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Characterization of endothelin receptor subtypes mediating Ca^{2+} mobilization and contractile response in rabbit iris dilator muscle

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1 We investigated the characteristics of endothelin (ET)-induced contraction and changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using the fura-2-loaded and non-loaded rabbit iris dilator. ET-1 and ET-2 (3–100 nM) and ET-3 (30–1000 nM) caused contraction in a concentration-dependent fashion.

2 The selective ET_B-receptor agonists, IRL1620 and sarafotoxin S6c produced only a small contraction or no contraction at a concentration of 1 μM. The rank order of potencies for the contraction (pD_2 value) was ET-1 = ET-2 > ET-3 >> sarafotoxin S6c = IRL1620.

3 The contractile response to ET-3 was antagonized by pretreatment with BQ-123 (10 nM), a selective ET_A receptor antagonist. The contractile responses to ET-1 and ET-2 were antagonized by pretreatment with BQ-123 (10 μM), but not at a concentration of 10 nM.

4 ETs increased $[Ca^{2+}]_i$ and sustained muscle contraction. ET-1 (100 nM), ET-2 (100 nM), and ET-3 (1 μM) induced an elevation of $[Ca^{2+}]_i$ consisting of two components: first a rapid and transient elevation to reach a peak, followed by a second, sustained elevation; a sustained contraction was produced without a transient contraction. The ET_B receptor-selective agonist, IRL1620 (1 μM) and sarafotoxin S6c (1 μM) also induced a rapid and transient elevation of $[Ca^{2+}]_i$ to reach a peak and a sustained elevation, together with only a small contraction or no contraction.

5 ET-1 (100 nM) induced a transient increase in $[Ca^{2+}]_i$ in a Ca^{2+}-free, 2 mM EGTA-containing physiological saline solution (Ca^{2+}-free PSS), and a small sustained contraction which was significantly different from that induced by ET-1 (100 nM) in normal PSS. The ET-1-induced increase in $[Ca^{2+}]_i$ and sustained contraction were not affected by the voltage-dependent Ca^{2+} channel blocker, nicardipine (10 μM). The ET-1-induced transient increase in $[Ca^{2+}]_i$ was significantly reduced by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (30 μM); however, the ET-1-induced sustained contraction was not affected by this agent.

6 The selective ET_A receptor antagonist, BQ-123 (100 nM) reduced the ET-3 (100 nM)-induced contraction, but did not affect the transient increase or elevation of the second phase of $[Ca^{2+}]_i$. However, this antagonist at 1 μM did not affect the ET-1 (100 nM)- and ET-2 (100 nM)-induced elevation of $[Ca^{2+}]_i$ and contractile response, or the IRL1620-induced elevation of $[Ca^{2+}]_i$.

7 The selective ET_B receptor antagonist, BQ-788 (1 μM) reduced the transient increase in $[Ca^{2+}]_i$ induced by ET-1 (30 nM), ET-2 (30 nM), ET-3 (100 nM) and IRL1620 (1 μM), but did not affect the sustained elevation of $[Ca^{2+}]_i$ and contractile responses produced by ET-1, ET-2 and ET-3.

8 Pretreatment with IRL1620 (1 μM) reduced the increase in $[Ca^{2+}]_i$ induced by IRL1620 (1 μM) and sarafotoxin S6c (1 μM), as well as the ET-1 (100 nM)-, ET-2 (100 nM)- and ET-3 (1 μM)-induced elevation of $[Ca^{2+}]_i$, whereas in the presence of IRL1620, ET-1-, ET-2- and ET-3-induced contractions were unaltered.

9 These results suggest that ET_A and ET_B receptor subtypes exist in the rabbit iris dilator muscle, and that the ET_A receptor is divided into: (1) BQ-123-sensitive ET_A subtypes activated by ET-1, ET-2 and ET-3, and (2) BQ-123-insensitive ET_A subtypes activated by ET-1 and ET-2, which cause the sustained increase of $[Ca^{2+}]_i$ and contraction; in contrast, ET_B receptor subtypes, are activated by ET-1, ET-2, ET-3, IRL1620 and sarafotoxin S6c and cause the transient and sustained increase in $[Ca^{2+}]_i$ which is not able to contract the smooth muscle.

