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BQ-123 IDENTIFIES HETEROGENEITY AND ALLOSTERIC INTERACTIONS AT THE RAT HEART ENDOTHELIN RECEPTOR

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Studies of the binding of endothelin-1 (ET-1) to its receptors in the rat heart, as well as kinetic measurements in the presence and absence of the specific antagonist BQ-123, appear to exclude a scheme of simple competitive inhibition and an interaction of ET-1 with a homogeneous population of receptors. Studies with BQ-123 established the presence of three subtypes of endothelin receptors and an allosteric interaction, suggesting the possible existence of a specific site for BQ-123 that interacts and/or interferes with the properties of endothelin-binding sites.

In an earlier study we employed cultures of primary myocytes prepared from the hearts of newborn rats to investigate the properties of receptors of the endothelin-sarafotoxin (ET/SRTX) family of peptides (1). Binding studies in intact cells revealed significantly higher affinities of ET-1 and SRTX-b than of ET-3 and SRTX-c towards these receptors (ET-R). Receptors exhibiting this rank order of affinities toward the peptides are termed ET_A-R, while those sharing similar affinities are termed ET_B-R. We have recently described a new subtype of endothelin receptor in brain and atrial preparations, with Kd values in the pM range, which is distinct from the ET_A-R in cardiomyocytes where Kd values are in the nM range (2).

Binding of [^{125}I]ET-1 in the presence of the antagonist BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]), a potent and selective antagonist of ET_A-R (3), suggested that newborn rat cardiomyocytes contain ET_A-R as a major component but also contain another subtype, yet to be identified, which is insensitive to BQ-123 (4). This possibility was investigated in the present study, in which binding studies were performed and phosphoinositide hydrolysis was carried out in the presence and absence of BQ-123, employing newborn rat cardiomyocytes as well as atrial preparations (homogenates and slices) from adult rats. The use of homogenates minimizes the possibility that radioligand will be trapped within the cells. Binding and kinetic studies were

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therefore carried out with homogenates, while for the induction of phosphoinositide hydrolysis atrial slices and/or cardiomyocytes were used.

Materials

ET-1 was purchased from American,Peptide Company (Santa Clara, CA). [^{125}I]ET-1 (2200 Ci/mmol) from Du Pont - New England Nuclear, [^{3}H]myo-inositol (18.8 Ci/mmol) from Amersham International (Buckinghamshire, England) and BQ-123 from Novabiochem (Switzerland).

Tissue preparations

Myocyte-enriched cultures (>95% myocytes) were prepared from the hearts of newborn (1- to 3-day-old) rats (CD strain) as described in detail previously (5). Atrial slices were excised from adult male rats and kept in buffer containing protease inhibitors (see below). When needed, excised atrial tissue was homogenized with a glass-teflon homogenizer (10 strokes). The homogenates were filtered through three layers of cheesecloth and the filtrate centrifuged twice at 30000 g for 20 min. The final pellet was resuspended in Tris buffer, pH 7.4, containing the protease inhibitors.

Binding experiments

For binding experiments, intact myocytes were incubated with 0.2 ml of modified Krebs Henseleit buffer (25 mM Tris-HCl, 118 mM NaCl, 4.67 mM KCl, 1.0 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11 mM glucose, pH 7.4), containing 5 units/ml aprotinin, 5 mg/ml pepstatin, 0.1 mM PMSF, 3 mM EDTA, 1 mM EGTA, and increasing concentrations of radiolabeled ligand in the absence and presence of 1 µM unlabeled peptide. Binding of [^{125}I]ET-1 to the receptor was measured at two concentration ranges. The first, designed to activate binding to sites with affinities in the pM range (90 min, 25°C), was between 5 and 500 pM [^{125}I]ET-1 at a specific activity of 2200 Ci/mmol. The second ranged between 0.5 and 10 nM (60 min, 25°C) at a specific activity of 22 Ci/mmol (100-fold isotopic dilution). Incubation was terminated by washing of each well three times with PBS. NaOH (1 N, 0.3 ml) was then added at room temperature and after about 60 min the cells were collected. Following the addition of 0.3 ml of 1 M Tris-HCl pH 7.4, [^{125}I]-counts were determined using a γ-counter (LKB).

A similar procedure was followed for binding to homogenates. Aliquots (50 µl) of homogenates were added to the reaction mixture containing the buffer, protease inhibitors and radiolabeled ligand (total volume 200 µl) as detailed above. Reactions were terminated by addition of 3 ml of ice-cold buffer and filtration through GF/C filters. The filters were washed twice and their radioactivity estimated using a γ-counter. All assays were performed in triplicate.

