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Pharmacological characterization of endothelin-induced contraction in the guinea-pig oesophageal muscularis mucosae

[1,2]Kohsuke Uchida, [1]Rika Yuzuki & [1]Yuichiro Kamikawa

[1]Department of Pharmacology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

1 In the oesophageal muscularis mucosae, we examined the effects of endothelin-1 (ET-1), endothelin-2 (ET-2), endothelin-3 (ET-3) and sarafotoxin S6c (SX6c) as agonists, and FR139317, BQ-123 and RES-701-1 as endothelin receptor antagonists.

2 All of the endothelins produced tonic contractions which were frequently superimposed on rhythmic motility in a concentration-dependent manner. The order of potency ($-\log EC_{50}$) was ET-1 (8.61) = SX6c (8.65) > ET-2 (8.40) > ET-3 (8.18).

3 FR139317 (1–3 μM) and BQ-123 (1 μM) caused parallel rightward shifts of the concentration-response curve to ET-1, but at higher concentrations caused no further shift. RES-701-1 (3 μM) caused a rightward shift of the concentration-response curve to ET-1, while RES-701-1 (10 μM) had no additional effect. RES-701-1 (0.1–1 μM) concentration-dependently caused a rightward shift of the concentration-response curve to SX6c. The contraction to ET-1 (10 nM) in preparations desensitized to the actions of SX6c was greatly inhibited by pretreatment with FR139317 (10 μM).

4 Modulation of the Ca^{2+} concentration in the Krebs solution caused the concentration-response curve to ET-1 or SX6c to shift to the right and downward as external Ca^{2+} concentrations decreased. Verapamil (30 μM) abolished rhythmic motility induced by ET-1 or SX6c. Ni^{2+} (0.1 mM) weakly inhibited ET-1- or SX6c-induced tonic contraction. SK&F 96365 (60 μM) completely inhibited ET-1-induced contractions.

5 We conclude that there are two types of ET-receptors, excitatory ET_A- and ET_B-receptors in the oesophageal muscularis mucosae. These receptors mediate tonic contractions predominantly by opening receptor-operated Ca^{2+} channels (ROCs) and partly by opening T-type Ca^{2+} channels, and mediate rhythmic motility by opening L-type Ca^{2+} channels.

Keywords: Guinea-pig oesophagus; muscularis mucosae; FR139317; RES-701-1; endothelin receptor subtype; ET_A receptor; ET_B receptor; SK&F 96365; ROCs; receptor-operated Ca^{2+} channels

Introduction

ET-1 is the most potent vasoconstrictor polypeptide of mammalian origin (Yanagisawa *et al*., 1988). ET-1 also has a potent contractile activity on non-vascular smooth muscles from the respiratory, gastro-intestinal and urogenital tracts (Rae *et al*., 1995). Two ET-receptor subtypes, ET_A and ET_B, have been cloned from mammalian tissues (Ohlstein *et al*., 1996). Previous studies have indicated that the external smooth muscles of gut wall have excitatory ET_A- or ET_B-receptor subtype linking with L-type Ca^{2+} channels (Rae *et al*., 1995). In addition, the coexistence of ET_A- and ET_B-receptors have been found in the ileal smooth muscle of the guinea-pig (Yoshinaga *et al*., 1992; Warner *et al*., 1993). In the gastro-intestinal tract, ETs probably have an important physiological role in motility of the external smooth muscle layers. On the other hand, an autoradiographic study has demonstrated that ^{125}I-ET binds in the mucosal layer of the rat stomach, intestine and colon (Koseki *et al*., 1989). ETs possibly produced by mucosal epithelial cells are present in the rat gastro-intestinal tracts and cause contraction of gastro-intestinal smooth muscles (Takahashi *et al*., 1990). ETs also stimulate electrogenic Cl^- secretion in the colonic mucosa, and ET-1 induces mucosal hypoxia and ulcer (Rae *et al*., 1995). Thus, ETs are thought to have important physiological and pathophysiological roles in both absorptive and secretory functions of the gastro-intestinal mucosa. Their exact physiological functions on the mucosal layer, however, have not yet been established.

The muscularis mucosae, a thin band of smooth muscle located at the base of the gastro-intestinal mucosa, has received very little attention when compared with the external smooth muscle layers. The muscularis mucosae probably has a great influence on the absorptive and secretory functions of the mucosa (King *et al*., 1922). We have investigated the autonomic innervation and receptor systems of the muscularis mucosae in the guinea-pig oesophagus. The oesophageal muscularis mucosae is innervated chiefly by excitatory cholinergic nerves and sparsely by inhibitory adrenergic nerves, but not by non-adrenergic and non-cholinergic nerves (Kamikawa & Shimo, 1979; Kamikawa *et al*., 1982). It is already known that the receptor systems in this tissue for catecholamines, 5-hydroxytryptamine, acetylcholine and histamine are different from those in the external smooth muscles (Uchida, 1983; Uchida *et al*., 1983; Kamikawa & Shimo, 1983; Kamikawa *et al*., 1985; Fujinuma *et al*., 1985). ET-1 elicits contractions of the isolated oesophageal muscularis mucosae, but the receptor mechanisms and subtypes involved in this effect are still uncharacterized. The present experiments were designed to clarify the receptor mechanisms underlying contractions induced by ETs in the muscularis mucosae isolated from the guinea-pig oesophagus.

Methods

Preparations

After adult male guinea-pigs (250–400 g) were euthanized by anaesthetizing with overdose sevoflurane (Maruishi Pharmaceutical Co.) and exsanguinized. Then, the oesophageal body

[2] Author for correspondence.

was excised. Briefly, the excised oesophagus was pinned on a cork mat immersed in an oxygenated (95% O_2 and 5% CO_2) Krebs solution. The oesophagus was cleaned of fat and connective tissue. The outer striated muscle coat was cut longitudinally, and gently peeled away leaving an inner tube (Uchida, 1983). The tube including longitudinal muscularis mucosae was about 10–15 mm long without a load and was immersed in a 10 ml organ bath filled with a modified Krebs solution of the following composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 14, disodium edetate (EDTA) 0.03 and ascorbic acid 0.12. The Krebs solution was bubbled with 95% O_2 and 5% CO_2, and maintained at 37°C.

