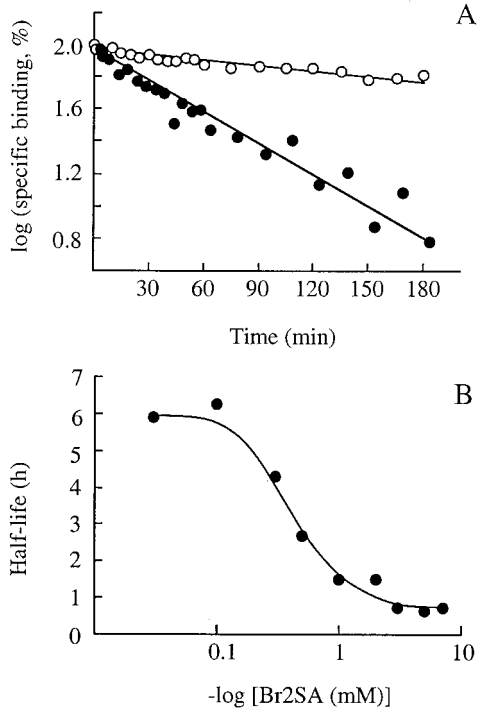

Fig. 3. Br2SA promoted dissociation of ^{125}I-ET-1 receptor complexes. A, first order plots of the dissociation of ^{125}I-ET-1 receptor complexes. Complexes were allowed to form for 4 h in the absence (○) or the presence (●) of 7 mM Br2SA. Dissociation kinetics were then initiated by the addition of 100 nM unlabeled ET-1. The means of duplicates are shown. B, concentration-response curve for the action of Br2SA on the dissociation of ^{125}I-ET-1 receptor complexes. Half-lives of ^{125}I-ET-1 receptor complexes were determined by fitting dissociation kinetics such as those presented in A according to a first order process.

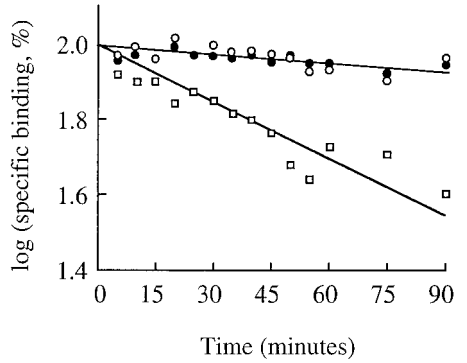

Fig. 4. Reversibility of actions of Br2SA. Dissociation of ^{125}I-ET-1 receptor complexes was followed in parallel using three pools of membrane (see *Materials and Methods*): ●, controls without Br2SA; □, controls with 7 mM Br2SA during the association and dissociation process; and ○, membranes first reacted with 7 mM Br2SA and washed.

receptor indicated that Br2SA did not act as a simple competitive antagonist. It recognized a site that was distinct from the ET-1-binding site.

Br2SA Modified the Properties of Interaction of ETA Receptors with Receptor Antagonists. The properties of the interaction of ETA receptor with two competitive antagonists, BQ-123 and bosentan, were defined using competitive binding assays. Figure 5A shows that BQ-123 prevented ^{125}I-ET-1 binding to ETA receptors with an IC_{50} value of 8.6 ± 1.5 nM (n = 3). Br2SA shifted the dose-response curve to larger concentrations. IC_{50} values for BQ-123 were 34 ± 12 and 130 ± 20 nM in the presence of 1 and 3 mM Br2SA, respectively. Similar results were obtained with bosentan (Fig. 5B). Bosentan inhibited ^{125}I-ET-1 binding with an IC_{50} value of 4.6 ± 1.5 nM (n = 5). This value increased to 190 ± 25 nM (n = 4) in the presence of 3 mM Br2SA. Thus, Br2SA decreased the apparent affinity of ETA receptors for BQ-123 and bosentan. This action was expected from an allosteric type of mechanism.

