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Characterization of Endothelin-1 Receptor Subtypes in Isolated Human Cardiomyocytes

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Summary: On cardiac membranes and isolated cardiomyocytes from the human heart, cell-type distribution and functional activities of endothelin-1 (ET-1) receptor subtypes were investigated by using binding methods and messenger RNA (mRNA) in situ hybridization. The ET-receptor antagonist BMS-182874 selectively and competitively inhibits ET_A receptors both on isolated myocytes and ventricular membranes with ~1,300 times greater affinity for ET_A than ET_B subtypes. The [^{125}I]-ET-1 specific binding revealed 42,851 ± 2,546 receptors/myocyte with a prevalent proportion of ET_A-receptor subtypes on both myocytes (84 ± 2%) and ventricular membranes (66 ± 3%). In situ hybridization studies revealed that mRNA for ET_A receptors was expressed on both myocytes and nonmyocyte cells, whereas mRNA for ET_B receptors was almost exclusively expressed on fibroblasts and endothelial cells. Specific binding of [^{125}I]-ET-1 to both myocytes and ventricular membranes in the presence of specific ET_A (BMS-182874) and ET_B (BQ-788)-receptor antagonists showed a displacement of [^{125}I]-ET-1 by unlabeled ET-1, which were significantly faster from ET_B than from ET_A. This suggests a clearance function of ventricular ET_B receptors. **Key Words:** Endothelin—Myocyte—Receptors—Growth factors—Receptor antagonists.

Endothelin-1 (ET-1) is a peptide that provides numerous biologic actions including potent and long-lasting vasoconstriction (1), and potent positive inotropic effect both in vivo (2) and on isolated cardiomyocytes (3,4). Cardiomyocytes express messenger RNA (mRNA) for pre-proET-1(5,6), a precursor of ET-1, which in turn acts in the heart as an autocrine factor able to induce hypertrophy of myocytes (7) and proliferation of fibroblasts (8). Recent studies demonstrated that ET-1 is involved in pressure overload–induced hypertrophy (9,10). Cardiac activities of ET-1 are mediated by two ET-1–receptor subtypes, ET_A and ET_B, both represented in human myocardium (11) with an average proportion in the left ventricle of 60:40 (ET_A/ET_B) (12). Myocytes almost exclusively express the ET_A-receptor subtype (>90%), whereas fibroblasts express both receptor subtypes (12–14). Due to the potential pathophysiologic and clinical importance of an increased ET-1 formation (15), a number of both nonselective and selective ET-1–receptor antagonists have been synthesized (16,17). Experimental studies have shown that short- or long-term administration of nonselective or selective ET-1–receptor antagonists can result in a favorable effect (18). BMS-182874 [5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-naphthalene sulfonamide] is an orally active, low-molecular-weight, nonpeptidic, ET_A receptor–selective antagonist (19). Although BMS-182874 has been found to be effective in a number of experimental settings (19–21), its capability to inhibit cardiac and human ET_A receptors has been assessed only in cardiac rat membranes and in rat and Chinese hamster ovary cell lines transfected with the human complementary DNAs (cDNAs) for ET_A and ET_B receptors (21,22). No study has been performed on human myocytes. Discrepancies have been reported in the binding affinities for other ET-1–receptor antagonists (BQ 3020) among human, rat, and pig hearts (23). In addition, recombinant rat and human ET_B receptors have shown different affinities when competing against several peptidic and nonpeptidic antagonists (24). Thus the extrapolation of data derived from animal studies to human subjects may not be correct (23). Moreover, the use of myocardial homogenates that contain both myocytes and a variety of other cell types does not allow to distinguishing the functional characteristics of ET_A and ET_B receptors expressed on myocytes (prevalently ET_A) from those of the receptors expressed on myocardial interstitial cells (both ET_A and ET_B). This differentiation may be important, as several experimental studies have raised the possibility that ET_A receptors mediate myocardial and coronary activities, whereas ET_B recep-

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tors act in the local clearance of ET-1 (25–27). This possibility suggests a different kinetics of [^{125}I]-ET-1 binding to ET_A- and ET_B-receptor subtypes. Therefore this study was planned to investigate the ET-1–binding inhibition of BMS-182874 in both isolated ventricular myocytes and cardiac membranes from the human heart and to analyze the kinetics of [^{125}I]-ET-1 binding to ET_A and ET_B receptors on both isolated human myocytes and human cardiac membranes.

