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Binding characterization of [^{3}H]S-0139, an antagonist of the endothelin ET_A receptor subtype

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Abstract

S-0139 (27-O-3-[2-(3-carboxy-acryloylamino)-5-hydroxyphenyl]-acryloyloxy myricerone, sodium salt) is a highly specific nonpeptide endothelin ET_A receptor antagonist. The binding of [^{3}H]S-0139 was compared to that of [^{125}I]endothelin-1 to characterize the binding of the antagonist in porcine aortic smooth muscle membranes. Scatchard analysis revealed a single class of [^{3}H]S-0139 binding sites with a K_d value of 0.61 ± 0.10 nM and a B_{max} of 0.72 ± 0.16 pmol/mg protein. These sites were saturable and reversible. [^{125}I]Endothelin-1 also showed binding with high affinity ($K_d = 0.12 \pm 0.02$ nM) to a homogenous population of binding sites, whose B_{max} (0.71 ± 0.20 pmol/mg protein) was almost the same as that for [^{3}H]S-0139. In both cases, the binding could be displaced by known endothelin receptor ligands and their IC_{50} values in each case showed a very close correlation ($r = 0.986$). The potency of seven endothelin receptor antagonists to displace [^{3}H]S-0139 binding also correlated highly to the potency for inhibiting the endothelin-1-induced increase in cytosolic Ca^{2+} concentration ($r = 0.949$). Myriceric acid A showed a more potent functional activity than expected from its binding affinity, but this seemed to result from the different assay conditions, such as incubation time. Together, the results suggest that S-0139 labels only endothelin ET_A receptor binding sites in porcine aortic smooth muscle. © 1998 Elsevier Science B.V.

Keywords: Endothelin; Endothelin ET_A receptor; S-0139; Receptor antagonist; Smooth muscle, aortic

1. Introduction

Endothelin-1 was isolated initially from a conditioned medium of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988) and has been shown to play a pathophysiologic role in cardiovascular disease (Rubanyi and Polokoff, 1994; Yanagisawa, 1994). The binding profile of endothelin-1 and related peptides has revealed two types of endothelin receptors, ET_A and ET_B (Sakurai et al., 1992). The hallmark of endothelin activity is its potent and long-lasting constrictor effect on the vasculature via endothelin ET_A receptors on smooth muscle cells. On the other hand, endothelin ET_B receptors on endothelial cells mediates transient vasodilation via synthesis of nitroxide and/or prostacyclin (Rubanyi and Polokoff, 1994). These findings suggested that endothelin ET_A receptor-selective antagonists might be useful as therapeutic agents for some cardiovascular diseases (Doherty, 1996; Webb et al., 1996).

S-0139 (27-O-3-[2-(3-carboxy-acryloylamino)-5-hydroxyphenyl]-acryloyloxy myricerone, sodium salt, formerly called 97-139) is a potent nonpeptide endothelin ET_A receptor-selective antagonist (Mihara et al., 1994). It was obtained by chemical modification of myriceric acid A (50-235), which had been isolated from the bark of the bayberry, *Myrica cerifera* (Fujimoto et al., 1992; Mihara and Fujimoto, 1993; Mihara et al., 1993; Maguire et al., 1994; Sakurawi et al., 1996). In the present study, we characterized the binding of [^{3}H]S-0139 in comparison to that of [^{125}I]endothelin-1 in porcine aortic smooth muscle membranes which contain only endothelin ET_A receptors.