Solubilization and Gel Filtration of ETA Receptors. ETA receptors were solubilized using CHAPS and digitonine. Conditions were chosen so that tight binding could be retained. The kinetics of dissociation of ^{125}I-ET-1-solubilized receptor complexes was defined. The half-life of ^{125}I-ET-1-solubilized receptor complexes was 6.5 ± 1.1 h (n = 5). This value was similar to that obtained for the membrane-bound receptor (5.9 ± 0.1 h). Thus, solubilized receptors retain their native properties.

Solubilized receptors were loaded onto an ACA34 gel filtration column which fractionates globular proteins in the 20,000 to 350,000 molecular weight range. Fractions were collected, and the specific ^{125}I-ET-1 binding was assessed on each fraction. Figure 6A compares the elution profiles of ETA receptors in the absence or the presence of Br2SA. ETA receptors eluted as a large peak that followed the void volume. Br2SA (2 mM) shifted the profile to lower molecular weights. The molecular weight at the peak was larger than the largest molecular weight standard used to calibrate the column (200,000). It could not be defined with more precision. An identical result was obtained in experiments in which receptors were exposed to Br2SA during or after solubilization.

In a second series of experiments, receptors were first solubilized, and preformed ^{125}I-ET-1 receptor complexes were loaded onto the column. Figure 6B shows that the label eluted as two distinct peaks. The second peak had the same retention time as free ^{125}I-ET-1. The first peak eluted with

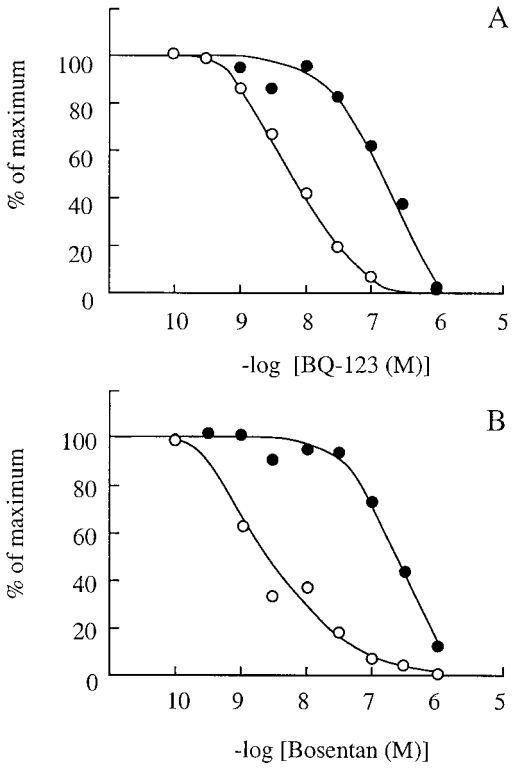

Fig. 5. Br2SA decreased the apparent affinity of ETA receptors for receptor antagonists. Inhibition by BQ-123 (A) or bosentan (B) of ^{125}I-ET-1 binding to ETA receptors. Experiments were performed in the absence (○) or the presence (●) of 3 mM Br2SA. Means of triplicates of a representative experiment are shown.

