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Coexpression Studies with Endothelin Receptor Subtypes Indicate the Existence of Intracellular Cross-Talk between ET_A and ET_B Receptors

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Human Girardi heart cells expressing endothelin ET_B receptors (GH_B cells) were transfected with human ET_A cDNA, and coexpression of ET_A and ET_B in the ratio of 4:6 was demonstrated by Scatchard analysis. [^{125}I]Endothelin (ET)-1 binding to ET_A-transfected GH cells (GH_{AB} cells) was displaced by an ET_A antagonist, BQ-123, in a biphasic manner. An ET_B agonist, BQ-3020, and an ET_B antagonist, BQ-788, inhibited [^{125}I]ET-1 binding to GH_{AB} cells in a monophasic manner with low affinities (IC_{50} = 2,800 and 890 nM, respectively); IC_{50} values for ET_B receptors seemed to be as weak as those for ET_A receptors. However, BQ-3020 and BQ-788 had a high affinity for ET_B receptors in a binding experiment using [^{125}I]ET-1 in the presence of 1 μM BQ-123, where ET_A receptors are masked (IC_{50} = 0.49 and 0.89 nM, respectively). The ET_B-mediated increase in intracellular calcium concentrations in GH_{AB} cells was not affected by 0.1 μM BQ-788 alone but was inhibited significantly by the same concentration of BQ-788 in combination with 10 μM BQ-123. ET-1 suppressed forskolin-stimulated accumulation of cAMP through the activation of ET_A and ET_B in GH_{AB} cells; 1 μM BQ-123 or BQ-788 inhibited the suppression by only 20%, whereas a mixture of BQ-123 and BQ-788 (1 μM each) completely inhibited the cAMP decrease. These findings suggest that the stimulation of ET_A receptors with ET-1 results in a lowering of the affinity of BQ-3020 and BQ-788 for ET_B receptors in GH_{AB} cells. We conclude that there is intracellular cross-talk between ET_A and ET_B receptors in GH_{AB} cells.

Key words: cross-talk, endothelin, endothelin receptor, Girardi heart cell, signal transduction.

Endothelin (ET)-1, the most potent vasoconstrictor peptide, was originally isolated from the medium of cultured endothelial cells (*1*). After its discovery, two related peptides, ET-2 and ET-3, were identified (*2*). There are two subtypes of ET receptors: ET_A, selective to endothelin-1 and endothelin-2, and ET_B, nonselective to the three ET isopeptides (*3, 4*).

We have previously reported on the ET_A antagonist, BQ-123 [*cyclo*(-D-Trp-D-Asp-Pro-D-Val-Leu-)] (*5*), the ET_B agonist, BQ-3020 (*N*-acetyl-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp) (*6*) and the ET_B antagonist, BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-N^{in}-methoxycarbonyltryptophanyl-D-norleucine) (*7*). BQ-123 and BQ-788 inhibit [^{125}I]ET-1 binding to ET_A receptors in human neuroblastoma-derived SK-N-MC cells with IC_{50} values of 24 and 1,300 nM, respectively, and to ET_B receptors in human Girardi heart cells with IC_{50} values of 9,700 and 1.2 nM, respectively (*7*). BQ-3020 inhibits [^{125}I]ET-1 binding to ET_A receptors in porcine aortic smooth muscle cells with an IC_{50} value of 940 nM and to ET_B receptors in porcine cerebellar membranes with an IC_{50} value of 0.2 nM (*6*).

In vascular systems, it was initially thought that vascular smooth muscle ET_A mediates vasoconstriction and endothelial ET_B mediates vasodilation through release of prostacyclin or nitric oxide (*8, 9*). However, it is now apparent that smooth muscle ET_B also mediates vasoconstriction (*10-12*) and airway constriction (*13*). Recent studies using some ET agonists or antagonists have demonstrated that endothelin-induced constrictions of swine pulmonary vein, rabbit pulmonary artery, rat stomach strip, and guinea pig ileum were not fully explained by only an ET_A and/or ET_B-mediated mechanism, thus suggesting the existence of unidentified receptor subtypes in these tissues (*14, 15*). Since such novel receptor subtypes have not been defined at the molecular level yet, their existence is still debatable.

Recently, Maggio *et al.* demonstrated possible cross-talk between muscarinic receptors using chimeric α_2-adrenergic/M_3 muscarinic receptors and various other mutant M_3 receptors, suggesting cross-talk between guanine-nucleotide-binding-protein (G-protein)-coupled receptors (*16*). Furthermore, some δ-opioid receptors are considered to interact with μ-receptors (*17, 18*). As ET receptors are also G-protein-coupled, ET_A and ET_B may interact with each other in tissues possessing both receptor subtypes, resulting in a change in affinity for agonists or antagonists. If such

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Abbreviations: BQ-123, *cyclo*(-D-Trp-D-Asp-Pro-D-Val-Leu-); BQ-3020, *N*-acetyl-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp; BQ-788, *N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-N^{in}-methoxycarbonyltryptophanyl-D-norleucine; DMEM, Dulbecco's modified Eagle's medium; ET, endothelin; GH_{AB} cells, ET_A-transfected human Girardi heart cells; GH_B cells, human Girardi heart cells; G-protein, guanine-nucleotide-binding-protein; IBMX, 3-isobutyl-1-methylxanthine; MEM, minimum essential medium; FBS, fetal bovine serum.

changes in affinities of agonists or antagonists occur, it would be difficult to determine the receptor subtypes based on the potencies of the agonists or antagonists toward each receptor subtype.

Human atrium-derived Girardi heart cells are a unique cell line that expresses ET_B but not ET_A (GH_B cells), and in which activation of the ET_B results in an increase in intracellular calcium levels (*19*). We tried to construct a cell line that coexpresses ET_A and ET_B (GH_{AB} cells) by means of transfection with human ET_A cDNA and to demonstrate possible intracellular cross-talk between the two endothelin receptor subtypes in receptor binding studies and by measuring intracellular calcium levels and cAMP contents.