2. Materials and methods

2.1. Binding of [^{3}H]S-0139 and [^{125}I]endothelin-1

Porcine aortic smooth muscle membranes were prepared as described previously (Mihara et al., 1988). Ligand binding experiments with [^{3}H]S-0139 and [^{125}I]endothelin-1 were performed with a slight modification of previously reported procedures (Fujimoto et al., 1992). [^{3}H]S-0139 binding was studied with concentrations of the radioligand as indicated and 360 μg of membrane protein in 1.0 ml Tris HCl buffer (50 mM, pH 7.4) containing 0.1 mM

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phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin A, 250 μg/ml of bacitracin and 10 μg/ml of soybean trypsin inhibitor in the absence or presence of 1 μM cold S-0139. For competition binding experiments, 1 nM [^{3}H]S-0139 was used with 3 or 4 concentrations of endothelin receptor ligands including concentrations both lower and higher than that inhibiting [^{3}H]S-0139 binding by 50% (IC_{50}). For saturation experiments, various concentrations (0.1–2.1 nM) of [^{3}H]S-0139 were used. The incubation was performed at 37°C for 60 min, unless otherwise noted, and terminated by dilution with 2.5 ml of ice-cold 50 mM Tris buffer (pH 7.4) and filtering through Whatman glass fiber filters (Whatman International, Maidstone, UK) presoaked in 1% polyethleneimine. The filters were washed four times (2.5 ml each) with the same buffer and counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 1 μM unlabeled S-0139.

[^{125}I]Endothelin-1 binding was carried out according to the above-mentioned method except that the membranes (4.4 μg) were incubated with 25 pM [^{125}I]endothelin-1 in 0.1 ml of the reaction mixture for competition experiments. Saturation experiments were performed with 3.6–320 pM [^{125}I]endothelin-1. Nonspecific binding was determined in the presence of 0.1 μM unlabeled endothelin-1.

Binding studies with membranes from rat cerebellum and CHO cells with cloned human endothelin receptors were performed basically as mentioned above. The fraction between $12\,000 \times g$ and $100\,000 \times g$ of the cerebellar homogenate prepared in 250 mM sucrose was used as the cerebellar membrane fraction. Membrane preparations of Chinese hamster ovary (CHO) cell lines with cloned human endothelin receptor subtypes were used according to the manufacturer's instructions.

2.2. Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured fluorometrically, using the Ca^{2+}-sensitive fluorescent dye, fura-2. Rat aortic smooth muscle A7r5 cells were obtained from Dainippon Seiyaku (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO), 10 mM HEPES buffer (pH 7.4), 50 μg/ml of streptomycin and 50 U/ml of penicillin G (GIBCO) in a 5% CO_2–95% air incubator at 37°C. Confluent A7r5 cells were dispersed with 0.025% trypsin/1 mM EDTA. Cell suspensions were washed once with the growth medium. Single cells were counted and suspended in HEPES (20 mM)-buffered Hanks' solution (pH 7.4). The cell suspensions (10^6/ml) were incubated with 2 μM fura-2-acetoxymethyl (fura-2-AM) at 37°C for 30 min. The fura-2-loaded cells thus obtained were resuspended in HEPES-buffered Hanks' solution at 5×10^5 cells/ml and the suspension (0.3 ml) was continuously stirred in a cuvette (50 × 7 mm diameter). Antagonists in 1 μl of dimethylsulfoxide were added at 1 min, unless otherwise mentioned, before the addition of endothelin-1 in 3 μl of 0.1% bovine serum albumin. Fluorescence was measured with a spectrofluorometer (CAF-100, Japan Spectroscopy, Tokyo, Japan) as described previously (Mihara et al., 1989).

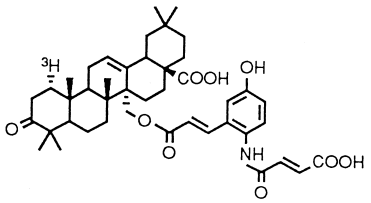

Fig. 1. Chemical structure of [^{3}H]S-0139.