METHODS

Tissue procurement

The characteristics of ET-1 binding and the inhibitory properties of BMS-182874 were investigated on isolated heart membranes and cardiomyocytes obtained from four nontailing organ donors with no history of cardiac disease who were not taking any drugs. These donors were initially considered for cardiac transplantation but subsequently were deemed unsuitable for transplantation either because of age or size incompatibility with the recipient.

Membrane isolation and cell separation

Membrane isolation. Three to five grams of left ventricular cardiac free wall were homogenized in an ice-cold buffer (20 m*M* $NaHCO_3$, 0.1 m*M* phenyl-methyl-sulphonyl-fluoride, pH 7.4), and centrifuged at 1,500 *g* for 15 min at 4°C. Supernatant was then centrifuged at 48,000 *g* for 15 min at 4°C. The pellet was resuspended in ice-cold buffer (50 m*M* Tris-HCl, 1 m*M* EDTA, 0.1 m*M* phenyl-methyl-sulphonyl-fluoride, pH 7.4) and recentrifuged at 48,000 *g* for 15 min. This procedure was repeated once. Protein concentration was assessed according to Bradford (28). 5′-Nucleotidase assay (Sigma Chemicals, St. Louis, MO, U.S.A.) in the final fraction showed an enrichment of at least fourfold compared with those of the 1,500 *g* supernatant.

Cardiomyocyte isolation. The selected coronary artery was cannulated and perfused with a calcium-free buffer (minimal essential medium, MEM) Eagle Joklik (Sigma Chemicals) with 21 m*M* HEPES, 4.4 m*M* $NaHCO_3$, 1.5 m*M* KH_2CO_4, 1.7 m*M* $MgCl_2$, 11.7 m*M* glucose, 2 m*M* L-glutamine, 21 U/ml insulin (pH 7.2: HEPES-MEM buffer) gassed with 95% O_2 and 5% CO_2 at 32°C for 10 min (blood washout). Then collagenase perfusion was carried out at 32°C with HEPES-MEM buffer gassed with 95% O_2 and 5% CO_2 and Worthington-type II collagenase, 100 U/ml (20 ml/min). The collagenase-perfused tissue was then minced and shaken in a resuspension buffer (HEPES-MEM buffer supplemented with bovine serum albumin 0.5%, 0.3 m*M* $CaCl_2$, 10 m*M* taurine) and Worthington type II collagenase, 100 U/ml, for 30 min at 37°C. After centrifugation for 4 min at 35 *g*, myocytes were enriched by centrifuging the resuspended pellet through Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Cell purity (>99% cardiomyocytes) was assessed by using anti-human myosin monoclonal antibodies (M8421; Sigma Chemicals).

Binding studies

Kinetic analysis. The kinetics of association of [^{125}I]-ET-1 (100 p*M*, 2,000 Ci/mmol, Amersham, Buckinghamshire, U.K.) to cardiac membranes (300 μg/ml) or isolated cardiomyocytes (10^5 cells/ml) was evaluated at selected times (30 s to 240 min), at 22°C. Nonspecific binding was obtained by adding unlabeled 1 μ*M* ET-1 2 h before the addition of [^{125}I]-ET-1. The content was then rapidly filtered through Whatman GF/C filters (Whatman International Ltd., Maidstone, U.K.) presoaked with polyethylene glycol (PEG, 6.6%). The kinetics of dissociation was evaluated by adding unlabeled ET-1 (1 μ*M*, final concentration (fc)) to the reaction mixture after 120 min of incubation. The kinetics of [^{125}I]-ET-1 binding to ET_A or ET_B subtypes was analyzed by using cardiac membranes or cardiomyocytes preincubated for 4 h with selective ET_B (BQ-788, 10 n*M*) or ET_A (BMS-182874, 1 μ*M*) antagonists, respectively. Kinetic constants (K_{obs}, K_{-1}, and K_1) were calculated according to Weiland and Molinoff (29).