MATERIALS AND METHODS

Materials—BQ-123, BQ-788, and BQ-3020 were synthesized in our laboratory. [^{125}I]ET-1 and [^{125}I]ET-3 were purchased from Amersham International (Buckinghamshire, England); ET-1 and ET-3, from Peptide Institute (Osaka); pertussis toxin, from Sigma Chemical (St. Louis, MO, USA); and G418, from Life Technologies (Grand Island, NY, USA). All other chemicals and reagents used were of the highest quality available.

Transfection and Stable Expression of the Cloned Human ET_A Receptors—The expression plasmid pCDNA-1neo containing the entire coding region of the human ET_A receptor was a gracious gift from Dr. Jwu-Sheng Tung, Merck Research Laboratories (Rahway, NJ, USA) (*20*). The plasmid was transfected into Girardi heart cells, and grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) using the calcium phosphate method (*21*). After growth (48 h), the cells were selected for stable integration of the endothelin receptor expression vectors by adding 5 mg/ml of G418 to the medium and were isolated by single cell cloning. The expression of ET_A receptors was confirmed by [^{125}I]ET-1 binding studies.

Cell Culture—GH_{AB} cells were grown in MEM containing 10% FBS and 5 mg/ml G418 at 37°C under atmospheric conditions of 95% air-5% CO_2. GH_B cells were grown in MEM containing 10% FBS under the same conditions. Confluent cells in 24- or 96-well plates were used for each assay.

Binding Experiments—Confluent cells were washed three times with Hank's balanced salt solution (HBSS) containing 0.1% glucose and 0.3% BSA and were incubated with [^{125}I]ET-1 or [^{125}I]ET-3 under atmospheric conditions of 95% air-5% CO_2. The cells were then washed three times with ice-cold buffer. Cell-bound radioactivity was determined with a gamma counter (Packard: COBRA 5002). Nonspecific binding was elucidated by adding 200 nM ET-1. Membrane fractions of GH_B or GH_{AB} cells were prepared as previously described (*22*) and were incubated with [^{125}I]ET-1 in 50 mM Tris-HCl (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 2 μM leupeptin, 1 mM 1,10-phenanthroline, 1 mM EDTA, 10 μM $CaCl_2$, 10 μM $MgCl_2$, and 0.1% BSA. Free and cell-bound [^{125}I]ET-1 were separated by filtration using Whatman GF/C glass fiber filters. Radioactivity was determined with the gamma counter. Nonspecific binding was elucidated by adding 200 nM ET-1.

Measurement of Intracellular Free Calcium Ion Concentration ($[Ca^{2+}]_i$)—$[Ca^{2+}]_i$ was fluorometrically measured using a Ca^{2+}-sensitive fluorescent dye, fura-2. GH_B cells and GH_{AB} cells were harvested using 0.25% trypsin and 0.02% EDTA. The cells were washed once with Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes and 0.3% BSA (pH 7.4) (DMEM/Hepes/BSA). The cells were then suspended in DMEM/Hepes/BSA and incubated with 2 μM fura-2 acetoxymethylester at 37°C for 30 min. The cell suspensions were diluted with a 10-fold volume of DMEM/Hepes/BSA and again incubated at 37°C for 20 min. The fura-2-loaded cells were centrifuged and resuspended in Krebs-Henseleit Hepes buffer containing 0.1% BSA (pH 7.4). Fluorescence intensity at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm was monitored with a JASCO CAF-110 spectrofluorometer (Tokyo).

Measurement of cAMP Accumulation in the Cells—After removal of the culture medium, the cells were washed three times with Locke's buffer, pH 7.4 (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 3.6 mM; glucose, 5.6 mM; Hepes, 10 mM; IBMX, 0.3 mM) and equilibrated in the buffer for 10 min at 37°C. After pretreatment with 10 μM forskolin, the cells were incubated with endothelin-1. The buffer was then changed to ice-cold 50 mM sodium acetate buffer, pH 4.0. In some experiments the cells were pretreated with 100 ng/ml pertussis toxin for 6 h at 37°C before the assays. The samples were quickly frozen with liquid N_2 and stored at −80°C until use. The frozen samples were thawed at room temperature and centrifuged at $2,000 \times g$ for 15 min. The supernatant was transferred to tubes and dried in a vacuum oven. The cAMP content was determined using the cAMP EIA System (Amersham, England).

Statistics—Data are expressed as mean±SE. Statistical comparison was performed using the analysis of variance followed by the Newman-Keuls test; $p < 0.05$ was taken as the criterion of significance.

RESULTS

Expression of ET_A in Transfected Girardi Heart Cells—To confirm the expression of transfected human ET_A receptors in GH_{AB} cells, we performed Scatchard plot analysis using [^{125}I]ET-1 or [^{125}I]ET-3. As shown in Fig. 1, A and B, Scatchard plot analysis using [^{125}I]ET-1 provided identical K_d values for GH_{AB} cells (33±6 pM) and GH_B cells (33±8 pM), and 1.8-fold different B_{max} values in GH_{AB} cells *vs.* GH_B cells (2.1±0.1 fmol/10^5 cells *vs.* 1.2±0.1 fmol/10^5 cells). In the case of [^{125}I]ET-3, the K_d and B_{max} values in GH_{AB} cells were 13±2 pM and 1.1±0.05 fmol/10^5 cells, respectively. Good agreement of B_{max} values for [^{125}I]ET-1 to GH_B cells and [^{125}I]ET-3 to GH_{AB} cells indicates that the density of ET_B receptors in GH_{AB} cells is about 1.2 fmol/10^5 cells, and that another portion of B_{max} (0.9 fmol/10^5 cells) involves ET_A. Therefore, we consider that GH_{AB} cells coexpress both ET_A and ET_B in the ratio of 4:6.

