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Differential inhibition by TAK-044 of the inotropic effects of endothelin-1 and endothelin-3

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Abstract

The influence of a nonselective antagonist of endothelin receptors, TAK-044 (cyclo-[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium), on the positive inotropic effect of endothelin-1 and endothelin-3 was investigated in isolated rabbit myocardium. While TAK-044 produced a concentration-dependent rightward shift of the concentration–response curve for endothelin-1 and endothelin-3, the effect of endothelin-3 was hundred times more sensitive to TAK-044 than that of endothelin-1. The combination of FR139317 ([2-(*R*)-[2(*R*)-[2(*S*)-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl] amino-3-(2-pyridyl)propionic acid]) and BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-leucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) mimicked the inhibitory action of TAK-044 on the positive inotropic effect of endothelin-3 but enhanced the effect of endothelin-1. In a receptor-binding assay, TAK-044 was four times more potent in antagonizing the specific binding of endothelin-1 than that of endothelin-3. Endothelin-1 may activate receptor subtypes that trigger both positive and negative inotropic effects, the latter being more susceptible to the antagonistic action of TAK-044, which may explain in part the differential antagonistic action of TAK-044 on the inotropic effect of endothelin-1 and endothelin-3.

Keywords: Positive inotropic effect; (Rabbit); Endothelin receptor; Endothelin receptor antagonist; TAK-044; FR139317

1. Introduction

Specific high-affinity binding sites for endothelin isopeptides are distributed throughout various organs and have been subclassified as endothelin ET_A and ET_B receptors. In early studies, it was claimed that endothelin ET_A receptors existed mainly on vascular smooth muscle cells and responded to endothelin-1 but less effectively to endothelin-3, whereas endothelin ET_B receptors were predominantly located on endothelial cells and responded equally well to endothelin-1 and endothelin-3. More recently, endothelin receptors have been found in various cell types in a more complex manner (see Masaki et al., 1991; Haynes et al., 1993; Rubanyi and Polokoff, 1994; Goto et al., 1996 for reviews). Endothelin receptors are also expressed in high density in mammalian myocardial cells (Endoh et al., 1998a).

In isolated rabbit right ventricular papillary muscle, endothelin-1 and endothelin-3 produce a positive inotropic effect with essentially identical potency and efficacy (Takanashi and Endoh, 1991). Detailed investigation, however, revealed that the concentration–response curve for endothelin-1 was biphasic: endothelin-1 elicited a positive inotropic effect reaching 20% of ISO_{max} (phase I) at a lower concentration range ($< 3 \times 10^{-10}$ M) over which endothelin-3 was ineffective (Kasai et al., 1994). The main portion of the concentration–response curve for the positive inotropic effect of endothelin-1 (phase II) elicited at 10^{-9}–10^{-7} M was affected neither by selective antagonists of endothelin ET_A receptors, such as BQ-123 (Kasai et al., 1994) and FR139317 (Endoh et al., 1996), nor by the nonselective antagonist of endothelin receptors, PD 145065 (AcD-Bhg-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp; Bhg = 5*H*-dibenzyl[*a*,*d*]cycloloheptene 10,11-dihydroglycine) (Norota and Endoh, 1996). In contrast, the positive inotropic effect of endothelin-3 and the phase I response to endothelin-1 were abolished by these selective and nonselective antagonists (Kasai et al., 1994; Endoh et al., 1996; Norota and Endoh, 1996),

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an indication that the response of the rabbit ventricular myocardium to endothelin isopeptides is atypical. On the basis of these findings, it has been suggested that the positive inotropic effect of endothelin-3 and the phase I response to endothelin-1 in the rabbit ventricular myocardium may be mediated by endothelin ET_{A1} receptors, while the main portion of the concentration–response curve for the positive inotropic effect of endothelin-1 (phase II response) may be due to activation of endothelin ET_{A2} receptors (Endoh et al., 1996, 1998a).

In a recent study, it was found that endothelin-1 was approximately 60 times more potent in rabbit single ventricular myocytes than in papillary muscles. Furthermore, an endothelin ET_A antagonist, BQ-485 (hexahydro-1*H*-azepinylcarbonyl-leucyl-D-tryptophanyl-D-tryptophan), and an endothelin ET_B antagonist, BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) (Ishikawa et al., 1994), enhanced the increase in cell shortening and Ca^{2+} transients induced by endothelin-1 at 10^{-8} M (maximal concentration in single myocytes), while a non-selective antagonist, TAK-044 (cyclo-[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl]) (Kikuchi et al., 1994), abolished the response to 10^{-8} M endothelin-1 (Talukder et al., 2001).

In the present study, we examined the influence of TAK-044 on the positive inotropic effects of endothelin-1 and endothelin-3 in rabbit papillary muscle in more detail. For comparison, we also investigated the effect of the combination of a selective antagonist of endothelin ET_A receptors, FR139317 ([2-(*R*)-[2(*R*)-[2(*S*)-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl] amino-3-(2-pyridyl)propionic acid]), and a selective antagonist of endothelin ET_B receptors, BQ-788.