Equilibrium studies and identification of endothelin-receptor subtypes. In equilibrium binding studies, cell membranes (300 μg/ml) or isolated cardiomyocytes (10^5 cells/ml) were incubated with [^{125}I]-ET-1, 100 p*M*, and increasing concentrations of displacer, unlabeled ET-1 (0–1 μ*M*) or BMS-182874 (0–1 m*M*) and BQ-788 (0–100 μ*M*), for 120 min at 22 C in a final volume of 0.2 ml in the same experimental conditions as described earlier. Data were analyzed according to Scatchard (30) and Cheng and Prusoff (31). Competition binding data were analyzed by iterative curve fitting to a one- or two-site binding model by using a nonlinear-fitting computer program (LIGAND) (32) to obtain the final estimation of K_d for ET-1, K_i for BMS-182874 and BQ-788, and the receptor density (B_{max}) values. To assess whether BMS-182874 is a competitive antagonist of ET_A receptors, [^{125}I]-ET-1 binding experiments were performed in the presence of three fixed concentrations of BMS-182874 (0, 50, and 100 n*M*).

In situ hybridization studies

The cDNA probes for ET_A- and ET_B-receptor subtypes were prepared from the phage clones of human endothelin receptors (ET_A: American Type Culture Collection, Rockville, MD, U.S.A.: ATCC 105194 and ET_B, ATCC 1250426). In situ hybridization studies were performed as previously described (33). Positive controls were obtained by using a cDNA probe for GAPDH (ATCC no. 57090). Myocytes were stained by using a specific anti-human myosin antibody (M8421; Sigma) and a secondary fluorescein-conjugated antibody (F4143; Sigma).

The specificity of the in situ hybridization signals was searched by testing the sections with hybridization mixture (a) without the probe, and (b) after incubation with RNAase A (1 Kunitz unit/L) for 1 h at 37°C before hybridization.

Statistical analysis

Each single experiment was performed in triplicate. If not otherwise indicated, all data given in the text are expressed as mean ± S.D.

RESULTS

In situ hybridization studies

Negative and positive controls showed that the hybridization in left ventricular tissue was specific for mRNA and that the mRNA was intact (Fig. 1B and A). mRNA for ET_A receptors in human left ventricle was expressed in both the myocytes and nonmyocyte cells (Fig. 1C). In contrast, mRNA for ET_B receptors was almost exclusively expressed in nonmyocyte cells (fibroblasts and endothelial cells) but not in myocytes (Fig. 1D–F).

Competition binding studies at equilibrium

The [^{125}I]-ET-1 specific binding to heart membranes reached saturation at ~1 n*M* with a B_{max} of 183 ± 19

