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Coupling of the Type A Endothelin Receptor to Multiple Responses in Adult Rat Cardiac Myocytes

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SUMMARY

In adult rat cardiac myocytes, endothelin (ET) receptors couple to multiple signaling pathways, including stimulation of phosphoinositide hydrolysis (pertussis toxin insensitive) and inhibition of adenylyl cyclase via G_i. We have used ET-1 and congeners to characterize the subtypes of ET receptors on isolated rat myocytes. The rank orders of potency for stimulating phosphoinositide hydrolysis, inhibiting hormone-sensitive adenylyl cyclase, and competing with ^{125}I-ET-1 for binding to myocytes are the same and show the pattern characteristic of an ET_A receptor interaction, i.e., ET-1 ~ ET-2 > sarafotoxin 6b > ET-3; the corresponding EC_{50} values for the effects of ET on signal transduction are approximately 0.5 nM (ET-1), 0.7 nM (ET-2), 7 nM (sarafotoxin 6b), and 60 nM (ET-3). The ET_A receptor antagonist BQ-123 abolishes the cellular responses to ET-1 and competes fully for ^{125}I-ET-1 binding in a concentration-dependent manner. Sarafotoxin 6c, an ET_B-specific agonist, does not diminish the responses to ET-1 or compete for ^{125}I-ET-1 binding; no specific binding of the ET_B-specific ligand ^{125}I-IRL-1620 is detectable on myocytes. Myocytes express approximately 4×10^5 ET-1 binding sites/cell. The association of ^{125}I-ET-1 with myocytes is largely irreversible, as are the biochemical responses to ET-1; thus, constants derived from analyses that assume reversible equilibria are in error. We conclude that the effects of ET on transmembrane signaling in rat ventricular myocytes result from occupation of ET_A receptors and that the responses are likely to be long lived, compared with those of the readily dissociable neurotransmitters released by the autonomic nervous system.

ET-1 is one of a family of 21-amino acid, vasoactive peptides and was initially identified and purified from porcine aortic endothelial cell cultures (1). The peptide is a potent vasoconstrictor that can evoke positive inotropic responses in the heart that are characterized by a slow onset and sustained duration (2–3). ET-1 also produces diverse biological effects in vascular and nonvascular tissue. For instance, ET-1 stimulates atrial natriuretic peptide secretion from atrial cells (4) and induces hypertrophy in neonatal heart cells (5) and mitogenesis in 3T3 cells, fibroblasts, vascular smooth muscle cells, and mesangial cells (6–8). Three forms of ET (ET-1, -2, and -3) have been cloned from a human genomic library (9) and, whereas they share agonist properties, their order of potency at various sites has helped define two receptor types, ET_A (ET-1 ≅ ET2 ≫ ET-3) and ET_B (ET-1 ≅ ET-2 ≅ ET-3). These two receptors have also been cloned recently (10–13).

A family of cardiotoxic peptides from snake venom (from the snake *Atractaspis engaddensis*) known as sarafotoxins (S6) share structural and functional homology with ETs (14, 15). These S6 forms (S6a, -b, -c, and -d) are also helpful in defining ET receptor subtypes; S6c is a selective agonist of the ET_B receptor (16). Recently, useful synthetic pharmacological agents have become available, such as the cyclic pentapeptide BQ-123, an ET_A-specific antagonist (17), and IRL-1620, an ET_B-specific agonist (18). Thus, tools are available for characterizing ET receptors and for defining the linkage of receptor subtypes to specific cellular responses. In general, the data in the literature indicate that the ET_A receptor is largely responsible for mediating the vasoactive and mitogenic responses (19, 20) of ET, whereas the ET_B receptor seems to couple to NO production and vasodilation (20, 21).

We have been studying the interaction of ET-1 with transmembrane signaling pathways in isolated adult rat ventricular myocytes. In these cells, ET-1 stimulates PI hydrolysis through a pertussis toxin-insensitive mechanism and inhibits hormone-sensitive adenylyl cyclase via a pertussis toxin-sensitive (G_i) pathway (22). As a means toward further dissection of the biochemical and physiological effects of ET-1 on cardiac myocytes, we have used pharmacological tools to characterize the ET receptors that couple to G_i and to PI hydrolysis.

Whether the two ET receptor subtypes coexist in ventricular myocytes and whether they may be linked to distinct signaling

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ABBREVIATIONS: ET, endothelin; S6, sarafotoxin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TCA, trichloroacetic acid; PI, phosphatidylinositol; MEM, minimum essential medium; BSA, bovine serum albumin.

pathways is not known. mRNA for both the ET_A and ET_B receptors has been detected in myocardium (23, 24). The biochemical responses to ET-1 do not suggest what subtypes may be present on myocytes. For instance, Aramori and Nakanishi (23) have recently demonstrated that, when mRNAs for the ET-1 receptors are transfected into Chinese hamster ovary cells, both ET_A and ET_B receptors are coupled to stimulation of PI hydrolysis, whereas occupation of the ET_A receptor leads to enhanced cAMP accumulation that is partially inhibited by pertussis toxin and occupation of the ET_B receptor causes inhibition of cAMP accumulation, apparently via the stimulation of a G_i pathway.

In this report, we present data on ligand binding and on receptor-effector coupling in adult rat cardiac myocytes. We detect no ET_B receptors and we find that activation of ET_A receptors accounts for both inhibition of cAMP synthesis and stimulation of PI hydrolysis in response to ET. Thus, we hypothesize that the positive inotropic effect of ET-1 on isolated cardiac myocytes (3) also results from stimulation of ET_A receptors, although the signaling process coupled to altered inotropism is still unknown.