2. Methods

2.1. Isolation of rabbit papillary muscle and measurements of inotropic effects

This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). Approval for the animal experiments was obtained from the Committee for Animal Experimentation, Yamagata University School of Medicine, prior to the experiments, and the study was carried out also in accordance with the Declaration of Helsinki.

The procedure for isolation of right ventricular papillary muscles from rabbits has been described previously (Endoh et al., 1996). In brief, adult male Japanese White rabbits (1.8–2.4 kg) were anesthetized with pentobarbital sodium (50 mg/kg, i.v.). One to three papillary muscles were excised from the right ventricle of each rabbit and mounted in a 20-ml organ bath that contained Krebs–Henseleit solution (with 0.057 mM ascorbic acid and 0.027 mM disodium EDTA to prevent autoxidation of the compounds examined). The composition of the solution (in mM) was as follows: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, and glucose 11.1. The solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 37 °C (pH 7.4). Papillary muscles were electrically stimulated by square-wave pulses of 5-ms duration at a voltage that was about 20% above the threshold, at 1.0 Hz, through bipolar platinum electrodes. Isometric tension was detected with strain-gauge transducers (Shinkoh UL-10 GR; Minebea, Tokyo, Japan) and recorded on a thermal pen-writing oscillograph (Recti-Horiz-8K; NEC San-ei Instruments, Tokyo, Japan). During the 60-min equilibration period, the muscles were initially stretched at a resting tension of 5 mN and the length was then adjusted to give 90% of the maximal developed tension. The inotropic response to endothelin-1 or endothelin-3 is expressed as a percentage of ISO_{max} for each preparation. Simultaneous blockade of β- and α-adrenoceptors was achieved by addition of 0.3 μM (±)-bupranolol and 0.3 μM prazosin, respectively, to avoid modulation by catecholamines released during the administration of endothelin isopeptides (Yang and Endoh, 1997). (±)-Bupranolol and prazosin were allowed to act for 30 min before the administration of endothelin isopeptides and both agents were present in the solution in the organ bath throughout the experiments. Selective or nonselective antagonists of specific subtypes of endothelin receptor were also allowed to act for 30 min before the determination of the concentration–response curve for each endothelin isopeptide, and the indicated antagonists were present in the organ bath throughout each experiment. The concentration–response curve for the positive inotropic effect of each endothelin isopeptide was constructed after cumulative administration of an isopeptide to a muscle preparation in the presence or absence of a receptor antagonist. At the end of each experiment, the maximum contractile force was determined for each muscle by cumulative administration of isoproterenol after washing out other drugs for at least 2 h. ISO_{max} was defined as the maximal contractile force in the presence of high concentrations (10^{-6}–10^{-5} M) of isoproterenol minus the basal force of contraction at the beginning of each experiment.

2.2. Radioligand-binding assays

Assays of binding of [^{125}I]endothelin-1 and of [^{125}I]endothelin-3 were carried out as described in detail elsewhere (Endoh et al., 1996; Yang and Endoh, 1997). In brief, pieces of right and left ventricular muscle, including free wall and septum, were excised from the hearts of rabbits that had been anesthetized with pentobarbital sodium, as described previously, and homogenized in 10 volumes of ice-cold buffer (0.25 M sucrose containing 5 mM Tris–HCl and

1 mM $MgCl_2$, pH 7.4) in a Polytron (PT-10; Kinematica, Lucerne, Switzerland) three times for 15 s each at setting 7. The homogenate was then centrifuged at 500 × *g* for 15 min at 4 °C. The supernatant was filtered through a single layer of cheesecloth and centrifuged at 50,000 × *g* for 20 min at 4 °C. The resulting pellet was washed twice with ice-cold incubation buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5) by repeated resuspension and recentrifugation. The final pellet was resuspended in ice-cold incubation buffer that contained 1 mg/ml bovine serum albumin.

The specific binding assay was performed in an incubation mixture that contained 150 μl of a suspension of membranes (approximately 50–100 μg of protein), 50 μl of l of a solution of [^{125}I]endothelin-1 or [^{125}I]endothelin-3 (specific activity of each, 2000 Ci/mmol) and 50 μl of incubation buffer that contained 1 mg/ml bovine serum albumin with or without various concentrations of TAK-044. The reaction was started by the addition of the solution of [^{125}I]endothelin-1 or [^{125}I]endothelin-3, and the reaction mixture was incubated for 90 min at 25 °C. The reaction was terminated by addition of 2 ml of ice-cold incubation buffer. Then the mixture was rapidly filtered through a GF/C glass filter (Whatman International, Maidstone, UK) in a cell harvester (M-24R; Brandel, Gaithersburg, MD, USA). Each filter was washed rapidly with 12 ml (3 × 4 ml) of ice-cold incubation buffer. After the filter had been dried for 1 h at 90 °C, radioactivity bound to the filter was quantitated. Nonspecific binding of [^{125}I]endothelin-1 or [^{125}I]endothelin-3 was defined as the binding detected in the presence of 1 μM unlabeled endothelin-1 or endothelin-3, respectively. Specific binding of [^{125}I]endothelin-1 or [^{125}I]endothelin-3 was defined as the total radioactivity minus the radioactivity due to nonspecific binding. Each binding assay was carried out in duplicate. Protein was quantitated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.3. Peptides and drugs