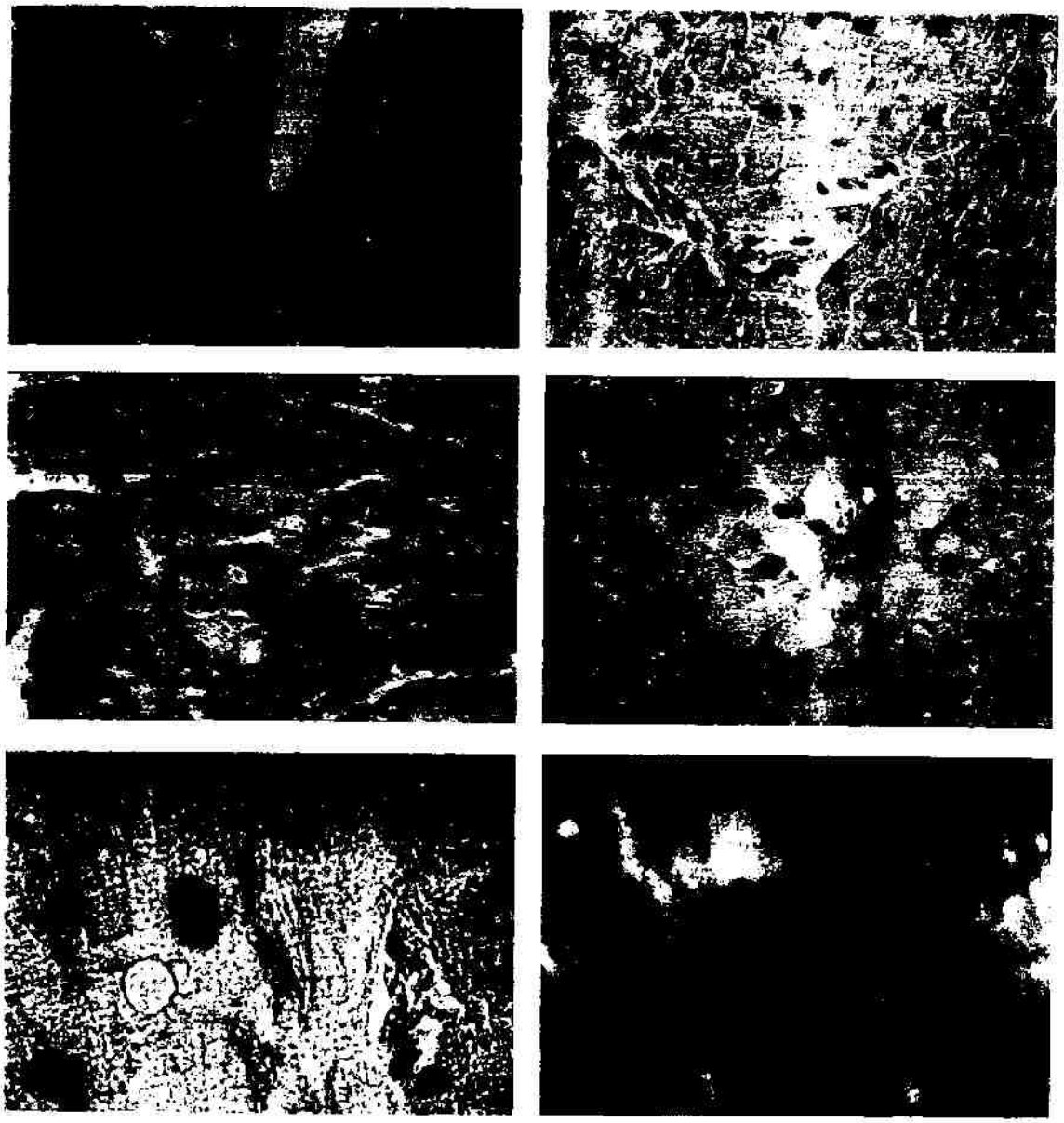

FIG. 1. In situ hybridization for GAPDH messenger RNA (mRNA) **(A)**, ET_A **(C)**, and ET_B **(D, E)** in left ventricular sections from human donors. **A:** Positive GAPDH mRNA signals in both myocytes and interstitial cells, and the same section after RNAase treatment (×400 magnification) **(B)**. **C:** Positive ET_A mRNA signals in both myocytes and nonmyocytes (×400). **D:** Positive ET_B mRNA signals in interstitial cells at ×400 **(D)** and ×1,000 **(E)**. In F, the same section stained for human myosin at fluorescent light (×1,000).

fmol/mg protein and a k_D of 0.36 ± 0.09 n*M* (Table 1). Unlabeled ET-1 displaced the [^{125}I]-ET-1 binding with a linear pattern of the Hill plot, indicating that ET-1 does not discriminate between receptor subtypes. Conversely, BMS-182874 (0–1 m*M*) inhibited the [^{125}I]-ET-1 specific binding to isolated membranes with a biphasic pattern (Fig. 2; Table 1) and a 1260 times greater affinity for ET_A than for the ET_B subtype (Table 1). The calculated average proportions of ET_A and ET_B receptors were 66 ± 3% and 34 ± 2% (Table 1).