Experimental Procedures

Materials. ^{125}I-ET-1 (2200 Ci/mmol) and ^{125}I-IRL-1620 were obtained from DuPont-NEN. BQ-123 was a generous gift from Dr. Theodore Torphy of SmithKline Beecham. All other chemicals and biochemicals were reagent grade from Sigma. ET-1 was the synthetic human-porcine ET-1. ET-2 was the synthetic human ET-2. ET-3 was the synthetic human-rat ET-3.

Isolation of adult ventricular myocytes and cell suspensions. Ventricular myocytes from 250–300-g adult Sprague-Dawley rats were prepared by a collagenase dissociation method (25). Four rat hearts were perfused at 32° for 45 min with MEM containing 0.1% collagenase (Boehringer-Mannheim), 0.1% BSA, and 25 μM Ca^{2+}. After removal of the atria, the ventricles were cut into several pieces and the myocytes were dispersed by gentle agitation through a wide-bore serological pipette. The cells were filtered through a nylon mesh and washed twice at 50 × *g* for 1 min. The cell pellet was suspended in MEM containing 0.1% BSA and Ca^{2+} (added slowly to a final concentration of 1 mM). The preparation provided 10–15 × 10^6 myocytes/heart with 80% viability, as assessed by rod-shaped morphology and exclusion of trypan blue.

For individual experiments, myocytes were suspended in MEM with 1 mM Ca^{2+} and 0.1% BSA, at densities ranging from 2 × 10^5 cells/ml (for cAMP assays) to 1 × 10^6 cells/ml (for PI hydrolysis). All incubations of whole cells were carried out at 32° in a gyrorotatory water bath (120 rpm).

ET binding studies. For ligand binding studies, myocytes (~50,000 cells/0.5 ml) were incubated with ^{125}I-ET-1 (~15 pM) or ^{125}I-IRL-1620, with or without increasing concentrations of unlabeled ET-1, ET-2, ET-3, S6b, S6c, or BQ-123, at 32° for 40 min. Incubation was terminated by filtering the cells through GF/C filters and washing them twice with 10 ml of cold (4°) buffer (25 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4), using a Brandel cell harvester, after which the filters were removed and radioactivity was quantified with a γ counter.

Membrane preparations. Frozen tissue from rat brain was powdered and homogenized on ice in a glass Dounce homogenizer. The homogenization buffer consisted of 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, and 10 μg/ml leupeptin, pH 7.4. The homogenate was centrifuged at 5000 × *g* for 10 min and the crude membrane pellet was resuspended in the same buffer. Aliquots of 100 μl containing 75–100 μg of membrane protein were used for binding studies with ^{125}I-IRL-1620.

PI hydrolysis. Myocytes (1 × 10^6 cells/ml) were labeled with *myo*-[^{3}H]inositol (12 μCi/ml, for 90 min), washed twice (50 × *g* for 1 min), and aliquoted into tubes containing 10 mM LiCl, 10 mg/ml leupeptin, and agonist. Incubation was terminated by addition of cold 10% TCA to the samples and centrifugation at 1200 × *g* for 15 min. The supernatants were extracted four times with 6 volumes of water-saturated ether and fractionated by anion exchange chromatography (26). Radioactivity in fractions corresponding to inositol monophosphate, inositol bisphosphate, and inositol trisphosphate was quantified by liquid scintillation counting, with an average efficiency of 30%. Data are expressed as cpm/aliquot of cells.

Assay of cAMP accumulation in myocytes. Equilibrated cell suspensions (2–3 × 10^5 cells/ml) were treated with agonists for 4 min at 32° (conditions giving a plateau in the β-adrenergic response), after which cold TCA (10% final concentration) was added. The samples were centrifuged at 1200 × *g* for 15 min and the TCA extracts were purified on Dowex AG50WX4 columns (200–400 mesh). cAMP content was determined by the protein binding method of Gilman (27). Acid-precipitable material was suspended in 0.4 N NaOH and the protein content was estimated by the method of Bradford (28). Data are expressed as picomoles of cAMP (corrected for recovery from Dowex columns) per milligram of myocyte protein.