The drugs and reagents used were endothelin-1 and endothelin-3 (Peptide Institute, Osaka, Japan); TAK-044 (cyclo-[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium) (Takeda Chemical Industries, Osaka, Japan); FR139317 (2-(*R*)-[2(*R*)-[2(*S*)-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl] amino-3-(2-pyridyl)propionic acid) (Fujisawa Pharmaceutical, Tsukuba, Japan); BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine sodium) (Banyu Pharmaceutical, Tsukuba, Japan); (−)-isoproterenol hydrochloride (Sigma, St. Louis, MO, USA); pentobarbital sodium (Tokyo Kasei Kogyo, Tokyo, Japan); (±)-bupranolol hydrochloride (Kaken Pharmaceutical, Tokyo, Japan); prazosin hydrochloride (Pfizer Taito, Tokyo, Japan); and [^{125}I]endothelin-1 and [^{125}I]endothelin-3 (specific activity of each, 2000 Ci/mmol; Amersham, Buckinghamshire, UK). Other reagents used were of the highest grade commercially available. TAK-044 was dissolved in distilled water and diluted with a 0.9% (w/v) solution of NaCl. The stock solution of isoproterenol was prepared in a 0.1% (w/v) solution of ascorbic acid, kept ice-cold, and diluted with a 0.9% (w/v) solution of NaCl just before use.

2.4. Statistical analysis of data

Data are presented as means ± S.E.M. The significance of differences was estimated by repeated measures analysis or by one-way analysis of variance followed by application of the Bonferroni/Dunn method. The significance of the difference between two mean values at corresponding concentrations of an agonist was estimated by Student's *t*-test. A *P* value of less than 0.05 was considered to be an indication of significance.

3. Results

3.1. Effects of TAK-044 on the positive inotropic effects of endothelin-1 and endothelin-3

Endothelin-1 elicited a positive inotropic effect in a concentration-dependent manner over a range of concentrations from 3×10^{-10} to 3×10^{-7} M in isolated rabbit papillary muscle. The maximum response was 53.7 ± 3.7% of ISO_{max} and the pD_2 ($-\log EC_{50}$) was 8.33 ± 0.16 ($n = 5$), as shown in Fig. 1.

TAK-044 at 10^{-9} M did not affect the positive inotropic effect of endothelin-1 (data not shown), but at 10^{-8} M it inhibited the effect of endothelin-1 at lower concentrations (e.g., at 3×10^{-10} M) and enhanced the effect of endothelin-1 at higher concentrations (e.g., at 3×10^{-8} M; see Fig. 1A). The maximal response to endothelin-1 (66.1 ± 4.2%) and the pD_2 for endothelin-1 (8.27 ± 0.12; $n = 5$; Fig. 1A) were unaltered.

TAK-044 at 10^{-7} M shifted the concentration–response curve for endothelin-1 only at concentrations of 3×10^{-9} M and lower, while the main portion of the concentration–response curve for endothelin-1 was not affected appreciably (Fig. 1B).

TAK-044 at 3×10^{-7}, 10^{-6} and 3×10^{-6} M shifted the concentration–response curve for endothelin-1 to the right in an essentially parallel and concentration-dependent manner (Fig. 1C,D,E). TAK-044 at 10^{-5} M almost completely inhibited the positive inotropic effect of endothelin-1 (Fig. 1F).

Endothelin-3 also elicited a positive inotropic effect in a concentration-dependent manner over a range of concentrations from 3×10^{-9} to 3×10^{-7} M. The maximum response was 64.4 ± 2.6% of ISO_{max} and the pD_2 was 8.06 ± 0.15 ($n = 5$), as shown in Fig. 2.