Measurement of contractile response

The preparation was suspended under a 0.5 g load and 60 min was allowed to elapse before experiments were started. During this equilibration period, the preparation was washed with a fresh Krebs solution every 20 min. Responses of the longitudinal muscularis mucosae were recorded by an isotonic transducer (TD-112S, Nihon Kohden Inc., Tokyo, Japan) connected to a chart recorder (RJG-4124, Nihon Kohden Inc., Tokyo, Japan). After the 60 min equilibration period, the preparation was maximally contracted with a single concentration of carbachol (10 μM) (Kamikawa *et al*., 1985) and was allowed to equilibrate for 30 min after washout. The concentration-response relationships for contractions to ETs were obtained by the cumulative dose technique. Contractions to ETs were measured as a percentage of 10 μM carbachol-induced contraction. Efficacy (E_{max}) and potency ($-\log EC_{50}$) of ETs were calculated from individual concentration-response curves. E_{max} (the maximal response of each agonist) was expressed as a percentage of 10 μM carbachol-induced contraction. EC_{50} (molar concentration eliciting 50% of E_{max}) was determined by linear interpolation for each curve. ET_A- and ET_B- antagonists, verapamil, Ni^{2+} and indomethacin were pretreated to the tissue for 20 min. The slope of Schild plot and pA_2 value were calculated by the method of Arunlakshana & Schild, (1959).

Statistics

Responses were averaged at each concentration of the agonist. The data obtained are expressed as means ± s.e.mean. Each experimental group consisted of 4–14 preparations taken from different animals. Student's *t*-test was used for statistical evaluation of the difference between two groups. Values of P less than 0.05 were considered to be significant.

Drugs

The following drugs were used: endothelin-1, endothelin-2, endothelin-3, sarafotoxin S6c (Peptide Institute, Osaka, Japan); FR139317 ((R)2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl)propionic acid) (Sogabe *et al*., 1993) (a gift from Fujisawa Pharmaceutical Co., Osaka, Japan); RES-701-1 (cyclic (Gly^1-Asp^9)(Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp)) (Tanaka *et al*., 1994) (a gift from Kyowa Hakko Kogyo Co., Tokyo, Japan); BQ-123 (cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-)) (Ihara *et al*., 1992) (Research Biochemicals Inc., Natick, U.S.A.); SK&F 96365 (BIOMOL Research Laboratories Inc., Plymouth Meeting, U.S.A.); verapamil hydrochloride (Eisai Co. Ltd., Tokyo, Japan); indomethacin, carbamylcholine chloride (carbachol) (Sigma Chemical Co., St. Louis, U.S.A.); nickel chloride hexahydrate, nicardipine hydrochloride (Wako Pure Chemical Industries Ltd., Osaka, Japan). ETs were dissolved in and diluted with 0.1% aqueous acetic acid solution. FR139317 was dissolved in equimolar 1N NaOH and diluted with physiological saline. RES-701-1 was dissolved in dimethyl sulphoxide and diluted with Krebs solution. BQ-123, nickel chloride and nicardipine were dissolved in and diluted with distilled water. SK&F 96365, verapamil and carbachol were dissolved in and diluted with physiological saline. Indomethacin was dissolved in distilled water containing equimolar Na_2CO_3 and diluted with physiological saline. We have ascertained that these solvents, at the concentrations used in the present experiments, did not affect the contractility of the muscularis mucosae by themselves. The molar concentrations of drugs described in this paper refer to the final bath concentrations.

Results

Responsiveness to ETs

Contractile responses to ETs of the muscularis mucosae isolated from the guinea-pig oesophagus consisted of a tonic contraction which was frequently superimposed on rhythmic motility (Figure 1). ET-1 (0.1–3 nM), ET-2 (0.3–3 nM), ET-3 (0.3–3 nM) and SX6c (0.1–3 nM) produced tonic contractions superimposed on rhythmic motility, but rather reduced rhythmic motility at higher concentration (10–300 nM) (Figure 1). All of ET-1, ET-2 and SX6c produced contractions of the muscularis mucosae in a concentration-dependent manner, and their cumulative concentration-response curves were sigmoid (Figure 2). The maximum contraction induced by the highest concentration of ET-1 (30 nM) gradually decreased even in the presence of this peptide (Figures 1 and 8), and nearly returned to the resting level after 150–180 min. SX6c-induced maximum contraction was more quickly restored to the resting level at 30–40 min after the drug application, and SX6c (30 nM) readily desensitized to the subsequent application of SX6c (Figures 1 and 8). When the contraction to SX6c (30 nM) had reached to the maximum, further addition of a higher concentration (100 nM) rather depressed the preceding one (data not shown). ET-3 also produced a concentration-dependent contraction of the oesophageal muscularis mucosae, but its concentration-response curve was bimodal sigmoid (Figures 1 and 2). The order of potency ($-\log EC_{50}$) was ET-1 = SX6c > ET-2 > ET-3, but their efficacies (E_{max}) were not significant (Table 1). On the other hand, ET-1 (0.1–30 nM) and SX6c (0.1–100 nM) did not produce significant relaxations of the oesophageal muscularis mucosae precontracted with carbachol (3 μM) ($n = 3$, data not shown).

Influence of ET-receptor antagonists

FR139317 (1–3 μM) and BQ-123 (1 μM) caused parallel rightward shifts of the concentration-response curve to ET-1, but at higher concentrations of FR139317 (10 μM) and BQ-123 (3 μM) the curve was no further shifted (Figure 3). RES-701-1 (3 μM) also caused a rightward shift of the curve to ET-1 with a slight reduction in the maximal response, but at a higher concentration of RES-701-1 (10 μM) the curve was no further shifted (Figure 4). Combined treatments with 10 μM FR139317 and 3 μM RES-701-1 more effectively caused a rightward shift of the concentration-response curve to ET-1

with a slight reduction in the maximal response (Figure 5). FR139317 (1–10 μM) caused only a slight rightward shift of the lower part (1–30 nM) of the concentration-response curve to ET-3, but not shift of the higher part (30–300 nM) (Figure 6A). In contrast, RES-701-1 (0.3–3 μM) caused a markedly rightward shift of the curve to ET-3 with a slight reduction in the maximal response (Figure 6B). However, the higher concentration of RES-701-1 (10 μM) caused no further shift (Figure 6B). RES-701-1 (0.1–1 μM) also caused a rightward shift of the concentration-response curve to SX6c with a slight reduction in the maximal response (Figure 7A). The pA_2 value of RES-701-1 against SX6c was estimated as 6.83, and the slope (1.05) of Schild plot for RES-701-1 was not significantly different from unity (Figure 7B).