Comparison of the Binding Affinities of BQ-3020 in GH_{AB} Cells and GH_B Cells—[^{125}I]ET-1 binding to GH_B cells was displaced by ET-1, ET-3, BQ-123, BQ-3020, and BQ-788 in a monophasic manner with IC_{50} values of 0.023±0.0002, 0.026±0.001, 9,900±1,700, 0.62±0.09, and 1.2±0.2 nM, respectively (Fig. 2A). In GH_{AB} cells,

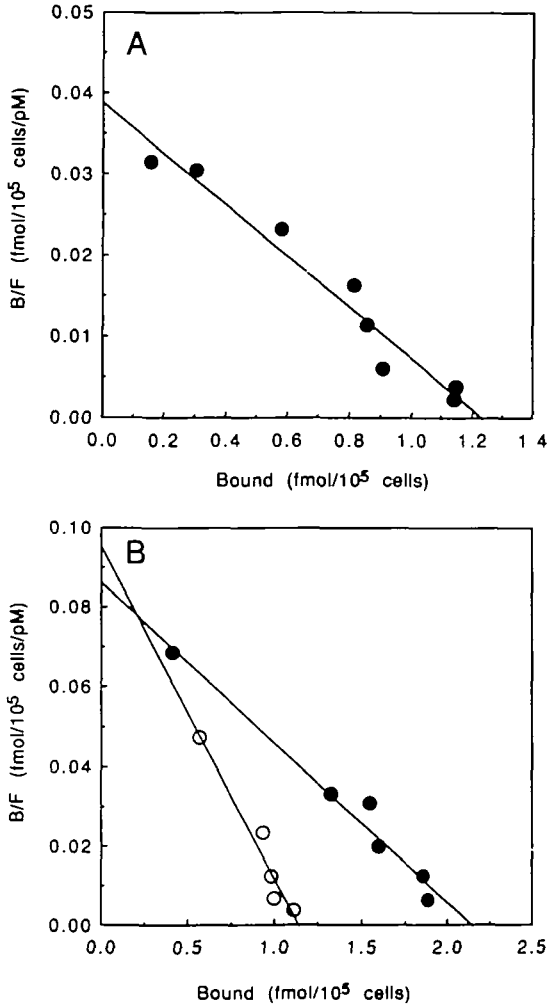

Fig. 1. **Scatchard plot analysis of (A) [^{125}I]ET-1 (●) binding to GH_B cells or (B) [^{125}I]ET-1 (●) or [^{125}I]ET-3 (○) binding to GH_{AB} cells.** Confluent cells were incubated with various concentrations of [^{125}I]ET-1 or [^{125}I]ET-3 at 37°C for 4 h. The results are presented as the mean of three individual experiments.

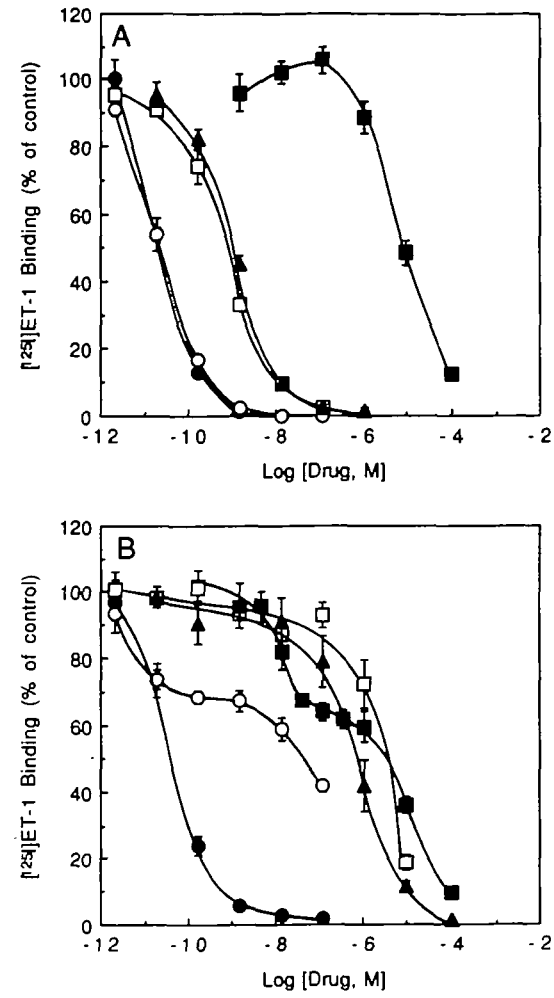

Fig. 2. **Effects of ET-1 (●), ET-3 (○), BQ-123 (■), BQ-3020 (□), and BQ-788 (▲) on [^{125}I]ET-1 binding to (A) GH_B cells or (B) GH_{AB} cells.** Confluent cells were incubated with 12 pM [^{125}I]ET-1 at 37°C for 4 h. Each point represents the mean ± SE (n=3).