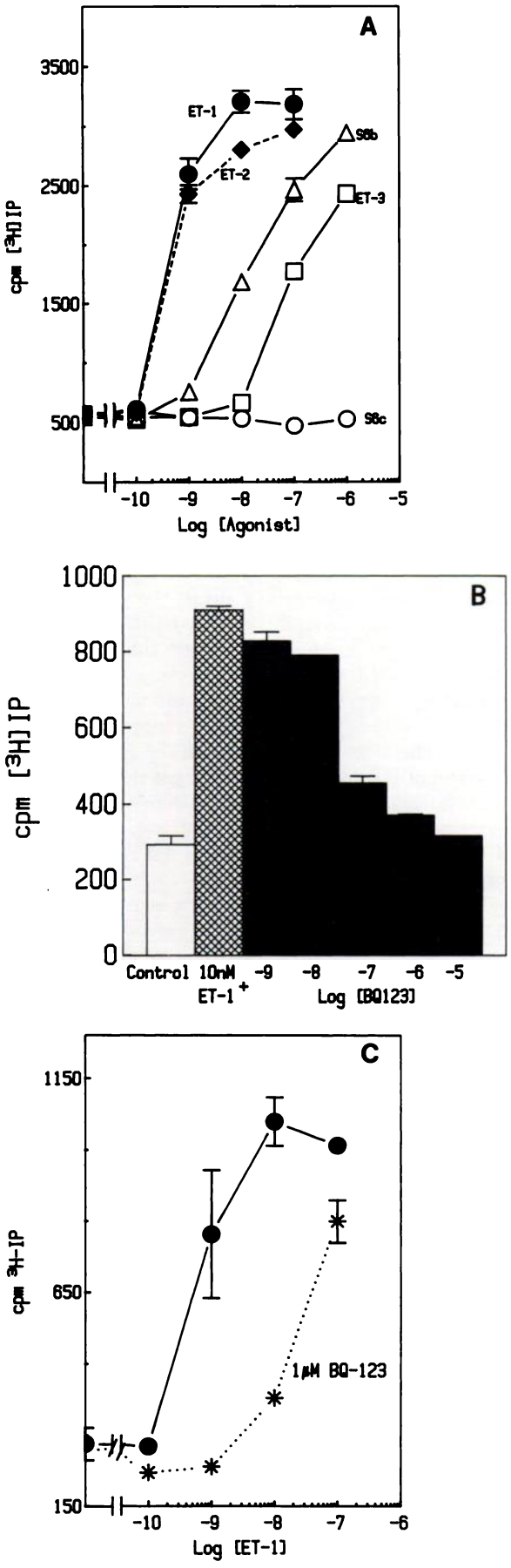
Analysis of data. Statistical comparisons were performed with the program InStat and curve fitting with the program InPlot, both a gift from Dr. Harvey Motulsky (GraphPAD Software, San Diego, CA). Data are expressed as the mean ± standard error of four or more experiments unless otherwise noted. Affinities of competitive inhibitors were estimated by the dose-ratio method of Schild (29), relying on the magnitude of shift in response produced by a known concentration of competitive antagonist.

Results

Effects of ET and congeners on PI turnover. A prominent effect of ET on cardiac myocytes is the stimulation of PI hydrolysis, with the accumulation of inositol monophosphate, inositol bisphosphate, and inositol trisphosphate (22). In cells labeled with [^{3}H]inositol, ET and its congeners stimulated [^{3}H]inositol phosphate accumulation with the order of potency ET-1 ≈ ET-2 > S6b > ET-3. EC_{50} values for these compounds were 0.4–1 nM (ET-1 and ET-2), 7 nM (S6b), and 60 nM (ET-3) (Fig. 1A). Likewise, S6c was inactive in stimulating PI hydrolysis. We conclude that this functional coupling of ET receptors is explained by activation of ET_A receptors.

The effects of a specific ET_A receptor antagonist substantiate the assignment of receptor type. Increasing concentrations of BQ-123 completely abolished the capacity of 10 nM ET-1 to stimulate PI hydrolysis (Fig. 1B). BQ-123, at 1 μM, shifted the concentration-dependence curve of PI turnover versus ET-1 concentration 2 orders of magnitude to the right; if analyzed as a competitive interaction, this shift yields an estimated K_i of 9.6 ± 1.4 nM (mean ± standard error of four determinations) for BQ-123, in good agreement with published values for the affinity of BQ123 for the ET_A receptor (17, 19) (see Basic properties of the interaction of ^{125}I-ET-1 with myocytes and Discussion for information on problems with the assumptions of reversibility of binding in experiments involving ET-1).

Effects of ET and congeners on cAMP accumulation. ET-1 inhibits cAMP production in cardiac myocytes, with the ET-1 receptor coupling to adenylate cyclase via G_i (tentatively identified as G_{i2}) (22). This effect of ET-1 also seemed to result from activation of an ET_A receptor. The order of potency was appropriate, i.e., ET-1 ≈ ET-2 > S6b > ET-3; S6c was without effect (Fig. 2A). The potencies (average EC_{50} values from two to five experiments: ET-1, 0.56 ± 0.17 nM; ET-2, 0.45 ± 0.25 nM; S6b, 7.70 ± 0.8 nM; ET-3, 68.0 ± 5.4 nM) agree quite well



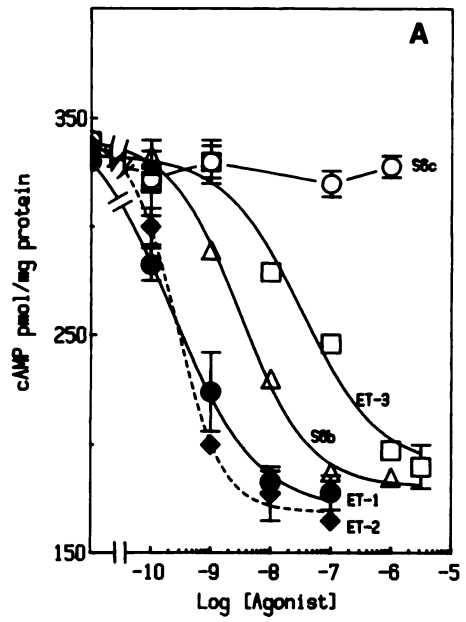
with values obtained from studies of inositol phosphate formation (see above and Fig. 1). Likewise, the effect of BQ-123, the specific ET_A receptor antagonist, was concentration dependent and complete in antagonizing the effect of ET-1 on cAMP production (Fig. 2B); the estimated inhibition constant calculated from this functional competition (Fig. 2C), 11.8 ± 1.1 nM (see legend to Fig. 2C), agrees precisely with that obtained from the effect of the drug on ET-stimulated PI hydrolysis (Fig. 1C) and with literature values (17, 19). In all assessed aspects, the effect of ET-1 to inhibit adenylate cyclase seems to result from activation of an ET_A receptor.