Dissociation of [^{125}I]ET-1 from the receptor (6)

Receptor/ligand complexes were formed by preincubation of membrane preparations for 1 h at 25°C with 5 nM [^{125}I]ET-1. The membranes were pelleted out by centrifugation (15,000 x g, 20 min), washed with ice-cold buffer and resuspended in the original volume of buffer (5 ml). Dissociation reactions were initiated by the addition of unlabeled peptide (final concentration 1 µM). Reactions were terminated either immediately upon addition of the unlabeled peptide (zero time) or at the indicated times.

Assays of phosphoinositide hydrolysis

The formation of [^{3}H]inositol phosphates (inositol phosphate, IP_1; inositol biphosphate, IP_2; inositol triphosphate, IP_3) in the atrial slices was assayed by the method of Berridge (7). Briefly, slices were prelabeled (1 h) with 2 µCi/ml of [^{3}H]inositol and washed three times with prewarmed to 37°C and oxygenated fresh buffer (123 mM NaCl, 5 mM KCl, 1.4 mM KH_2PO_4, 1.3 mM $MgSO_4$, 0.8 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4). The slices were then suspended in 200 µl of buffer, which was supplemented by 50 µl of the

same buffer containing 10 mM LiCl in the presence or absence of ET-1. The vials were oxygenated capped tightly, and incubated for 30 min at 37°C. Reactions were terminated by the addition of 1 ml chloroform-methanol (1:2). The water-soluble products of phosphoinositide hydrolysis were extracted and separated by ion-exchange chromatography. Samples of the chloroform phase were dried and counted in parallel with the fractions eluted from the columns.

Results and Discussion

As indicated above, binding of [^{125}I]-labeled ET-1 was measured at two concentration ranges, the one designed to achieve binding to the pM-sites and the other to the nM-sites. The specific saturable binding of radio-labeled ET-1 to cardiomyocyte homogenates exhibited a Kd of 50±5 pM and a maximal density of 205±20 fmol/mg protein. Binding of [^{125}I]ET-1 to the second site exhibited a Kd of 0.8±0.2 nM and a Bmax of 730±70 fmol/mg protein. In atrial homogenates the values were 70±7 pM (240±20 fmol/mg protein) and 1.2±0.1 nM (840±50 fmol/mg protein), respectively.

We then examined the effect of BQ-123 on the [^{125}I]ET-1 binding isotherm (Fig. 1). The resulting binding curves were characterized by a rightward shift relative to controls as well as by an apparent tendency in the pM sites to reach saturation at values lower than Bmax values. Unlike in

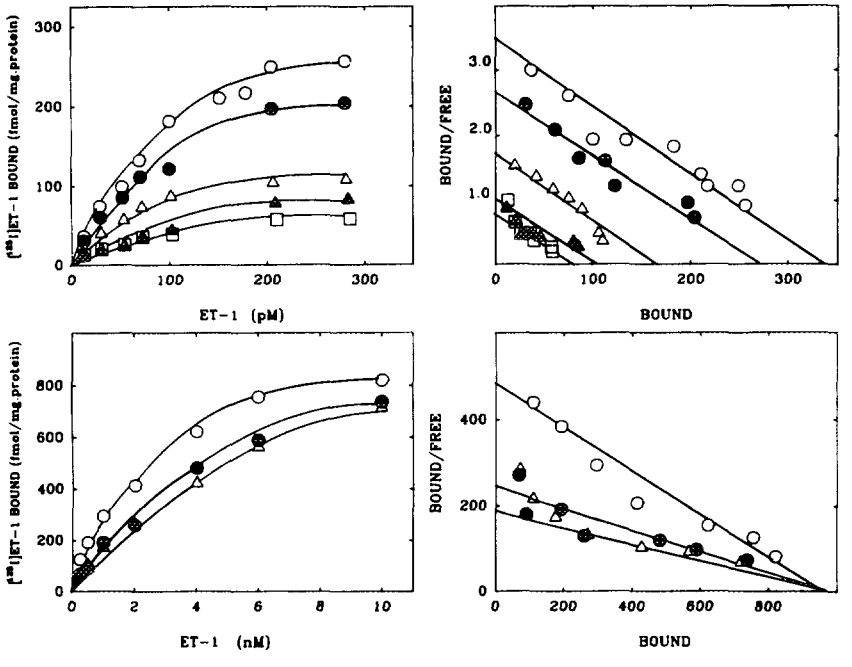

Figure 1.
Concentration-dependent inhibition of binding of [^{125}I]ET-1 by various concentrations of BQ-123. The upper panels represent binding isotherms and Scatchard plots of binding to the pM sites (●, 10 nM; △, 25 nM; ▲, 50 nM; □, 100 nM; o, control), while the corresponding lower panels represent binding to the nM sites (●, 50 nM; △, 100 nM; o, control).

the pM sites, in the nM sites Bmax values are attainable though at higher ligand concentrations. Scatchard plots of the binding isotherm show that BQ-123 reduces the Bmax values of the pM sites to 20-24% of the value of the control with no change in Kd (72±8 pM). In contrast, at the nM sites BQ-123 does not affect the Bmax values but decreases the affinity of ET-1 binding from Kd=1.2 nM to Kd=4.6±0.4 nM.