2.3. Materials

S-0139 was supplied by Dr. Toshiro Konoike and tritiated by Dr. Toru Nagasaki in our laboratories (Fig. 1). Specific activity of [^{3}H]S-0139 was 162 GBeq/mmol. Myriceric acid A (50-235) was supplied by Dr. Kensuke Sakurawi (Sakurawi et al., 1996). BQ-123 (cyclo[D-Trp–D-Asp–Pro–D-Val–Leu]) (Ihara et al., 1992) was synthesized by Kunio Watanabe in our laboratories. Bosentan (4-tert-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide) (Clozel et al.,1994), SB209670 ((+)-1*S*,2*R*,3*S*)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid]) (Nambi et al.,1994) and L-749329 (3′,4′-methylenedioxy-1-(2-propyl-4-carboxyphenoxy)-*N*-(4-isopropyl-phenylsulfonyl)-benzene acetamide) (Walsh, 1995) was synthesized by Dr. Toshiro Konoike and Dr. Teruo Yamamori in our laboratories. Endothelin-1 and endothelin-3 were purchased from the Peptide Institute (Osaka, Japan). BQ-788 (*cis*-2,6-dimethyl-piperidinocarbonyl-methylleucyl-D-Trp(1-CO_2CH_3)-D-Nle-ONa) (Ishikawa et al., 1994) were from the American Peptide Company (Sunnyvale, CA). [^{125}I]Endothelin-1 (81.4 TBeq/mmol) was obtained from Amersham. Fura-2-AM was from Dojin (Kumamoto, Japan). Membranes of CHO cells with cloned human endothelin ET_A and ET_B receptors were obtained from Du Pont/NEN Research Products.

3. Results

3.1. Binding of [^{3}H]S-0139 and [^{125}I]endothelin-1

The binding of [^{3}H]S-0139 reached a plateau within 15 min and remained stable for at least 1 h (Fig. 2A). The observed association rate constant (k_{obs}), determined from the slope of the pseudo-first-order plot (Fig. 2B), was found to be 0.249 ± 0.039 min^{-1} ($n = 3$). The dissociation rate constant was determined from the time course of the displacement of [^{3}H]S-0139 from its binding site induced by 1 μM S-0139. A rapid and exponential decrease in the