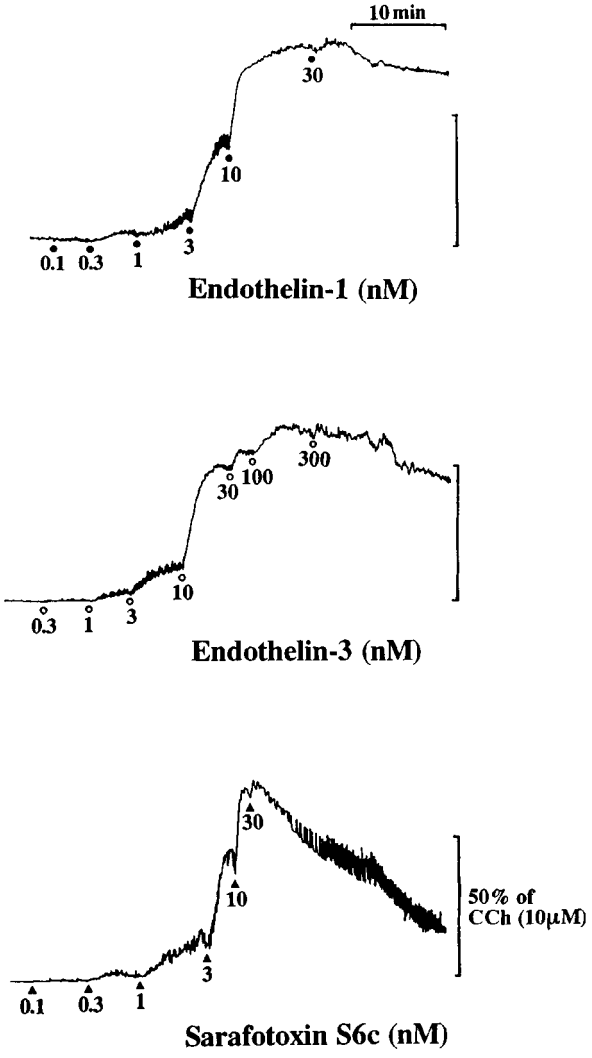

Figure 1 Typical tracings of endothelin-1-, endothelin-3- and sarafotoxin S6c-induced contractions of the guinea-pig oesophageal muscularis mucosae. Endothelins and sarafotoxin S6c were applied to the muscularis mucosae with the cumulative dose technique. Vertical calibrations show 50% contraction induced by 10 μM carbachol (CCh). Horizontal calibration is 10 min.

Responses to ET-1 in SX6c-desensitized preparations

As shown in Figure 8, SX6c (30 nM)-induced contraction of the oesophageal muscularis mucosae was transient and returned to the resting level after 30 min. When SX6c (30 nM)-induced contraction returned to the resting level, a subsequent application of SX6c (30 nM) did not produce any contraction (Figure 8A). In contrast, an application of ET-1 (10 nM) still produced a sustained contraction of SX6c-desensitized preparation (Figure 8B). The contraction to ET-1 in SX6c-desensitized preparation was greatly inhibited by pretreatment with 10 μM FR139317 (Figure 8C). Such desensitization to SX6c or pretreatment with FR139317 did not affect carbachol (10 μM)-induced contraction of the muscularis mucosae (Figure 8).

Role of Ca^{2+} on ET-1 or SX6c-induced contraction

When the oesophageal muscularis mucosae was incubated for 30 min in a Krebs solution containing various Ca^{2+} concentrations (1.25, 0.625, 0 mM), the concentration-response

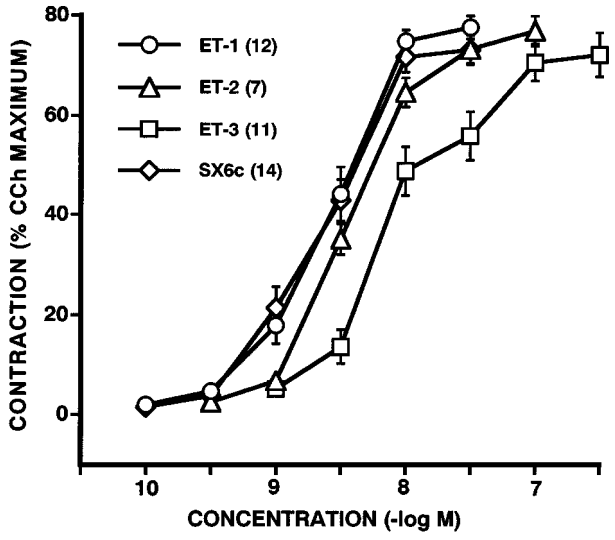

Figure 2 Cumulative concentration-response curves to endothelins in the guinea-pig oesophageal muscularis mucosae. Endothelin-1 (ET-1,○), endothelin-2 (ET-2,△), endothelin-3 (ET-3,□) and sarafotoxin S6c (SX6c,◇). Ordinate scale shows the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM). Each point represents means±s.e.mean. Numbers of observations indicate in parenthesis.