[^{125}I]ET-1 binding was displaced by ET-1 in a monophasic manner (IC_{50} = 0.054 ± 0.009 nM), but by ET-3 and BQ-123 in a biphasic manner. The high affinity site for BQ-123 (IC_{50} = 16 ± 3 nM) represented 40% of the receptor population, whereas the low affinity site (IC_{50} = 17,000 ± 2,600 nM) represented 60% of the population, suggesting the existence of ET_A and ET_B receptors in a ratio of 4:6 (Fig. 2B). This receptor subtype ratio was consistent with the result obtained in Fig. 1B. In tissues possessing both ET_A and ET_B, BQ-3020 is known to inhibit [^{125}I]ET-1 binding in a biphasic manner (*23*). Therefore, we suspected that BQ-3020 would inhibit [^{125}I]ET-1 binding to GH_{AB} cells in a biphasic manner. Contrary to our expectations, BQ-3020 showed monophasic inhibition with a low affinity (IC_{50} = 2,800 ± 140 nM) (Fig. 2B), although it has a high affinity for ET_B in GH_B cells, as shown in Fig. 2A. Furthermore, BQ-788, having a high affinity for ET_B in GH_B cells, also displaced [^{125}I]ET-1 binding to GH_{AB} cells in a monophasic manner with a low affinity (IC_{50} = 890 ± 350 nM) (Fig. 2B). The resultant IC_{50} values for BQ-3020 and BQ-788 to inhibit [^{125}I]ET-1 binding to GH_{AB} cells were comparable to those previously reported for ET_A (*6, 7*), suggesting that the affinities of BQ-3020 and BQ-788 for ET_B in GH_{AB} cells are more than 700 times lower than those in GH_B cells.

Binding Affinities of BQ-3020 and BQ-788 for ET_B Receptors in GH_{AB} Cells—We next conducted competitive binding experiments using [^{125}I]ET-1 in the presence of 1 μM BQ-123 or [^{125}I]ET-3 to clarify the affinities of BQ-3020 and BQ-788 for ET_B in GH_{AB} cells. ET-1, ET-3, BQ-123, BQ-3020, and BQ-788 inhibited [^{125}I]ET-1 binding in the presence of 1 μM BQ-123, and [^{125}I]ET-3 binding to GH_{AB} cells in a monophasic manner (data not shown); the IC_{50} values are summarized in Table I. The resultant IC_{50} values for each ligand were comparable to those required to inhibit [^{125}I]ET-1 binding to ET_B receptors in GH_B cells, indicating that the affinities of BQ-3020 and BQ-788 for ET_B in GH_{AB} cells are not different from those in GH_B cells.

Binding Studies Using a Membrane Preparation of GH_{AB} Cells—The experimental difference between the results shown in Fig. 2B and Table I is only the binding of ET to ET_A. If a certain ET_A-mediated intracellular signal were required to reduce the affinities of BQ-788 and BQ-3020 for

ET_B, such a reduction should not be observed in our [^{125}I]-ET-1 binding experiments using a membrane preparation of GH_{AB} cells because intracellular signal transductions ought no longer to be observable in the membrane preparations. We further investigated the effects of these ligands on [^{125}I]ET-1 binding to membrane preparations of GH_{AB} cells or GH_B cells. In membranes derived from GH_B cells, [^{125}I]ET-1 binding was displaced by ET-1, ET-3, BQ-123, BQ-3020, and BQ-788 in a monophasic manner with IC_{50} values of 0.042±0.001, 0.031±0.004, 29,000±3,600, 0.19±0.02, and 1.7±0.2 nM, respectively (Fig. 3A). The IC_{50} values for the ligands were comparable to those observed in the cell binding experiment (Fig. 2A), indicating that the affinity of ET_B for each ligand was similar in the whole cells and in membrane preparations. Displacement curves for ET-1 and BQ-123 in membranes derived from GH_{AB} cells were the same as those observed in whole cells; IC_{50} values for ET-1 and BQ-123 were 0.045±0.004, and 4.0±0.6 (high-affinity site; ET_A), and 53,000±6,100 nM (low-affinity site; ET_B), respectively. The displacement profiles of BQ-3020 and BQ-788 in membrane preparations were very different from those in the whole cells; BQ-3020 displaced [^{125}I]ET-1 binding to membranes in a biphasic

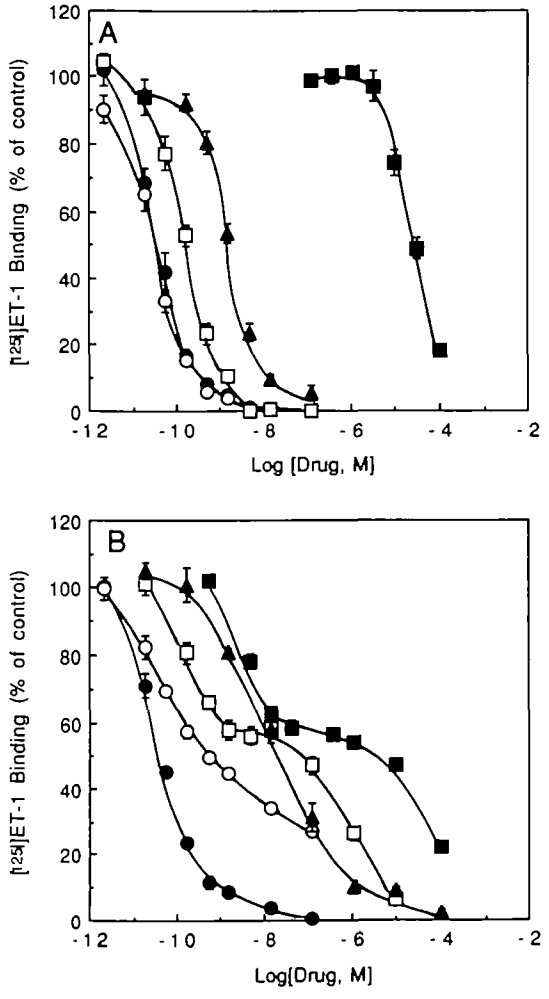

Fig. 3. **Effects of ET-1 (●), ET-3 (○), BQ-123 (■), BQ-3020 (□), and BQ-788 (▲) on [^{125}I]ET-1 binding to membrane preparations of (A) GH_B cells or (B) GH_{AB} cells.** The membranes were incubated with 12 pM [^{125}I]ET-1 at 25°C for 4 h. Each point represents the mean±SE (n=3).

