British Journal of Pharmacology (1997) 121, 253–263

Comparison of the contractile effects and binding kinetics of endothelin-1 and sarafotoxin S6b in rat isolated renal artery

Peter S.S. Devadason & [1]Peter J. Henry

Department of Pharmacology, University of Western Australia, Nedlands, Australia 6907

1 To date, only two mammalian endothelin (ET) receptors, termed ET_A and ET_B, have been cloned, sequenced and characterized. However, several functional studies of isolated blood vessels suggest that ET-1-induced contractions may be mediated by multiple ET_A receptors. In this study, the ET_A receptors in renal arteries isolated from Wistar rats were characterized by isometric tension recording and radioligand binding techniques.

2 ET-1, sarafotoxin S6b (StxS6b) and ET-3 produced concentration-dependent contraction with similar response maxima in endothelium-denuded arteries, whereas the ET_B receptor-selective agonist StxS6c was inactive. ET-1 and StxS6b were equipotent and 30 times more potent than ET-3. This agonist profile, together with the findings that the ET_A receptor-selective antagonists, BQ-123 and FR-139317 caused concentration-dependent, rightward shifts of the concentration-effect curves to each agonist indicated that ET-1-induced contractions in rat renal artery were mediated via ET_A receptors.

3 BQ-123 and FR-139317 were both significantly more potent inhibitors of contractions induced by StxS6b or ET-3 than of responses to ET-1, raising the possibility that a component of ET-1-induced contraction was mediated through atypical, BQ-123 (or FR-139317)-insensitive ET_A receptors. However, in competition binding studies, specific [^{125}I]-ET-1 and [^{125}I]-StxS6b binding to rat renal artery sections was completely abolished by BQ-123 in a manner consistent with an action at a single site. Thus, competition binding studies did not provide any supportive evidence of the existence of a BQ-123-insensitive ET_A receptor.

4 Additional studies revealed marked differences in the kinetics of [^{125}I]-ET-1 and [^{125}I]-StxS6b binding. Following a 3 h period of association of [^{125}I]-ET-1 with its receptors, no significant dissociation of receptor-bound [^{125}I]-ET-1 was observed during a 4 h washout period. In stark contrast, dissociation studies revealed that specific [^{125}I]-StxS6b binding to ET_A receptors was reversible ($t_{0.5diss}$, 100 min). A series of association binding studies were also consistent with the specific binding of [^{125}I]-ET-1 and [^{125}I]-StxS6b being irreversible and reversible processes, respectively.

5 Thus, differences in BQ-123 potency against ET-1 and StxS6b-induced contractions in rat renal arteries might be due to differences in the kinetics of agonist binding, rather than due to the existence of atypical ET_A receptors.

Keywords: Endothelin-1; endothelin receptors; sarafotoxin S6b; smooth muscle contraction; radioligand binding; BQ-123; FR-139317; renal artery; kinetics

Introduction

In mammalian cells and tissues, endothelin-1 (ET-1) exerts its actions via specific endothelin receptors of which two subtypes, termed ET_A and ET_B receptors, have been cloned, sequenced and characterized. The prominent vasoconstrictor actions of ET-1 are generally mediated via the activation of the ET_A receptor subtype, the involvement of which is typically based on several lines of evidence; the potency order of the endothelin isopeptides (ET-1 = ET-2 > ET-3), the inhibition of ET-1-induced responses by ET_A receptor-selective antagonists such as BQ-123 (Ihara *et al*., 1991) and FR-139317 (Sogabe *et al*., 1993), and the failure of ET_B receptor-selective agonists to elicit a response.

Although the development of subtype-selective endothelin receptor agonists and antagonists has greatly aided the process of endothelin receptor characterization, a number of studies in a range of tissues, have found significant differences in the potency of endothelin receptor antagonists against various agonists. In preparations containing ET_A receptors, for example, BQ-123 is frequently more potent in inhibiting contractions induced by sarafotoxin S6b (StxS6b) and ET-3, than similar responses to ET-1. Such agonist-dependent antagonist potency has been observed in rat aorta (Sumner *et al*., 1992), goat cerebral artery (Salom *et al*., 1993), human saphenous vein (Bax *et al*., 1993), human coronary artery (Godfraind, 1993), human umbilical artery (Bodelsson & Sjernquist, 1993) and human omental vein (Riezebos *et al*., 1994), as well as in rat vas deferens (Eglezos *et al*., 1993). These findings have prompted speculation concerning the possible existence of a population of BQ-123-insensitive ET_A receptors (see Bax & Saxena, 1994 for review). However, the use of functional data alone to characterize endothelin receptors is associated with several potential problems, including the presence of multiple/interacting signal transduction systems (see Sokolovsky, 1995), or the rapid desensitization/down-regulation (Clozel *et al*., 1993) of receptors which might result in an altered response to various endothelin peptides (Le Monnier de Gouville *et al*., 1990). Furthermore, the formation of ligand-receptor complexes with different dissociative behaviours depending on the ligand used, may result in complicated receptor kinetics (Galron *et al*., 1991; Vigne *et al*., 1993). It also should be noted that endothelin peptides can be internalized after binding to the receptor (Resink *et al*., 1990) and hence may not be available for further receptor interaction. Despite the intense speculation that surrounds the existence of putative atypical ET_A receptors (Bax & Saxena, 1994), there has been little supporting evidence for these receptor subtypes from molecular biology or radioligand binding studies.

Recently, Clark & Pierre (1995) showed that ET-1-induced contractions of renal arteries from Sprague-Dawley rats were not mediated by ET_B receptors, yet were only poorly inhibited

[1] Author for correspondence.

by an ET_A receptor-selective antagonist BQ-123. In contrast, BQ-123 antagonized ET-3 with a potency consistent with its affinity for typical ET_A receptors. These workers concluded that ET-1-induced contractions were mediated via a population of BQ-123-insensitive ET_A receptors. However, Seo & Lüscher (1995) showed the presence of typical, FR-139317-sensitive ET_A receptors in renal artery preparations from Wistar rats. The findings of the two studies might be reconciled if renal arteries from Sprague-Dawley rats contain a mixture of both typical and atypical ET_A receptors, whereas Wistar rats contain only typical ET_A receptors.

The aim of the current study was to characterize pharmacologically the ET_A receptors in the renal artery of the Wistar rat by use of both isometric tension recording and radioligand binding techniques. In contraction studies, the potencies of BQ-123 and FR-139317 were determined against contractions induced by ET-1, StxS6b and ET-3 to investigate whether antagonist potency was agonist-dependent. Radioligand binding experiments were also performed to establish whether BQ-123-insensitive ET_A receptors could be identified and to investigate the kinetics of specific [^{125}I]-ET-1 and [^{125}I]-StxS6b binding to ET_A receptors in rat isolated renal artery.

Methods

Functional studies

Preparation of isolated renal artery Male Wistar rats of 7 to 8 weeks of age (Animal Resources Centre, Perth, Australia) were killed either with a lethal dose of sodium pentobarbitone (Nembutal; 150–200 mg kg^{-1}) or by a sharp blow to the head. The kidneys were removed together with the renal arteries and abdominal aorta and immediately immersed in cold Krebs bicarbonate solution (KBS). Both main renal arteries were cleaned of surrounding connective tissue under a dissection microscope and cut into rings approximately 2 mm in length. A toal of 4 renal artery rings were obtained from each rat. Rings were denuded of endothelium by gentle rubbing with a metal wire, placed in 3 ml organ baths containing KBS at 37°C and bubbled with 5% CO_2 in O_2. Rings were suspended under 0.5 g resting tension and changes in isometric tension were recorded via FT03 force displacement transducers (Grass Instruments), linked to custom built preamplifiers and a computer-controlled data acquisition programme. Rings were allowed to equilibrate for 40 min with regular washings. During the equilibration period it was usual for preparations to lose tone spontaneously and this was offset by readjusting the resting tension back to 0.5 g. KCl (60 mM) was then added to test the contractile viability of the preparations. Upon reaching a plateau contractile response, the tissues were washed, allowed to relax and the tension was readjusted to 0.5 g. Approximately 20 min later, a second 60 mM dose of KCl was added and it was this contractile response to which all subsequent agonist-induced contractions in that preparation were compared (100% KCl response). Preparations were washed and rested for a further 15 min before the addition of an antagonist (BQ-123 or FR-139317) or its vehicle. The peptidase inhibitor phosphoramidon (1 μM) was also added at this time. Thirty min later, a concentration-response curve to a selected agonist (ET-1, StxS6b or ET-3) was constructed cumulatively at 0.5 log concentration increments in each of the four arterial rings. For each set of 4 rings (from a single rat), one preparation was always used as a control (no antagonist) and the remaining three were exposed to different concentrations of antagonist. Only one concentration-effect curve was completed in each ring preparation and only one agonist was used in each set of rings.