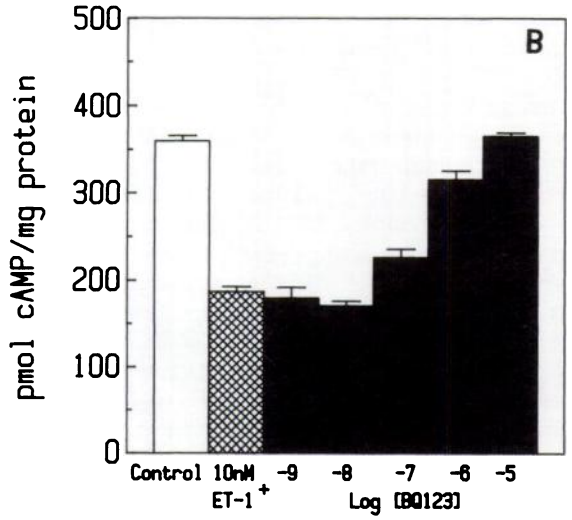
Basic properties of the interaction of ^{125}I-ET-1 with myocytes. ^{125}I-ET-1 associated rapidly with myocytes. At 32°, binding reached a steady state level by 40 min and remained at a plateau for at least an additional 100 min. Under our assay conditions, specific binding (i.e., total binding minus binding occurring in the presence of 0.3 μM ET-1) comprised 80–85% of the total signal and was a linear function of the number of cells added to the binding reaction. Based on the data from control experiments, we routinely used 50,000 cells/tube in binding assays and employed 15 pM labeled ligand. We found that using siliconized surfaces and including BSA were important to prevent adherence of ET-1 to the surfaces of pipettes and reaction vessels. Under these conditions, (15 pM ^{125}I-ligand and 50 × 10^3 cells in 0.5 ml, with 40-min reaction time), fractional binding of added ^{125}I-ET-1 did not exceed 10%.

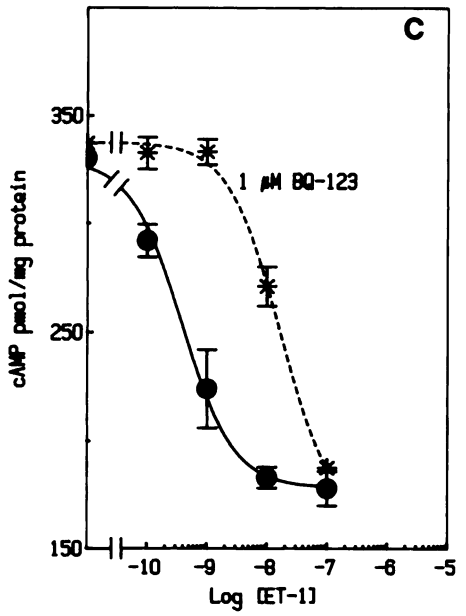
Although the interaction of ^{125}I-ET-1 with myocytes was specific and saturable (both in time and with respect to ligand concentration), the association was not readily reversible. Addition of 0.3 μM unlabeled ET-1 to equilibrium binding reactions prevented further binding of radiolabeled ligand but failed to lower the signal (Fig. 3). This irreversibility developed quickly, being apparent by 10 min of exposure to the ligand. It is possible that the rate of dissociation is too small to produce a noticeable decline, although our experiments would have detected a 10% decline in 100 min (k_{-1} = 0.10/100 min or 0.001/min). This aspect of binding is considered below as data on functional consequences of receptor occupancy are presented, as well as in the Discussion.

Competition binding studies (Fig. 4) produced data that were consistent with ^{125}I-ET-1 binding to a single population of high affinity receptors on the myocytes, with an apparent K_d of ~0.34 ± 0.07 nM and a B_{max} of ~4.4 ± 0.7 × 10^5 sites/cell (mean ± standard error, four or more experiments) (Fig. 4). Due to the irreversible nature of the binding, however, binding constants obtained from Fig. 4 cannot be considered reliable,

Fig. 1. Stimulation of PI hydrolysis by ETs and congeners. A, Concentration-dependent activation of PI hydrolysis by ET-1 (●), ET-2 (♦), ET-3 (□), S6b (△), and S6c (○). Cells were labeled with 12 μCi/ml *myo*-[^{3}H] inositol for 90 min at 32°. The cells were then washed and suspended in MEM with 10 mM LiCl, and aliquots (5 × 10^5 cells) were stimulated for 20 min with ET or congeners. Incubations were terminated by addition of 5% ice-cold TCA. Inositol phosphates (*IP*) were isolated as described in Experimental Procedures. *Points*, average ± range of duplicates from a representative experiment. B, Inhibition by BQ-123 of ET-1-stimulated PI hydrolysis. The experiment was conducted as described for A; BQ-123 and ET-1 were added simultaneously. C, Quantitation of antagonistic effect of BQ-123 on ET-1-stimulated PI hydrolysis. Experiments were conducted as described for A with ET-1 alone (●) or in the presence of 1 μm BQ-123 (*). The dose shift due to 1 μM BQ-123 would correspond to an apparent K_i of 9.6 ± 1.4 nM in a system displaying reversible ligand interactions. See Figs. 4 and 5, accompanying text, and Discussion concerning the apparent lack of reversibility in this system.