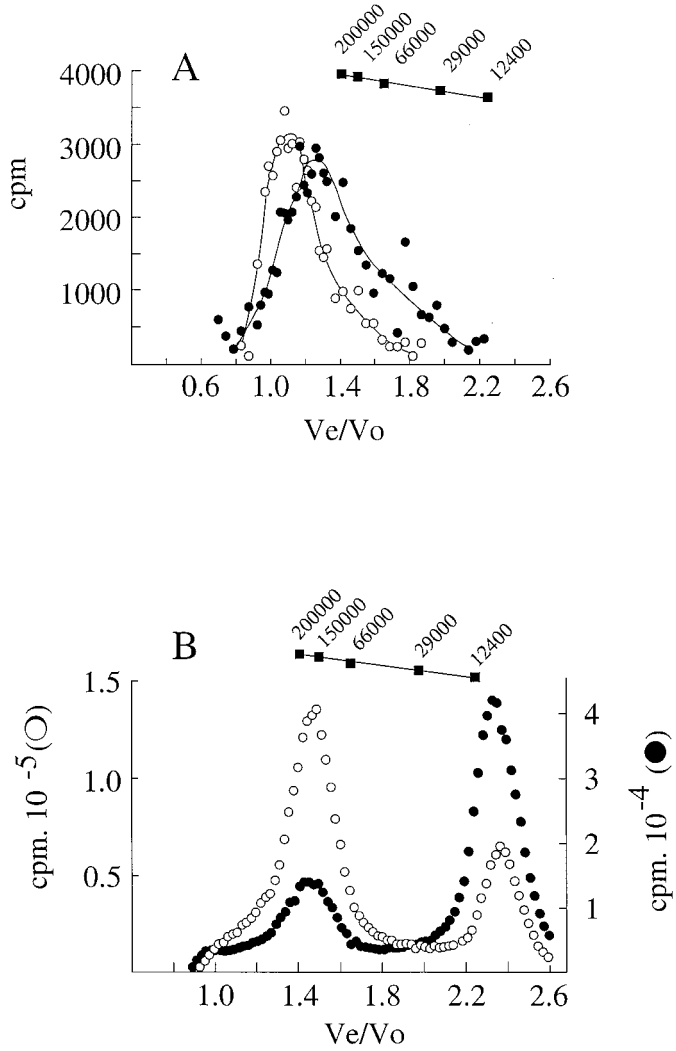

Fig. 6. Gel filtration of ETA receptors. Solubilized ETA receptors were loaded onto an ACA34 gel filtration column. A, elution profiles of free receptors in the absence (○) or the presence (●) of 2 mM Br2SA. The specific ^{125}I-ET-1 binding was assessed on each fraction. Means of triplicates are shown. Nonspecific binding represented less than 10% of the total binding component. B, elution profiles of preformed ^{125}I-ET-1 receptor complexes in the absence (○) or the presence (●) of 2 mM Br2SA. Calibration of the column is shown on the top of the figures. The abscissa shows elution volumes (Ve) divided by the void volume of the column (Vo). The void volume was determined using trypan blue.

an apparent molecular weight of 169,000 ± 6,000 (n = 7), which was smaller than that observed in the previous experiments. Figure 6B also shows that Br2SA (2 mM) did not modify the elution of ^{125}I-ET-1 receptor complexes. Identical elution profiles were observed in experiments in which ^{125}I-ET-1 receptor complexes were formed before or after solubilization and in experiments performed at different ionic strengths (0.15 or 0.5 mM NaCl) of the solubilization buffer. Guanosine 5′-[γ-thio]triphosphate, which did not prevent ^{125}I-ET-1 binding to ETA receptors, did not modify the elution profile of ETA receptors. Finally, Fig. 6B shows that the relative heights of the two peaks differed in the two experiments. A larger fraction of the label eluted as free ^{125}I-ET-1 when the experiments were performed in the presence of Br2SA. This was an indication that a larger fraction of ^{125}I-ET-1 receptor complexes dissociated on the column in the presence of Br2SA, i.e., that Br2SA accelerated dissociation of solubilized ^{125}I-ET-1 receptor complexes.

Thus, free ETA receptors, Br2SA receptor complexes, and ET-1 receptor complexes have different mobilities on a gel filtration column. The results further suggest that Br2SA and ET-1 partially dissociate ETA receptors from associated proteins. Actions of Br2SA and ET-1 were not additive.