The selective ET_B antagonist (BQ-788: 0–100 μ*M*) showed also a biphasic pattern of inhibition of the [^{125}I]-ET-1 specific binding to isolated membranes confirming the presence of a balanced proportion of ET_A (59 ± 2%) and ET_B receptors (41 ± 2%) in isolated membranes (Table 1).

The [^{125}I]-ET-1 specific binding to isolated cardiomyocytes showed the presence of 42,851 ± 2,546 receptors/myocyte (Table 1). Unlabeled ET-1 displaced [^{125}I]-ET-1 specific binding with a linear pattern.

BMS-182874 inhibited the [^{125}I]-ET-1 specific binding to isolated cardiomyocytes with a nonlinear pattern of inhibition (Fig. 3; Table 1), indicating that both receptor subtypes are represented in cardiomyocytes, al-

TABLE 1. *Competition studies of [^{125}I]-ET-1 binding to human isolated heart membranes and cardiomyocytes by unlabeled ET-1 and selective ET_A (BMS-182874) or ET_B (BQ-788) receptor antagonists*

	ET-1	BMS-182874	BQ-788
Cardiac membranes			
B_{max} (fmol/mg)	183 ± 19	—	—
K_D (n*M*)	0.36 ± 0.09	—	—
n_H	0.90	0.44	0.43
ET_A/ET_B (%)	—	66 ± 3/34 ± 2	59 ± 2/41 ± 2
ET_A B_{max} (fmol/mg)	—	119 ± 10	126 ± 8
K_i	—	57 ± 4.3 nM	266 ± 73 nM
ET_B B_{max} (fmol/mg)	—	64 ± 8	56 ± 9
K_i	—	72 ± 12 μM	0.96 ± 0.16 nM
Cardiomyocytes			
B_{max} (fmol/mg)	42 ± 7	—	—
K_D (n*M*)	0.34 ± 0.05	—	—
n_H	0.97	0.57	0.49
ET_A/ET_B (%)	—	84 ± 2/16 ± 2	86 ± 3/14 ± 2
ET_A B_{max} (fmol/mg)	—	35 ± 1	37 ± 2
K_i	—	73 ± 4.3	318 ± 81 nM
ET_B B_{max} (fmol/mg)	—	7 ± 1	6 ± 1
K_i	—	95 ± 32.1 μM	0.60 ± 0.37 nM

ET, endothelin; B_{max}, maximal binding; K_D, equilibrium dissociation constant; n_H, Hill coefficient; K_i, inhibitory constant.

though ET_A is prevalent (84 ± 2%). Displacement studies of [^{125}I]-ET-1 specific binding to cardiomyocytes by unlabeled ET-1, performed in the absence and in the presence of increasing concentrations of BMS-182874 (0, 50, and 100 n*M*), showed a progressive increase in the K_D values of [^{125}I]-ET-1 from 0.28 to 0.39 and 0.45 n*M*, respectively, whereas the B_{max} remained unchanged (39, 42, and 43 fmol/mg protein, respectively; Fig. 4), indicating a competitive interaction between BMS-182874 and [^{125}I]-ET-1 for binding to receptor sites.

BQ-788 caused a lower inhibition of [^{125}I]-ET-1 specific binding than BMS-182874 (Fig. 3; Table 1) indicating the presence of a low number (14 ± 2%) of ET_B receptors on isolated cardiomyocytes.