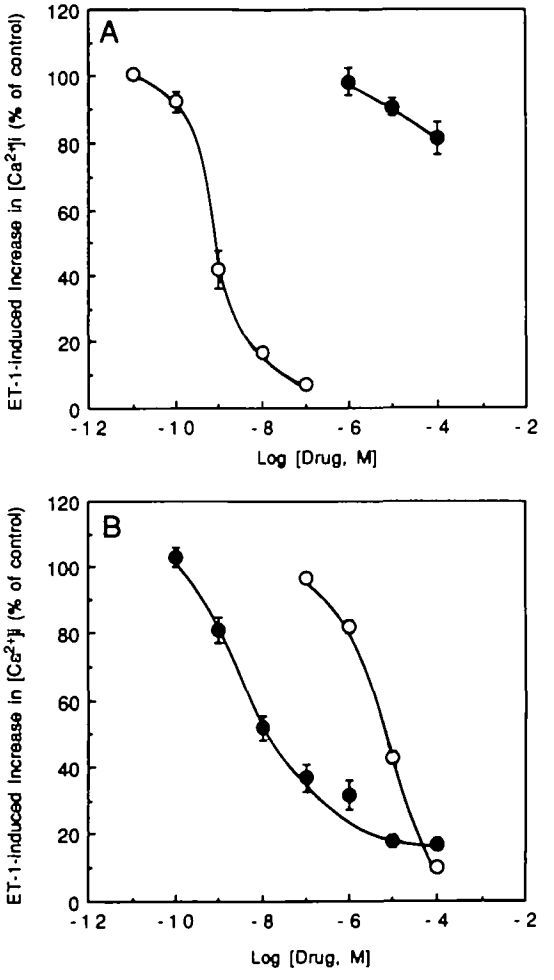

Fig. 4. **Effects of BQ-123 (●) and BQ-788 (○) on the ET-1-induced increase in $[Ca^{2+}]_i$ in (A) GH_B cells or (B) GH_{AB} cells.** The cells were treated with the indicated concentrations of each antagonist for 5 min and then stimulated with 100 nM ET-1. Each point represents the mean±SE (n=3).

TABLE I. **IC_{50} values for [^{125}I]ET-1 or [^{125}I]ET-3 binding to ET_B receptors in GH_{AB} cells.** Confluent cells were incubated with 12 pM [^{125}I]ET-1 in the presence of 1 μM BQ-123 or 12 pM [^{125}I]ET-3 at 37°C for 4 h. The results are presented as the mean±SE of three individual experiments.

Ligand	IC_{50} (nM)				
	ET-1	ET-3	BQ-123	BQ-788	BQ-3020
[^{125}I]ET-1[a]	0.038±0.001	0.017±0.004	ND[b]	0.89±0.1	0.49±0.1
[^{125}I]ET-3	0.031±0.001	0.016±0.004	12,000±4,600	2.0 ±1.0	0.27±0.05

[a]ET_A receptors were blocked by 1 μM BQ-123. [b]Not determined.

manner (IC_{50}: 0.17±0.04 nM, ET_B; 810±210 nM, ET_A) and BQ-788 displaced the binding with an IC_{50} of 24±3.3 nM (Fig. 3B). These results suggest that the binding of ET-1 to ET_A leads to characteristic changes in ET_B in GH_{AB} cells, and that the changes may occur because of ET_A-mediated intracellular signaling.

Effect of Possible Cross-Talk between ET_A and ET_B on $[Ca^{2+}]_i$ Response—We designed experiments to determine whether a reduction of the affinities of BQ-788 and BQ-3020 is observed in functional analysis. In GH_B cells, both ET-1 and ET-3 produced a dose-dependent increase of up to 100 nM in $[Ca^{2+}]_i$ with similar EC_{50} values of 0.31±0.03 and 0.28±0.02 nM, respectively (data not shown). The ET-1-induced increase in $[Ca^{2+}]_i$ was substantially inhibited by BQ-788 (IC_{50}=0.58±0.04 nM), but not by BQ-123 at concentrations of up to 100 nM (Fig. 4A), thus suggesting an ET_B-mediated response. On the other hand, in GH_{AB} cells, ET-1 increased $[Ca^{2+}]_i$ by up to 100 nM with an EC_{50} of 1.5±0.2 nM, and ET-3 and BQ-3020 increased $[Ca^{2+}]_i$ by up to 27±0.3 and 24±0.7 nM at the concentration of 100 nM with EC_{50} values of 4.5±2.7 and 4.0±0.2 nM, respectively (data not shown). The ET-1-induced increase in $[Ca^{2+}]_i$ was inhibited by BQ-123 with a high affinity (IC_{50}=17±4.4 nM) and by BQ-788 with a low affinity (IC_{50}=6,800±530 nM). About 20% of the $[Ca^{2+}]_i$ increase was insensitive to BQ-123 (Fig. 4B), suggesting that ET-1-induced increases in $[Ca^{2+}]_i$ are mediated by both ET_A and ET_B in a ratio of 8:2 in GH_{AB} cells.

We expected that 100 nM BQ-788 would inhibit 20% of the ET-1-induced increase in $[Ca^{2+}]_i$ in GH_{AB} cells, since 20% of the response was mediated by ET_B in GH_{AB} cells and 100 nM BQ-788 almost completely inhibited the 100 nM ET-1-induced increase in $[Ca^{2+}]$ in GH_B cells (Fig. 4A). Contrary to our expectations, 100 nM BQ-788 had no effect on the ET-1-induced increase in $[Ca^{2+}]_i$ in GH_{AB} cells. However, the same concentration of BQ-788 significantly inhibited the ET-1-induced $[Ca^{2+}]_i$ response in the presence of 10 μM BQ-123 (Fig. 5). As shown in Fig. 4B, BQ-123 at this concentration completely blocked the ET_A-mediated increase in $[Ca^{2+}]_i$, but did not affect the ET_B-mediated increase in $[Ca^{2+}]_i$. In addition, the ET-3- and BQ-3020-induced increases in $[Ca^{2+}]_i$ were also inhibited completely by 100 nM BQ-788 (data not shown). These results indicate that BQ-788 has a high affinity for ET_B unless ET_A is stimulated by ET-1, and that ET_A stimulation may result in a reduction of the affinity of BQ-788 for ET_B, as observed in our binding experiments.