Control studies: effects of ACh, StxS6c and sodium nitroprusside After the two doses of KCl were added, some endothelium-denuded preparations were washed and rested for 45 min and then contracted with 0.03 μM U-46619. At contraction plateau, preparations were exposed to 1 μM ACh, 0.3 μM StxS6c or 0.1 μM sodium nitroprusside and the response measured.

Radioligand binding experiments

Left and right main renal arteries were isolated as described above and were submerged in Macrodex (6% dextran 70 in 5% glucose) and frozen into small blocks by immersion in isopentane, quenched with liquid nitrogen. Each block contained 12 renal arteries from 6 rats. Transverse sections of 10 μm thickness were cut at −23°C, and thaw-mounted onto gelatine chrom-alum-coated glass microscope slides. Four sections were mounted onto each slide (i.e. 48 renal artery sections per slide) and approximately 40–50 slides obtained from each tissue block. Mounted sections were given 2 × 20 min prewashes in incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 0.25% bovine serum albumin, pH 7.4) containing the protease inhibitor phenylmethylsulphonyl fluoride (10 μM) and phosphoramidon (1 μM). Mounted tissue sections were then incubated at 22°C with [^{125}I]-ET-1 or [^{125}I]-StxS6b as indicated below. After incubation with radioligand, mounted tissue sections were washed twice for 15 min in incubation buffer. Sections were then wiped from slides with glass fibre filter paper (Whatman, GF/A) and the radioactivity counted in a Packard gamma counter (Model 5650). Tissue-associated radioactivity was expressed as amol of radioligand per slide (amol per slide).

Association binding studies Slide-mounted tissue sections were prepared as described above. After the prewashes, mounted tissue sections were incubated with [^{125}I]-ET-1 (0.08, 0.32 or 0.73 nM) or [^{125}I]-StxS6b (0.32 or 0.73 nM) for 10, 20, 40, 80, 160 or 240 min. All procedures after the incubation period were as described above.

Dissociation binding studies Slide-mounted tissue sections were incubated with 0.32 nM [^{125}I]-ET-1 or [^{125}I]-StxS6b for 3 h, washed and then incubated in buffer containing 0.1 μM unlabelled ET-1 to prevent rebinding of radiolabel during this dissociation phase. At hourly intervals for a further 4 h, samples of slides were washed, wiped and measured for radioactivity.

Competition binding studies Slide-mounted tissue sections were incubated with 0.32 nM of radioligand ([^{125}I]-ET-1 or [^{125}I]-StxS6b) for 3 h, in the absence or presence of ET-1 (1 pM–3 μM), BQ-123 (10 pM–3 μM) or StxS6b (3 pM–1 μM). After the incubation period, sections were processed as described above. Specific binding recorded in tissue sections in the absence of competing ligand was taken as 100% binding.

Determination of non-specific binding In association, competition and dissociation experiments with [^{125}I]-ET-1, non-specific binding was determined in the combined presence of BQ-123 (ET_A receptor selective ligand; 1 μM) and StxS6c (ET_B receptor selective ligand; 100 nM) (Goldie *et al*., 1994), and separately with unlabelled ET-1 (10 nM). The levels of non-specific [^{125}I]-ET-1 binding determined with these two methods were not significantly different in any experiment. In experiments with [^{125}I]-StxS6b, non-specific binding was determined either by including a combination of BQ-123 and StxS6c (as above), unlabelled ET-1 (10 nM), or unlabelled StxS6b (300 nM). The levels of non-specific [^{125}I]-StxS6b binding obtained with these three methods were not significantly different.

Data and statistical analyses

Functional studies Contractions induced by ET-1, StxS6b and ET-3 were standardized to a reference level of contraction induced by 60 mM KCl in each preparation. The concentration of agonist that produced 40% KCl response (EC_{40}) was estimated from linear interpolation of each

concentration-effect curve. The 40% level of response was used in preference to higher levels to maximize the number of antagonist concentrations for which dose-ratio values could be calculated. Dose-ratios were calculated as the EC_{40} (in presence of antagonist)/EC_{40} (in absence of antagonist) for each set of ring preparations. Mean log (dose-ratio − 1) values were plotted against the log [antagonist] and two way analysis of variance (SigmaStat) used to compare the log (dose-ratio − 1) values obtained with the two agonists, ET-1 and StxS6b. Since none of the concentrations of antagonists used against ET-3 were common to those used against ET-1 and StxS6b, the log (dose-ratio − 1) values obtained using ET-3 and ET-1 could not be compared by two-way analysis of variance. Rather, the method chosen to compare ET-3 with ET-1 and StxS6b involved a comparison of regressions, with residual sum of squares (Mead *et al.*, 1993). Mean log (EC_{40}) values were estimated and used as a measure of agonist potency. Arithmetic means are expresed as mean ± s.e.mean, whereas geometric means are presented as mean (95% confidence limits) for *n* preparations. *P* values of less than 0.05 were considered statistically significant.

Radioligand binding studies In all experiments, mean total and non-specific binding were determined from triplicate measurements and specific binding was calculated from the difference between total and non-specific binding. As indicated by McPherson (1985), for a simple model of reversible bimolecular association, the amount of ligand associated to a saturable binding site at time t (RL_t) is dependent on the pseudo-first order association rate constant (k_{OBS}) and bound receptor concentration at equilibrium (RL_E), according to;

$$RL_t = RL_E\ (1 - e^{-k_{OBS}.t}) \tag{1}$$

where RL_E and k_{OBS} are further defined by equations (2) and (3).

$$RL_E = \frac{B_{max}}{1 + (k_{-1}/k_1.L)} \tag{2}$$

$$k_{OBS} = k_1.L + k_{-1} \tag{3}$$

where, L is the concentration of free ligand, B_{max} is the total receptor concentration and k_1 and k_{-1} are the rate constants of association and dissociation, respectively, Thus, by substituting equations (2) and (3) into equation (1) (Karlsson & Neil, 1988);

$$RL_t = \frac{B_{max}}{1 + (k_{-1}/k_1.L)}.(1 - e^{-(k_1.L + k_{-1}).t}) \tag{4}$$

Reversible binding; estimation of K_D *and* B_{max} *from equilibrium binding studies* For reversible interactions, the equilibrium dissociation constant (K_D) of the ligand for the receptor is;

$$K_D = \frac{k_{-1}}{k_1} = \frac{R.L}{RL_E} \tag{5}$$

The free receptor concentration R is the difference between B_{max} and RL_E and thus equation (2) becomes;

$$K_D = \frac{(B_{max} - RL_E).L}{RL_E} \tag{6}$$

The Scatchard plot of RL_E/L against RL_E provided estimates of B_{max} (intercept on the abcissa scale) and K_D (negative reciprocal of the slope).

Reversible binding; estimation of K_D *from kinetic studies* Association binding curves obtained for reversibly binding ligands were fitted to equation (1) and estimates for k_{OBS} and RL_E obtained from nonlinear least squares regression analysis (SigmaStat). In dissociation experiments, free radiolabel was removed (i.e. L = 0) and equations 1 and 3 reduce to;

$$RL_t = RL_E(1 - e^{-(k_{-1}).t}). \tag{7}$$

Rearranging,

$$\ln(\frac{RL_E - RL_t}{RL_E}) = -(k_{-1}).t \tag{8}$$

where $RL_E - RL_t$ is the concentration of dissociated ligand. Thus, the slope of the linear plot $\ln(\frac{RL_E - RL_t}{RL_E})$ against t provided an estimate of k_{-1}.

The estimates of k_{OBS} and k_{-1} obtained from association and dissociation studies, respectively, were used to calculate k_1 (from equation 3) and K_D (from equation 5). The half-life of dissociation ($t_{0.5diss}$) was calculated from;

$$t_{0.5diss} = \frac{\ln(2)}{k_{-1}} \tag{9}$$

Pseudo-irreversible binding: estimation of k_1 *and* B_{max} If a ligand binds irreversibly to its receptor, then little dissociation of the receptor-ligand complex occurs over time and $k_1 \gg k_{-1}$. Consequently, equation (4) reduces to;

$$RL_t = B_{max}.(1 - e^{-k_1.L.t}) \tag{10}$$

Thus, association binding curves obtained for irreversibly binding ligands were fitted to equation (10) and estimates of B_{max} and k_1 obtained from nonlinear least squares regression analysis.

Competition binding curves Competition binding data were fitted to a one-site model based on the logistic equation;

$$\%\ \text{specific binding} = \frac{100}{1 + (x/IC_{50})^n}$$

where x is the concentration of competing ligand, IC_{50} is the concentration of competing ligand at which 50% of the specific binding was inhibited, and n is the slope of the line tangent to IC_{50}. The equation of the two-part model was;

$$\%\ \text{specific binding} = p\frac{100}{1 + (x/IC_{50H})^n} + (1 - p)\frac{100}{1 + (x/IC_{50L})^m}$$

where p is the fraction of the binding attributed to the higher affinity site and (1 − p) is the fraction of the binding attributed to the lower affinity site, IC_{50H} and IC_{50L} are the IC_{50} values estimated for the high and low affinity sites, and n and m are the slopes of these portions of the curves. The *F* ratio test was used to determine whether the competition binding data was significantly better described by the two-part model than the one-part model.