Br2SA Inhibited ET-1-Induced Intracellular Ca^{2+} Mobilization. A major action of ET-1 acting via ETA receptors is to activate phospholipase C. Actions of Br2SA and I2SA on ET-1-induced intracellular Ca^{2+} mobilization were analyzed to define functional consequences of the allosteric regulation of ETA receptors. Experiments were performed using B7 cells that express endogenous ETA receptors. Figure 7A shows that ET-1 (100 nM) induced a transient increase in the indo-1 fluorescence ratio that peaked at 10 to 20 s and then declined. It also shows that Br2SA (1 mM) almost completely abolished this action of ET-1. An identical result was obtained with I2SA or with SA. The concentration-response curves for the inhibitions by Br2SA, I2SA, and SA of the Ca^{2+} mobilizing action of 30 nM ET-1 are shown in Fig. 7B. Half-maximum inhibitions were observed at 0.19 ± 0.04 mM (n = 3) Br2SA and 0.14 ± 0.05 (n = 3) I2SA. SA (IC_{50} = 6.7 ± 1.6 mM) was 35 times less potent than Br2SA.

Actions of Br2SA on the Isolated Rat Aorta. ET-1 induces long-lasting contractions of isolated rat aortic rings. This action of ET-1 is mediated by ETA receptors (Marsault et al., 1993). Figure 8A presents a typical recording of the contractile action of ET-1 on an isolated rat aortic ring. Once maximum tension had been reached, increasing doses of Br2SA were added at 10-min intervals. Figure 8A shows that Br2SA reversed the contractile action of ET-1 in a concentration-dependent manner. The cumulative concentration-response curve is presented in Fig. 8B. Half-maximum relaxations were observed at 1 mM Br2SA. The concentration-response curves for the relaxing actions of SA or of I2SA were determined using the same cumulative protocol. Figure 8B shows that SA was 15 times less potent than Br2SA. I2SA was 2 times more potent than Br2SA.

Figure 9 compares the relaxing actions of Br2SA and a competitive receptor antagonist, BQ-123. Rings were precontracted with ET-1. BQ-123 or Br2SA was then added without washing the preparation. Figure 9 shows that BQ-123 produced complete relaxations that developed slowly. The half-time for the relaxations induced by BQ-123 (10 μM) was 42 ± 6 min (n = 6). Relaxations induced by 0.2 mM Br2SA were slower. To quantitate the difference, we compared the two types of relaxations in aortic rings prepared from the same animals. At the time at which relaxations induced by BQ-123 were complete, Br2SA produced only a 50.2 ± 8.0% relaxation (n = 5). At the time at which BQ-123 produced a 50% reduction of tension, Br2SA produced a 21.8 ± 7.5% (n = 5) decrease in tension. Thus, Br2SA-induced relaxations were about 2 times slower than BQ-123-induced relaxations.

Br2SA Accelerated Relaxing Actions of BQ-123. A major interest of an allosteric inhibitor of ETA receptors is the possibility to accelerate actions of receptor antagonists (Talbodec et al., 2000). Figure 10 shows the results of an experiment in which a rat aortic ring was first contracted with ET-1 and then exposed to 0.2 mM Br2SA for 30 min. Under these conditions, Br2SA induced a 20.4 ± 5.4% (n = 6) relaxation. Figure 10 shows that further addition of 10 μM BQ-123 induced a rapid and complete relaxation. Data were quantitated by measuring the half-time of the relaxations induced by BQ-123 and Br2SA. Table 2 shows that bosentan and BQ-123 relaxed aortic rings with identical kinetics. Br2SA acted in a concentration-dependent manner. It was inactive at 0.1 mM and accelerated relaxations 5-fold at 0.3 mM. Note that a mixture of 0.3 mM Br2SA and 10 μM BQ-123 reversed half of ET-1 contractions in 7 min only. Table 2 also shows that I2SA (0.1 mM) and SA (10 mM) potentiated actions of BQ-123 to the same extent as 0.2 mM Br2SA. Thus, the rank order of potency for the potentiation by SA derivatives of the relaxing action of BQ-123 was I2SA ≥ Br2SA ≫ SA. It was identical to that obtained in other functional experiments and in binding experiments.