Effect of Possible Cross-Talk between ET_A and ET_B on cAMP Response—In GH_B cells, ET-1 (100 nM) had no effect on the level of cAMP. Forskolin increased cAMP from 3.3±0.4 pmol/10^6 cells (basal level) to 27±0.2 pmol/10^6 cells, which was reduced by ET-1 to 15±0.6 pmol/10^6 cells. The inhibitory effect of ET-1 on forskolin-stimulated cAMP accumulation was completely abolished by BQ-788 and pertussis toxin, but not by BQ-123 (Fig. 6A), suggesting a role of ET_B in the inhibition of adenylate cyclase activity by ET-1 through pertussis toxin-sensitive G-proteins. In GH_{AB} cells, ET-1 itself could not alter cAMP,

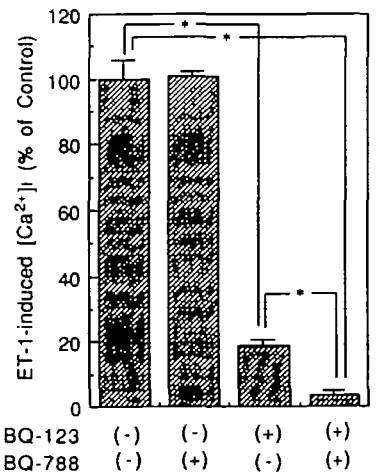

Fig. 5. **Effects of BQ-788 on the ET-1-induced increase in $[Ca^{2+}]_i$ in GH_{AB} cells in the presence or absence of BQ-123.** The cells were treated with 100 nM BQ-788 for 5 min in the presence or absence of 10 μM BQ-123 and then stimulated with 100 nM ET-1. The values of BQ-788 (−) are shown as control values. Each column represents the mean±SE (n=3). *p-values <0.05.

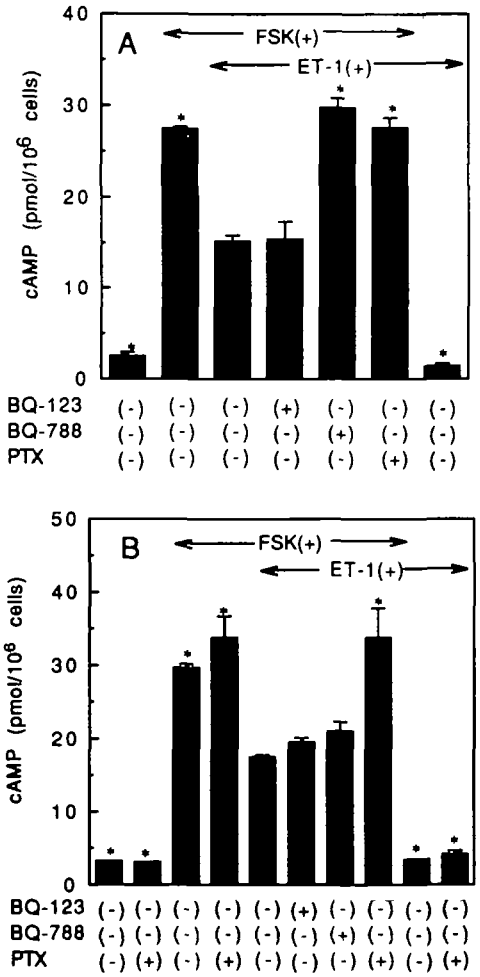

Fig. 6. **Effects of BQ-123, BQ-788, and PTX on ET-1-induced inhibition of FSK-stimulated accumulation of cAMP in (A) GH_B cells or (B) GH_{AB} cells.** Confluent cells treated with or without 100 ng/ml PTX for 6 h were incubated with 10 μM FSK in the presence or absence of 10 μM BQ-123 or 10 μM BQ-788 for 5 min, and then exposed to 100 nM ET-1 for 5 min. Each column represents the mean±SE (n=3). *p-values <0.05 compared with (A, B) FSK(+) and ET-1(+) or (C) FSK(+) and BQ-3020 (+).

but significantly suppressed the forskolin-induced increase in cAMP from 30±0.5 to 16±0.6 pmol/10^6 cells. Neither BQ-123 nor BQ-788 could inhibit the ET-1-induced suppression completely at the high concentration of 10 μM (Fig. 6B). BQ-3020 itself had no direct effect but significantly inhibited the forskolin-induced increase in cAMP to the same extent as ET-1 (19±0.6 pmol/10^6 cells). The inhibitory effect of BQ-3020 was abolished completely by BQ-788 but scarcely by BQ-123 (data not shown), implicating ET_B receptors. In addition, the dose-response curves for ET-1, ET-3, and BQ-3020 to inhibit forskolin-induced cAMP accumulation suggested the involvement of ET_A receptors. As shown in Fig. 7A, these agonists inhibited forskolin-induced cAMP accumulation in the rank order of ET-1>ET-3=BQ-3020 with EC_{50} values of 0.07, 0.9, and 0.9 nM, respectively. ET-1 was 10-fold more potent than ET-3 or BQ-3020, suggesting a contribution of ET_A receptors. These results indicate that both ET_A and ET_B receptor subtypes are involved in the suppression of cAMP in GH_{AB} cells. In addition, pertussis toxin treatment of the cells completely abolished the effects of ET-1 and BQ-3020 on the forskolin-induced increase in cAMP, suggesting that both ET_A and ET_B couple to pertussis toxin-sensitive G-proteins.