To determine whether the apparent reduction in Bmax of the pM sites BQ-123 treatment is reversible, membranes were exposed (for 60 min) to the highest concentration of BQ-123 and then washed three times with inhibitor-free buffer. Binding experiments yielded the same Bmax values for treated and untreated preparations, indicating that the apparent decrease in the Bmax value of the pM sites is reversible. Similar results were obtained with cardiomyocytes (not shown). Thus, BQ-123 appears to act as a competitive inhibitor at the nM sites and as a non-competitive inhibitor at the pM sites (Fig. 1). These observations are reminiscent of those of Vigne et al. (8), who showed that in brain capillary endothelial cells BQ-123 acts either as a competitive or as a non-competitive antagonist depending on the experimental conditions. Since BQ-123 is a specific inhibitor of ET_A-R, it would seem that the majority of pM sites are of the ET_A-R subtype. In addition to these two sites, there are sites which are not affected by BQ-123. In rat cardiomyocytes these BQ-123-insensitive sites represent 20-24% of the total (4) while in atrial preparations they represent 15-20%. It should be noted that incomplete inhibition of the pressor effects of ET-1 in anesthetized rats with BQ-123 also provided evidence for the existence of more than one vasoconstrictor receptor (i.e., in addition to ET_A-R) (9).

We have previously shown (1) that stimulation of atrial slices or of cultured rat heart myocytes with ET-1, ET-3, SRTX-b or SRTX-c induces a time- and dose-dependent hydrolysis of phosphoinositides, resulting in the accumulation of [^{3}H]-inositol phosphates (IP_1, IP_2, and IP_3). We now investigated the effect of 100 nM BQ-123 on ET-1 (1 µM)-induced phosphoinositide hydrolysis by measuring the generation of inositol phosphates during a period of 15 min at 37°C in the presence of 10 mM LiCl. Under these experimental conditions maximal effect of ET-1 is achieved. As shown in Figure 2, the presence of 100 nM BQ-123 induces a rightward shift of the dose-dependent curve, yielding an IC_{50} value of 70±5 nM as compared to the control value of 25±3 nM. The effect is completely reversed at ET concentrations higher than 5×10^{-7} M. Similar results were obtained with cardiomyocytes, with IC_{50} values of 10 nM and 40 nM in the absence and presence, respectively, of 100 nM BQ-123. Thus, both the binding and the IC_{50} values of phosphoinositide hydrolysis showed a decrease of 3- to 4-fold.

The decreased Bmax values of the pM sites (Fig. 1) are not reflected in the phosphoinositide hydrolysis (Fig. 2). This is to be expected, since the

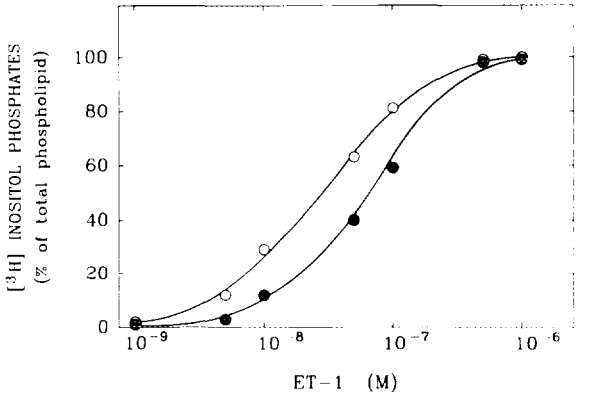

Figure 2.
Concentration-dependent induction of [^{3}H]-inositol phosphates accumulation by ET-1 in rat atrial slices in the presence (●) and absence (o) of 100 nM BQ-123. Data are presented as a percentage of the maximal attainable formation of [^{3}H]-inositol phosphates induced by 1 μM of ET-1. Assays were performed for 15 min. Values are means of three separate experiments. Standard deviation was 5-7% of the measured values.

pM sites do not activate phospholipase C (PLC) (2). Note the excellent agreement between the approximately 4-fold change in Kd values in the presence of BQ-123 and the change in IC_{50} of the phosphoinositide hydrolysis.

As indicated above, the decrese in Bmax values of the pM sites is reversible. This can most probably be explained in terms of a diminished affinity of the ligand to the receptor due to interaction of BQ-123 with a secondary site, and these low-affinity sites can no longer be detected.