Drugs

Drugs used were; [^{125}I]-ET-1 (2000 Ci mmol^{-1}), [^{125}I]-StxS6b (2000 Ci mmol^{-1}), ET-1, StxS6b, BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]), StxS6c (Auspep, Melbourne, Australia), phosphoramidon, ACh, bovine serum albumin (Sigma Chemical Company, St. Louis, U.S.A.), phenylmethylsulphonyl fluoride (Calbiochem, La Jolla, U.S.A.), and U-46619 (Cayman Chemical Company, Ann Arbour, U.S.A.). FR-139317 ((**R**)2-[(**R**)-2-[(**S**)-2-[1-(hexahydro-1H-azepinyl)]carbonyl]amino - 4 - methylpentanoyl]amino -3 -[3 - (1 -methyl-1H-indoyl)]proprionyl]amino-3-(2-pyridyl)proprionic acid) was a generous gift from Fujisawa Pharmaceutical Company (Osaka, Japan). The composition of Krebs bicarbonate solution (KBS) was (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgS0_4.7H_2O$ 0.57, $CaCl_2$ 2.5 and glucose 11.1.

In the functional experiments, 50 μM stock solutions of ET-1, ET-3, StxS6b and StxS6c were prepared in 0.1 M acetic acid

and stored at −20°C. Dilutions were made in 0.9% NaCl solution daily. ACh was prepared fresh as required by dissolving in saline to obtain a stock solution of 1 mM. In the radioligand experiments, the drugs were dissolved and diluted in incubation buffer, as required. Drugs were kept on ice and protected from light.

Results

Functional experiments

Agonist profile As shown in Figures 1 and 2, the cumulative addition of ET-1, StxS6b and ET-3 produced concentration-dependent contractions of endothelium-denuded rat isolated renal artery preparations. When expressed as a percentage of the response to 60 mM KCl, the maximal response to ET-1 was 177±10% ($n=12$), and the concentration of ET-1 that produced 40% of the KCl-induced contraction (EC_{40}) was 1.3 nM (95% confidence limits, 0.9–1.9 nM). ET-1 was equipotent with StxS6b (EC_{40}; 1.5 nM (0.9–2.8 nM), $n=6$) and 30 fold more potent than ET-3 (EC_{40}; 37 nM (26–54 nM), $n=6$, $P<0.05$). At the highest concentration of agonist used (0.3 μM), ET-1, StxS6b and ET-3 evoked a similar contractile response. In contrast, the ET_B receptor-selective agonist StxS6c failed to induce any contractile response under conditions of basal or U46619-induced tone ($n=4$, data not shown).

In order to eliminate any possible modulatory influence that activation of endothelium-dependent ET_B receptors might have exerted on the contractile responses to ET-1, StxS6b and ET-3, all preparations used in the current study were gently rubbed to denude the vessel lumen of endothelial cells. This protocol appeared to remove the endothelium successfully without significantly damaging the underlying smooth muscle since U46619-contracted rings relaxed in response to an endothelium-independent vasodilator, sodium nitroprusside (100% relaxation, $n=4$) but were unresponsive to StxS6c and to the endothelium-dependent vasodilator ACh (0–2% relaxation, $n=4–6$).

Antagonist studies with BQ-123 The ET_A receptor-selective antagonist BQ-123 (0.1, 0.5 and 3 μM) caused concentration-dependent rightward shifts of the concentration-effect curves to ET-1 (Figure 1a) and StxS6b (Figure 1b), with no significant reduction in the maximal effect. However, as shown in Figure 1d, the Schild plot for BQ-123 against ET-1 was significantly to the right of that against StxS6b, indicating that BQ-123 was significantly more potent as an inhibitor of contractions induced by StxS6b than those induced by ET-1. ET-3 (1 nM–0.3 μM) failed to elicit any contractile response in the presence of 0.1, 0.5 or 3 μM BQ-123 (data not shown). This lack of contractile response to ET-3 was most likely due to the combined influences of the relatively low potency of ET-3, the limited availability of ET-3 (concentrations of ET-3 > 0.3 μM were not used) and the high potency of BQ-123 against ET-3-induced contractions. At the lower concentrations of 0.01 and 0.03 μM, BQ-123 shifted the ET-3 concentration-effect curve by 0.33±0.09 and 0.71±0.07 log concentration units, respectively (Figure 1c). The Schild plot for BQ-123 against ET-1 was also significantly to the right of that against ET-3, indicating that BQ-123 was significantly more potent as an inhibitor of contractions induced by ET-3 and StxS6b than those induced by ET-1 (Figure 1d).

Antagonist studies with FR-139317 A similar profile of inhibitory effects was seen with FR-139317 (Figure 2) as that described above for BQ-123. FR-139317 caused concentration-dependent rightward shifts of the concentration-effect curves to ET-1, StxS6b and ET-3 (Figures 2a–c), but was significantly less potent as an inhibitor of contractions induced by ET-1 (Figure 2d).

Radioligand binding experiments with [^{125}I]-ET-1

Association binding studies The rate of association of [^{125}I]-ET-1 to binding sites in rat isolated renal artery sections was strictly concentration-dependent (Figure 3a–d). At the lowest concentration used (0.08 nM), specific [^{125}I]-ET-1 binding in-

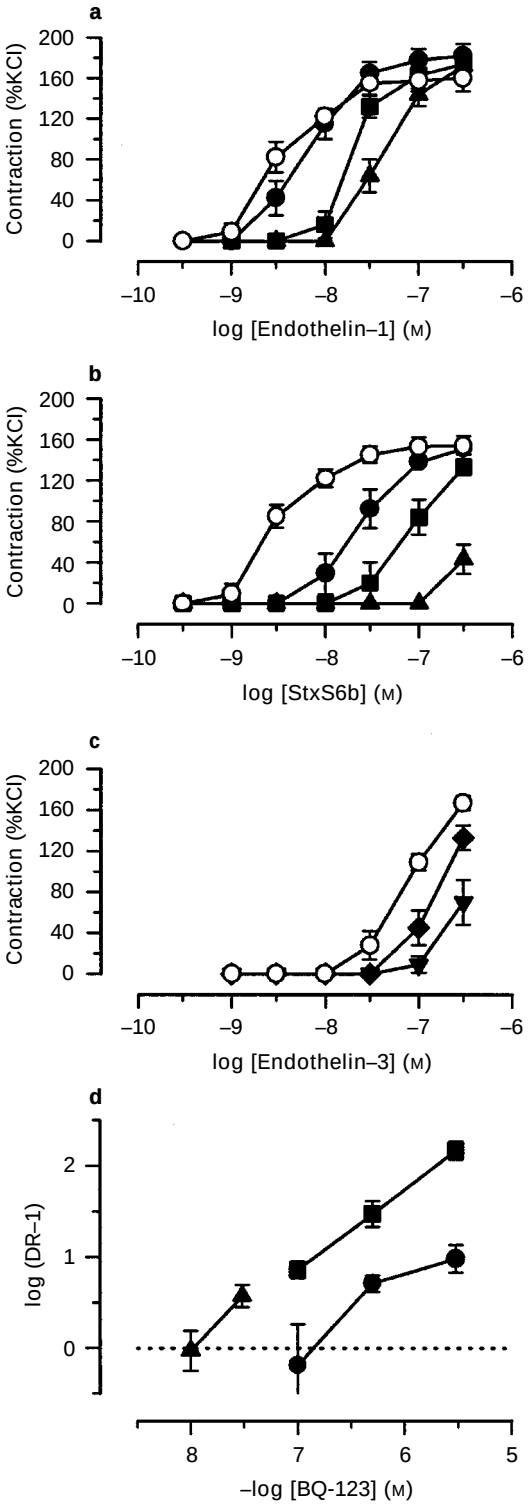

Figure 1 Concentration-effect curves to (a) endothelin-1, (b) StxS6b and (c) endothelin-3 in the absence (○) and in the presence of 0.01 μM (◆), 0.03 μM (▼), 0.1 μM (●), 0.5 μM (■) and 3 μM (▲) BQ-123. (d) A Schild plot analysis of the data presented in (a)–(c), illustrating that BQ-123 was a more potent inhibitor of contractions induced by StxS6b (■) and ET-3 (▲) than those induced by ET-1 (●). Data are expressed as the arithmetic mean±s.e.mean (vertical lines) of 5–6 values.

creased in a time-dependent manner, but a plateau level of binding was not observed within 4 h (Figure 3a). However, when a higher concentration of 0.32 nM [^{125}I]-ET-1 was used, a plateau level of specific binding was achieved but only after 3 to 4 h (Figure 3b). At the highest concentration of [^{125}I]-ET-1 used (0.73 nM), a plateau level of specific binding was observed within 1 h (Figure 3c). Despite these differences in the rates at which plateau levels of binding were achieved, the plateau level of binding obtained at 4 h with 0.73 nM [^{125}I]-ET-1 (432 ± 72 amol per slide) was not significantly different from the level obtained with 0.32 nM [^{125}I]-ET-1 (517 ± 43 amol per slide). Subsequent experiments (see below) established that the specific binding of [^{125}I]-ET-1 to its receptors involved an irreversible process and thus association binding data were fitted to equation (10), which describes the first order binding of an irreversible ligand and estimates of B_{max} and k_1 were obtained (Table 1). Mean estimates of B_{max} (475 amol per slide) and k_1 (5.2×10^9 M^{-1} min^{-1}) were then applied to equation (10) to generate a series of association binding curves for selected concentrations of [^{125}I]-ET-1, which graphically illustrate the influence of ligand concentration on the observed rate of ligand binding (Figure 3d).