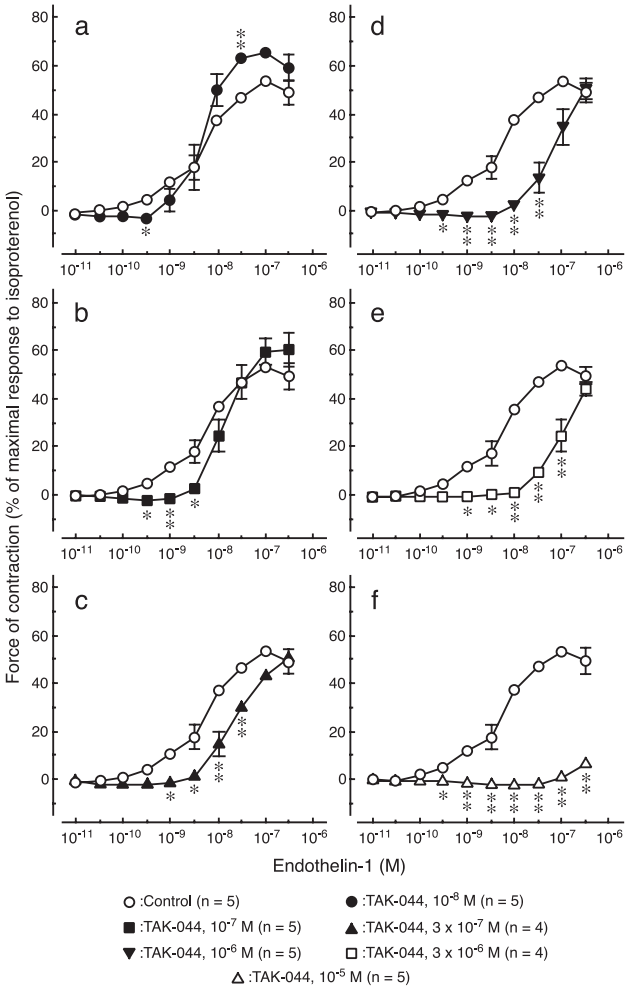

Fig. 1. Influence of increasing concentrations of TAK-044 on the positive inotropic effect of endothelin-1 in isolated rabbit papillary muscles. TAK-044 was allowed to act for 30 min and the concentration–response curves for endothelin-1 were determined from the data obtained in the presence of TAK-044 at different concentrations: 10^{-8} M (A, $n=5$); 10^{-7} M (B, $n=5$); 3×10^{-7} M (C, $n=4$); 10^{-6} M (D, $n=5$); 3×10^{-6} M (E, $n=4$); and 10^{-5} M (F, $n=5$). Five experiments were performed to generate the control concentration–response curve for endothelin-1. The basal force of contraction was 6.2 ± 1.7 mN/mm^2 in the control endothelin-1 group ($n=5$) and 6.7 ± 1.0 mN/mm^2 in the TAK-044-treated groups ($n=28$). The maximum force of contraction, as determined by application of isoproterenol, was 30.6 ± 6.0 mN/mm^2 in the control group ($n=5$) and 36.5 ± 4.0 mN/mm^2 in the TAK-044-treated groups ($n=28$). *$P<0.05$; **$P<0.01$ vs. the values for the corresponding concentrations of endothelin-1 in the absence of TAK-044 (control).

TAK-044 at 10^{-9} M did not affect the positive inotropic effect of endothelin-3, but at 3×10^{-9} M and higher it inhibited the positive inotropic effect of endothelin-3 in a concentration-dependent manner (Fig. 2). At 10^{-7} M, at which the main portion of the concentration–response curve for endothelin-1 was unaffected (Fig. 1B), TAK-044 abolished the positive inotropic effect of endothelin-3 (Fig. 2D).

Because of the complex nature of the antagonistic effects of TAK-044 on the positive inotropic effects of endothelin-1 and endothelin-3, it was difficult to determine exactly the potency of TAK-044 and to discriminate between the ability of TAK-044 to antagonize the positive inotropic effect of endothelin-1 and endothelin-3. However, it was evident that TAK-044 antagonized the positive inotropic effect of endothelin-3 more effectively than that of endothelin-1 (Figs. 1 and 2). We tried to make a rough estimate of the relative potency of TAK-044 by calculating the ratio of concentrations at the level that corresponded to 20% of the maximal response to isoproterenol. The calculated pA_2 value (defined as-log K_i value) for TAK-044 was 7.0 against endothelin-1, whereas that against endothelin-3 was 9.0, an indication that the ability of TAK-044 to antagonize the positive inotropic effect of endothelin-1 was hundred-fold lower than its ability to inhibit the positive inotropic effect of endothelin-3.

3.2. Effects of FR139317 on the positive inotropic effect of endothelin-1

In previous investigations of the influence of FR139317 on the positive inotropic effect of endothelin-1, FR139317 at concentrations up to 10^{-5} M did not shift the main