Table 1 Responsiveness to endothelins of the muscularis mucosae isolated from the guinea-pig oesophagus

Endothelins	n	$-log\ EC_{50}$	E_{max} (% of 10 μM CCh)
endothelin-1	12	8.61±0.07	77.61±2.29
endothelin-2	7	8.40±0.04*	76.90±2.98^{NS}
endothelin-3	11	8.18±0.06***	72.18±4.35^{NS}
sarafotoxin S6c	14	8.65±0.06^{NS}	73.17±2.28^{NS}

Each $-\log EC_{50}$ and E_{max} value represents means±s.e.mean. *$P<0.05$; ***$P<0.001$; these were significantly different from the value to endothelin-1. NS, not significant. CCh, carbachol.

curves to ET-1 and SX6c were shifted to the right and downward as external Ca^{2+} concentrations decreased (Figure 9). The maximum contraction to ET-1 (30 nM) and SX6c (30 nM) were completely abolished after only a 10 min incubation with a Ca^{2+}-free EGTA (1 mM)-containing medium ($n=3$, data not shown). Pretreatment with 30 μM verapamil completely inhibited rhythmic motility superimposed on ET-1 or SX6c-induced tonic contraction, but caused only a slight rightward shift of the curves to ET-1 and SX6c (Figure 10). Ni^{2+} (0.1–0.3 mM) did not inhibit rhythmic motility, but weakly caused a shift to the right and a slight downward (Figures 10 and 11). In contrast, SK&F 96365 (60 μM) completely reversed Ni^{2+} (0.3 mM)- and nicardipine (1 μM)-resistant contraction to ET-1 (Figure 11).

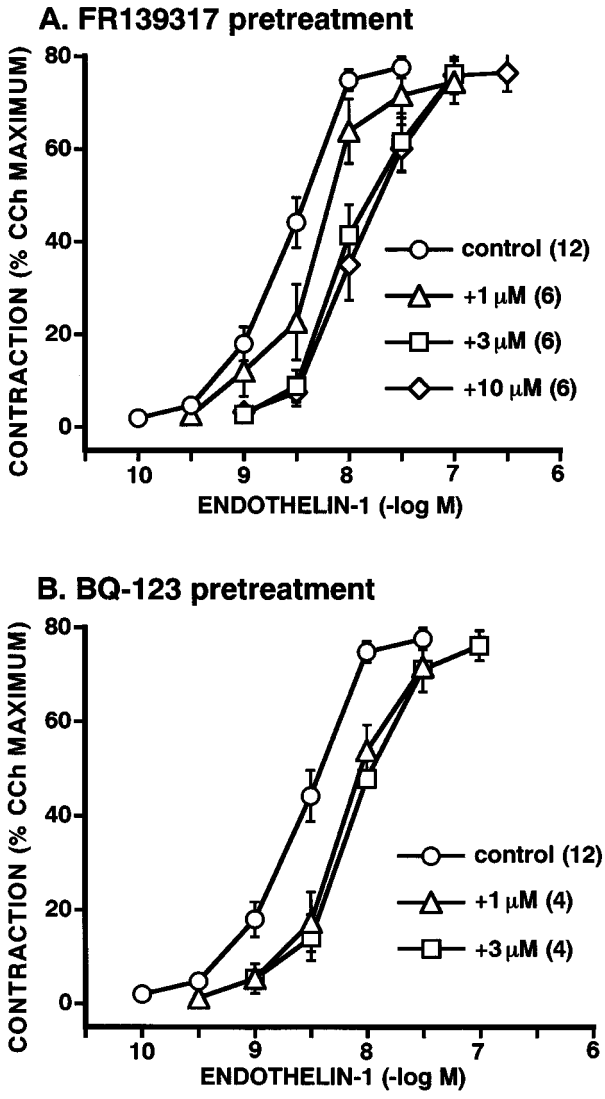

Figure 3 Effects of FR139317 (A) and BQ-123 (B) on the cumulative concentration-response curve to endothelin-1 of the guinea-pig oesophageal muscularis mucosae. Preparations were pretreated with given concentrations of FR139317 or BQ-123 for 20 min before the addition of endothelin-1. Ordinate scales show the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM) in the absence of antagonist. Each point represents means ± s.e.mean. Numbers of observations indicate in parenthesis. (A) control,(○); +FR139317(1 μM,△); +FR139317(3 μM,□); +FR139317(10 μM,◇). (B) control,(○); +BQ-123(1 μM,△); +BQ-123(3 μM,□).

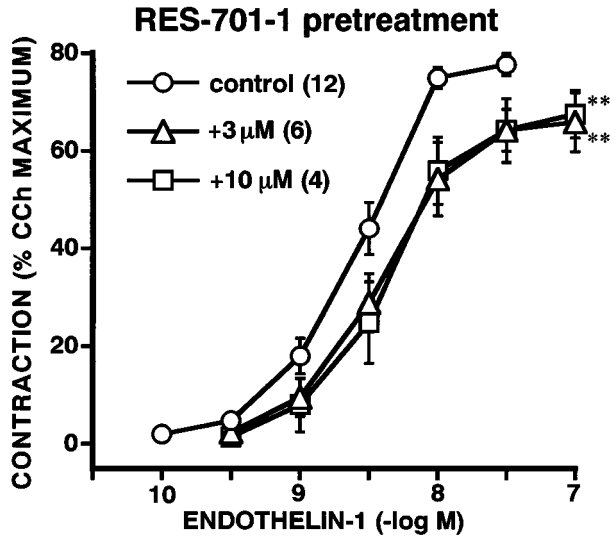

Figure 4 Effect of RES-701-1 on the cumulative concentration-response curve to endothelin-1 of the guinea-pig oesophageal muscularis mucosae. Preparations were pretreated with RES-701-1 for 20 min before the addition of endothelin-1. Ordinate scale shows the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM) in the absence of RES-701-1. Each point represents means ± s.e.mean. Numbers of observations indicate in parenthesis. Control,(○); +RES-701-1(3 μM,△); +RES-701-1(10 μM,□). **$P<0.01$; compared with the maximal response induced by endothelin-1 in the absence of RES-701-1.