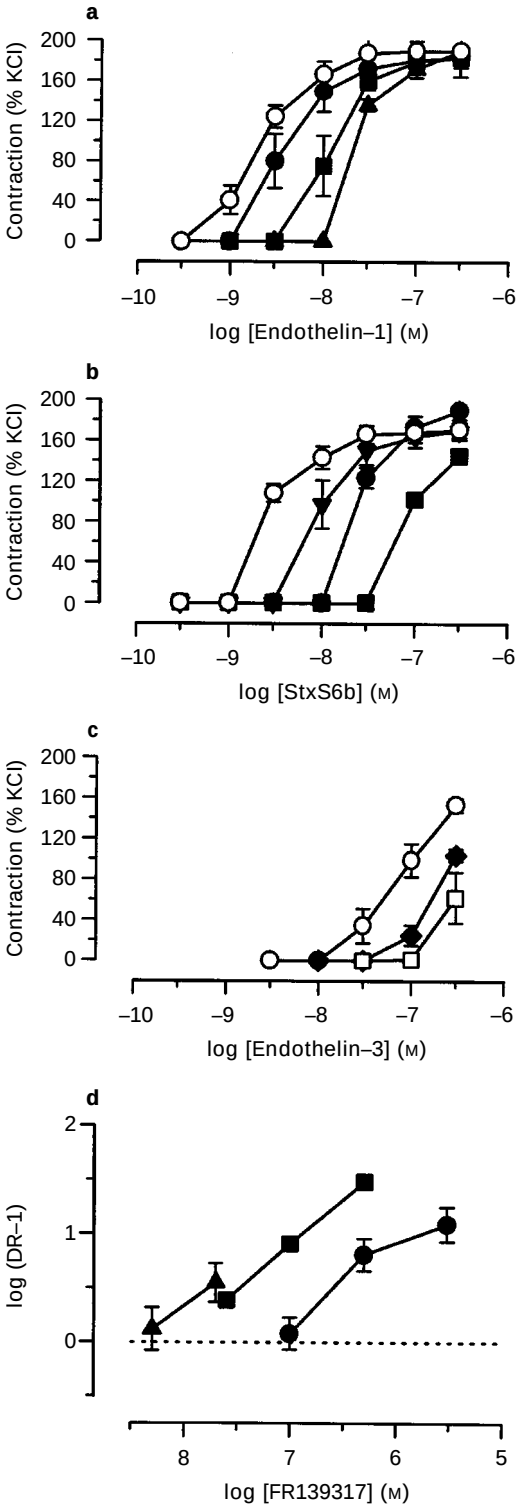

Figure 2 Concentration-effect curves to (a) endothelin-1, (b) StxS6b and (c) endothelin-3 in the absence (○) and in the presence of 0.005 μM (◆), 0.02 μM (□), 0.025 μM (▼), 0.1 μM (●), 0.5 μM (■) and 3 μM (▲) FR-139317. (d) A Schild plot analysis of the data presented in (a)–(c), illustrating that FR-139317 was a more potent inhibitor of contractions induced by StxS6b (■) and ET-3 (▲) than those induced by ET-1 (●). Data are expressed as the arithmetic mean ± s.e.mean (vertical lines) of 3–8 values.

Dissociation binding studies Following a 3 h period of association, the replacement of 0.32 nM [^{125}I]-ET-1 in the incubation media with 1 μM unlabelled ET-1 caused a small (approximately 15%), but not a statistically significant reduction in the level of specific [^{125}I]-ET-1 binding to tissue sections over the next 4 h (Figure 3e).

Competition binding studies ET-1 (3 pM to 10 nM) completely inhibited the specific binding of 0.32 nM [^{125}I]-ET-1 in a monophasic manner, with an IC_{50} of 0.21 nM (Figure 3f, Table 2). BQ-123 also inhibited specific [^{125}I]-ET-1 binding in a concentration-dependent and monophasic manner (Figure 3f), although it was 10 times less potent than ET-1. In neither case did a two-site model provide a significantly better fit than the corresponding one-site model. StxS6b (10 pM to 1 μM) completely inhibited specific [^{125}I]-ET-1 binding. However, the data were better described by a two-site model ($P < 0.025$, F-test, Figure 3f). The first of the two sites, inhibited by low concentrations of StxS6b, accounted for approximately 80% of specific [^{125}I]-ET-1 binding, whereas the remaining 20% of specific [^{125}I]-ET-1 binding was only inhibited by higher concentrations of StxS6b (0.1 to 1 μM).

Radioligand binding experiments with [^{125}I]-StxS6b

Association binding studies At the lower concentration used (0.32 nM), specific [^{125}I]-StxS6b binding increased in a time-dependent manner and a plateau level of binding was achieved after 3–4 h (Figure 4a). At a higher concentration of 0.73 nM [^{125}I]-StxS6b, a plateau level of specific binding was also observed after 3–4 h (Figure 4b). However, the plateau level of binding obtained at 4 h with 0.73 nM (357 ± 7 amol per slide) was significantly greater than that obtained with 0.32 nM (257 ± 11 amol per slide; $P < 0.01$). Subsequent dissociation experiments (see below) established that specific [^{125}I]-StxS6b binding was reversible and thus the levels of specific [^{125}I]-StxS6b binding determined at equilibrium (4 h) were used to generate estimates of K_D and B_{max} from Scatchard analyses (Figure 4d, Table 1). Furthermore, association binding data were fitted to equation (1) and an estimate of k_{OBS} was obtained (Table 1). Together, the association and dissociation studies (described below) provided estimates of B_{max} (508 amol per slide), k_1 (4.6×10^7 M^{-1} min^{-1}) and k_{-1} (0.00694 min^{-1}) which were then inserted into equation (4) to generate a series of association binding curves for selected concentrations of [^{125}I]-StxS6b (Figure 4c). Estimates of K_D for [^{125}I]-StxS6b binding determined by kinetic (k_{-1}/k_1) and equilibrium (Scatchard) approaches were similar (1.5×10^{-10} M and 3.3×10^{-10} M, respectively; Table 1). In addition, the B_{max} value of 508 amol per slide estimated from the Scatchard analysis of specific [^{125}I]-StxS6b binding was similar to that of 475 amol per slide determined from the analysis of specific [^{125}I]-ET-1 association binding data (Table 1).

Dissociation binding studies Following a 3 h period of association, the replacement of 0.32 nM [^{125}I]-StxS6b in the incu-

bation media with 1 μM unlabelled ET-1 caused a significant reduction in the level of specific [^{125}I]-StxS6b binding to rat renal artery sections (Figure 4e). Approximately 30% of specific [^{125}I]-StxS6b binding had dissociated by 1 h, and the radioligand continued to dissociate such that only 21% remained after 4 h (Figure 4e). The estimated half-life of dissociation ($t_{0.5diss}$) was 100 min.

Competition binding studies StxS6b, BQ-123 and ET-1 potently inhibited specific [^{125}I]-StxS6b binding (Figure 4f) and