Keywords: Endothelins; BQ-123; BQ-788; cytosolic Ca^{2+} level; contraction; smooth muscle (rabbit iris dilator); IRL1620; sarafotoxin S6c; endothelin receptor subtypes

Introduction

Endothelins (ETs) have a wide variety of biological effects in many different tissues, iris (Ishikawa *et al.*, 1993), blood vessels (Yanagisawa *et al.*, 1988), uterus, trachea, vas deferens and others (Borges *et al.*, 1989). At least two ET receptor subtypes (ET_A and ET_B) have recently been identified. ET_A receptors are activated by ET-1 and ET-2 at concentrations lower than ET-3 (Arai *et al.*, 1990) and their responses are blocked by the ET_A-selective antagonist, BQ-123 (Ihara *et al.*, 1992; Moreland *et al.*, 1992). ET_B receptors are activated by ET-1, ET-2 and ET-3 at similar concentrations (Sakurai *et al.*, 1990), and activated by ET_B-selective agonists, sarafotoxins and IRL1620 {Suc-[Glu^9, Ala^{11,15}]-ET-1(8-21)} (Kloog *et al.*, 1988; Takai *et al.*, 1992; Shetty *et al.*, 1993; Karaki *et al.*, 1993). In the ocular tissues, application of ET-1 into the anterior chamber of the cat eye caused a reduction in pupil size (MacCumber *et al.*, 1991; Granstam *et al.*, 1992) and produced contraction of the

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isolated iris sphincter muscle of the pig (Geppetti *et al.*, 1989) and the rabbit (Abdel-Latif, 1991; Osborne & Barnett, 1992). In rat iris sphincter, ET interacts with two different receptor subtypes, ET_A and ET_B which exist post- and presynaptically at cholinergic neuroeffector junctions of the rat iris (Shinkai *et al.*, 1994). In rabbit iris sphincter, ET-1 activates phospholipase C which leads to the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Xuan *et al.*, 1989; Hay, 1990). Elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in smooth muscle cells causes contraction and other intracellular signal transductions. However, there is no report on the relation between $[Ca^{2+}]_i$ and contraction produced by ET receptors, including subtypes in the rabbit iris dilator muscle.

In the present experiments in which simultaneous measurements of $[Ca^{2+}]_i$ and contraction were made using fura-2-loaded muscle, we have identified the ET receptor subtypes related to the contraction of smooth muscle, and suggest (1) that the activation of BQ-123-sensitive and -insensitive ET_A receptors caused increase of $[Ca^{2+}]_i$ and sustained contraction, and (2) that the activation of ET_B receptors caused the transient increase in $[Ca^{2+}]_i$; this did not contract the rabbit iris dilator muscle.

Methods

Measurement of mechanical response

The dilator muscle strips were dissected from male albino rabbits, weighing 2–3 kg according to the method of Takayanagi & Kaneko (1982). They were suspended in 2 ml organ baths filled with a physiological saline solution (PSS) gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. The solution contained (in mM): NaCl 118, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 5.0 and glucose 11.0 dissolved in distilled water. After equilibration in normal PSS for 60 min, the responses to an agonist were recorded isometrically under an initial tension of 100 mg. The solution contained propranolol (1 μM), yohimbine (300 nM), desmethylimipramine (100 nM), and normetanephrine (1 μM), to block β- and α_2-adrenoceptors and to inhibit neural and non-neural uptake of catecholamines, respectively. The responsiveness of each preparation was first tested by at least 3 applications of noradrenaline (100 μM) for the dilator effect to confirm that the same amplitude of contraction would be obtained each time. Only one cumulative concentration-response curve to ET was determined. The responses were expressed as percentages of the contraction, where noradrenaline-induced contraction was defined as 100%. The agonist activity of drugs was expressed in terms of pD_2 values. The antagonist activity of BQ-123 (an ET receptor antagonist) was expressed as the pA_2 value which was calculated as described by Van Rossum (1963). When we determined the effects of BQ-123 on the ET-induced contraction, the strip was incubated for 30 min before application of the agonist (for references, see Hisayama & Takayanagi, 1988; Ihara *et al.*, 1992). Two iris preparations were prepared from the same animals, and one of them was used as control.