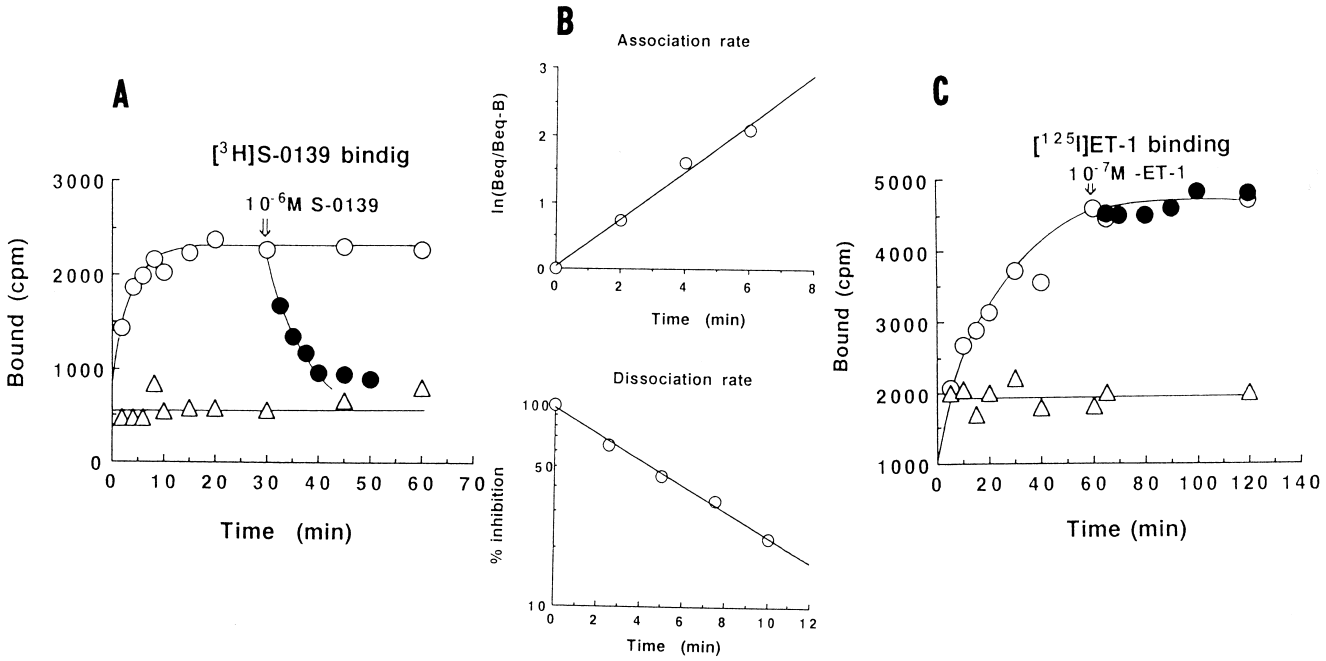

Fig. 2. Time courses of association and dissociation of [^{3}H]S-0139 binding (A), which were linearized (B), and [^{125}I]endothelin-1 binding (C) to porcine aortic smooth muscle membranes. For [^{3}H]S-0139 binding, the membranes were incubated with 1 nM [^{3}H]S-0139 and aliquots of reaction mixtures were pipetted off at the indicated times for the binding assay (○). At 60 min, 10^{-6} M S-0139 was added to initiate dissociation (●). Nonspecific binding was obtained in the presence of 10^{-6} M S-0139 (△). For the [^{125}I]endothelin-1 binding, 25 pM [^{125}I]endothelin-1 and 10^{-7} M endothelin-1 were used in place of [^{3}H]S-0139 and 10^{-6} M S-0139.

binding of [^{3}H]S-0139 was observed with time (Fig. 2A). As shown in Fig. 2B, linear transformation of the data yielded a k_{-1} of 0.132 ± 0.027 min^{-1} ($n = 3$). The association rate constant (k_{+1}) determined from the equation $k_{+1} = (k_{obs} - k_{-1})/[L]$ was found to be 0.113 ± 0.014 nM^{-1} min^{-1}. The kinetically determined dissociation constant (K_d) given by $K_d = k_{-1}/k_{+1}$ was found to be 1.2 ± 0.3 nM.

The binding of [^{125}I]endothelin-1 reached a plateau in 1 h and remained stable for at least 2 h (Fig. 2C). In contrast to the displacement of [^{3}H]S-0139 binding, excess cold endothelin-1 did not induce the displacement of [^{125}I]endothelin-1 from its binding site.

Equilibrium binding studies were performed by incubating the aortic membranes with increasing concentrations of [^{3}H]S-0139 and [^{125}I]endothelin-1. Scatchard analysis of the whole of binding data showed that the membranes had a single class of specific binding sites for each ligand with an apparent K_d of 0.61 ± 0.10 nM and maximal binding capacity (B_{max}) of 0.72 ± 0.16 pmol/mg protein ($n = 3$)