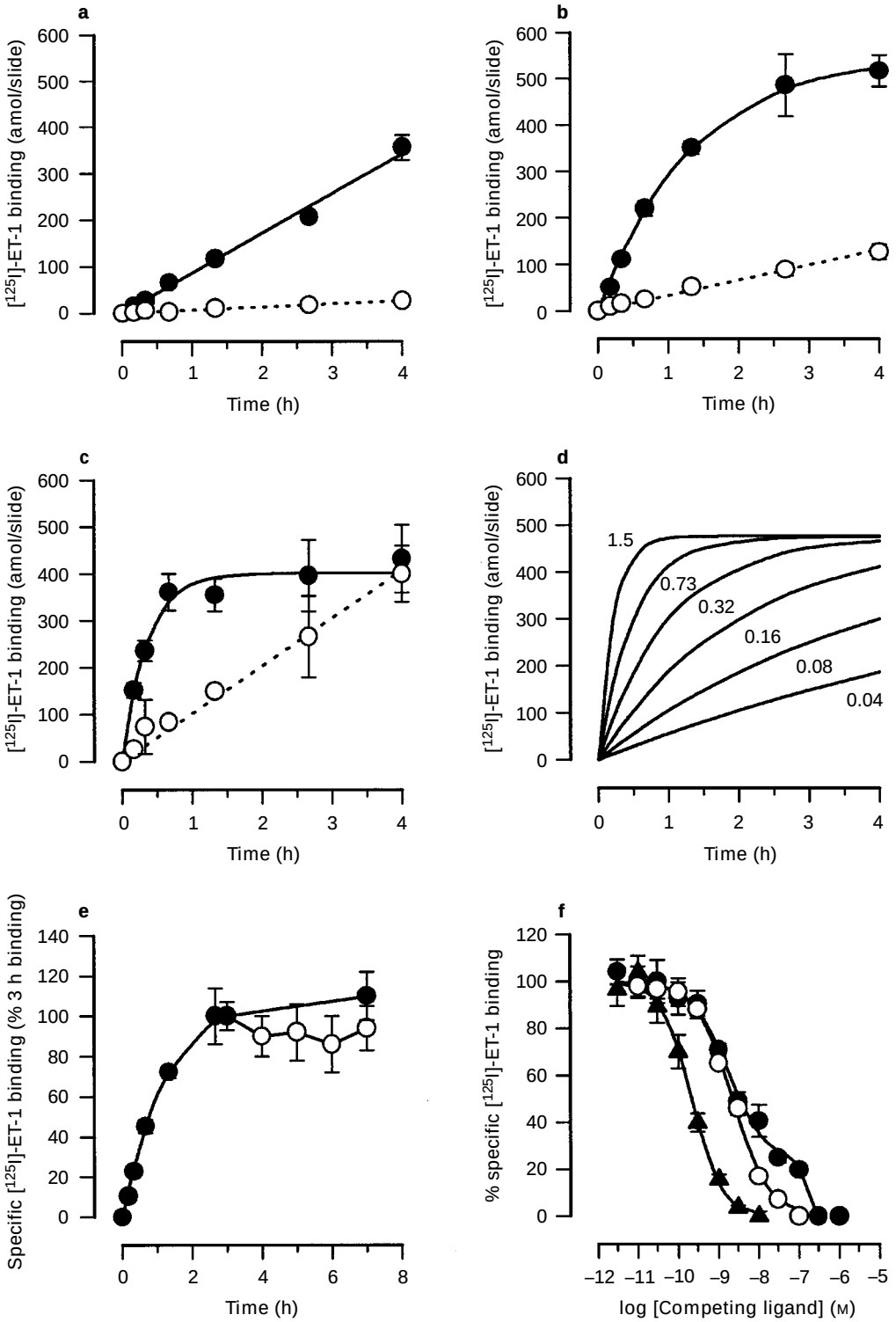

Figure 3 Association (a–d), dissociation (e) and competition (f) binding curves for [^{125}I]-ET-1 binding to slide-mounted sections of rat renal artery. The association of specific (●) and non-specific (○) binding is shown for (a) 0.08 nM, (b) 0.32 nM and (c) 0.73 nM [^{125}I]-ET-1. To illustrate the effect of ligand concentration on the observed rate of binding, a series of association binding curves was generated from equation (10), where B_{max} (475 amol per slide) and k_1 (5.2×10^7 M^{-1} min^{-1}) were estimated from the data shown in (a)–(c) and L was varied between 0.04 and 1.5 nM (d). As shown in (e), the specific binding of [^{125}I]-ET-1 was essentially irreversible. Following a 3 h period of association between 0.32 nM [^{125}I]-ET-1 and tissue (●), the replacement of free radiolabel with 1 μM unlabelled ET-1 in selected slides (○) resulted in no significant reduction in specific tissue binding (e). (f) Competition binding curves for ET-1 (▲), BQ-123 (○) and StxS6b (●) against 0.32 nM [^{125}I]-ET-1. Each data point is the mean ± s.e.mean (vertical lines) of triplicate values obtained from 2–3 experiments.

Table 1 Estimates of the binding parameters derived from specific [^{125}I]-StxS6b and [^{125}I]-ET-1 binding data by kinetic and equilibrium approaches

	Kinetic analyses		*Equilibrium analyses*	
	Estimate	*Source*	*Estimate*	*Source*
[^{125}I]-StxS6b				
k_{obs}	0.0218 min^{-1}	Eq. (1)		
k_1	4.6×10^{7} M^{-1} min^{-1}	Eq. (3)		
k_{-1}	0.00694 min^{-1}	Eq. (8)		
$t_{0.5diss}$	100 min	Eq. (9)		
K_D	1.50×10^{-10} M	Eq. (5)	3.3×10^{-10} M	Eq. (6), Figure 4d
B_{max}			508 amol per slide	Eq. (6), Figure 4d
[^{125}I]-ET-1				
k_1	5.2×10^{7} M^{-1} min^{-1}	Eq. (10)		
k_{-1}	NE			
B_{max}	475 amol per slide	Eq. (10)		

NE, not estimated since no significant reversal of specific [^{125}I]-ET-1 binding.

Table 2 Binding constants (IC_{50}) and slopes (n) for inhibition of specific [^{125}I]-ET-1 and [^{125}I]-StxS6b binding by ET-1, BQ-123 and StxS6b by use of a one-site model

Competing ligand	*[^{125}I]-ET-1*		*[^{125}I]-StxS6b*	
	IC_{50} (nM)	*n*	IC_{50} (nM)	*n*
ET-1	0.21±0.02	1.16±0.09	0.30±0.03	0.96±0.08
BQ-123	2.2±0.1	0.97±0.05	5.0±0.5	0.96±0.07
StxS6b	4.5±0.8	0.64±0.06*	3.4±0.4	1.02±0.11

*Significantly less than one.

each of the competition binding curves were well described by a one-site model (Table 2). In no case did a two-site model provide a significantly better fit than the corresponding one-part model. The potency of ET-1 for inhibiting specific [^{125}I]-StxS6b binding (IC_{50}, 0.30±0.03 nM) was about 10 times greater than that of either StxS6b (3.4±0.4 nM) or BQ-123 (5.0±0.5 nM).

Discussion

In this study, a combination of isometric tension recording and radioligand binding techniques were used to characterize ET_A receptors in rat isolated renal arteries, and in particular, to establish whether this preparation contained putative atypical ET_A receptors. In functional studies, BQ-123 was significantly more potent as an inhibitor of contractions induced by StxS6b and ET-3 than by ET-1, raising the possibility that ET-1 mediated its response, at least in part, via a population of atypical, BQ-123-insensitive receptors. However, competition binding experiments did not provide any evidence for such a binding site. Rather, the atypical responses may be the result of differences in the binding kinetics of the ligands, since subsequent studies revealed significant differences in the dissociation rates for specific [^{125}I]-ET-1 and [^{125}I]-StxS6b binding to rat isolated renal artery sections.

Pharmacological characterization of ET_A receptors in rat renal artery

ET-1-induced contractions in the rat renal artery appear to be linked to activation of ET_A receptors since, (a) ET-1 and StxS6b were 30 fold more potent than ET-3, (b) the ET_A receptor-selective antagonists, BQ-123 and FR-139317, significantly inhibited these contractions and (c) an ET_B receptor-selective agonist StxS6c was inactive. This profile is similar to that previously found in numerous blood vessels including rat thoracic aorta, guinea-pig aorta and rabbit carotid artery (Sumner *et al*., 1992; Schoeffter & Randriantsoa, 1993; White *et al*., 1993).

However, the potencies of BQ-123 and FR-139317 were agonist-dependent. Both antagonists were significantly more potent as inhibitors of contractions induced by StxS6b and ET-3 than ET-1. The observed agonist-dependency of antagonist potency may be an indication of a mixed receptor population in this tissue since in a homogeneous receptor population the potency of an antagonist would be expected to be independent of the agonist used (Kenakin, 1984). Thus, one possible explanation for the functional data is that StxS6b and ET-3 contract rat renal artery solely via typical, BQ-123-sensitive ET_A receptors, whereas ET-1 may also stimulate a novel BQ-123-insensitive endothelin receptor subtype. This explanation has previously been offered by Clark & Pierre (1995) who demonstrated a similar, BQ-123-resistant component of ET-1-induced contraction in renal arteries isolated from a different strain of rat, the Sprague-Dawley. The similarity in the responses obtained in the current study and by Clark & Pierre (1995) suggests that the potency of BQ-123 is agonist-dependent in the renal arteries from both Wistar and Sprague-Dawley rats. Furthermore, in the current study, agonist dependency of antagonist potency was not limited to BQ-123, but was also observed with another ET_A receptor antagonist, FR-139317. It is therefore unlikely that the seemingly contradictory results of Clark & Pierre (1995) and Seo & Lüscher (1995) can be attributed to differences in the selectivity of the antagonists or in the strain of rat studied.