Measurement of cytosolic Ca^{2+} and tension in fura-2-loaded preparation

The experiments were carried out as described previously (Sato *et al.*, 1988; Hisayama *et al.*, 1990). The dilator strips were incubated with normal PSS gassed with 100% O_2, which contained (mM): NaCl 145, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 20, N,N'-tetrakis (2-pyridylmethyl)ethylendiamine (TPEN) 10, glucose 11 and ethylenediamine N,N'-tetraacetic acid (EDTA·2Na) 10 μM (pH 7.4 at 37°C). A high-K^+ solution was made by substituting NaCl with equimolar KCl. The strips were incubated with 5 μM fura-2/AM in normal PSS for 3–4 h at room temperature in the presence of 0.2% Cremophor EL, then rinsed with the solution for 15 min. Thereafter, experiments were performed with a double wavelength excitation fluorimeter (CAF 100, Japan Spectroscopic Co., Tokyo, Japan) where the fura-2-loaded strips were fixed horizontally in a bath that was bubbled with 100% O_2 at 37°C. After equilibration in normal PSS, the strips were challenged with 72.7 mM high-K^+ solution three times. Tension generated by the last challenge was used as a reference value to normalize the size of the response to ETs. In some experiments, we used Ca^{2+}-free PSS (made by omitting Ca^{2+} from normal PSS and adding 2 mM ethyleneglycol*bis* (β-aminoethylether), N,N'-tetraacetic acid (EGTA)). Simultaneously, 500 nm fluorescence emitted by 340 nm and 380 nm excitation (F_{340} and F_{380}, respectively) were measured by successive alternating illuminations (48 Hz), and the ratio ($R_{340/380}$) of F_{340} to F_{380} was automatically calculated. In the muscle strips that were successfully loaded with fura-2, the increase in $[Ca^{2+}]_i$ resulted in a symmetrical increase in F_{340} and decrease in F_{380}, and increase in $R_{340/380}$. Relative changes in $[Ca^{2+}]_i$ were determined by measuring the $R_{340/380}$ (Himpens & Somlyo, 1988; Sato *et al.*, 1988; Hisayama *et al.*, 1990).

Statistics

Numerical results are expressed as means ± s.e., and statistical significance was calculated by Student's *t* test or Duncan's new multiple range test. A P value < 0.05 was considered to indicate a significant difference.

Drugs

The following drugs were used: endothelin-1, endothelin-2, endothelin-3, IRL1620 (suc-[Glu^9,$Ala^{11,15}$]endothelin-1-[11-21]) and sarafotoxin S6c (Peptide Institute, Osaka, Japan), BQ-788 (N-cis-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) (Calbiochem-Novabiochem AG, Löufelfingen, Switzerland), fura-2-pentaacetoxymethylester (fura-2/AM), ethyleneglycol*bis* (β-aminoethylether)N,N'-tetraacetic acid (EGTA), ethylenediamine *N,N'*-tetraacetic acid (EDTA·2Na), N,N'-tetrakis (2-pyridylmethyl)ethylendiamine (TPEN) and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (Dojindo Laboratories, Kumamoto, Japan), noradrenaline hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), Cremophor EL (Nacalai Tesque, Kyoto, Japan), cyclopiazonic acid, nicardipine hydrochloride, diltiazem hydrochloride, verapamil hydrochloride, desmethylimipramine hydrochloride, (±)-normetanephrine hydrochloride, (±)-propranolol hydrochloride, yohimbine hydrochloride, flurbiprofen, prazosin and tetrodotoxin (Sigma Chemical Co., St. Louis, MO, U.S.A.), all in powder form. BQ-123 (cyclo[-Asp-Pro-Val-Leu-Trp-]) was a gift from Tsukuba Research Institute of Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan. Other chemicals used were of analytical grade.

Results

Endothelin-produced contraction

The cumulative application of noradrenaline contracted isolated strips of rabbit iris dilator in a concentration-dependent manner. The pD_2 value for noradrenaline was 5.48 ± 0.09 ($n=8$). A single application of ETs induced a slowly developing tonic-contraction in dilator muscles which reached maximum tension (ET-1; 150.3 ± 10.8 mg, $n=6$; ET-2: 139.4 ± 14.2 mg, $n=6$; ET-3: 145.9 ± 20.6 mg, $n=6$) within 30 min; this was similar to or larger than the noradrenaline-induced maximum contraction. Relative maximum effect (E_{max}) values (% noradrenaline E_{max}) for ET-1, ET-2 and ET-3 were 106.4 ± 4.72% ($n=5$), 99.1 ± 6.58% ($n=5$) and 102.4 ± 5.37% ($n=5$), respectively. The ETs also caused concentration-dependent contraction (Figure 1). The pD_2 values