Kinetic studies

Specific binding of [^{125}I]-ET-1 to heart membranes reached steady-state by 120 min (Fig. 5). The binding was only partially displaceable by unlabeled ET-1 (21% after 2 h) with a half-life of dissociation of 21 min (Table 2). When ET_A receptors were blocked by preincubation with BMS-182874, specific binding of [^{125}I]-ET-1 was 35% of maximal binding, and the [^{125}I]-ET-1 displacement was more rapid with a half-life of dissociation of 13 min. In the presence of ET_B-receptor blockade, specific binding was 65% of maximal binding and the half-life of dissociation increased to 28 min (Table 2), thus indicating that [^{125}I]-ET-1 binds more tightly to ET_A than to ET_B receptors.

The [^{125}I]-ET-1 specific binding to isolated cardiomyocytes reached a steady state after 120 min (Fig. 6) and was barely displaced by the addition of a large amount of unlabeled ET-1 (14% after 2 h with half-life of 20 min). The addition of BMS-182874 significantly decreased the [^{125}I]-ET-1 specific binding to 15% of the maximal binding and caused a more rapid dissociation of [^{125}I]-ET-1 (half-life of 15 min; Fig. 6; Table 2). On the contrary, when ET_B receptors were blocked by preincubation with BQ-788 [^{125}I]-ET-1, the specific binding was reduced to 80% of maximal binding, and dissociation of

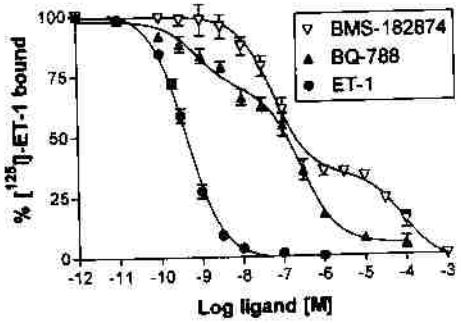

FIG. 2. Displacement of [^{125}I]-endothelin-1 specific binding by endothelin-1, BMS-182874, and BQ-788 from isolated cardiac membranes. Each point represents the mean ± SD.

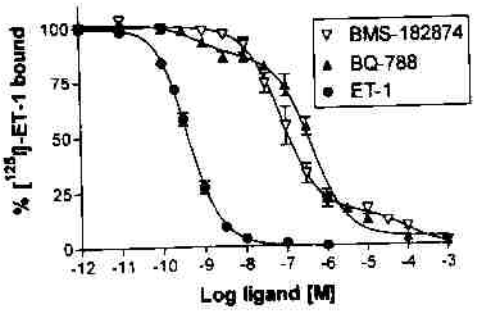

FIG. 3. Displacement of [^{125}I]-endothelin-1 specific binding by endothelin-1, BMS-182874, and BQ-788 from isolated cardiomyocytes. Each point represents the mean ± SD.

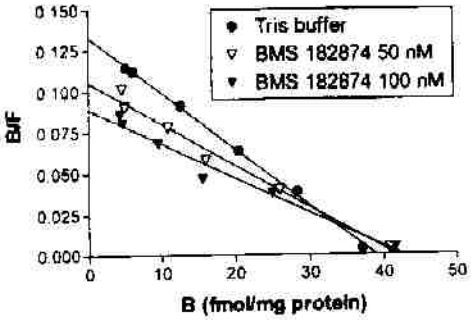

FIG. 4. Scatchard plot of [^{125}I]-endothelin-1 specific binding to isolated myocytes in the absence or presence of 50 or 100 n*M* BMS-182874. Each point represents the mean ± SD.

[^{125}I]-ET-1 became slower, with a half-life of 36 min (Table 2).