One possible explanation of the functional data is that ET-1 binds to two ET_A receptor subtypes but StxS6b, ET-3 and BQ-123 bind to only one of these subtypes. However, competition binding experiments demonstrated that BQ-123 fully inhibited the specific binding of [^{125}I]-ET-1 in a manner consistent with an action at a single site and with no indication of a residual BQ-123-insensitive component to the [^{125}I]-ET-1 binding. Thus, there was no supportive evidence from these competition binding studies for the existence of a second, BQ-123-insensitive ET_A receptor. This conclusion of course, is based on the assumption that the binding conditions employed in this study resulted in the labelling of the putative BQ-123-insensitive ET_A receptor by [^{125}I]-ET-1. Compared with the concentrations of ET-1 used in the functional studies, low concentrations of [^{125}I]-ET-1 are routinely used in radioligand binding studies and this may have led to low levels of receptor labelling if the putative BQ-123-insensitive receptor has a very low affinity for [^{125}I]-ET-1. Thus, the combination of low radioligand concentration and low receptor affinity may have led to this receptor subtype being incompletely labelled and remaining undetected during competition binding studies. The possibility cannot be excluded that a low affinity receptor might only be activated at high concentrations of ET-1, such as

used during functional experiments. However, all endothelin receptors investigated so far have displayed very high affinity for ET-1 and thus it is unlikely that ET receptors would have escaped detection in the current radioligand binding studies. In summary, assuming that [^{125}I]-ET-1 has a similar affinity for endothelin receptors on the rat renal artery, the existence of atypical ET_A receptors is not supported by the competition binding studies performed in the present study.

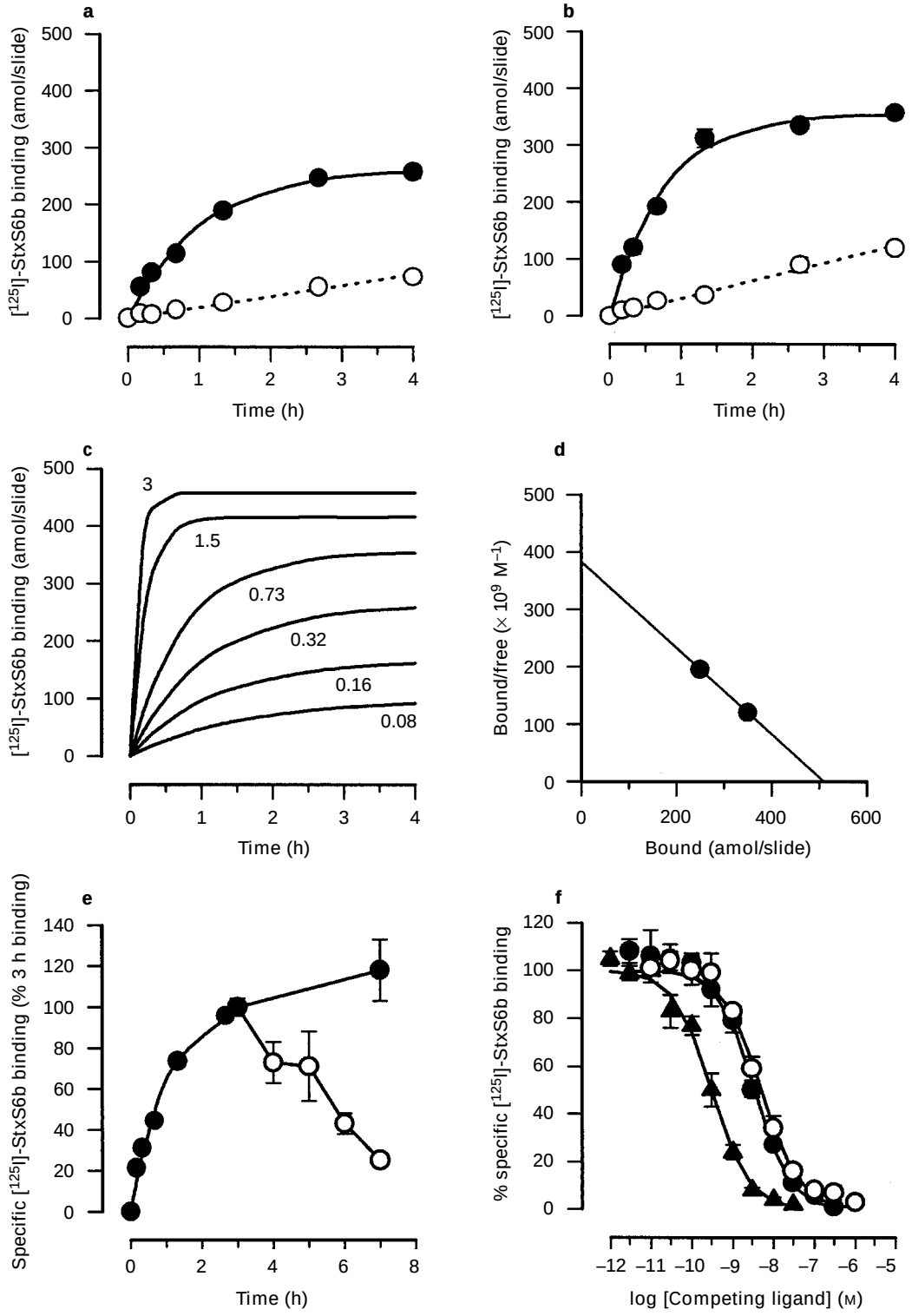

Figure 4 Association (a–c), Scatchard (d), dissociation (e) and competition (f) binding curves for [^{125}I]-StxS6b binding to slide-mounted sections of rat renal artery. The association of specific (●) and non-specific (○) binding is shown for (a) 0.32 nM and (b) 0.73 nM [^{125}I]-StxS6b. To illustrate the effect of ligand concentration on the rate of binding, a series of association binding curves was generated from equation (4), where B_{max} (508 amol per slide), k_1 (4.6×10^7 M^{-1} min^{-1}) and k_{-1} (0.00694 min^{-1}) were estimated (see Table 1) and L was varied between 0.08 and 3 nM (c). (d) A two-point Scatchard plot derived from the specific [^{125}I]-StxS6b binding data at equilibrium (4 h time-point data from (a) and (b)). As shown in (e), the specific binding of [^{125}I]-StxS6b was reversible. Following a 3 h period of association between 0.32 nM [^{125}I]-StxS6b and tissue (●), the replacement of free radiolabel with 1 μM unlabelled ET-1 in selected slides (○) resulted in significant reduction in specific tissue binding over the next 4 h (e). (f) Competition binding curves for ET-1 (▲), BQ-123 (○) and StxS6b (●) against 0.32 nM [^{125}I]-StxS6b. Each data point is the mean ± s.e.mean (vertical lines) of triplicate values obtained from 2–3 separate experiments.

Kinetics of [^{125}I]-ET-1 and [^{125}I]-StxS6b binding

However, there are other possible explanations of the atypical responses observed in the functional studies. For example, the various endothelin analogues have vastly different binding characteristics from ET-1 and this might contribute to the differences in antagonist potencies against these agonists. The possibility was considered that differences in the rates of dissociation of ET-1 and StxS6b from the ET_A receptor may contribute to the differing abilities of BQ-123 to antagonize their contractile responses, whilst competing with equal affinity against each in the competition studies. ET-1 is known to bind with high affinity to its receptor and to dissociate very slowly (Hemsen *et al*., 1991; Waggoner *et al*., 1992; Ihara *et al*., 1992; 1995). If bound ET-1 does not dissociate, the opportunity for BQ-123 to compete for that receptor is attenuated. Indeed, differences in the binding characteristics and kinetics of the various endothelin analogues may have important functional consequences. For example, Wu-Wong and co-workers have shown that the ability of antagonists to inhibit labelled ET-1 binding to either the ET_A or ET_B receptor is critically dependent on the incubation time, because antagonist binding is more readily reversed than ET-1 binding (Wu-Wong *et al*., 1994a,b; 1995).

In sections of rat renal artery, the rates of dissociation of [^{125}I]-ET-1 and [^{125}I]-StxS6b from ET_A receptors were markedly different. Dissociation binding studies demonstrated that specific [^{125}I]-ET-1 binding was irreversible over a 4 h washout period. This is similar to several earlier studies including one by Hirata and coworkers (1988), where less than 20% of [^{125}I]-ET-1 binding had dissociated after 24 h. Thus, the dissociation binding studies were indicative of the binding of [^{125}I]-ET-1 to its receptors being an irreversible process. The results from association binding studies were also consistent with the irreversible nature of [^{125}I]-ET-1 binding. Firstly, the observed rate of [^{125}I]-ET-1 binding was strictly concentration-dependent; the higher the concentration of [^{125}I]-ET-1, the more rapid the binding process. Secondly, the plateau levels of binding obtained with 0.32 nM [^{125}I]-ET-1 and 0.73 nM [^{125}I]-ET-1 were not significantly different. The concentration-dependency of the rate of binding and the concentration-independency of the plateau level of binding are characteristic features of a ligand binding irreversibly. By virtue of the fact that the binding of [^{125}I]-ET-1 was irreversible, it was not appropriate to derive estimates of K_D and B_{max} from the binding data with methods such as the Scatchard Plot which depend on the equilibrium assumption. Instead, the association binding curves for [^{125}I]ET-1 were fitted to a model describing an irreversible first-order reaction process and provided estimates of the tissue endothelin receptor concentration ($B_{max} = 475$ amol per slide) and association rate constant ($k_1 = 5 \times 10^9$ M^{-1} min^{-1}).