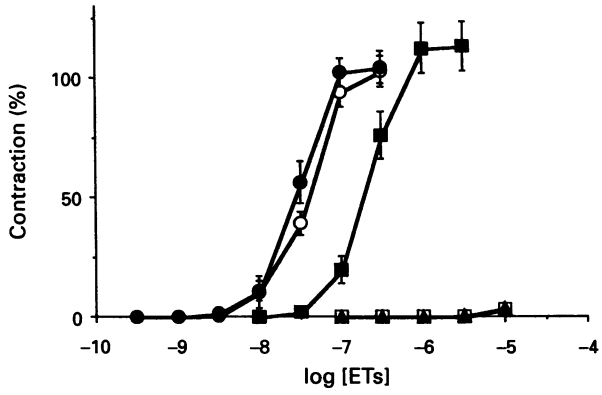

Figure 1 Concentration-response curves for the ET-1, ET-2, ET-3, IRL1620 and sarafotoxin S6c in the rabbit iris dilator: (●) ET-1; (○) ET-2; (■) ET-3; (□) IRL1620; (▲) sarafotoxin S6c. Ordinate scale: % of the maximum contraction to noradrenaline (100 μM). Abscissa scale: the negative logarithm of the molar concentration of the ETs and ET-related peptides. Each value is presented as a mean ± s.e. of 4–6 experiments.

obtained from the concentration-response curves for ET-1, ET-2 and ET-3 were 7.58 ± 0.28 (n = 4), 7.54 ± 0.06 (n = 5) and 6.73 ± 0.07 (n = 4), respectively. However, the selective ET_B receptor agonists, IRL1620 (1 μM) and sarafotoxin S6c (1 μM) produced only small contractions (Figure 1). The rank order of potencies for the contraction (pD_2 values) was ET-1 = ET-2 > ET-3 > > IRL1620 = sarafotoxin S6c, suggesting that ETs produce the muscle contraction by activating ET_A receptors. Moreover, these concentration-response curves were not affected by pretreatment with flurbiprofen (1 μM) or prazosin (1 μM) (data not shown).

As shown in Figure 2, the concentration-response curves for ETs were shifted to the right in a parallel manner by the selective ET_A receptor antagonist, BQ-123 at 10 μM for ET-1 and ET-2, or at 10 nM for ET-3. The apparent pA_2 values for BQ-123 against ET-1, ET-2 and ET-3 were 5.78 ± 0.12 (n = 6), 5.98 ± 0.11 (n = 4) and 8.32 ± 0.15 (n = 4), respectively.

ET-induced elevation of $[Ca^{2+}]_i$ and contraction

In smooth muscle of rabbit iris dilator, neither $[Ca^{2+}]_i$ nor muscle tension changed spontaneously. High K^+ (72.7 mM)-induced a sustained increase in $[Ca^{2+}]_i$ and force (Figure 3), which lasted at least 30 min. ET-1 (100 nM), ET-2 (100 nM) and ET-3 (1 μM) produced a transient increase in $[Ca^{2+}]_i$, however, they induced only a slowly developing sustained contraction (Figure 3a, b and c). The amount of $[Ca^{2+}]_i$ and the magnitude of contraction induced by ETs were much greater than those stimulated by high K^+. On the other hand, the selective ET_B agonists, IRL1620 (1 μM) and sarafotoxin S6c (1 μM), induced transient and sustained increases in $[Ca^{2+}]_i$. These responses were similar to and their amplitudes were smaller than those induced by ET-1, ET-2 and ET-3; but they did not cause contraction or produced only a small contractile response (Figure 3d and e). To examine the characteristics of ET-induced increase in $[Ca^{2+}]_i$ and contraction, the influence of Ca^{2+}-free PSS and the effects of nicardipine (10 μM), diltiazem (10 μM) and cyclopiazonic acid (30 μM) in normal PSS on the increase in $[Ca^{2+}]_i$ and the contraction induced by ET-1 (100 nM) were tested. The transient increase in $[Ca^{2+}]_i$ induced by ET-1 (100 nM) was reduced to 50% in Ca^{2+}-free PSS, approximately. In contrast, the sustained increase in $[Ca^{2+}]_i$ was completely abolished and the ET-1-induced contraction was significantly reduced with the decrease of sustained $[Ca^{2+}]_i$ (Figure 4b). Neither the transient and sustained increase of $[Ca^{2+}]_i$ nor the contraction induced by ET-1 (100 nM) was affected by a voltage-dependent Ca^{2+} channel blocker, nicardipine at 10 μM, which is sufficient to block voltage-dependent Ca^{2+} channels (Huang *et al.*, 1990; Xuan *et al.*, 1994) (Figure 4c). The ET-1-induced contraction