DISCUSSION

These results indicate that (a) BMS-182874 is able to antagonize [^{125}I]-ET-1 binding to ET_A receptors on human cardiomyocytes; (b) in left ventricular human myocytes, ET_A receptors are largely prevalent in respect to ET_B subtypes; and (c) the two receptor subtypes are characterized by a different ET-1–binding tightness. BMS-182874 binds to ET_A receptors on human myocytes with high selectivity because the absolute difference in the affinity for the two receptor subtypes is of three orders of magnitude (1,300 times), resulting in a clear differentiation between ET_A and ET_B receptors. The characteristics of [^{125}I]-ET-1 displacement in the absence and in the presence of different concentrations of BMS-182874 are consistent with a competitive interaction at the ET-1 binding site. The high selectivity of BMS-182874 for cardiac human myocyte ET_A receptors makes BMS-182874 suitable for defining the presence and relative proportion of the two ET-1–receptor subtypes expressed on human heart myocytes. In human left ventricular myocytes, BMS-182874 reveals a 84:16 ET_A/ET_B receptor ratio. This high prevalence of ET_A receptors on myocytes is confirmed also by in situ hybridization studies, which clearly showed a positive signal of mRNA of ET_A but not of mRNA for ET_B receptors. Also on human heart membranes, BMS-182874 binds to ET_A receptors with high selectivity (~1,260 times greater for the ET_A than the ET_B subtype). The selectivity of BMS-182874 for human membrane ET_A receptors is in the same order of magnitude as those reported for peptidic, nonorally active ET_A-receptor antagonists such as BQ-123 (12) and FR-139317 (23). The affinity of BMS-182874 for human ET_A subtypes is fourfold lower (K_i = 57 n*M*) than that previously reported for rat heart membranes (K_i = 227 n*M*) (21). Similar discrepancies regarding the affinity of ET_A-receptor blocking agents between human and rat heart membranes have been reported by using BQ-123 (an ET_A-selective antagonist) (23,34). Minor discrepancies also were found for the affinity of the peptidic ET_B antagonist used in our study (BQ-788), in comparison with the values previously reported in rats (35). These human–rat binding differences may be explained by the 7–9% and 12% species difference in the primary sequences of ET_A and ET_B receptors, respectively (34,36,37).

The use of selective ET antagonists in time-course experiments allowed us to give an accurate estimate of binding characteristics of the two ET-receptor subtypes. Kinetic studies demonstrated that [^{125}I]-ET-1 binds more tightly to the ET_A receptor than to the ET_B subtype. The half-life for dissociation of ET-1 from cardiac membranes when ET_A receptors were blocked was significantly shorter than that calculated in the presence of ET_B inhibition. It seems unlikely that the different kinetics for ET_B and ET_A receptors may be due to the interference between nonpeptidic ET_A (BMS-182874) and peptidic ET_B (BQ-788) antagonists at receptor sites, because the concentrations of selective antagonists used in kinetic studies were far from inhibiting the other receptor class, as shown by competition studies.

Furthermore, mutational studies of both ET_A and ET_B receptors revealed that the sites required for antagonist binding and agonist-induced signal transduction are shared by both peptidic and nonpeptidic compounds (38–41). In particular, high-affinity binding of chemically distinct peptidic and nonpeptidic ET_A antagonists is largely dependent on the residue at position 129, because high-affinity binding of BQ-123, SB-209670, bosentan, and BMS-182874 is not retained by Tyr-129 mutants (38,40,41). Conversely, binding of both peptidic and nonpeptidic ET_B-receptor antagonists is dependent on Lys-182 on the ET_B receptor sequence (39). Therefore no cross-interference seems to exist between both peptidic and nonpeptidic ET_A and ET_B antagonists.

The peculiar kinetic pattern of interaction of ET-1 at the two receptor subtypes observed in this study may account for the role played by ET_B receptors in the cardiac clearance of ET-1, because the faster kinetics of ET_B than ET_A may cause a preferential ET-1 association or dissociation to or from ET_B subtype receptors. Thus

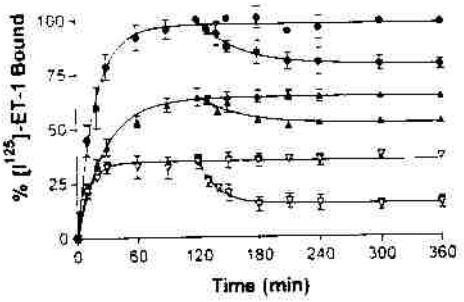

FIG. 5. Kinetic analysis of the [^{125}I]-endothelin-1 specific binding to isolated cardiac membranes in the absence (solid circles) and presence of BMS-182874, 1 μ*M* (open triangles), or BQ-788, 10 n*M* (solid triangles). Each point represents the mean ± SD.