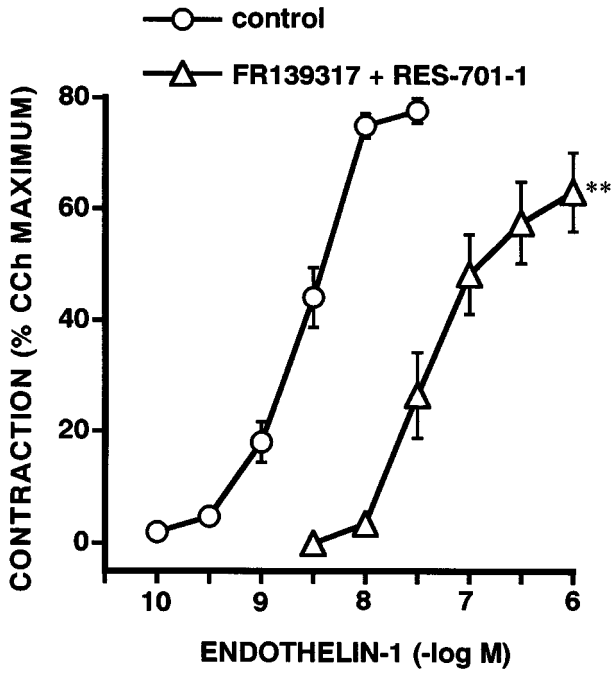

Figure 5 Effect of combined treatments with FR139317 (10 μM) and RES-701-1 (3 μM) on the cumulative concentration-response curve to endothelin-1 of the guinea-pig oesophageal muscularis mucosae. Preparations were pretreated with both antagonists for 20 min before the addition of endothelin-1. Ordinate scale shows the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM) in the absence of antagonists. Each point represents means ± s.e.mean. Control,(○,$n=12$); FR139317+RES-701-1,(△,$n=8$). **$P<0.01$; compared with the maximal response induced by endothelin-1 in the absence of antagonists.

Effect of indomethacin on ET-1- or SX6c-induced contraction

Pretreatment with 2 μM indomethacin did not modify the concentration-response curves to ET-1 and SX6c (Figure 10).

Discussion

Previously, Eglen *et al.* (1989) have reported that ET-1 is a potent contractile agonist with a pD_2 value of approximately 8.4 in the guinea-pig oesophageal muscularis mucosae and is a partial agonist with respect to carbachol. Our present study confirmed these findings. In general, ET receptors can be divided into at least two types by the affinity rank order of three isopeptides (Masaki *et al.*, 1994). ET_A-receptor is more selective to ET-1 than ET-3, ET_B-receptor shows equal affinity for all the isopeptides. In the present experiments using the muscularis mucosae isolated from the guinea-pig oesophagus, the order of potency was ET-1 > ET-2 > ET-3 suggesting the involvement of ET_A-receptor. However, the potency of SX6c, a selective ET_B-agonist, was almost the same with that of ET-1 in this tissue. Moreover, the order of potency of ETs was ET-1 (8.02) = ET-3 (8.08) in the presence of FR139317 (10 μM), a selective ET_A-antagonist (Sogabe *et al.*, 1993), and the rank order was ET-1 (8.37) > ET-3 (7.48) in the presence of RES-701-1 (10 μM), a selective ET_B-antagonist (Tanaka *et al.*, 1994). Combined treatments with FR139317 and RES-701-1 more effectively antagonized ET-1-induced contractions. Contractions to ET-1 in SX6c-desensitized preparations were greatly inhibited by FR139317. In addition, the

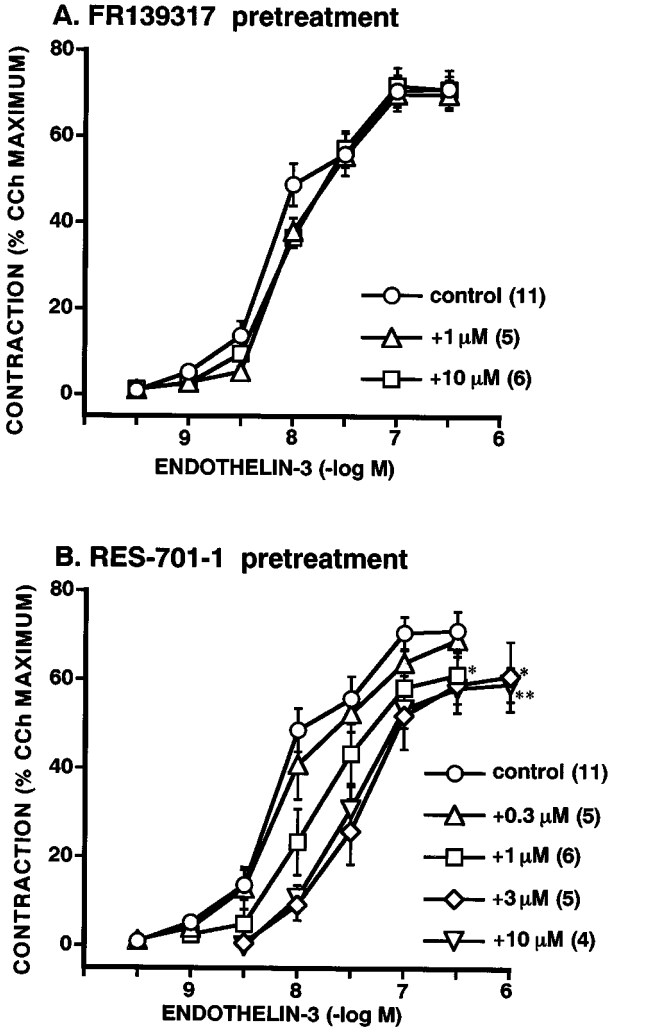

Figure 6 Effects of FR139317 (A) and RES-701-1 (B) on the cumulative concentration-response curve to endothelin-3 of the guinea-pig oesophageal muscularis mucosae. Preparations were pretreated with given concentrations of FR139317 or RES-701-1 for 20 min before the addition of endothelin-3. Ordinate scales show the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM) in the absence of antagonist. Each point represents means ± s.e.mean. Numbers of observations indicate in parenthesis. (A) control,(○); + FR139317(1 μM,△); + FR139317(10 μM,□). (B) control,(○); + RES-701-1(0.3 μM,△); + RES-701-1(1 μM,□); + RES-701-1(3 μM,◇); + RES-701-1(10 μM,▽). $^*P<0.05$; $^{**}P<0.01$; compared with the maximal response induced by endothelin-3 in the absence of antagonist.