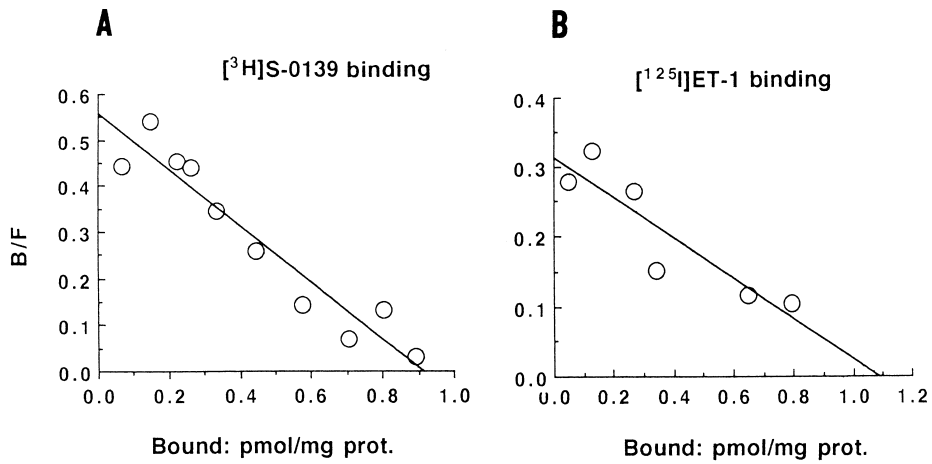

Fig. 3. Scatchard plot analysis of [^{3}H]S-0139 (A) and [^{125}I]endothelin-1 binding (B) to porcine aortic smooth muscle membranes. Plots, which were transformed from the saturation curve of each specific binding, represent the mean values of duplicate determinations and are representative of those from three separate experiments.

Table 1
Binding of [^{3}H]S-0139 and [^{125}I]endothelin-1 in porcine aortic smooth muscle membranes and rat cerebellar membranes

	Binding, fmol/mg protein	
	[^{3}H]S-0139 (1 nM)	[^{125}I]endothelin-1 (25 pM)
Aortic smooth muscle	379 ± 37	87 ± 8
Cerebellum	N.D.	309 ± 54

Results are means ± S.D. from three experiments. N.D., not detected.

for [^{3}H]S-0139 and an apparent K_d of 0.12 ± 0.02 nM and B_{max} of 0.71 ± 0.20 pmol/mg protein ($n = 3$) for [^{125}I]endothelin-1 (Fig. 3).

3.2. Endothelin ET_A receptor selectivity of [^{3}H]S-0139

S-0139 is a highly selective endothelin ET_A receptor antagonist as described previously (Mihara et al., 1994). To confirm this, we studied [^{3}H]S-0139 binding in rat cerebellum, which is known to have only endothelin ET_B binding sites (Elshourbagy et al., 1992). Under conditions where [^{125}I]endothelin-1 binding in cerebellar membranes was about 4-fold greater than that in aortic smooth muscle, no [^{3}H]S-0139 binding was detected in cerebellar membranes (Table 1). This conclusion was confirmed with binding studies using cloned human endothelin receptor subtypes. In short, [^{3}H]S-0139 bound to membranes prepared from CHO cell lines with cloned human endothelin ET_A receptors with an apparent K_d of 0.68 ± 0.13 nM ($n = 3$), but did not bind at all to membranes prepared from CHO cell lines with cloned human endothelin ET_B receptors (data not shown).

3.3. Inhibition of [^{3}H]S-0139 and [^{125}I]endothelin-1 binding by endothelin receptor ligands

Endothelin-1 completely inhibited [^{3}H]S-0139 binding with an IC_{50} of 0.49 ± 0.16 nM, while S-0139 completely inhibited [^{125}I]endothelin-1 binding with an IC_{50} of 2.2 ± 0.8 nM (Fig. 4). Several endothelin receptor ligands were examined for their ability to inhibit the specific binding of [^{3}H]S-0139 and [^{125}I]endothelin-1 to membranes (Table 2). The IC_{50} values of ligands for inhibition of [^{3}H]S-0139 binding were very close to the corresponding IC_{50} values