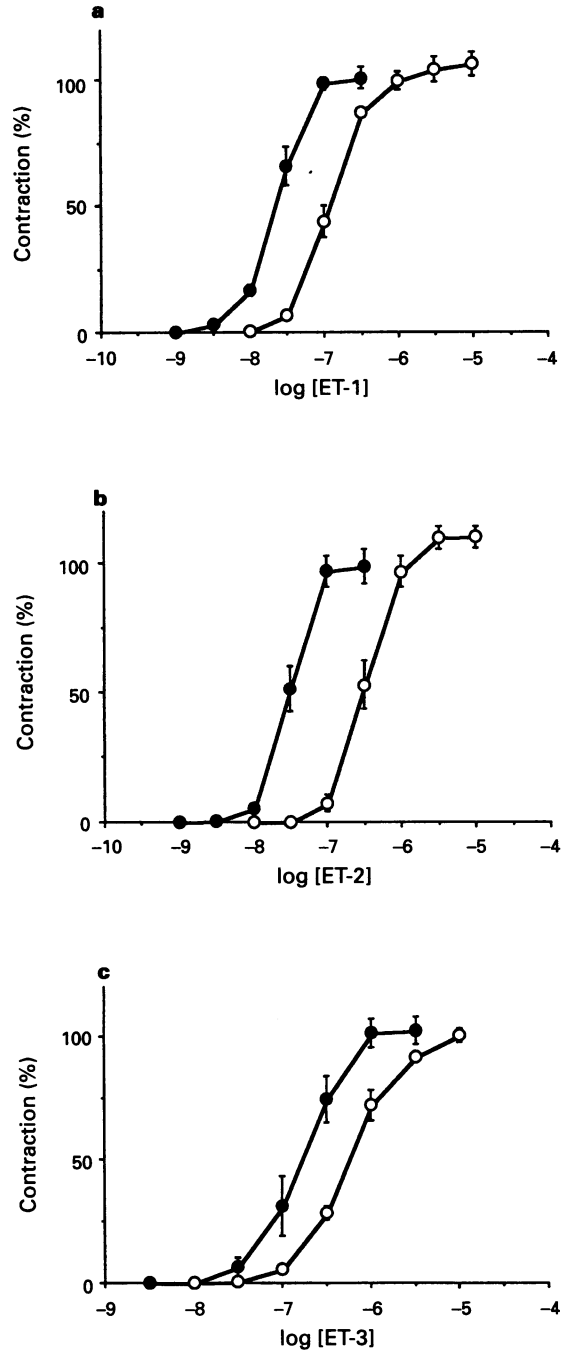

Figure 2 Effect of BQ-123 on the concentration-response curves for ET-1 (a), ET-2 (b) and ET-3 (c) in the rabbit iris dilator. In (a): (●) ET-1 alone; (○) after treatment with BQ-123 (10 μM, 30 min). In (b), (●) ET-2 alone; (○) after treatment with BQ-123 (10 μM, 30 min). In (c), (●) ET-3 alone; (○) after treatment with BQ-123 (10 nM, 30 min). The concentration-response curves for peptides were shifted to the right after treatment with the selective ET_A receptor antagonist, BQ-123. Ordinate scale: % of the maximum contraction to noradrenaline (100 μM). Abscissa scale: the negative logarithm of the molar concentration of ETs. Each value is presented as a mean ± s.e. of 4–6 experiments.

was also not affected by treatment with the Ca^{2+}-ATPase inhibitor, cyclopiazonic acid at 30 μM, which is sufficient to inhibit Ca^{2+}-ATPase (Uyama *et al.*, 1992; Henry, 1994); whereas the transient increase in $[Ca^{2+}]_i$ induced by ET-1 was significantly reduced by treatment with cyclopiazonic acid. The transient increase in $[Ca^{2+}]_i$ induced by IRL1620 (1 μM) was not affected by nicardipine (10 μM) or diltiazem (10 μM) in normal PSS, but significantly reduced by treatment with cyclopiazonic acid (30 μM) (Table 1). ET-1 and IRL1620 pro-

duced a transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free PSS. These observations suggested that the transient increase in $[Ca^{2+}]_i$ caused by ETs was induced by Ca^{2+} release from intracellular Ca^{2+} stores such as the sarcoplasmic reticulum (SR) and that the sustained increase in $[Ca^{2+}]_i$ caused by ET-1 was due to Ca^{2+} influxes induced by activation of the non voltage-dependent Ca^{2+} channels, which developed the sustained contraction.

To determine the relation of ET receptor subtypes (ET_A and ET_B) to the increase in $[Ca^{2+}]_i$ and the mechanism of contraction, we used a selective ET_A receptor antagonist BQ-123 and examined the response of ETs in preparations pretreated with the selective ET_B agonist, IRL1620 (tachyphylaxis studies).