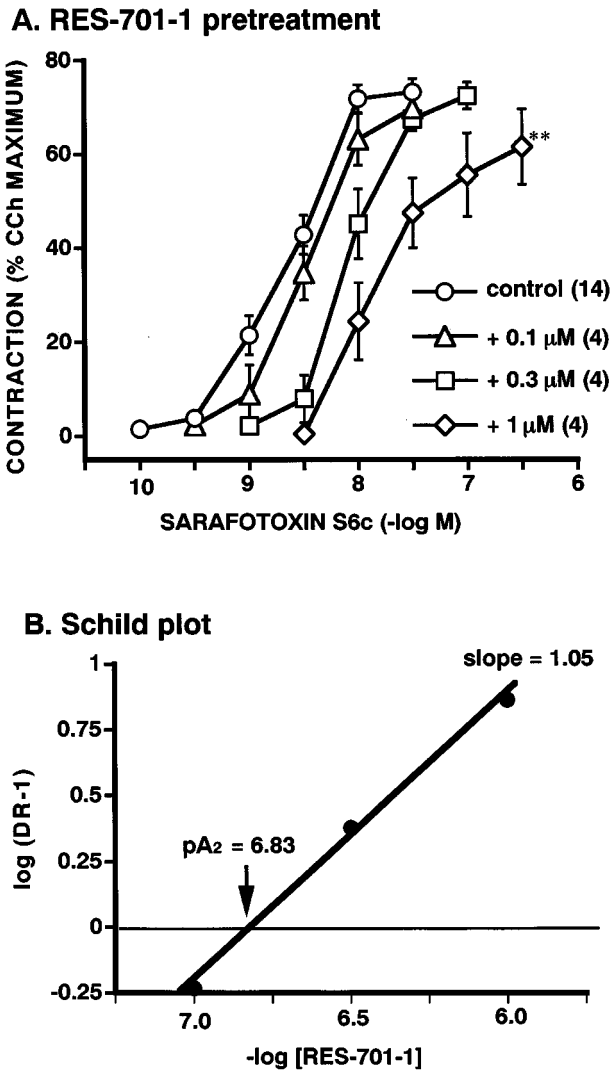

Figure 7 (A) Effect of RES-701-1 on the cumulative concentration-response curve to sarafotoxin S6c of the guinea-pig oesophageal muscularis mucosae. Preparations were pretreated with RES-701-1 for 20 min before the addition of sarafotoxin S6c. Ordinate scale shows the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM) in the absence of RES-701-1. Each point represents means ± s.e.mean. Numbers of observations indicate in parenthesis. (A) control,(○); + RES-701-1(0.1 μM,△); + RES-701-1(0.3 μM,□); + RES-701-1(1 μM,◇). (B) Schild plot for RES-701-1. $^{**}P<0.01$; compared with the maximal response induced by sarafotoxin S6c in the absence of RES-701-1.

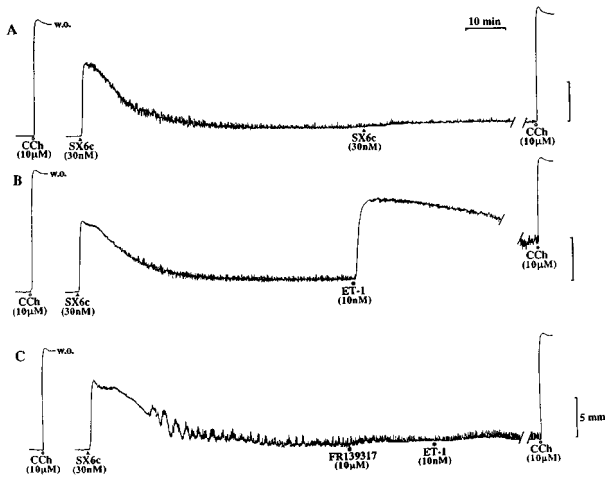

Figure 8 Typical tracings of contractions induced by endothelin-1 (ET-1) in the sarafotoxin S6c (SX6c)-desensitized guinea-pig oesophageal muscularis mucosae. (A) SX6c (30 nM) readily desensitized to the subsequent application of SX6c, but carbachol (CCh, 10 μM)-induced contraction was unaffected by such pretreatment. (B) ET-1 (10 nM) still produced a sustained contraction in the SX6c-desensitized preparation. (C) ET-1-induced contraction in the SX6c-desensitized preparation was greatly inhibited by pretreatment with FR139317 (10 μM), but carbachol (10 μM)-induced contraction was not affected by such pretreatment. Vertical calibrations show a 5 mm shortening of the preparation. Horizontal calibration is 10 min. W.O.; wash out of drug.