In stark contrast to the irreversible nature of [^{125}I]-ET-1 binding, the binding of [^{125}I]-StxS6b was readily reversible. About 80% of specific [^{125}I]-StxS6b binding had dissociated from rat renal artery sections within 4 h, with an estimated half-life of dissociation ($t_{0.5diss}$) of 100 min. Whereas the dissociation of [^{125}I]-StxS6b binding was not as rapid as that previously found for endothelin antagonists such as BQ-123 ($t_{0.5diss}$, 1.4 min), it was more rapid than the routinely used β-adrenoceptor radioligand, [^{125}I]-cyanopindolol ($t_{0.5diss}$, 470 min; Buxton *et al*., 1987) and of [^{125}I]-ET-1 ($t_{0.5diss}$, 40–200 h; Waggoner *et al*., 1992). Consistent with [^{125}I]-StxS6b being a reversibly binding ligand, the level of binding observed at equilibrium (plateau) was concentration-dependent; the plateau level of binding observed with 0.32 nM [^{125}I]-StxS6b was significantly lower than that observed with 0.72 nM [^{125}I]-StxS6b. In view of the fact that [^{125}I]-StxS6b bound reversibly, it was possible to estimate B_{max} by Scatchard analysis. Interestingly, the estimate of B_{max} obtained with [^{125}I]-StxS6b by use of the Scatchard (equilibrium) approach (508 amol per slide) was similar to that obtained from the studies with [^{125}I]-ET-1 by use of the irreversible binding model (475 amol per slide). Furthermore, and as would be predicted, the estimates of the K_D for [^{125}I]-StxS6b binding were similar whether determined from the Scatchard or kinetic analyses. However, it should be stressed that each of these estimates was obtained from the use of just two concentrations of [^{125}I]-StxS6b and [^{125}I]-ET-1 and that confirmation of the closeness of these estimates must await further, more extensive studies.

In a very recent study, Gresser and coworkers (1996) showed that although both ET-1 and ET-3 activate phospholipase C in fibroblasts expressing cloned ET_A receptors of bovine, rat and human origins, BQ-123 was 10 times more potent at inhibiting the effects of ET-3 than ET-1 (Gresser *et al*., 1996). Their view that agonist dependency of BQ-123 potency is an intrinsic property of ET_A receptors and not necessarily indicative of additional receptor subtypes, is consistent with the findings of the current study. Similarly, Battistini and coworkers have argued that the reversibility of receptor binding of ET-1 is different from ET-3 and StxS6b, and this could account for the differences in antagonist potencies against these agonists (Battistini *et al*., 1994). In contrast, a recent study from Maguire and coworkers (1996) showed that an additional population of receptors may well explain their findings that BQ-123 was a much more potent inhibitor of contractile responses to StxS6b than to ET-1 in human isolated saphenous vein. This conclusion was based on several lines of evidence. In saturation binding experiments with homogenates of human saphenous vein, [^{125}I]-ET-1 consistently labelled a significantly higher density of receptors than [^{125}I]-StxS6b. Furthermore, in competition binding experiments, StxS6b inhibited [^{125}I]-ET-1 binding in a biphasic manner, suggestive that ET-1 is binding to two receptor populations which can be distinguished by StxS6b. Interestingly, and in contrast to our findings, Maguire and coworkers showed that the dissociation rate of [^{125}I]-ET-1 and [^{125}I]-StxS6b from ET_A receptors (from slide-mounted tissue sections of human aorta) was similar (very slow), and concluded that the agonist-dependency of antagonist potency could not be explained by differences in agonist kinetics of binding. However, the failure of BQ-123 to distinguish between [^{125}I]-ET-1 and [^{125}I]-StxS6b binding in their studies may be indicative of other factors, in addition to additional receptor subtypes and kinetic differences, contributing to the agonist-dependency of BQ-123 potency.

The postulate that agonist dependency of antagonist potency might be a reflection of the kinetics of ligand binding rather than due to the existence of receptor subtypes requires further testing by studies with additional agonists and antagonists. Although the current study indicates that ET-3 has a functional profile similar to StxS6b, the completion of kinetic binding studies may not be possible due to the low affinity of [^{125}I]-ET-3 for ET_A receptors. Further studies with more potent ET_A receptor agonists such as ET-2 may prove beneficial, although presently there is a paucity of published studies describing the functional and radioligand binding characteristics of this ET isopeptide in the same cell or tissue type. Nevertheless, the binding of ET-2 to Swiss 3T3 fibroblasts has been shown to be more readily reversible than the binding of ET-1 (Devesly *et al*., 1990) and the contractions induced by ET-1 and ET-2 of rabbit iris sphincter muscle were similarly more resistant to inhibition by BQ-123 than those induced by ET-3 or StxS6b (Ishikawa *et al*., 1996). If this profile of responses to ET-2 (i.e. reversible binding kinetics, but similar functional profile to ET-1), were demonstrable in the same cell or tissue, such as the rat renal artery, then it is unlikely that agonist-dependency of antagonist potency can be explained by differences in binding kinetics. Thus, functional and kinetic studies performed in the same cell or tissue type with ET-2 and other agonists, such as vasoactive intestinal contractor (VIC), are likely to prove useful in the further testing of this postulate.

Although there was no supportive evidence from binding studies of a BQ-123-insensitive ET_A receptor, competition binding curves for StxS6b against [^{125}I]-ET-1 were consistently biphasic, raising the possibility of a small population of StxS6b-insensitive endothelin receptors similar to that found in

homogenates of human saphenous vein (Maguire *et al*., 1996). However, the existence of these StxS6b-insensitive receptors is unlikely to explain the functional responses since these sites were not insensitive to BQ-123; [^{125}I]-ET-1 binding was completely and monophasically inhibited by BQ-123, consistent with binding to a single homogeneous class of receptors. Whether these StxS6b-insensitive sites are linked to contraction is not known, but it is obvious from the competition binding studies that ET-1 has equal affinity for this site as it has for other endothelin receptors.

In the current study, we have shown that the endothelium-denuded main branch renal artery preparation from Wistar rats does not contain ET_B receptors mediating either relaxation or contraction. In this tissue, the contractile effects of ET-1, StxS6b and ET-3 appear to be mediated by ET_A receptors. However, BQ-123 was markedly more potent at inhibiting contractions induced by StxS6b and ET-3, raising the possibility that a component of ET-1-induced contraction was mediated through atypical, BQ-123-insensitive ET_A receptors. Subsequent competition binding experiments revealed that BQ-123 completetly abolished specific [^{125}I]-ET-1 and [^{125}I]-StxS6b binding in a manner consistent with an action at a single site. Thus, binding studies did not provide any evidence in support of the existence of atypical, BQ-123-insensitive ET_A receptors. On the other hand, additional studies of [^{125}]-ET-1 and [^{125}I]-StxS6b binding revealed marked differences in the kinetics of their binding. Whereas [^{125}I]-StxS6b binding was fully reversible, the binding of [^{125}I]-ET-1 was irreversible. Hence, differences in BQ-123 potency against ET-1 and StxS6b/ET-3-induced contractions in rat renal arteries might be due to differences in the kinetics of agonist binding, rather than due to the existence of atypical ET_A receptors.

This research is supported by the National Health and Medical Research Council of Australia. The authors would also like to thank the Fujisawa Pharmaceutical Company, Osaka, Japan, for the generous gift of FR139317 and to acknowledge the expert technical assistance provided by Mr Glenn Self during the radioligand binding studies.

References

BATTISTINI, B., O'DONNELL, L.J.D., WARNER, T.D., FOURNIER, A., FARTHING, M.J.G. & VANE, J.R. (1994). Characterization of endothelin (ET) receptors in the isolated gall bladder of the guinea-pig: evidence for an additional ET receptor subtype. *Br. J. Pharmacol*., **112,** 1244 – 1250.

BAX, W.A., BOS, E. & SAXENA, P.R. (1993). Heterogeneity of endothelin/sarafotoxin receptors mediating contraction of the human isolated saphenous vein. *Eur. J. Pharmacol*., **239,** 267 – 268.

BAX, W.A. & SAXENA, P.R. (1994). The current endothelin receptor classification: time for reconsideration. *Trends Pharmacol. Sci*., **15,** 379 – 386.

BODELSSON, G. & STJERNQUIST, M. (1993). Characterization of endothelin receptors and localization of [^{125}I]-endothelin-1 binding sites in human umbilical artery. *Eur. J. Pharmacol*., **249,** 299 – 305.