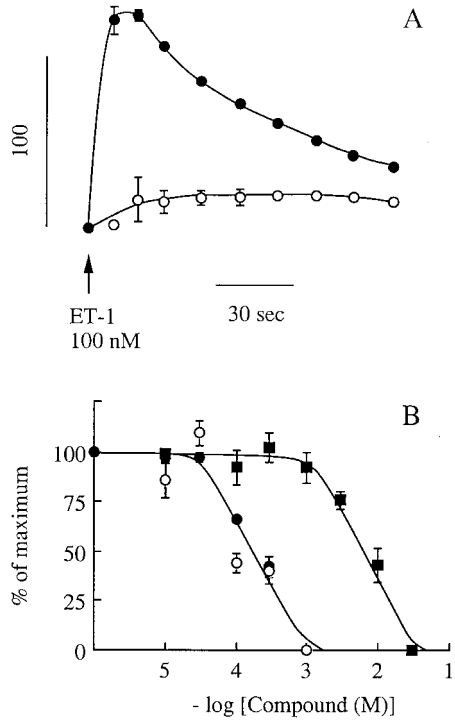

Fig. 7. Br2SA inhibited ET-1-induced intracellular Ca^{2+} mobilization. A, intracellular Ca^{2+} transiently induced by 100 nM ET-1 in the absence (●) or the presence (○) of 1 mM Br2SA. Indo-1 fluorescence ratios are expressed in arbitrary units. B, concentration-response curve for the inhibitory actions of I2SA (○), Br2SA (●), and SA (■) on the peak Ca^{2+} level. The concentration of ET-1 used was 30 nM. Means ± S.E. (n = 3) are shown.

Table 2 also shows that I2SA (0.1 mM) and SA (10 mM) potentiated actions of BQ-123 and bosentan to similar extents. Their action was thus independent of the nature of the antagonist used. Finally, Table 2 shows that relaxations induced by mixtures of I2SA and bosentan were independent of the order of application of the two drugs.

Discussion

Inhibition by Salicylates of ^{125}I-ET-1 Binding. The results (Table 1) show that the hydroxyl group of salicylates contributes little to the effect for related molecules in the benzoic acid, and SA series are equally potent. Substitution of the aromatic ring with halogens dramatically improves activity. Actions of halogens in the benzoic acid series follow the following rank order of potency: Br > Cl > F. All dihalogenated derivatives of SA are equipotent. 3,5-Diisopropylsalicylic acid is almost as potent as dihalogenated derivatives. These indicate that bulky groups at positions 3 and 5 of the aromatic ring of benzoic acid or of SA favor activity of the compounds.

Anti-platelet and anti-inflammatory actions of aspirin involve different molecular targets. The anti-platelet action of aspirin is due to the inhibition of cyclo-oxygenases (Patrono, 1994). Anti-inflammatory actions of salicylates are due to an inhibition of tumor necrosis factor-induced nuclear factor-κB activation. The mechanism of this inhibition is not yet clear. It may involve an inhibition of I-κB kinase (Yin et al., 1998) or an activation of p38 mitogen-activated protein kinase (Schwenger et al., 1997, 1998; Alpert et al., 1999). Salicylates have also been reported to inhibit or activate c-Jun N-terminal kinases depending on the cell type (Schwenger et al., 1997, 1999). The inhibition of cyclo-oxygenases by aspirin is due to the acetylation of a critical serine residue (Patrono, 1994). It is not observed with SA. The structure activity relationship for the inhibition of I-κB kinase by salicylates has not been defined in detail. It was noticed however that 5-aminosalicylic acid, which is an important anti-inflammatory agent used for the management of inflammatory bowel disease, is a potent inhibitor of I-κB kinase (Yan and Polk,