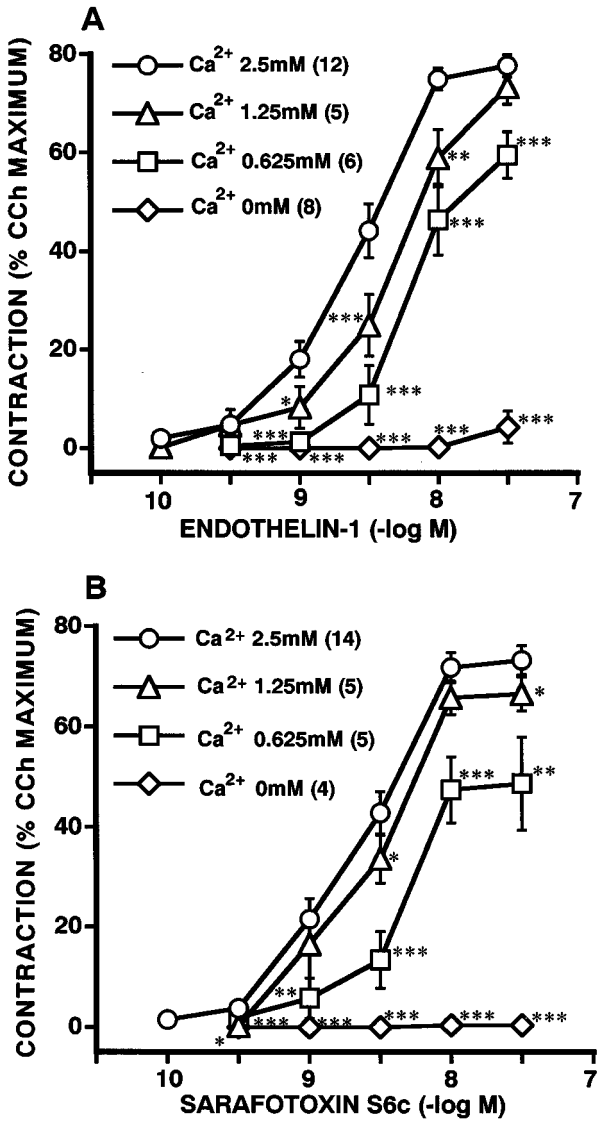

Figure 9 Cumulative concentration-response curves to endothelin-1 (A) and sarafotoxin S6c (B) in the guinea-pig oesophageal muscularis mucosae after incubation with Krebs solution containing various Ca^{2+} concentrations. Ca^{2+} 2.5 mM,(○); Ca^{2+} 1.25 mM,(△); Ca^{2+} 0.625 mM,(□); Ca^{2+} 0 mM,(◇). Ordinate scales show the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM) in normal Krebs solution (Ca^{2+}, 2.5 mM). Each point represents means ± s.e.mean. Numbers of observations indicate in parenthesis. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; significantly different from the response in normal Krebs solution (Ca^{2+}, 2.5 mM).

concentration-response curve to ET-3 was bimodal sigmoid, suggesting two different affinity sites, and ET-3-induced contractions were weakly antagonized with FR139317 but markedly antagonized with RES-701-1. These findings indicate that in the muscularis mucosae of the guinea-pig oesophagus ET-1 activates both ET_A- and ET_B-receptors, ET-3 seems to weakly activate ET_A-receptors and moderately activate ET_B-receptors, and SX6c acts as a selective and potent ET_B-receptor agonist. Recently, He *et al.* (1995) reported that synthetic RES-701-1 was a non-selective ET-receptor antagonist. In the present experiments, contractions to SX6c were concentration-dependently antagonized with RES-701-1, and the slope of Schild plot for RES-701-1 (0.1 – 1 μM) was not significantly different from unity, indicating the competitive antagonism. However, high concentration of RES-701-1 reduced maximum contractions to ETs, suggesting the weak non-specific inhibitory action. We conclude that the oesophageal muscularis mucosae of the guinea-pig has both excitatory ET_A- and ET_B-receptors and ET_B-receptors are readily desensitized by the application of SX6c. ETs might regulate the absorptive and secretory functions of the gut through movements of the muscularis mucosae.

The coexistence of excitatory ET_A- and ET_B-receptors have been suggested in the pulmonary artery and vein (Cardell *et al.*, 1993; Sudjarwo *et al.*, 1995). In the isolated gall bladder of the guinea-pig, Battistini *et al.* (1994) suggested that ET-1 activated two ET-receptor subtypes, an ET_B-receptor and an atypical ET-receptor. Our present study is the first report showing heterogeneity of excitatory ET_A- and ET_B-receptors in the muscularis mucosae of the gastrointestinal tract, but atypical ET-receptors are not involved in contractions induced by ET-1 and SX6c. Previous studies have shown that the ileal smooth muscle of the guinea-pig has both excitatory and inhibitory ET_A- and ET_B-receptors (Yoshinaga *et al.*, 1992; Warner *et al.*, 1993; Karaki *et al.*, 1994; Shan *et al.*, 1996). The present study indicates that the oesophageal muscularis mucosae did not have inhibitory ET_A- and ET_B-receptors, because ET-1 and SX6c had no relaxations in the preparation precontracted with carbachol (3 μM). More recently, the existence of ET_{A1}-, ET_{A2}-, ET_{B1}- and ET_{B2}-receptor subtypes has been suggested from pharmacological studies, but these subtypes were not cloned (Ohlstein *et al.*, 1996). ET_A-receptors have been subclassified as a BQ-123-sensitive ET_{A1} and a BQ-123-insensitive ET_{A2} subtypes, and ET_B-receptors are thought to subclassify as ET_{B1} and ET_{B2} subtypes based on the sensitivity to ET_B antagonist, RES-701-1 or IRL 1038 (Sudjarwo *et al.*, 1994; Hori *et al.*, 1994). In the present study, ET-1-induced contractions were antagonized with BQ-123, a selective

ET_A-antagonist (Ihara *et al*., 1992), and SX6c-induced contractions were concentration-dependently antagonized with RES-701-1. These results suggest that contractile responses to ETs in the oesophageal muscularis mucosae are mediated *via* both ET_{A1}- and ET_{B1}-receptor subtypes.