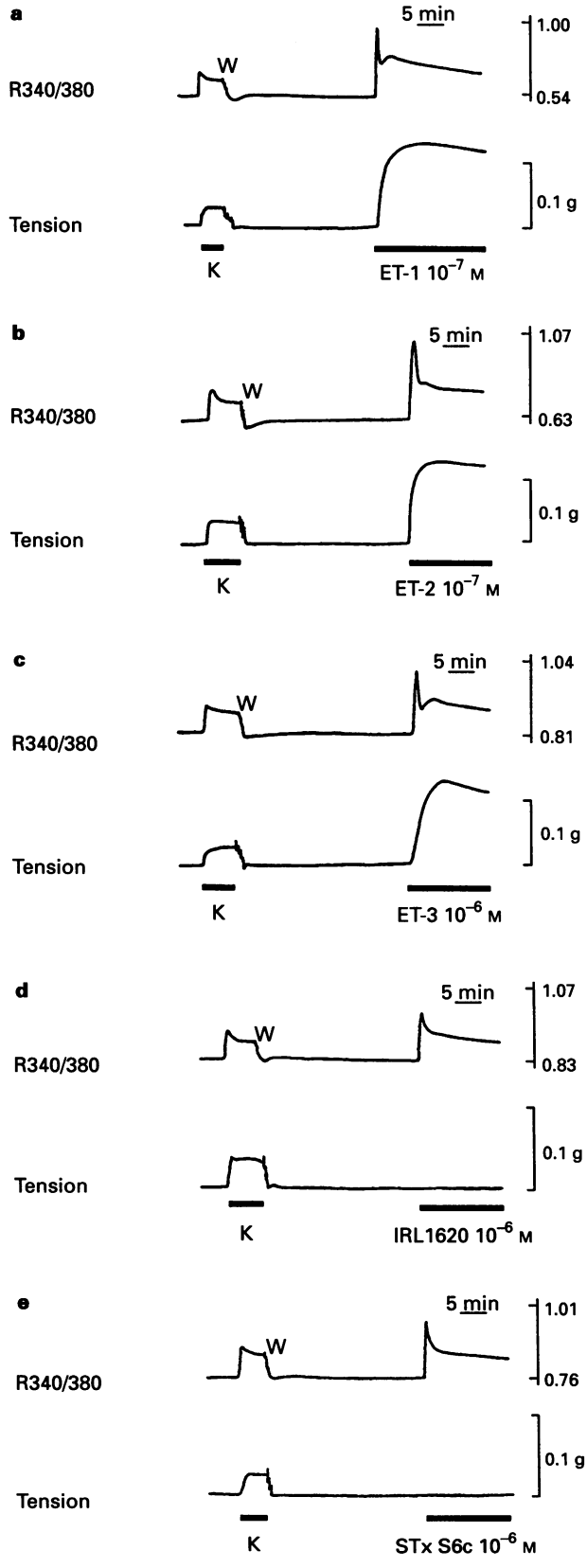

Figure 3 Typical tracings of cytosolic Ca^{2+} level (upper trace, expressed as $R_{340/380}$) and muscle tension (lower trace) induced by 100 nM ET-1 (a), 100 nM ET-2 (b), 1 μM ET-3 (c), 1 μM IRL1620 (d) and 1 μM sarafotoxin S6c (STx S6c) (e) in rabbit iris dilator. Each trace shown is typical of 3–6 experiments.

Effects of BQ-123, BQ-788 and treatment with IRL1620 on ET-1-, ET-2-, ET-3- and sarafotoxin S6c-induced increase in $[Ca^{2+}]_i$ and contraction

BQ-123 (1 μM) did not affect ET-1 (30 nM)- or ET-2 (30 nM)-induced elevation of $[Ca^{2+}]_i$ (transient and sustained) or contraction (Figure 5a and b) and IRL1620 (1 μM) induced increase in $[Ca^{2+}]_i$ (transient and sustained) (Figure 5d). BQ-123 (100 nM) did not affect the ET-3 (100 nM)-induced increase in $[Ca^{2+}]_i$, but almost completely eliminated the sustained contractile response (Figure 5c).

BQ-788 (1 μM) reduced the transient elevation of $[Ca^{2+}]_i$ induced by ET-1 (30 nM), ET-2 (30 nM), ET-3 (100 nM) and IRL1620 (1 μM), but did not affect the sustained elevation of $[Ca^{2+}]_i$ induced by these agonists (Figure 6). In addition ET-1-, ET-2- and ET-3-induced contractions were not affected by this antagonist (Figure 6a, b and c).