Dissociation of bound [^{125}I]ET-1 from its preformed receptor-ligand complexes was initiated by unlabeled ET-1 (1 μM). As shown in Figure 3, the dissociation rate is very slow, with $t_{1/2} \simeq$ 45-60 min, and it is a multi-exponential process. For quantitative analysis of the curves, measurements should be extended over at least 3-4 hr. Our kinetic studies were conducted for not more than 90 min, after which both the atrial and the cardiomyocyte preparations became unstable as a result of extensive proteolysis (despite the presence of protease inhibitors). In cardiomyocyte preparations, desensitization processes would also complicate the system. Therefore only qualitative consideration will be given. As shown in Figure 3, about two-thirds of the sites of the control preparation dissociate more slowly than the rest. Since the experiments were performed in such a way (5 nM [^{125}I]ET-1, 60 min) that the two sites (pM and nM) are completely occupied, it would seem that the larger population of sites, i.e. the nM sites, representing 60-70% of the total, is the slower component.

When dissociation was initiated in the presence of 1 μM BQ-123 (Fig. 3, right panel) the rate of dissociation was decreased ($t_{1/2} >$ 90 min), possibly reflecting a change in the proportion of the slower and faster disso-

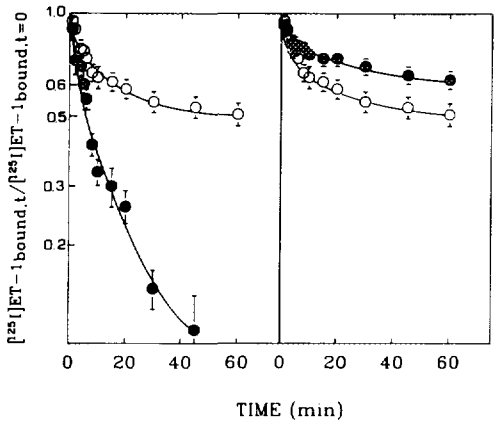

Figure 3.
First-order plots of dissociation of [^{125}I]ET-1 receptor complexes. Dissociation of the preformed complexes was initiated by the addition of 1 μM unlabeled ET-1. Left panel o - no pretreatment with BQ-123; ●, pretreatment with 1 μM BQ-123 for 30 min prior to the addition of unlabeled ET-1. Right panel, 1 μM ET-1 was added with (●) and without (o) BQ-123 (1 μM). $B_{t=o}$ is the amount of radioligand bound at zero time and B_t, the amount bound at time t. Data are the mean values from three experiments, in which individual data points were obtained in triplicate.

ciating complexes, in favor of the slower component. Such a situation could arise in the case of allosteric inhibition, i.e. where BQ-123 acts via a secondary site. Alternatively, it might result from a two-step isomerization pattern (10) leading to an increase in the proportion of the more slowly dissociating complexes. The possibility that ETs induce an isomerization process in atrial preparation seems to be les likely since in experiments in which ligand receptor complexes were allowed to be formed over shorter (5 min, 10 nM ligand) and longer (60 min) time periods, the dissociation pattern was the same. If an isomerization pattern exists, different rates of dissociation would be expected (10). However, induction of an isomerization-like process by BQ-123 rather than by ETs represents an attractive possibility that should be further investigated. On the other hand, measurement of dissociation rates from the pM sites, where complexes were preformed with 30 pM [^{125}I]ET-1, yielded similar curves and $t_{1/2}$ values with and without BQ-123.

As pointed out above, there is a population of sites which are BQ-123-insensitive (4). The kinetic properties of this type of receptor can therefore be investigated via the formation and dissociation of ligand-receptor complexes in the presence of BQ-123. Accordingly, membranes were incubated with 5 nM [^{125}I]ET-1 for 1 hr, then pretreated with 1 μM BQ-123 for 30 min. After washing of the membranes (see Methods), dissociation was initiated by the addition of 1 μM ET-1. As shown in Figure 3 (left panel), dissociation of the ligand-receptor complexes was accelerated ($t_{1/2} \simeq 8$ min). Note, the

density of ligand-receptor complexes in this type of experiment represents about 40% of the control, (340 fmol/mg protein vs. 870 fmol/mg protein of the control). Thus, in this type of experiment, the more rapidly dissociating component(s) represents only the fraction which is BQ-123 resistant. The effect induced by the antagonist can best be explained if one assumes an allosteric interaction.

In conclusion, the results presented here clearly demonstrate that the BQ-123-induced inhibition of ET-1 binding to rat heart preparation does not conform to a scheme of simple competitive inhibition or to interaction of the ligand with a homogeneous population population of ET-R. Thus, BQ-123 represents a valuable tool for identification of heterogeneity in rat heart ET-R. Three subtypes were characterized in these preparations. BQ-123 confirmed an allosteric interaction, and indicated the possible existence of a specific site that interacts with and/or influences the binding properties of ET sites.

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