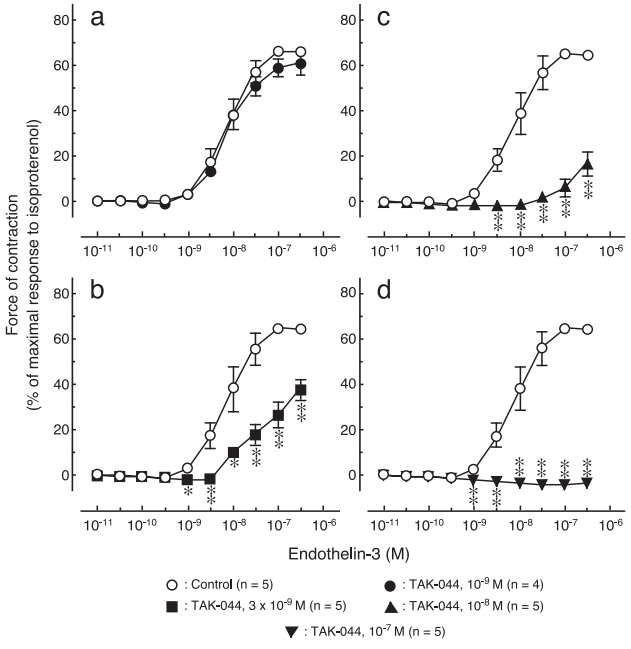

Fig. 2. Influence of increasing concentrations of TAK-044 on the positive inotropic effect of endothelin-3 in isolated rabbit papillary muscles. TAK-044 was allowed to act for 30 min and the concentration–response curve for endothelin-3 was determined from the data obtained in the presence of TAK-044 at different concentrations: 10^{-9} M (A, $n=4$); 3×10^{-9} M (B, $n=5$); 10^{-8} M (C, $n=5$); and 10^{-7} M (D, $n=5$). Five experiments were performed to obtain the control concentration–response curve for endothelin-3. The basal force of contraction was 8.2 ± 3.7 mN/mm^2 in the control endothelin-3 group ($n=5$) and 7.8 ± 1.6 mN/mm^2 in the TAK-044-treated groups ($n=19$). The maximum force of contraction, as determined by application of isoproterenol, was 47.8 ± 20.8 mN/mm^2 in the control group ($n=5$) and 34.0 ± 5.5 mN/mm^2 in the TAK-044-treated groups ($n=19$). *$P<0.05$; **$P<0.01$ vs. the values for the corresponding concentrations of endothelin-3 in the absence of TAK-044 (control).

portion of the concentration–response curve for the positive inotropic effect of endothelin-1 (Endoh et al., 1996); however, it inhibited the positive inotropic effect of endothelin-1 at 3×10^{-10} M and lower, and it enhanced the positive inotropic effect of endothelin-1 at concentrations of 10^{-8} M and higher. To clarify whether FR139317 could inhibit the positive inotropic effect of endothelin-1, very high concentrations of FR139317 were applied. At a concentration of 10^{-4} M, FR139317 was able to shift the concentration–response curve for endothelin-1 to the right (Fig. 3).

3.3. Effects of FR139317 and BQ-788 in combination on the positive inotropic effects of endothelin-1 and endothelin-3

FR139317 at 10^{-6} M inhibited the positive inotropic effect of endothelin-1 at lower concentrations but enhanced the positive inotropic effect of endothelin-1 at higher concentrations (Fig. 4A), which is consistent with previous findings (Endoh et al., 1996), while BQ-788 at 10^{-6} M shifted the concentration–response curve for the positive inotropic effect of endothelin-1 to the left (Fig. 4B). FR139317 and BQ-788 in combination shifted the concentration–response curve for endothelin-1 to the left and upward to an even greater extent (Fig. 4C), an indication that the enhancing effects of FR139317 and BQ-788 on the positive inotropic effect of endothelin-1 might be additive.

In contrast, FR139317 at 10^{-6} M shifted the concentration–response curve for endothelin-3 to the right (Fig. 4D), while BQ-788 at 10^{-6} M had no effect on the positive inotropic effect of endothelin-3 (Fig. 4E). The effects of FR139317 and BQ-788 in combination on the positive inotropic effect of endothelin-3 were the same as those of FR139317 by itself (Fig. 4F).

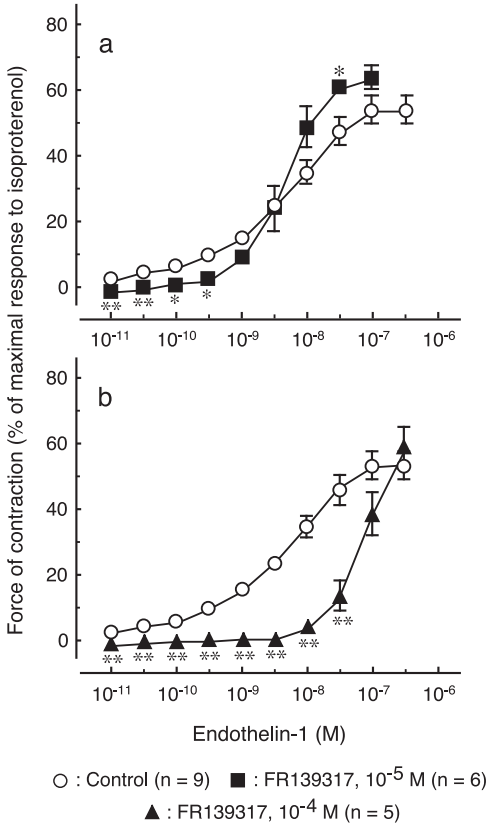

Fig. 3. Influence of very high concentrations of FR139317 on the positive inotropic effect of endothelin-1 in isolated rabbit papillary muscles. FR139317 was allowed to act for 30 min and the concentration–response curve for endothelin-1 was determined in the presence of FR139317 at 10^{-5} M (A, $n=6$) or at 10^{-4} M (B, $n=5$). The basal force of contraction was 4.8 ± 1.2 mN/mm^2 in the control endothelin-1 group ($n=9$) and 7.1 ± 1.6 mN/mm^2 in the FR139317-treated groups ($n=11$). The maximum force of contraction, as determined by application of isoproterenol, was 29.2 ± 5.6 mN/mm^2 in the control group ($n=9$) and 37.9 ± 5.5 mN/mm^2 in the FR139317-treated groups ($n=11$). *$P<0.05$; **$P<0.01$ vs. the values for the corresponding concentrations of endothelin-1 in the absence of FR139317 (control).