BUXTON, B.F., JONES, C.R., MOLENAAR, P. & SUMMERS, R.J. (1987). Characterization and autoradiographic localization of β-adrenoceptor subtypes in human cardiac tissues. *Br. J. Pharmacol*., **92,** 299 – 310.

CLARK, K.L. & PIERRE, L. (1995). Characterization of endothelin receptors in rat renal artery *in vitro*. *Br. J. Pharmacol*., **114,** 785 – 790.

CLOZEL, M., LOFFLER, B.M., BREU, V., HILFIGER, L., MAIRE, J.P. & BUTSCHA, B. (1993). Down-regulation of endothelin receptors by autocrine production of endothelin-1. *Am. J. Physiol*., **265,** C188 – C192.

DEVESLY, P., PHILLIPS, P.E., JOHNS, A., RUBANYI, G. & PARKER-BOTELHO, L.H. (1990). Receptor kinetics differ for endothelin-1 and endothelin-2 binding to Swiss 3T3 fibroblasts. *Biochem. Biophys. Res. Copmmun*., **192,** 126 – 134.

EGLEZOS, A., CUCCHI, P., PATACCHINI, R., QUARTARA, L., MAGGI, C.A. & MIZRAHI, J. (1993). Differential effects of BQ-123 against endothelin-1 and endothelin-3 on the rat vas deferens: evidence for an atypical endothelin receptor. *Br. J. Pharmacol*., **109,** 736 – 738.

GALRON, R., BDOLAH, A., KOCHVA, E., WOLLBERG, Z, KLOOG, Y. & SOKOLOVSKY, M. (1991). Kinetic and cross-linking studies indicate different receptors for endothelins and sarafotoxins in the ileum and cerebellum. *FEBS Lett*., **283,** 11 – 14.

GODFRAIND, T. (1993). Evidence for heterogeneity of endothelin receptor distribution in human coronary artery. *Br. J. Pharmacol*., **110,** 1201 – 1205.

GOLDIE, R.G., GRAYSON, P.S., KNOTT, P.G., SELF, G.J. & HENRY, P.J. (1994). Predominance of endothelin$_A$ (ET_A) receptors in ovine airway smooth muscle and their mediation of ET-1-induced contraction. *Br. J. Pharmacol*., **112,** 749 – 756.

GRESSER, O., CHAYARD, D., HERBERT, D., COUSIN, M.A., MOULLEC, J.M.L., BOUATTANE, F., GUEDIN, D. & FRELIN, C. (1996). Et-1 and Et-3 actions mediated by cloned ET_A endothelin receptors exhibit different sensitivities to BQ-123. *Biochem. Biophys. Res. Commun*., **224,** 169 – 171.

HEMSEN, A., LARSSON, O. & LUNDBERG, J.M. (1991). Characteristics of endothelin A and B binding sites and their vascular effects in pig peripheral tissues. *Eur. J. Pharmacol*., **208,** 313 – 322.

HIRATA Y., YOSHIMI, H., YAKAICHI, S., YANAGISAWA, M. & MASAKI, T. (1988). Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett*., **239,** 13 – 17.

IHARA, M., FUKURODA, T., SAEKI, T., NISHIKIBE, M., KOJIRI, K., SUDA, H. & YANO, M. (1991). An endothelin receptor (ET_A) antagonist isolated from Streptomyces misakiensis. *Biochem. Biophys. Res. Commun*., **178,** 132 – 137.

IHARA, M., SAEKI, T., FUKURODA, T., KIMURA, S., OZAKI, S., PATEL, A.C. & YANO, M. (1992). A novel radioligand [^{125}I]-BQ-3020 selective for endothelin (ET_B) receptors. *Life Sci*., **51,** PL47 – PL52.

IHARA, M., YAMANAKA, R., OHWAKI, K., OZAKI, S., FUKAMI, T., ISHIKAWA, K., TOWERS, P. & YANO, M. (1995). [^{3}H]BQ-123, a highly specific and reversible radioligand for the endothelin ET_A receptor subtype. *Eur. J. Pharmacol*., **274,** 1 – 6.

ISHIKAWA, H., HARANO, I., HARADA, Y., YOSHITOMI, T., ISHIKAWA, S. & KATORI, M. (1996). Pharmacological characterization of endothelin receptors in the rabbit iris sphincter muscle: suggestion for the presence of atypical receptors. *Curr. Eye Res*., **15,** 73 – 78.

KARLSSON, M.O. & NEIL, A. (1988). Estimation of binding parameters by kinetic data analysis: differentiation between one and two binding sites. *Eur. J. Pharmacol*., **148,** 115 – 121.

KENAKIN, T. (1984). The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev*., **36,** 165 – 222.

LE MONNIER DE GOUVILLE, A.C., LIPPTON, H., COHEN, G., CAVERO, I. & HYMAN, A. (1990). Vasodilator activity of endothelin-1 and endothelin-3: rapid development of cross-tachyphylaxis and dependence on the rate of endothelin administration. *J. Pharmacol. Exp. Ther*., **254,** 1024 – 1028.

MAGUIRE, J.J., KUC, R.E., ROUS, B.A. & DAVENPORT, A.P. (1996). Failure of BQ-123, a more potent antagonist of sarafotoxin 6b than of endothelin-1, to distinguish between these agonists in binding experiments. *Br. J. Pharmacol*., **118,** 335 – 342.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods*, **14,** 213 – 228.

MEAD, R., CURNOW, R.N. & HASTED, A.M. (1993). *Statistical Methods in Agriculture and Experimental Biology*. pp. 213 – 232. London: Chapman & Hall.

RESINK, T.J., SCOTT-BURDEN, T., BOULANGER, C., WEBER, E. & BUHLER, F.R. (1990). Internalization of endothelin by cultured human vascular smooth muscle cells: characterization and physiological significance. *Mol. Pharmacol*., **38,** 244 – 252.

RIEZEBOS, J., WATTS, I.S. & VALLANCE, P.J.T. (1994). Endothelin receptors mediating functional responses in human small arteries and veins. *Br. J. Pharmacol*., **111,** 609 – 615.

SALOM, J.B., TORREGROSA, G., BARBERA, M.D., JOVER, T. & ALBORCH, E. (1993). Endothelin receptors mediating contraction in goat cerebral arteries. *Br. J. Pharmacol.*, **109,** 826–830.

SEO, B. & LÜSCHER, T.F. (1995). ET_A and ET_B receptors mediate contraction to endothelin-1 in renal artery of aging SHR. *Hypertension*, **25,** 501–506.

SCHOEFFTER, P. & RANDRIANTSOA, A. (1993). Differences between endothelin receptors mediating contraction of guinea-pig aorta and pig coronary artery. *Eur. J. Pharmacol.*, **249,** 199–206.

SOGABE, K., NIREI, H., SHOUBO, M., NOMOTO, A., AO, S., NOTSU, Y. & ONO, T. (1993). Pharmacological profile of FR139317, a novel, potent endothelin ET_A receptor antagonist. *J. Pharmacol. Exp. Ther.*, **264,** 1040–1046.

SOKOLOVSKY, M. (1995). Endothelin receptor subtypes and their role in transmembrane signalling mechanisms. *Pharmacol. Ther.*, **68,** 435–471.

SUMNER, M.J., CANNON, T.R., MUNDIN, J.W., WHITE, D.G. & WATTS, I.S. (1992). Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction. *Br. J. Pharmacol.*, **107,** 858–860.

VIGNE, P., BREITTMAYER, J.P. & FRELIN, C. (1993). Competitive and non-competitive interactions of BQ-123 with endothelin ET_A receptors. *Eur. J. Pharmacol.*, **245,** 229–232.

WAGGONER, W.G., GENOVA, S.L. & RASH, V.A. (1992). Kinetic analyses demonstrate that the equilibrium assumption does not apply to [^{125}I]-endothelin binding data. *Life Sci.*, **51,** 1869–1876.

WHITE, D.G., CANNON, T.R., GARRATT, H., MUNDIN, J.W., SUMNER, M.J. & WATTS, I.S. (1993). Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction. *J. Cardiovasc. Pharmacol.*, **22** (Suppl 8), S144–S148.

WU-WONG J.R., CHIOU, W.J., DIXON, D.B. & OPGENORTH, T.J. (1995). Dissociation characteristics of endothelin ET_A receptor agonists and antagonists. *J. Cardiovasc. Pharmacol.*, **26,** S380–S384.

WU-WONG, J.R., CHIOU, W.J., HUANG, Z., VIDAL, M.J. & OPGENORTH, T.J. (1994a). Endothelin receptors in human smooth muscle cells: antagonist potency differs on agonist-evoked responses. *Am. J. Physiol.*, **267,** C1185–C1195.

WU-WONG, J.R., CHIOU, W.J., NAUGLES, K.E. JR. & OPGENORTH, T.J. (1994b). Endothelin receptor antagonists exhibit diminishing potency following incubation with agonist. *Life Sci.*, **54,** 1727–1734.

(Received November 22, 1996
Revised February 5, 1997
Accepted February 10, 1997)