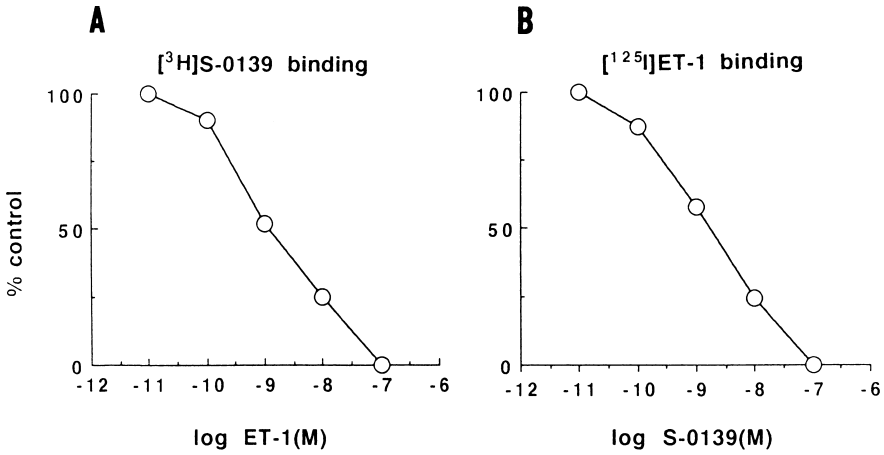

Fig. 4. Effect of endothelin-1 and S-0139 on [^{3}H]S-0139 (A) and [^{125}I]endothelin-1 binding (B) to porcine aortic smooth muscle membranes, respectively. Symbols represent the mean values of duplicate determinations and are representative of those from three separate experiments.

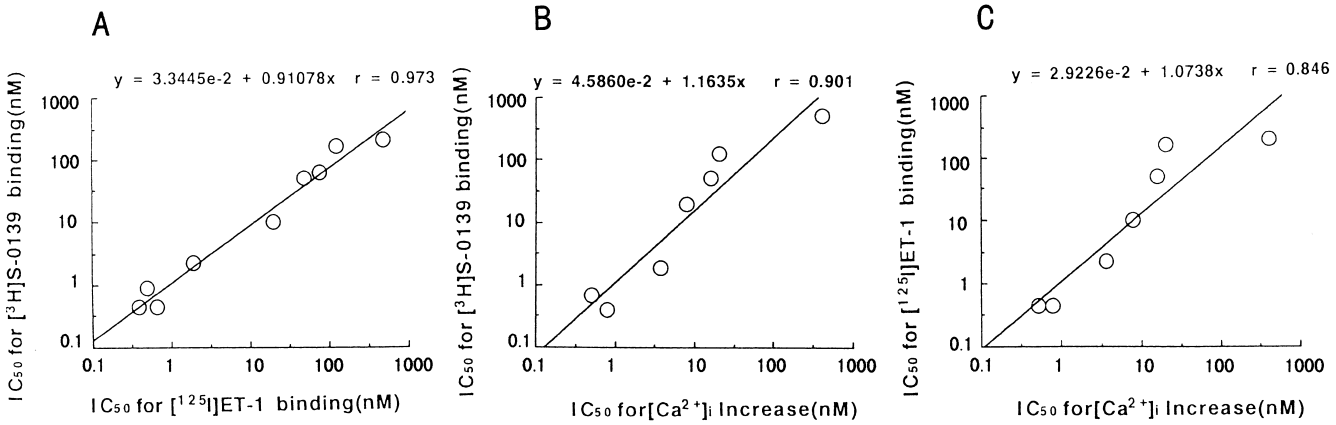

Fig. 5. Correlation of IC_{50} values of antagonists for the inhibition of [^{3}H]S-0139 binding and their IC_{50} values for the inhibition of [^{125}I]endothelin-1 binding (A) or endothelin-1 induced Ca^{2+} mobilization (B) and correlation between their IC_{50} values for the inhibition of [^{125}I]endothelin-1 binding and for that of endothelin-1-induced Ca^{2+} mobilization (C).