3.4. Competitive displacement of the specific binding of [^{125}I]endothelin-1 and [^{125}I]endothelin-3

Fig. 5 shows the displacement curves for the ability of TAK-044 to compete with [^{125}I]endothelin-1 and [^{125}I]endothelin-3 for specific binding to membrane fractions isolated from the rabbit ventricular myocardium. Both displacement curves fitted best to a model with a single binding site. TAK-044 completely prevented the specific binding of [^{125}I]endothelin-1, acting in a concentration-dependent manner with a K_i of 1.44 ± 0.32 nM ($n=3$), while it interfered with the binding of [^{125}I]endothelin-3 with a K_i of 5.17 ± 0.89 nM ($n=3$). Thus, the results of the receptor binding assay indicated that TAK-044 was about four times more potent in antagonizing the specific binding of [^{125}I]endothelin-1 than it was in antagonizing the specific binding of [^{125}I]endothelin-3.

4. Discussion

In isolated rabbit right ventricular papillary muscle, TAK-044 antagonized the positive inotropic effect of endothelin-1 in a concentration-dependent manner (Fig. 1). In previous studies, all antagonists of endothelin receptors examined, including selective antagonists of endothelin ET_A receptors, such as BQ-123 (Kasai et al., 1994) and FR139317 (Endoh et al., 1996), and a nonselective antagonist of endothelin receptors, PD 145065 (Norota and Endoh, 1996), abolished the positive inotropic effect of endothelin-3 but failed to affect the main portion of the concentration–response curve for endothelin-1 at concentrations that have been shown to have a selective antagonistic effect on the responses mediated by endothelin receptors in various other tissues (Douglas et al., 1994; Haynes et al., 1993; Rubanyi and Polokoff, 1994).

The ability of TAK-044 to inhibit the positive inotropic effect of endothelin-1 was, however, 100 times less than that to block the positive inotropic effect of endothelin-3