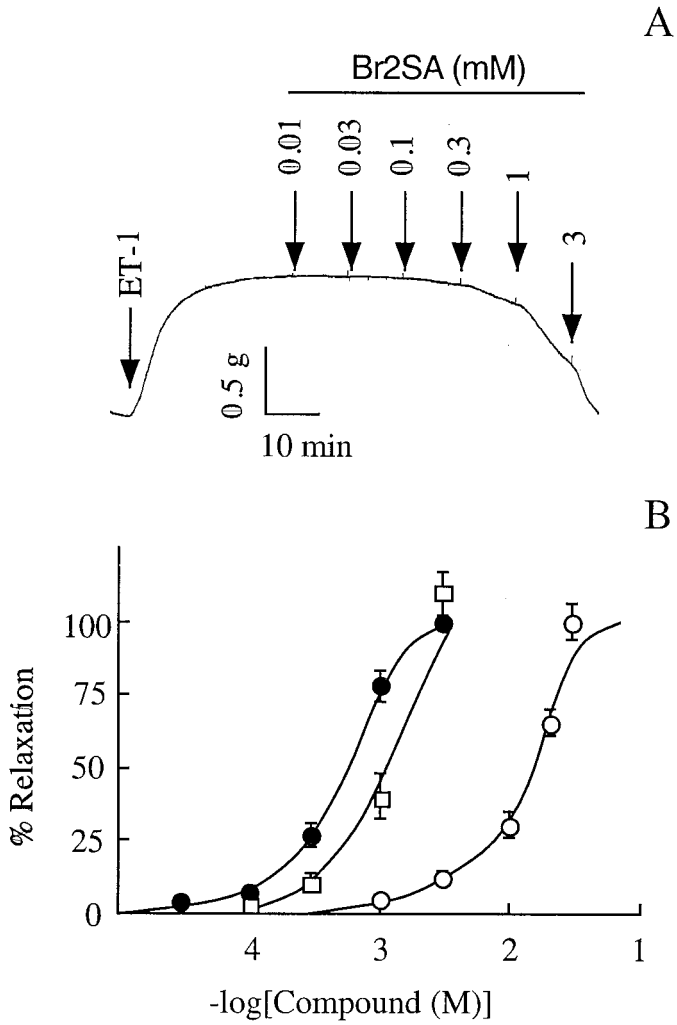

Fig. 8. Br2SA reversed the contractile action of ET-1 in rat aortic rings. A, representative trace showing the contractile action of 100 nM ET-1 and the relaxing actions of the indicated concentrations of Br2SA. B, cumulative concentration-response curves for the relaxing actions of I2SA (●), Br2SA (□), and SA (○). Means ± S.E. (n = 6) are shown.