Table 2
Inhibition by endothelin receptor ligands of [^{3}H]S-0139 and [^{125}I]endothelin-1 binding and endothelin-1-induced increase in $[Ca^{2+}]_i$

	IC_{50} (nM)		$[Ca^{2+}]_i$
	Binding		
	[^{3}H]S-0139	[^{125}I]endothelin-1	
Endothelin-1	0.49 ± 0.16	0.90 ± 0.20	—
Endothelin-3	80 ± 22	57 ± 17	—
S-0139	1.9 ± 0.4	2.2 ± 0.8	3.6 ± 0.4
Myriceric acid A	120 ± 50	160 ± 50	19 ± 4
SB209670	0.39 ± 0.08	0.44 ± 0.03	0.79 ± 0.05
BQ-123	54 ± 4	52 ± 24	16 ± 5
Bosentan	18 ± 5	10 ± 3	7.9 ± 1.9
L-749329	0.68 ± 0.10	0.45 ± 0.05	0.51 ± 0.04
BQ-788	480 ± 90	210 ± 50	390 ± 17

Results are means ± S.D. from three experiments.

for inhibition of [^{125}I]endothelin-1 binding and both values correlated well with each other ($r = 0.986$) (Fig. 5A).

3.4. Endothelin-1-induced Ca^{2+} mobilization

As described previously (Mihara et al., 1994), endothelin-1 induced a concentration-dependent increase in $[Ca^{2+}]_i$ in rat aortic smooth muscle A7r5 cells. Seven endothelin receptor antagonists suppressed the endothelin-1 (1 nM)-induced increase in $[Ca^{2+}]_i$ concentration dependently; the IC_{50} values are listed in Table 2. The rank order of potency for this inhibition, L-749329 = SB209670 > S-0139 > Bosentan > BQ-123 > myriceric acid A > BQ-788, was similar to the orders of the affinities of these antagonists for binding sites of [^{3}H]S-0139 and [^{125}I]endothelin-1 (Table 2). The correlation coefficient between IC_{50} values for [^{3}H]S-0139 binding and endothelin-1-induced Ca^{2+} mobilization was 0.949 (Fig. 5B). A correlation was also found between IC_{50} values for [^{125}I]endothelin-1 binding and endothelin-1-induced Ca^{2+} mobilization ($r = 0.920$, Fig. 5C). However, the correlation coefficient between binding affinity and functional activity was not as high as that between binding affinities.

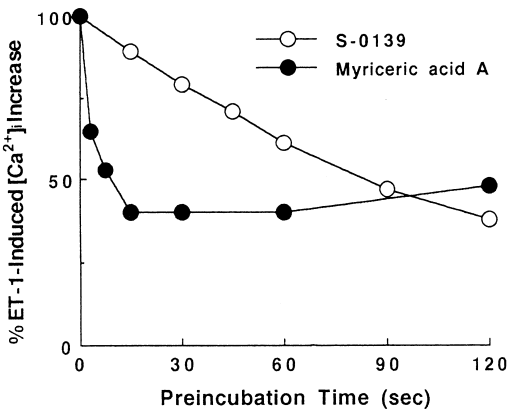

Fig. 6. Preincubation duration-dependent inhibition of endothelin-1-induced Ca^{2+} mobilization by S-0139 (○) and myriceric acid A (●). Endothelin-1 (10^{-8} M) was added at the times indicated, before the addition of S-0139 (10^{-8} M) or myriceric acid A (10^{-7} M).

As shown in Table 2, myriceric acid A inhibited the endothelin-1-induced increase in $[Ca^{2+}]_i$ more effectively than expected from the binding affinity (about 6-fold), while S-0139 was 2-fold less effective to displace the binding of radiolabeled ligands than to inhibit Ca^{2+} mobilization. The reason for this discrepancy was not clear, but we thought that it might depend on the different assay conditions, such as the incubation time (1 min for Ca^{2+} mobilization versus 60 min for binding). Thus we studied the effect of preincubation time on the inhibitory activity of antagonists for the endothelin-1-induced Ca^{2+} transient. Fig. 6 shows that the inhibitory activity of myriceric acid A reached a maximum very rapidly (15 s), while that of S-0139 still had not reached its maximum at 120 s.

4. Discussion

Endothelin receptors in porcine aortic smooth muscle membranes were only of the ET_A-type because endothelin-3 and S-0139 gave monophasic displacement curves against [^{125}I]endothelin-1 binding. There were neither high-affinity binding sites for endothelin-3 nor low-affinity binding sites for S-0139.