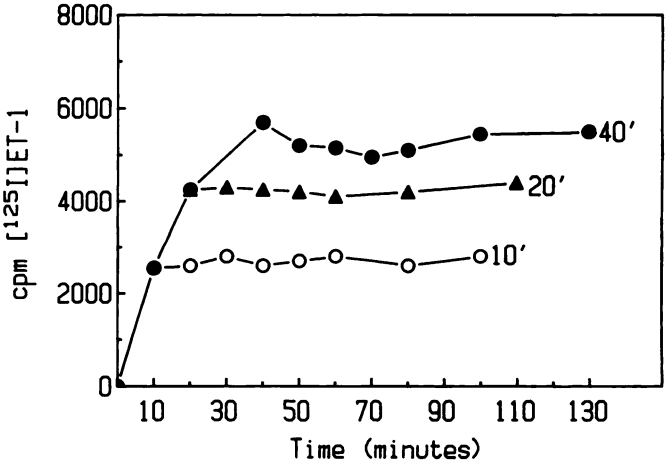


Fig. 3. Interaction of ^{125}-ET-1 with myocytes. Cells were incubated with ^{125}I-ET-1 (~15 pM), and at 10, 20, and 40 min excess unlabeled ET-1 (0.3 μM) was added. Cell suspensions were periodically sampled after addition of ET-1 and processed to assess ^{125}I-ET-1 binding. Free ligand and cell-associated ligand were separated by filtration, as described in Experimental Procedures. The data are mean of triplicate determinations within a single representative experiment.

because they were obtained by using analyses that assume reversible mass action equilibria. Comparable analyses in other studies of ET binding presumably share this defect, although it is rarely noted. Precise determination must await a detailed understanding of the kinetics of ET binding. What is certain and germane to the presence of ET receptor types on cardiac myocytes is the order of potency data; the ETs compete with ^{125}I-ET-1 binding with a rank order of potency of ET-1 ≅ ET-2 > S6b > ET-3. In typical experiments (e.g., Fig. 4), IC_{50} values were as follows: ET-1, 0.35 ± 0.07 nM; ET-2, 0.69 ± 0.13 nM; ET-3, 62.9 ± 8.6 nM; S6b, 9.4 ± 4.4 nM. This order of potency is characteristic of the ET_A receptor.

The relative potencies and efficacies of additional compounds confirm the exclusive presence of ET_A receptors on these cells. S6c, an ET_B-selective agonist, did not compete for ^{125}I-ET-1 binding sites on the myocytes (Fig. 4). When added to the myocytes simultaneously with the radioligand, the ET_A-selective antagonist BQ-123 competed with ^{125}I-ET-1 binding sites completely, with an IC_{50} of ~9 ± 3.1 nM (Fig. 4), similar to its potency in functional assays (Figs. 1C and 2C), and in the same rank order with respect to the three ETs.

To test whether our methods could detect ET_B receptors, we used the radiolabeled ET_B agonist ^{125}I-IRL-1620. We readily detected specific high affinity ^{125}I-IRL-1620 binding in small

Fig. 2. Inhibition of cAMP accumulation by ET and congeners. Myocytes were incubated with 0.5 mM isobutylmethylxanthine for 15 min and then stimulated with 1 μM isoproterenol in the presence or absence of ET or congeners for 4 min at 32°. For each *point*, duplicate samples were extracted and assayed for cAMP as described in Experimental Procedures. A, Effect of ET and congeners. ●, ET-1; ♦, ET-2; □, ET-3; △, S6b; ○, S6c. Data are mean ± standard error of five experiments. B, Antagonism by BQ-123 of ET-1 inhibition of cAMP accumulation. The concentration of ET-1 was 10 nM. Data are mean ± standard error of three experiments. C, Quantitation of antagonistic effect of BQ-123 on ET-1-inhibited cAMP production. Cells were exposed to isoproterenol (1 μM) and ET-1, in the absence (●) and presence (*) of BQ-123 (1 μm). All agents were added simultaneously. The dose shift due to 1 μM BQ-123 would correspond to an apparent K_i of 11.8 ± 1.1 nM (mean ± standard error of four experiments) in a system in which ligand binding was fully reversible. See note in the legend to Fig. 1C concerning the problem of apparent irreversibility in the interaction of radioligand and myocytes.

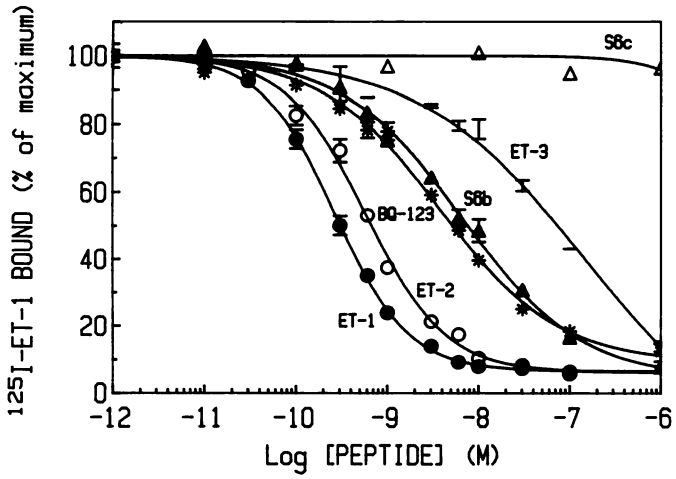


Fig. 4. Competitive binding of ^{125}I-ET-1 and congeners. Myocytes (50,000 cells/tube) were incubated at 32° for 40 min with ^{125}I-ET-1 (~15 pM) alone or in the presence of increasing concentrations of ET-1 (●), ET-2 (○), ET-3 (|), S6b (▲), S6c (△), or BQ-123 (*). Incubations were terminated by filtration, as described in Experimental Procedures. Data are mean of triplicate determinations within a single representative experiment. See text for discussion of the problem of deriving precise kinetic constants from these data because binding of radiolabeled ET-1 is not readily reversible.