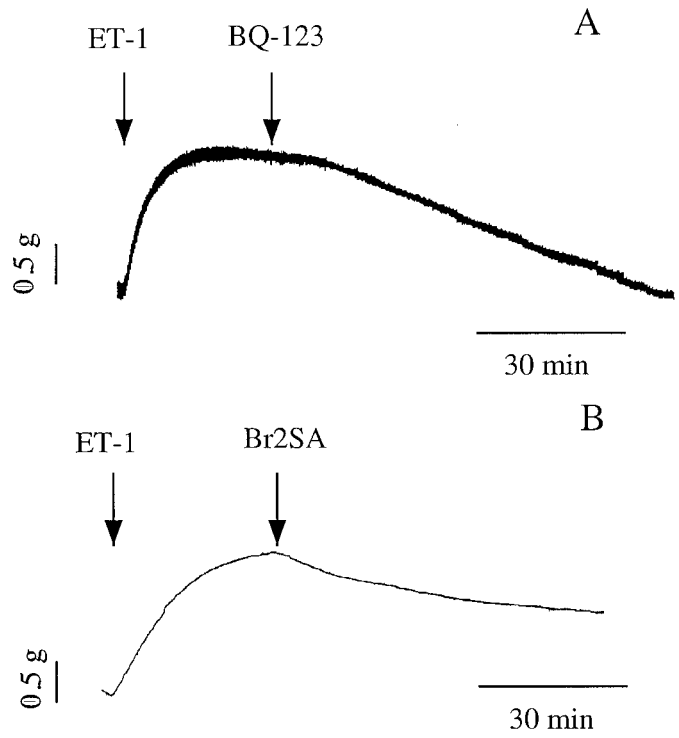

Fig. 9. Comparison of relaxing actions of BQ-123 and Br2SA. Representative traces showing the contractile action of 100 nM ET-1 and the relaxing actions of 10 μM BQ-123 and of 0.2 mM Br2SA are shown.

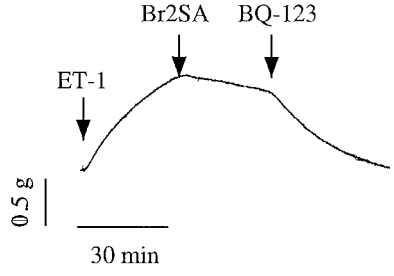

Fig. 10. Br2SA and I2SA potentiated relaxing actions of BQ-123. A representative trace showing the effect of the combined addition of BQ-123 and Br2SA on ET-1-induced contractions is shown. An aortic ring was first exposed to 100 nM ET-1. Once maximum tension had been reached, 0.2 mM Br2SA was added for 30 min. BQ-123 (10 μM) was then added, and the relaxation was followed until completion.

1999). 5-Aminosalicylic acid was included in our screening, but it was less active than SA or aspirin (Table 1). Thus, structure activity relationships for the actions of SA derivatives on cyclo-oxygenase, I-κB kinase, and ETA receptors are different.

Br2SA Is an Allosteric Inhibitor of ETA Receptors. All the results of binding experiments are consistent with a simple type of allosteric mechanism in which Br2SA binds to a site that is distinct from the ET-1-binding site. First, Br2SA does not modify the association of ^{125}I-ET-1 to its receptors. Second, Br2SA accelerates the dissociation of ^{125}I-ET-1 receptor complexes. Finally, The dose-response curve for the action of Br2SA on the dissociation of ^{125}I-ET-1 receptor complexes is saturable. Dissociation kinetics were monoexponential as expected if the dissociation kinetics of the allosteric ligand are fast relative to those of ^{125}I-ET-1 (Lazareno and Birdsall, 1995). The equilibrium dissociation constant of ET-1 receptor complexes, defined from kinetic experiments, is 1.7 pM in the absence of Br2SA. It is 13.6 pM in the presence of a near-saturating concentration of Br2SA. A decrease in the apparent affinity of ^{125}I-ET-1 for its receptors is also documented by a Scatchard analysis of equilibrium binding data. The ratio of the K_d values, measured in the presence and absence of a near saturating concentration of Br2SA, is an estimate of the allosteric constant ($\alpha = 8$). As expected for an allosteric inhibitor, Br2SA also decreases the apparent affinities of ETA receptor for bosentan and BQ-123, two receptor antagonists that bind to the same binding site as ET-1 (Clozel et al., 1993; Sakamoto et al., 1993).

Anti-ET-1 Actions of Br2SA. SA derivatives antagonize actions of ET-1 in B7 cells and in isolated aortic rings. The rank order of their potency (I2SA = Br2SA ≫ SA) is similar to that obtained in binding experiments. This suggests that anti-ET-1 properties of SA derivatives are related to the allosteric inhibition documented in binding experiments.