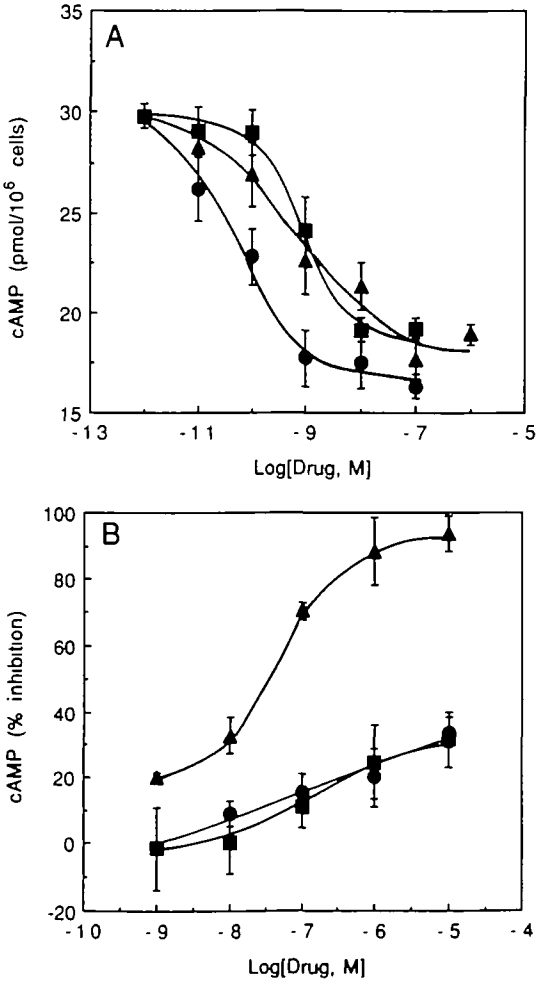

Fig. 7. **(A) Dose-response curves of ET-agonists for the inhibition of FSK-stimulated cAMP accumulation in GH_{AB} cells.** The cells were incubated with the indicated concentrations of ET-1 (●), ET-3 (■), or BQ-3020 (▲). (B) Effects of ET-antagonists on ET-1-induced inhibition of FSK-stimulated cAMP accumulation in GH_{AB} cells. The cells were treated with the indicated concentrations of BQ-123 (●), BQ-788 (■), or a mixture of equal concentrations of these antagonists (▲) for 5 min in the presence of 10 μM FSK, and then exposed to 100 nM ET-1 for 5 min. Each point represents the mean±SE (n=3).

Figure 7B indicates that BQ-123 and BQ-788 at concentrations of 0.1-10,000 nM had little effect on ET-1-induced inhibition of forskolin-stimulated cAMP accumulation. The IC_{50} values for the antagonists were estimated to be higher than 10 μM. However, a mixture comprising equal amounts of the two antagonists clearly inhibited the ET-1 response with an IC_{50} value of 28 nM each. The potencies of the antagonists seemed to be enhanced when they were used as a mixture. For example, BQ-123 or BQ-788 at a concentration of 1 μM produced 20% inhibition of the ET-1-induced suppression of cAMP, whereas a mixture of 1 μM of each antagonist completely inhibited the ET-1 response. Blockade of either ET_A or ET_B barely inhibited the ET-1 response, but blockade of both receptors inhibited the effect of ET-1 on forskolin-stimulated cAMP accumulation, suggesting a synergistic action of ET_A and ET_B to inhibit adenylate cyclase activity.

DISCUSSION

In the present study, the competition binding study of GH_{AB} cells using [^{125}I]ET-1 showed that the affinities of BQ-3020 and BQ-788 for ET_B receptors in GH_{AB} cells were more than 700 times lower than those in GH_B cells. However, when ET_A was free from ET-1, the affinities of these ligands to ET_B in GH_{AB} cells were almost comparable to those in GH_B cells. Therefore, we considered that the stimulation of ET_A with ET-1 may reduce the affinities of BQ-3020 and BQ-788 for ET_B. In function studies, 20% of the ET-1-induced increase in $[Ca^{2+}]_i$ was mediated by ET_B in GH_{AB} cells; BQ-788 (100 nM) had no effect on this $[Ca^{2+}]_i$ increase, but significantly inhibited it in the presence of BQ-123. ET-1 decreased cAMP through ET_A and ET_B in GH_{AB} cells, and the ability of BQ-788 to inhibit the ET-1-induced cAMP decrease was significantly enhanced by BQ-123. Therefore, the affinity of BQ-788 for ET_B seems to be reduced by the stimulation of ET_A with ET-1. Furthermore, in contrast to the results in Fig. 2B, BQ-3020 increased $[Ca^{2+}]_i$ and decreased forskolin-stimulated accumulation of cAMP in GH_{AB} cells as potently as ET-3 did, suggesting a high affinity of BQ-3020 to ET_B when ET_A is not stimulated by ET-1. Thus, we believe that there is cross-talk between ET_A and ET_B in GH_{AB} cells. Stimulation of ET_A receptors with ET-1 may induce a characteristic change in ET_B receptors.

Receptors added to the membranes prepared from cells are not linked to the intracellular signal transduction cascades. Therefore, we conducted binding experiments using a membrane preparation of GH_{AB} cells to examine whether intracellular signaling was involved in the cross-talk between ET_A and ET_B receptors. Under this experimental condition, we could not observe a lowering of the affinity of BQ-3020 or BQ-788 for ET_B receptors. Therefore, we conclude that stimulation of ET_A receptors induces a characteristic change in ET_B receptors through ET_A-mediated intracellular signaling, resulting in a lowering of

the affinities of BQ-3020 and BQ-788 for ET_B receptors.