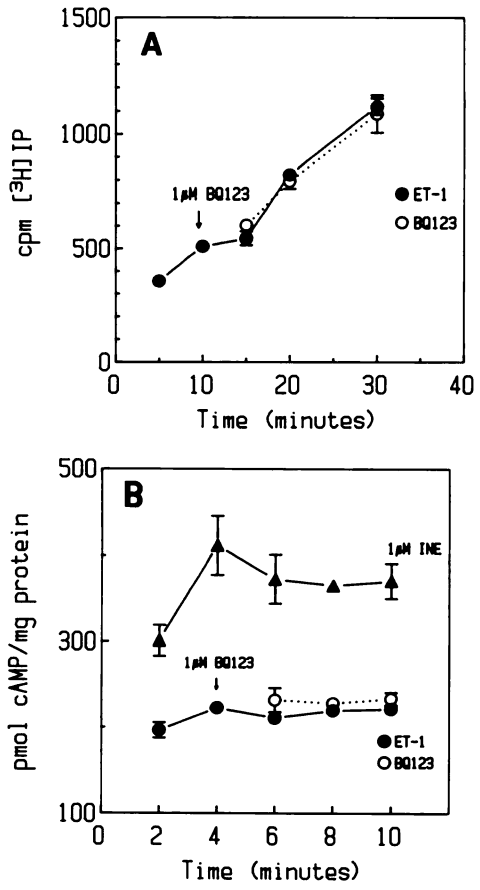


Fig. 5. Irreversibility of the functional effects of ET-1 on myocytes. After initiation of responses to ET-1 (1 nM), sufficient BQ-123 (1 μM) was added to fully antagonize the effects of ET-1 on PI turnover (Fig. 1) and cAMP production (Fig. 2). A, PI hydrolysis. BQ-123 was added 10 min after the addition of ET-1 and sampling continued for 20 min. The experiment was otherwise conducted as described for Fig. 1. Data are mean ± range of duplicate aliquots. ●, ET-1 alone; ○, ET-1 plus BQ-123. *IP*, inositol phosphates. B, cAMP accumulation. BQ-123 was added 4 min after the addition of ET-1 and isoproterenol, and sampling was continued for 6 min. The experiment was otherwise conducted as described for Fig. 2. Data are mean ± range of duplicate aliquots. ▲, Isoproterenol (*INE*) alone; ●, isoproterenol plus ET-1; ○, isoproterenol plus ET-1 plus BQ-123.

quantities of membranes from rat brain; incubation of ^{125}I-IRL-1620 (~20 pM) with rat brain membranes (~100 μg of protein) for 40 min at 32° resulted in 7000 cpm of specific binding, with which ET-1 competed with an EC_{50} of 0.2 nM. The ET_B receptor agonist S6c competed fully for the binding, whereas the ET_A receptor-specific antagonist BQ-123 (at 30 μM) was ineffective as a competitor for the sites interacting with IRL-1620. Thus, we could detect ET_B binding sites in brain tissue. Applying the same technique to cardiac myocytes (50,000 cells/tube, about 200 μg of protein), we were not able to detect a specific signal, that is, we were unable to demonstrate any specific binding to ET_B receptors on myocytes. Thus, all binding data are consistent with the presence of ET_A receptors, but not ET_B receptors, on ventricular myocytes.

Functional irreversibility. The most notable aspect of ET-1 binding to intact myocytes was the irreversibility of the interaction (Fig. 3). We tested whether this characteristic could be observed functionally by comparing time courses of ET-1 actions obtained with ET-1 alone or with the addition of the antagonist BQ-123 (added simultaneously with ET-1 or shortly after the addition of ET-1). As noted in Fig. 3, ET-1 binding is already irreversible at 10 min; thus, one would predict that the addition of BQ-123 at this time would have no effect on a response already in progress if the response results from the irreversible component of binding. Using ET-1 at 1 nM and BQ-123 at 1 μM (sufficient to fully inhibit the effects of 1 nM ET-1 and the binding of radiolabeled ligand) (see Figs. 1C, 2C, and 4), we observed the predicted responses; when BQ-123 was added 4–10 min after the agonist, the antagonist did not reverse either the stimulation of inositol phosphate production or the inhibition of cAMP accumulation, whereas when it was added simultaneously with ET-1, BQ-123 fully antagonized the effects of ET-1 (Fig. 5). We conclude that the measured irreversibility of ET-1 binding is not artifactual but is reflected in the interactions of ET-1 with ET_A receptor-regulated processes in isolated myocytes. Whether such irreversibility occurs in whole animals remains to be determined but would be of obvious interest in considerations of the physiological actions of ETs and the pharmacological alteration of those actions.