IRL1620 (1 μM)-induced a transient and sustained increase in $[Ca^{2+}]_i$, but did not elicit contraction of the smooth muscle. IRL1620-induced transient sustained increase in $[Ca^{2+}]_i$ was completely abolished by exposure to IRL1620 (1 μM) for 30 min (Figure 7a). Thereafter, the transient increase in $[Ca^{2+}]_i$ and sustained contraction induced by ET-1 (100 nM) were smaller than those obtained in the normal preparations. ET-2 (100 nM) and ET-3 (1 μM) also produced similar responses to those obtained by ET-1 in the preparations treated with the successive applications of IRL1620 (1 μM). The elevation in $[Ca^{2+}]_i$ induced by sarafotoxin S6c (1 μM) in the preparation treated with IRL1620 was significantly less than that in the untreated preparation (Figures 3e and 7e), but there was still a small residual response.

Discussion

ET-1, ET-2 and ET-3 were found to produce contractile responses in a concentration-dependent manner but IRL1620 and sarafotoxin S6c did not contract the iris dilator muscle of the rabbit. The rank order of potency was ET-1 = ET-2 > ET-3 > > IRL1620 = sarafotoxin S6c. From the observation that ET_B-selective agonists did not cause contraction of smooth muscle, it seems that ETs produced the contraction by the activation of ET_A receptor subtypes in rabbit iris dilator. The same findings were observed by Ishikawa *et al.* (1993) in rabbit iris sphincter and dilator and by EL-Mowafy & Abdel-Latif (1993) in rabbit iris sphincter. As shown in Figure 2, the concentration-response curves to ET-1, ET-2 and ET-3 were shifted to the right by an ET_A-selective antagonist, BQ-123 (Ihara *et al.*, 1992) with no effect on the maximum response to these peptides. The antagonistic effect of BQ-123 against ET-3 was consistent with those (7.3–7.96 nM) reported previously (Ihara *et al.*, 1992; Nakamichi *et al.*, 1992). However, the antagonistic effect of BQ-123 against ET-1 and ET-2 were much weaker than those reported by Ihara *et al.* (1992) and Nakamichi *et al.* (1992). These findings suggest the existence of BQ-123-insensitive ET receptors activated by ET-1 and ET-2 in addition to BQ-123-sensitive ET_A receptors.

As shown in Figure 3, all three ETs produced an increase in $[Ca^{2+}]_i$ and muscle tension, and increase in $[Ca^{2+}]_i$ without tension development was produced by IRL1620 and sarafotoxin S6c. This latter increase was transient and sustained,