Interestingly, in contrast with the results for BQ-3020 and BQ-788 in Fig. 2B, ET-3 displaced [^{125}I]ET-1 binding to GH_{AB} cells in a biphasic manner, suggesting that ET-3 possesses high affinity for ET_B even when ET-1 binds to ET_A in GH_{AB} cells. Therefore, we thought it important to evaluate the binding profile of another ET_B-selective ligand to examine whether the affinity-lowering of ET_B-selective ligands was applicable only to BQ-3020 and BQ-788.

Recently, Ogawa *et al.* have reported a novel ET_B-selective antagonist, RES-701-4, obtained from the culture broth of *Streptomyces* sp. (*24*). The IC_{50} values of RES-701-4 for ET_A and ET_B have been reported to be >1,000 nM (bovine lung) and 8 nM (bovine cerebellum), respectively. Therefore, we next examined the inhibition profile of this antagonist in GH_{AB} cells. RES-701-4 was found to have a high affinity for ET_B in GH_B cells (IC_{50}; 36 nM), while it inhibited [^{125}I]ET-1 binding to GH_{AB} cells in a monophasic manner with a low affinity (IC_{50}; 20,000 nM, data not shown), suggesting that the ET-1-induced affinity-lowering of ET_B-selective ligands for ET_B was not specific to BQ-3020 and BQ-788.

Recent point-mutagenesis studies in ET receptors revealed that a single amino acid replacement within hET_A or hET_B differentially affects the binding affinity of some ET-ligands (*25, 26*). Lee *et al.* demonstrated that replacement of Lys^{182} in hET_B by Arg, Ala, Met, Asp, or Glu does not alter the affinity of ET-1 for mutant receptors, but results in a decrease in binding affinity of ET-2 (11-48-fold), ET-3 (70-280-fold), sarafotoxin s6c (88-880-fold), and IRL 1736 (4,900-30,000-fold) for the mutant receptors (*27*), suggesting that various ET peptides do not quantitatively use the same set of receptor-ligand interactions when bound to hET_B.

One possible speculation to explain the difference between ET-3 and other ET_B-selective ligands tested in our present study is that stimulation of ET_A receptors with ET-1 induces a partial conformational change in ET_B receptors, lowering the affinity for ET_B of some ET_B-selective ligands, but not ET-3. However, the reason why the ET-1 differently alters the affinity of ET-3 and other ET_B-selective ligands for ET_B in GH_{AB} cells remains unclear.

Avissar *et al.* have demonstrated that rat heart and brain muscarinic receptors take a dimeric form by using photoaffinity labeling (*28*). Potter *et al.* reached a similar conclusion based on systematic analysis of the agonist binding properties of muscarinic receptors in various rat tissues (*29*). Recently, Maggio *et al.* demonstrated a functional interaction between muscarinic receptor subtypes by using chimeric receptors (*16*). Furthermore, some δ-opioid receptors are considered to be able to interact with μ-receptors (*18*). Rothman *et al.* demonstrated that a μ-receptor antagonist, β-funaltrexamine, attenuates the ability of δ-antagonists and naloxone to reverse δ-receptor-mediated physiological effects (*17*). Jiang *et al.* have confirmed, using the mouse tail-immersion test, that at sub-antinociceptive doses δ-opioid receptor agonists modulate antinociceptive responses to μ-opioid receptor agonists (*30*). A similar paradigm in which ET_A and ET_B receptors form a complex may explain the characteristic changes in ET_B receptors observed in this study.

Some studies have demonstrated subtype-selective short-term desensitization of receptors. Liggett *et al.* found that agonist stimulation of β_2-adrenergic receptors results in desensitization of the receptors, which depend on the agonist-induced phosphorylation of the C-terminal tail of the receptors, while β_3 receptors do not have such a property of desensitization (*31*). A_2-Adenosine receptors and α_2C10- and α_2C2-adrenergic receptors, but not A_1 receptors and α_2C4 receptors, also undergo agonist-promoted desensitization, which is thought to depend on cAMP accumulation and phosphorylation of the third intracellular loop of the receptors (*32, 33*). We hypothesize that certain intracellular signaling derived from ET_A may induce partial desensitization of ET_B receptors, resulting in a lowering of the affinities of BQ-3020 and BQ-788 for ET_B receptors. However, the mechanism of interaction between ET_A and ET_B receptors remains to be clarified.

Recent pharmacological studies in isolated vascular and non-vascular tissues have suggested the possible existence of another receptor subtype (*34*). Swine pulmonary vein, rabbit pulmonary artery, and guinea pig ileum possessing both ET_A and ET_B are considered to have the third receptor subtype, since the responses of these tissues cannot be fully explained by the activity of the known receptors, ET_A and ET_B (*14, 15*). The present study demonstrated that the stimulation of ET_A results in a change in the affinity of BQ-3020 and BQ-788 for ET_B receptors in GH_{AB} cells possessing both receptor subtypes. If such cross-talk between ET_A and ET_B occurred in the tissues mentioned above, receptor subtypes responsible for contraction could not be defined based on the potencies of known ET_A and ET_B ligands. Furthermore, only ET_A and ET_B, but not a third receptor subtype, were detected in rabbit pulmonary artery and guinea pig ileum in [^{125}I]ET-1 binding experiments using membrane preparations of these tissues (*12, 35*). Therefore, the receptor interaction might be a sufficient alternative explanation for these pharmacological events.

In the present study, we created Girard heart cells coexpressing ET_A and ET_B by transfecting Girardi heart cells with human ET_A cDNA and demonstrated a possible characteristic change in ET_B upon stimulation of ET_A. Our results suggest that some of the vascular or nonvascular contraction considered to be mediated by novel endothelin receptor subtypes can be explained by an interaction between ET_A and ET_B receptors.

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