Discussion

ET has a variety of immediate and long term effects on the heart. ET is a potent positive inotropic agent, an arrhythmogenic agent with complex effects on ionic fluxes in various cell types in the heart, a secretagogue for atrial natriuretic peptide from myocytes, and a stimulant of myocyte hypertrophy. These responses result from occupancy of one or more of the several known ET receptor types, heptaspans belonging to the superfamily of G protein-linked receptors. Although both ET_A and ET_B receptor types are expressed in cardiac tissue (10–12, 24, 30), the cellular localization of these two types of ET receptor has not been clear. Ligand binding studies do not provide a uniform picture. Data from homogenates of chick heart (31) and bovine atrium (32) seem consistent with the presence of

two ET receptors; only the ET_A receptor is detectable in rat atrium (11) and only the ET_B receptor was detected in human Girardi heart cells (33). *In situ* ligand binding studies in human ventricle indicate that both ET receptor types are present, with about 62% as ET_A (24). Using Northern analysis, Nambi and colleagues (34) found that the distribution of mRNAs for ET receptors differs markedly among different tissues; the mRNA for the ET_A receptor is prevalent in heart (ET_A:ET_B = 9:2). Thus, despite the variability of the exact data, the general consensus is that both types of ET receptors are present in the heart and the ET_A receptor predominates.

Prior studies of cellular responses to ET have not made it clear whether a specific ET subtype generally couples to a particular response. For instance, ET has been reported to stimulate cAMP accumulation in bovine tracheal cells (35), whereas we find that ET acts via G_i to inhibit adenylate cyclase activity in rat cardiac myocytes. Aramori and Nakanishi (23) have proposed that such disparate effects are due to the predominance of one or another subtype, with ET_A and ET_B subtypes being linked to provide opposing regulation of adenylate cyclase. In support of this hypothesis, those authors presented data on the expression of ET_A and ET_B receptors in Chinese hamster ovary cells; activation of ET_A receptors caused enhanced cAMP accumulation, whereas activation of ET_B receptors reduced cAMP accumulation. Curiously, however, coupling of ET_A receptors to stimulation of adenylate cyclase was partially inhibited by pertussis toxin. Overexpression of receptor subtypes is these experiments may provide "nonphysiological" receptor coupling. In any event, our data on freshly isolated normal cells must be viewed against this varied background.

In our experiments on adult rat ventricular myocytes, we have assessed two biochemical responses of the cells to ET, i.e., the activation of PI hydrolysis and the inhibition of adenylate cyclase (via G_i). Although it was tempting to hypothesize that a separate ET receptor type might couple to each of these responses, the data in this report indicate otherwise. The orders of potency (ET-1 $\approx$ ET-2 > ET-3) for activation of the two biochemical responses and for radioligand binding are the same, corresponding to the selectivity expected of an ET_A receptor. The effectiveness of S6b, but not of S6c, and the inhibition by the cyclic pentapeptide BQ-123 also indicate that occupation of ET_A receptors seems to account for both stimulation of PI breakdown and inhibition of cAMP synthesis. The lack of detectable ET_B receptors is confirmed directly by the absence of a specific interaction between cardiac myocytes and ^{125}I-IRL-1620 (an ET_B-specific ligand that exhibits 120,000-fold selectivity for ET_B receptors over ET_A receptors) (18).

Our data demonstrate that the ET receptors detectable on adult ventricular myocytes are of the ET_A type, as opposed to the ET_B type. Our data also show that ET_A receptors can couple to two G protein-linked pathways, as do numerous other types of receptors, such as α-adrenergic and muscarinic cholinergic receptors (26, 36). However, our data do not necessarily indicate that a single ET_A receptor couples to the two different G protein-linked responses that we measured, although such coupling is possible. In the future, synthetic ligands may be devised that will compete for a fraction of the ET_A receptors on myocytes and antagonize a single biochemical response. In the instances of α-adrenergic and muscarinic cholinergic receptors, chemicals have been found that permit substantial subclassification (37–39), although the physiological pattern of the coupling of these receptors to G proteins is still a matter of disagreement and may vary among different tissues. By analogy, similar developments may permit eventual subclassification of ET_A receptors. Thus, our data do not rule out the possibility that subclasses of ET_A receptor may be found, corresponding to specific receptor-G protein interactions, such that a unitary linkage will be defined between receptor and effector. Currently available pharmacological probes do not provide that level of discrimination. These limitations notwithstanding, the ligands we used and the order of potency data we obtained indicate that ventricular myocytes have receptors and responses that are clearly ascribable to ET_A rather than ET_B receptors.

Data derived from molecular cloning do not rule out the possibility of multiple subtypes of ET_A receptors with different G protein interactions. Both ET_A and ET_B receptors have been cloned and show substantial differences (9–12). Even within the set of putative ET_A receptor clones the deduced amino acid sequences differ, especially in the amino-terminal regions and in the first extracellular loop, which has been recognized to play a role in ET-1 binding (10, 11, 40, 41). Such differences may represent variations among species; these differences might also reflect a multiplicity of forms that subsequent cloning studies will reveal. There are both cloning and binding data (11, 20) to suggest other subtypes not corresponding to ET_A and ET_B in terms of the expected order of potency of ET congeners and S6 forms. Thus, it seems premature to rule out the possibility of the existence of subclasses of ET_A receptors. Nonetheless, the pharmacological data we have presented indicate that ET_A receptors, but not ET_B receptors, are expressed in adult rat ventricular myocytes and interact with multiple G protein-linked pathways.

ET-1 binds to approximately 4.4×10^5 sites/myocyte. By comparison with densities of surface receptors on other cell types, typically 10^3 to 10^4/cell in cultured cells (about 1–42/μm^2 for β_1-adrenergic receptors) (see discussion of this point in Ref. 42), this number may seem high. However, considering the large size of a myocyte (an irregular cylinder, roughly 100 μm $\times$ 20 μm) (25), the density of ET-1 receptors is about 70/μm^2, comparable to the densities of α_1-adrenergic, muscarinic, and β_1-adrenergic receptors on rat ventricular myocytes (8×10^4 to 2.5×10^5 sites/cell or about 13–40 receptors/μm^2).