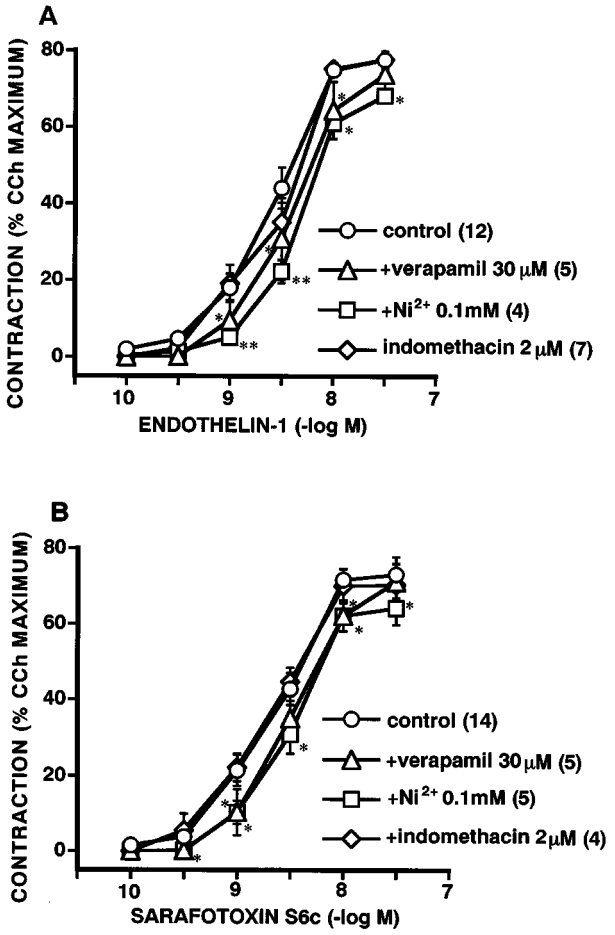

Figure 10 Effects of verapamil, Ni^{2+} and indomethacin on the cumulative concentration-response curves to endothelin-1 (A) and sarafotoxin S6c (B) of the guinea-pig oesophageal muscularis mucosae. Ordinate scales show the amplitude of contraction as a % of the maximum contraction induced by carbachol (CCh, 10 μM). Each point represents means ± s.e.mean. Numbers of observations indicate in parenthesis. Control,(○); +verapamil(30 μM,△); +Ni^{2+}(0.1 mM,□); +indomethacin(2 μM,◇). $^*P<0.05$; $^{**}P<0.01$; significantly different from the value of control for same concentration.

L- and T-type voltage-dependent Ca^{2+} channels (VDCs) have been found in a variety of smooth muscles (Bolton *et al*., 1988; Spedding & Paoletti, 1992). It has been reported that ET-1 produced contractions mainly by opening L-type Ca^{2+} channels in gastro-intestinal smooth muscles (Rae *et al*., 1995). Although ET-1- and SX6c-induced contractions of the oesophageal muscularis mucosae were dependent on extracellular Ca^{2+} concentrations, verapamil, a selective L-type Ca^{2+} channel blocker (Spedding & Paoletti, 1992), abolished only rhythmic motility, but not tonic contractions induced by ETs. Ni^{2+}, a T-type Ca^{2+} channel blocker at low concentration (Hagiwara *et al*., 1988), weakly inhibited ETs-induced tonic contractions, while SK&F 96365, both VDCs and ROCs blocker (Merritt *et al*., 1990; Li *et al*., 1997), strongly inhibited Ni^{2+}- and nicardipine-resistant contraction induced by ET-1. We have recently reported that SK&F 96365 concentration-dependently inhibited the ET-1- and SX6c-induced tonic contractions of the oesophageal muscularis mucosae (Uchida *et al*., 1998). From these findings, ETs are thought to produce tonic contractions of the oesophageal muscularis mucosae predominantly by opening ROCs and partly by opening T-type VDCs, but produce rhythmic motility by opening L-type VDCs. Previously, Blackburn & Highsmith, (1990) have reported that Ni^{2+} markedly inhibited ET-1-induced tonic contractions of porcine coronary artery, suggesting the activation of T-type Ca^{2+} channels. Since ETs-induced tonic contractions were weakly inhibited by Ni^{2+} in the present experiments, T-type Ca^{2+} channels may have a minor role on the oesophageal muscularis mucosae. Recently, Sudjarwo *et al*. (1995) have suggested that ET_A-receptor is coupled to Ca^{2+} release and Ca^{2+} influx but ET_B-receptor is coupled to Ca^{2+} influx in the pulmonary vein. From our results, both ET_A- and ET_B-receptors are mainly coupled to Ca^{2+} influx in the oesophageal muscularis mucosae.

There are some reports showing that contractions to ET-1 of the isolated airway smooth muscle were inhibited by indomethacin (Filep *et al*., 1991) and SK&F 96365 inhibited prostanoid formation (Leis *et al*., 1995). Our previous studies on the oesophageal muscularis mucosae (Uchida, 1983; Uchida *et al*., 1983) also indicate that catecholamine-induced contractions were probably mediated by endogenous prostaglandin production. In the present study, however, indomethacin had no effect on ETs-induced contractions. The role of endogenous prostaglandins on ETs-induced contractions

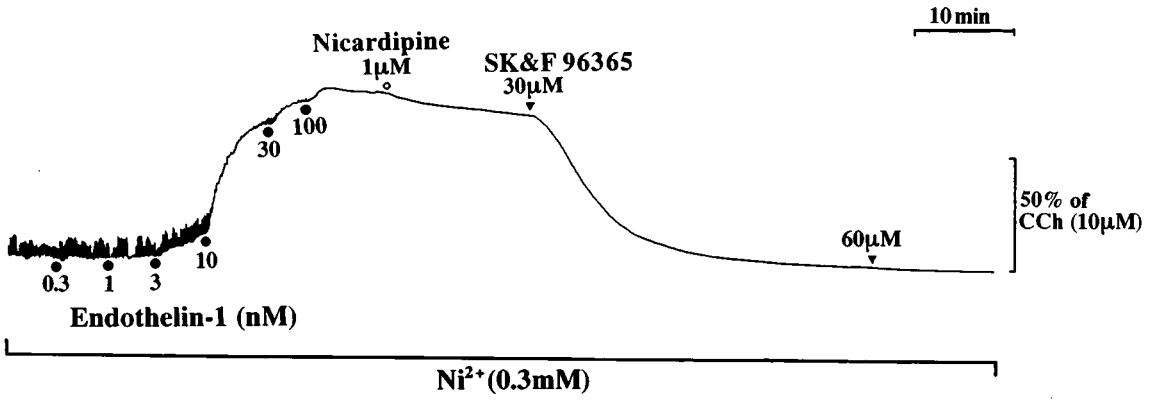

Figure 11 Effects of nicardipine and SK&F 96365 on endothelin-1-induced contraction of the guinea-pig oesophageal muscularis mucosae in the presence of Ni^{2+} (0.3 mM). Vertical calibration shows 50% contraction induced by 10 μM carbachol (CCh). Horizontal calibration is 10 min. SK&F 96365 completely reversed Ni^{2+}- and nicardipine-resistant contraction induced by endothelin-1.

might be negligible in the muscularis mucosae of the guinea-pig oesophagus.

In conclusion, the muscularis mucosae of the guinea-pig oesophagus has both ET_A- and ET_B-receptors which mainly link with ROCs and partly link with VDCs (L- and T-type). Our results represent the first report of the involvement of ROCs in ET-induced contractions of gastro-intestinal smooth muscle.

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