[^{3}H]S-0139 labeled only functional endothelin receptor binding sites in porcine aortic smooth muscle membranes, as shown by the following results. First, Scatchard analysis revealed that there was a single class of saturable and reversible [^{3}H]S-0139 binding sites whose B_{max} (0.72 ± 0.16 pmol/mg protein) was almost the same as that for [^{125}I]endothelin-1 (0.71 ± 0.20 pmol/mg protein). Second, [^{3}H]S-0139 binding was completely displaced by endothelin-1, while [^{125}I]endothelin binding was completely displaced by S-0139. Third, the K_d value of [^{3}H]S-0139 (0.61 ± 0.10 nM) was close to the K_i value of S-0139 for [^{125}I]endothelin binding (1.9 ± 0.7 nM), which was obtained from the IC_{50} values in Table 1. Fourth, binding in both cases was displaced by known endothelin receptor ligands and the two IC_{50} values showed a very close correlation. Finally, the IC_{50} values of seven endothelin receptor antagonists for [^{3}H]S-0139 binding showed correlation to their potencies to inhibit the endothelin-1-induced increase in cytosolic Ca^{2+} concentration. These results indicate that [^{3}H]S-0139 binds to the endothelin binding sites on the functional endothelin receptor.

As mentioned above, the affinity of antagonists for binding sites showed a significant correlation with their potency to inhibit the endothelin-1-induced increase in $[Ca^{2+}]_i$, but the correlation ($r = 0.949$ and $r = 0.920$) was not higher than that between their affinities for binding sites of [^{3}H]S-0139 and binding sites of [^{125}I]endothelin-1 ($r = 0.986$). In particular, myriceric acid A showed a highly potent functional activity compared with its binding affinity. This may have resulted from the differences in assay conditions. Binding affinity is the ratio of association and dissociation rate constants obtained under equilibrium conditions. Functional activity is obtained when cells are

preincubated with an antagonist for 1 min before the challenge with endothelin-1. Therefore, it is influenced more by the association rate constant because of the short preincubation time. The conditions for $[Ca^{2+}]_i$ experiments may favor antagonists with high association rate constants, while the conditions for binding studies may be advantageous for antagonists with low dissociation rate constants. To examine these possibilities, we studied the effect of the preincubation period on the functional activity of S-0139 and myriceric acid A (Fig. 6). The finding that the inhibitory activity of myriceric acid A for the endothelin-1-induced Ca^{2+} transient reached a maximum more rapidly than that of S-0139 suggests that myriceric acid A has a high association rate constant, which is consistent with the result that myriceric acid A inhibited the endothelin-1-induced increase in $[Ca^{2+}]_i$ more effectively than expected from its binding affinity. S-0139 was obtained by introducing a carboxy-acryloylamino group into myriceric acid A (Fujimoto et al., 1992). This modification probably gave rise to a lower dissociation rate from the receptor binding site, which is suggested by the higher binding affinity of S-0139 for the endothelin ET_A receptor.

The dissociation rate constant of an antagonist is an important factor for estimating whether it is short-acting or long-acting. Kinetic studies on the interaction of a radiolabeled antagonist with the receptor should yield an accurate dissociation rate constant. However, if there is no radiolabeled antagonist available, comparison of an antagonist's potency for inhibiting an endothelin-1-induced increase in $[Ca^{2+}]_i$ with its binding affinity may be useful for estimating its dissociation rate constant.

In conclusion, S-0139 is a potent nonpeptide endothelin antagonist with high selectivity for the endothelin ET_A receptor subtype. The antagonist displays a higher affinity than the parent compound, myriceric acid A, after introduction of a carboxy-acryloylamino group. This may be a consequence of the slower rate of dissociation of S-0139 from the endothelin ET_A receptor.

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