What is unusual about the association of ET-1 with cardiac myocytes is the rapid development of irreversibility (Fig. 3). Although others have noted this phenomenon in passing (43), it has not been taken into account in the analysis of ligand binding studies or in studies of the actions of ETs. Thus, if other systems share this aspect of ET binding, then published analyses of binding isotherms and dose-response data are in error. In our experiments, not only is the binding of ET-1 irreversible, but so are the activation of PI turnover and the inhibition of adenylate cyclase in response to ET-1 (Fig. 5). Thus, we believe that our data permit interpretation of irreversibility as a relevant physiological phenomenon, not merely as a test-tube contrivance. A possible binding scheme may be described as:

$$ET + ET_A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ET\text{-}ET_A \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} ET\text{-}ET_A^*$$

where ET is the ligand, ET_A is the receptor, the forward rate

constants k_{+1} and k_{+2} are large, and the reverse rate constants are small (k_{-1}) or essentially zero (k_{-2}). If ET-ET_A* is the form that interacts with G proteins, such a scheme would help explain the very slight effects of GTP on equilibrium binding and the failure of GTP to cause dissociation of bound ^{125}I-ET.[1] The irreversible association of ET with a putative ET_A receptor adds difficulty to the analysis of binding isotherms and concentration-dependence curves, because these analyses depend on equilibrium binding that is reversible. Although order of potency data may be reliable (our orders of potency are similar in assays varying in duration from 4 min to 40 min), the model described above could yield time-dependent changes in constants derived from competition experiments. We are currently designing experiments to determine these rate constants and to study the mechanism by which ET-1 interacts with cardiac cell membranes.

In preliminary experiments, we have found that simultaneous addition of dithiothreitol (10 mM) or β-mercaptoethanol (15 mM) with the ligand substantially inhibits binding of radiolabeled ligand to whole cells but that subsequent addition of these sulfhydryl reagents does not cause the dissociation of previously bound ^{125}I-ET-1 or the reversal of the effect of ET on cAMP accumulation. Thus, a role for the disulfide bonds of ET-1 in the interaction of ET-1 with the cell surface is possible but remains to be clarified. In other preliminary investigations, radioligand bound to whole cells at steady state (40 min) was largely recovered in a low-speed pellet of homogenates (plasma membrane fraction); the majority of ligand associated with whole cells was not removed by acid stripping. The finding that irreversible binding to myocyte membranes also occurs may simplify future studies.

This sort of irreversible association is not unknown, even for receptors linked to G proteins. A variety of eicosanoids that stimulate adenylyl cyclase interact with cultured cells and their membranes in a similar manner, with irreversibility developing relatively rapidly (44). The α-adrenergic receptor antagonist phenoxybenzamine interacts first reversibly and then irreversibly with α-adrenergic receptor proteins. The binding of lophotoxin, a cyclic diterpenoid isolated from coral, to nicotinic cholinergic receptors also proceeds irreversibly, leading to blockade of agonist-stimulated receptor function (45). Thus, irreversible association is a not uncommon feature of the interaction of ligands with membrane-bound receptors. In the case of a physiological agonist such as ET, it seems plausible that such binding could have relevance to the physiological response and might also precede endocytosis of the ligand.

We are currently designing and carrying out experiments to probe these matters further. In the context of the present report, however, we conclude that the apparent irreversible nature of ET binding is appropriate in terms of the observed signal transduction. If the interactions of ET-1 with the heart in whole animals are similarly irreversible, then ET would cause prolonged alteration of cardiac performance. The effects of ET differ in this respect from those of the neurotransmitters of the autonomic nervous system, the effects of which are rapid in onset and readily and rapidly reversed by dissociation, uptake, and metabolism of the neurotransmitter.

The positive inotropic effect of ET must also differ from the classical β-adrenergic paradigm involving increased cAMP. Smith and colleagues (3), in careful work on isolated rat myocytes, reported that ET produces a positive inotropic effect that is sensitive to pertussis toxin. Our current data indicate that an ET_A receptor mediates this effect. However, a positive inotropic effect seems unlikely to occur via the G_i-mediated, pertussis toxin-sensitive pathway that we have previously described, i.e., inhibition of adenylate cyclase and reduction of cellular cAMP (22). Consequently, we hypothesize that ET_A receptors of ventricular myocytes either couple to other positive contractile signals via G_i or couple to other G proteins that are substrates for pertussis toxin and thence to other critical regulators of contractility.

In summary, adult rat ventricular myocytes express one pharmacological class of surface receptor for ET. These receptors are of the ET_A class and couple to two processes believed to be G protein regulated, i.e., activation of PI turnover and inhibition of adenylate cyclase. Both the binding of ET and the subsequent regulation of these two signal-transducing pathways are essentially irreversible. Thus, it seems probable that the effects of ET on cardiac myocytes are long lasting, rather than of the short term, beat-to-beat type generally associated with other hormones that work via cyclic nucleotides and inositol phosphates, such as acetylcholine and catecholamines. With a population of ET_A receptors linked to two well characterized biochemical responses, and as normal cells in which problems of receptor overexpression and ensuing alterations in receptor-G protein subunit equilibria do not occur, isolated adult cardiac myocytes should prove a useful and physiologically important system for further studies of the actions of ET.

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