Actions of ET-1 develop at large nanomolar concentrations. The EC_{50} value for ET-1-induced intracellular Ca^{2+} mobilization in B7 cells is 10 nM (Vigne et al., 1993). It is 15 nM for ET-1-induced contractions of isolated aortic rings (Marsault et al., 1991). These are 3 orders of magnitude larger than the K_d value of ET-1 receptor complexes. It was therefore surprising that Br2SA inhibited actions of large nanomolar concentrations of ET-1, whereas it decreased the affinity of ETA receptors for ET-1 from 1.7 pM to only 13.6 pM. One reason for this discrepancy is provided by the modeling work of Ehlert (1988), which showed that a negative allosteric ligand can be effective against large concentrations of a highly efficacious agonist if the allosteric drug exhibits a large cooperativity factor and reduces the intrinsic efficacy of the agonist receptor complex by a large factor. Thus, anti-ET-1 actions of salicylates are consistent with the proposed allosteric mechanism.

Influence of ET-1 and Br2SA on the Molecular Form of Solubilized Receptors. Affinity labeling experiments have previously been used to estimate the molecular mass of ET receptors. The results show molecular masses in the 32- to 70-kDa range (Sokolovsky, 1995). The results of gel filtration experiments indicated i) that solubilized ETA receptors form complexes of a much larger mass (>200 kDa) and ii) that binding of ET-1 alters the apparent receptor size. Similar observations have been made for other G protein-coupled receptors such as β-adrenergic receptors (Limbird and Lefkowitz, 1978). This study further shows that Br2SA decreased the apparent molecular weight of ETA receptors, which could suggest a partial dissociation of the receptor from associated proteins. Such an action would be consistent with the possible decreased efficacy of the receptors discussed above. More importantly, these results indicate that an action of Br2SA on ETA receptors does not require the presence of ET-1.

SA Derivatives Accelerated Relaxing Actions of BQ-123 and Bosentan. We described previously that SA accelerates relaxing actions of bosentan (Talbodec et al., 2000). This study extends these observations to I2SA, Br2SA, and BQ-123 (Table 2). This acceleration is another consequence of the allosteric type of mechanism. Binding of ET-1 to its receptors is almost irreversible. SA derivatives relieve this irreversibility. They increase the probability that a bound ET-1 molecule leaves its binding site and allows antagonists to bind to the site. These results fully support the hypothesis that actions of ET receptor antagonists are limited by the slow rate of dissociation of ET-1 receptor complexes. They suggest that relieving tight ET-1 binding by allosteric inhibitors may be of therapeutic interest.

Taken together, these results provide strong evidence for the existence of an allosteric modifier site on ETA receptors that recognizes selected derivatives of SA. The main interest of these compounds is to potentiate actions of receptor antagonists.

TABLE 2

Br2SA, I2SA, and SA potentiated relaxing actions of BQ-123 and bosentan

Rat aortic rings were exposed to 100 nM ET-1. After 30 min, maximum tension was reached and rings were exposed to a first drug for 30 min. The second drug was then added. Relaxations were followed until completion, and the half-times of the relaxations were determined graphically. Three aortic rings were prepared and tested for each animal. Means ± S.E. and the number of animals used are indicated.

First Drug	Second Drug	Half-Time of the Relaxations
		min
BQ-123 (10 μM)	None	42.0 ± 6.0 (n = 6)
Br2SA (0.1 mM)	BQ-123 (10 μM)	37.2 ± 3.5 (n = 5)
Br2SA (0.2 mM)	BQ-123 (10 μM)	18.7 ± 2.8 (n = 6)
Br2SA (0.3 mM)	BQ-123 (10 μM)	7.1 ± 1.7 (n = 3)
I2SA (0.1 mM)	BQ-123 (10 μM)	21.6 ± 4.9 (n = 9)
SA (10 mM)	BQ-123 (10 μM)	15.9 ± 3.6 (n = 6)
Bosentan (10 μM)	None	38.0 ± 4.0 (n = 13)
I2SA (0.1 mM)	Bosentan (10 μM)	20.6 ± 1.8 (n = 6)
Bosentan (10 μM)	I2SA (0.1 mM)	20.1 ± 1.5 (n = 6)
SA (10 mM)	Bosentan (10 μM)	12.2 ± 0.7 (n = 13)

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