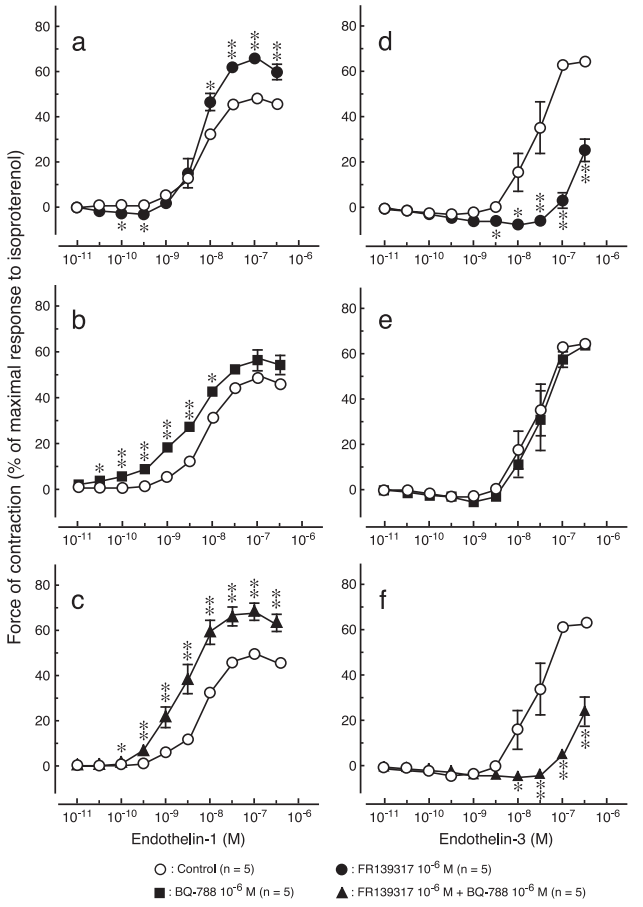

Fig. 4. Influence of FR139317 and of BQ-788, alone and in combination, on the positive inotropic effects of endothelin-1 and endothelin-3 in isolated rabbit papillary muscles. FR139317 and/or BQ-788 were allowed to act for 30 min and the concentration–response curve for endothelin-1 or for endothelin-3 was determined from the data obtained in their presence, as follows: FR139317 10^{-6} M (A, endothelin-1; $n=5$); BQ-788 10^{-6} M (B, endothelin-1; $n=5$); FR139317 10^{-6} M + BQ-788 10^{-6} M (C, endothelin-1; $n=5$); FR139317 10^{-6} M (D, endothelin-3; $n=5$); BQ-788 10^{-6} M (E, endothelin-3; $n=5$); FR139317 10^{-6} M + BQ-788 10^{-6} M (F, endothelin-3; $n=5$). Five experiments were performed to generate the control concentration–response curves for endothelin-1 or endothelin-3, respectively. The basal force of contraction in the endothelin-1 series was 8.0 ± 1.9 mN/mm^2 in the control endothelin-1 group ($n=5$), 6.3 ± 1.3 mN/mm^2 in the FR139317-treated group ($n=5$), 11.4 ± 4.5 mN/mm^2 in the BQ-788-treated group ($n=5$) and 5.6 ± 1.5 mN/mm^2 in the FR139317 + BQ-788-treated group ($n=5$); and in the endothelin-3 series it was 8.5 ± 1.6 mN/mm^2 in the control endothelin-3 group ($n=5$), 9.5 ± 2.0 mN/mm^2 in the FR139317-treated group ($n=5$), 10.4 ± 2.2 mN/mm^2 in the BQ-788-treated group ($n=5$) and 8.1 ± 1.6 mN/mm^2 in the FR139317 + BQ-788-treated group ($n=5$). The maximum force of contraction, as determined by application of isoproterenol, in the endothelin-1 series was 33.4 ± 4.8 mN/mm^2 in the control endothelin-1 group ($n=5$), 32.2 ± 2.7 mN/mm^2 in the FR139317-treated group ($n=5$), 36.1 ± 10.1 mN/mm^2 in the BQ-788-treated group ($n=5$) and 28.2 ± 7.7 mN/mm^2 in the FR139317 + BQ-788-treated group ($n=5$) and in the endothelin-3 series it was 36.5 ± 4.2 mN/mm^2 in the control endothelin-3 group ($n=5$), 40.0 ± 4.7 mN/mm^2 in the FR139317-treated group ($n=5$), 45.5 ± 6.1 mN/mm^2 in the BQ-788-treated group ($n=5$) and 36.6 ± 4.1 mN/mm^2 in the FR139317 + BQ-788-treated group ($n=5$). $^*P<0.05$; $^{**}P<0.01$ vs. the values at corresponding concentrations for the agonist in the absence of selective antagonists of endothelin receptors (control).

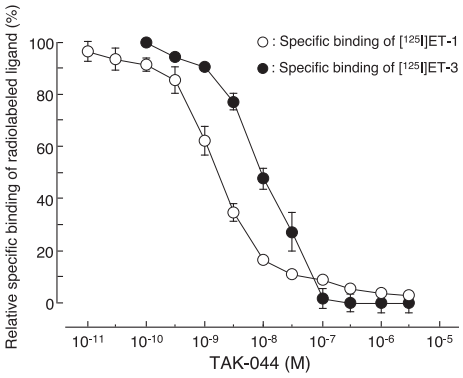

Fig. 5. Displacement curves showing competition between TAK-044 and [^{125}I]endothelin-1 and [^{125}I]endothelin-3 for specific binding to receptors in membrane fractions isolated from the rabbit ventricular myocardium. Values are presented as means ± S.E.M. of data from three experiments, in each of which the experiments were carried out in duplicate. Ordinate: binding of [^{125}I]endothelin-1 or [^{125}I]endothelin-3, expressed as a percentage of the specific binding in the absence of the antagonist. Abscissa: molar concentration of TAK-044 on a logarithmic scale. The concentrations of [^{125}I]endothelin-1 and [^{125}I]endothelin-3 used in the experiments were 200 and 40 pM. Relative levels of specific binding in this series were $96.2 \pm 0.5\%$ for [^{125}I]endothelin-1 and $53.4 \pm 4.7\%$ for [^{125}I]endothelin-3 ($n=3$, each).

(Fig. 2), indicating that the response to endothelin-1 is less sensitive to TAK-044 than the response to endothelin-3. We examined the effects of a very high concentration of FR139317, a selective antagonist of endothelin ET_A receptors, and found that, at 10^{-4} M, FR139317 shifted the concentration–response curve for endothelin-1 to the right (Fig. 3), an indication that the response to endothelin-1 is approximately 100 times less sensitive to FR139317 than is the response to endothelin-3 (Endoh et al., 1996).

A question arises about the nature of the factors that contribute to the effects of endothelin-1 in rabbit papillary muscle and that render the inotropic response to endothelin-1 so much less sensitive to TAK-044 and FR139317. It is likely that the molecular structures of endothelin receptors in the rabbit differ from those in other species, such as the human and the rat, that have been cloned (Arai et al., 1990; Sakurai et al., 1990; Goto et al., 1996). The cloning of rabbit endothelin receptors revealed that the deduced amino acid sequences of rabbit endothelin receptors are largely similar to those of other species, such as the human and the rat (Yomogida et al., 2004). Moreover, with respect to the binding characteristics of endothelin receptors, the radioligand-binding assay revealed that antagonists of endothelin receptors, including BQ-123, FR139317 and other unlabeled ligands, are able effectively to interfere with the specific binding of [^{125}I]endothelin-1, having a high affinity for its receptors (Kasai et al., 1994; Endoh et al., 1996). Likewise, TAK-044 interfered with the specific binding of [^{125}I]endothelin-1 to its receptors, exhibiting an even higher affinity than it showed for the binding of [^{125}I]endothelin-3 (Fig. 5).