TABLE 2. *Kinetic analysis of [^{125}I]-ET-1 binding to human isolated heart membranes and cardiomyocytes in the absence and in the presence of selective ET receptor antagonists*

	Buffer	BMS 182874	BQ-788
Cardiac membranes			
K_{obs} (min)	0.046 ± 0.004	0.089 ± 0.016	0.036 ± 0.003
K_{-1} (min)	0.030 ± 0.007	0.052 ± 0.013	0.026 ± 0.011
K_1 (n*M*/min)	0.163 ± 0.057	0.367 ± 0.09	0.104 ± 0.03
K_D (n*M*)	0.183 ± 0.064	0.142 ± 0.025	0.25 ± 0.051
$t_{1/2}$ association (min)	14	8	19
$t_{1/2}$ dissociation (min)	21	13	28
Cardiomyocytes			
K_{obs} (min)	0.051 ± 0.002	0.077 ± 0.021	0.028 ± 0.002
K_{-1} (min)	0.034 ± 0.001	0.046 ± 0.023	0.019 ± 0.008
K_1 (n*M*/min)	0.159 ± 0.025	0.309 ± 0.082	0.089 ± 0.011
K_D (n*M*)	0.217 ± 0.033	0.149 ± 0.086	0.215 ± 0.021
$t_{1/2}$ association (min)	14	9	25
$t_{1/2}$ dissociation (min)	20	15	36

ET, endothelin; K_{obs}, observation constant; K_{-1}, kinetic constant for dissociation; K_1, kinetic constant for association; K_D, equilibrium dissociation constant; $t_{1/2}$ association, half time of association; $t_{1/2}$ dissociation, half time of dissociation.

this receptor subtype might act as a clearance receptor. Recent studies both in isolated rat hearts (27) and in humans (42) showed that infusion of ET_B (BQ-788), but not of ET_A-selective antagonists (PD-155080 or BQ-123), caused an increase in ET-1 plasma concentrations. These results indirectly support the hypothesis that ET_B receptors act as clearance or buffer receptors for ET-1. The slower dissociation of ET-1 from ET_A than from ET_B receptor may have a functional consequence, because ET_A is the receptor subtype that prevalently mediates the cellular effects of ET-1 (43,44). The in situ hybridization studies showed that mRNA for ET_A receptor was expressed in both cardiomyocytes and interstitial cells, whereas mRNA for ET_B receptor was expressed almost exclusively in the interstitial cells. As a consequence, in conditions of an increased ET-1 local concentration, as occurs in heart failure, the biologic effects of ET-1 especially target the myocytes, which are void of ET_B-subtype receptors.

In conclusion, BMS-182874 antagonizes with high selectivity ET_A-receptor subtypes on both human isolated myocytes and ventricular membranes. The different binding tightness of ET-1 to ET_A versus ET_B ventricular receptor subtypes, revealed by specific antagonists, supports the hypothesis that ET_B plays a main role in the cardiac local clearance of ET-1.

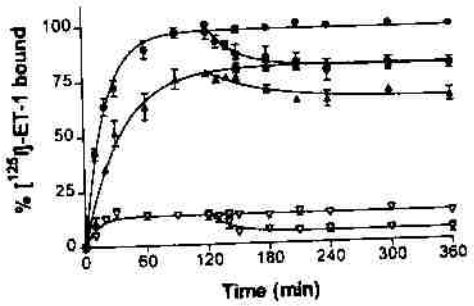

FIG. 6. Kinetic analysis of the [^{125}I]-endothelin-1 specific binding to isolated cardiomyocytes in the absence (solid circles) and presence of BMS-182874, 1 μ*M* (open triangles), or BQ-788, 10 n*M* (solid triangles). Each point represents the mean ± SD.

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