The finding that TAK-044 antagonized the effect of endothelin-1 whereas the combination of FR139317 and BQ-788 failed to mimic the inhibitory action of TAK-044 could be explained by differences in potency and differences in the balance between endothelin ET_A and ET_B receptor blockade between the two treatments. In the receptor-binding studies, the IC_{50} value of TAK-044 for endothelin ET_A and ET_B receptors was 0.082 and 120 nM, respectively (Kikuchi et al., 1994), while the K_i value of FR139317 for endothelin ET_A receptors was 1 nM (Aramori et al., 1993) and that of BQ-788 for endothelin ET_B receptors was 1.2 nM (Ishikawa et al., 1994). In the current study, the IC_{50} value of TAK-044 to antagonize the positive inotropic effect of endothelin-1 was 100 nM, while the combination of FR139317 at 1 μM and BQ-788 at 1 μM was without an inhibitory effect. Based on the comparison of these values, it is more likely that the low potency of FR139317 is the reason for the failure of the combined treatment rather than the difference in balance between endothelin ET_A and ET_B receptor blockade between the two treatments. Actually, FR139317 alone at 100 μM shifted the concentration–response curve for endothelin-1 to the right (Fig. 3).

The activation of endothelin receptors by endothelin-1 triggers a series of signal transduction processes that are coupled to inhibition of the endothelin-1-induced positive inotropic effect, because the maximum inotropic response to endothelin-1 was enhanced by a low concentration of TAK-044 at 10^{-8} M and by FR139317 at 10^{-6} M. The positive inotropic effect of endothelin-1 was also enhanced by the selective endothelin ET_B receptor antagonist BQ-788 and, to a greater extent, by FR139317 and BQ-788 in combination (Fig. 4). These results imply that endothelin-1 might activate the endothelin ET_B receptors that are associated with the negative inotropic effect. However, since BQ-788 had no effect on the positive inotropic effect of endothelin-3, it is unlikely that endothelin-3 activates the subtype of receptor that is coupled to the negative inotropic effect, which does not fit the general characteristics of endothelin ET_B receptors (Masaki et al., 1991; Rubanyi and Polokoff, 1994). It is more likely that BQ-788 potentiated the response to endothelin-1, because the endothelin ET_B receptor is a clearance receptor for endothelin-1: the free concentration of endothelin-1 stimulating the endothelin ET_A receptor may be higher in the presence of endothelin ET_B receptor blockade, and thereby the positive inotropic response may be enhanced. Inhibition of the process that is coupled to the negative inotropic effect induced by endothelin-1 mediated by endothelin ET_{A1} receptors and the clearance endothelin ET_B receptor may contribute, in part, to the resistance of the endothelin-1-induced positive inotropic effect to antagonists such as TAK-044 and FR139317 or BQ-123 (Kasai et al., 1994), and combined FR139317 and BQ-788 (present study).

The isolated papillary muscle includes not only myocardial cells but also non-myocardial cells, such as endocardial and vascular endothelial cells, and fibroblasts. Thus, it is possible that endothelin receptors that respond to endothelin-1 and mediate the inhibitory effect might exist in non-myocardial cells. Comparison of the concentration–response curve for the positive inotropic effect of endothelin-1 in isolated cardiomyocytes with that in papillary muscles provides some insight into differences in signaling processes involved in this phenomenon. In this context, it is noteworthy that the concentration–response curve for endothelin-1 in single myocytes was shifted several 10 times to the left of the curve obtained with isolated papillary muscles (Talukder et al., 2001; Endoh et al., 1998a,b). The difference between the potency of endothelin-1 in single cells and in papillary muscles implies a major contribution of non-myocardial cells to the inotropic response to endothelin-1 in the rabbit papillary muscle. However, it is unlikely that a difference in accessibility to receptors plays a key role because the CRCs for endothelin-3 and isoproterenol were not very different when single-cell and multicellular preparations were examined (Talukder et al., 2001). Furthermore, even in single myocytes, endothelin receptor antagonists, such as BQ-485 (endothelin ET_A receptor selective) and BQ-788 (endothelin ET_B receptor selective) enhanced the increase in cell shortening and Ca^{2+} transients induced by endothelin-1 (Talukder et al., 2001).

The density of endothelin receptors, as determined by the specific binding of [^{125}I]endothelin-1 and of [^{125}I]endothelin-3, is extremely high (several hundred fmol/mg protein) and is even higher than that of adrenoceptors in the rabbit ventricular myocardium (Takanashi and Endoh, 1991; Yang and Endoh, 1997). The presence of spare receptors and inhibitory regulatory mechanisms might be responsible for the characteristic biphasic profile of the concentration–response curve for the positive inotropic effect of endothelin-1 and the apparent resistance of the endothelin-1-mediated positive inotropic effect to TAK-044 in rabbit papillary muscle.

In summary, TAK-044 inhibits the positive inotropic effect induced by endothelin-1 in the rabbit ventricular myocardium. A concentration of TAK-044 that was 30–100 times higher than that required to inhibit the positive inotropic effect of endothelin-3 was necessary to inhibit the effect of endothelin-1. The subcellular mechanisms for the resistance of the endothelin-1-induced positive inotropic effect, however, remain for future study.

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