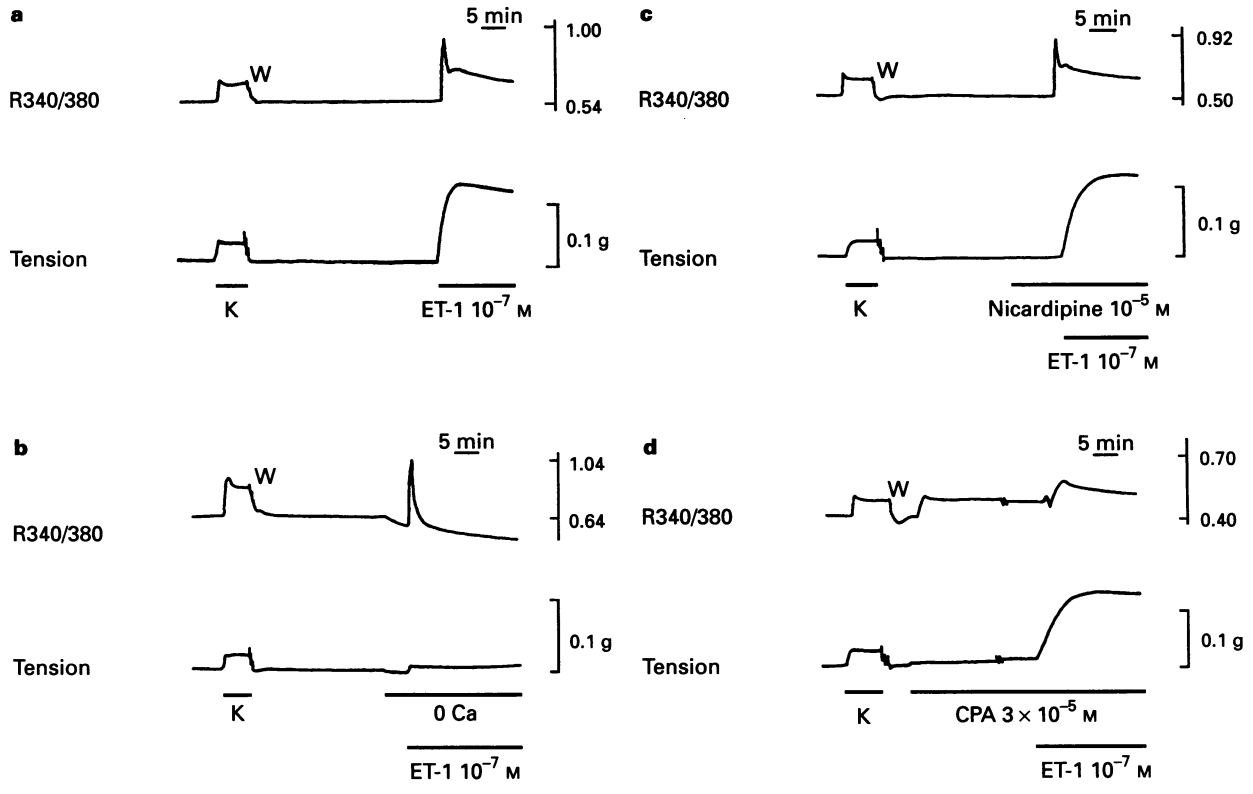

Figure 4 Effects of treatment with Ca^{2+}-free PSS (0 Ca), nicardipine and cyclopiazonic acid (CPA) on 100 nM ET-1-induced cytosolic Ca^{2+} level (upper trace, expressed as $R_{340/380}$) and muscle tension (lower trace) in rabbit iris dilator. (a) Normal PSS, (b) Ca^{2+}-free PSS, (c) 10 μM nicardipine (10 min), (d) 30 μM CPA (60 min). Each trace shown is typical of 4–6 experiments.

Table 1 Effects of treatment with Ca^{2+}-free PSS, nicardipine (10 μM) and cyclopiazonic acid (30 μM) on endothelin-1 (100 nM) and IRL1620 (1 μM)-induced cytosolic Ca^{2+} level and contraction in rabbit iris dilator

	n	$[Ca^{2+}]_i$ (transient)	$[Ca^{2+}]_i$ (sustained)	Contraction (%)
ET-1				
Normal PSS	6	412.7 ± 40.8	208.1 ± 18.8	422.6 ± 63.4
Ca^{2+}-free PSS	4	212.4 ± 13.3*	0	42.3 ± 6.8*
Nicardipine	6	433.9 ± 40.6	256.3 ± 48.5	440.6 ± 44.3
Cyclopiazonic acid	4	15.3 ± 2.7*	122.0 ± 37.8*	390.1 ± 64.4
IRL 1620				
Normal PSS	4	258.2 ± 17.5	160.0 ± 14.4	NC
Ca^{2+}-free PSS	3	170.2 ± 15.2*	0	NC
Nicardipine	4	267.9 ± 45.0	152.0 ± 23.8	NC
Cyclopiazonic acid	6	15.4 ± 5.7*	60.9 ± 14.8*	NC

%: expressed as percentage of Ca^{2+} level and contraction induced by 72.7 mM KCl.
NC: no contraction.

reverting to the baseline within 30 min; the increases induced by the three ETs also had two phases (transient and sustained) which lasted more than 30 min. In the absence of extracellular Ca^{2+}, ET-1 produced only a transient increase in $[Ca^{2+}]_i$ which was not accompanied by corresponding transient contractions; the sustained contractions produced in the Ca^{2+}-free PSS were significantly reduced by elimination of the sustained increase in $[Ca^{2+}]_i$. Neither the increase in $[Ca^{2+}]_i$ nor the contraction was affected by L-type Ca^{2+}-channel blockers, whereas after treatment with the Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, the transient increase in $[Ca^{2+}]_i$ induced by ET-1 was decreased without affecting the contraction. These findings strongly suggest that when Ca^{2+} was released from intracellular Ca^{2+} stores, the transient increase in $[Ca^{2+}]_i$ did not relate to the contraction, and instead, the subsequent increase in $[Ca^{2+}]_i$ produced by Ca^{2+}-influx from extracellular Ca^{2+} through non L-type (non voltage-dependent) Ca^{2+} channels caused the sustained contraction. Kurata *et al.* (1993) reported a similar observation on eicosanoids in the rat thoracic aorta: in the presence or absence of extracellular Ca^{2+}, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the thromboxane agonist, U-46619, produced a transient increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from the intracellular Ca^{2+} stores or the SR; the transient increase was not accompanied by corresponding transient contractions. Further, the contraction began to develop and was well sustained in the descending phase of the transient increase in $[Ca^{2+}]_i$. They suggested that the signal-transducing pathway utilized for development of the sustained contraction is different from that for the intracellular Ca^{2+} release. The L-type Ca^{2+} channel blockers, nicardipine, diltiazem and verapamil, at concentrations sufficient to block Ca^{2+} channels, did not influence the contractile responses to noradrenaline in rabbit

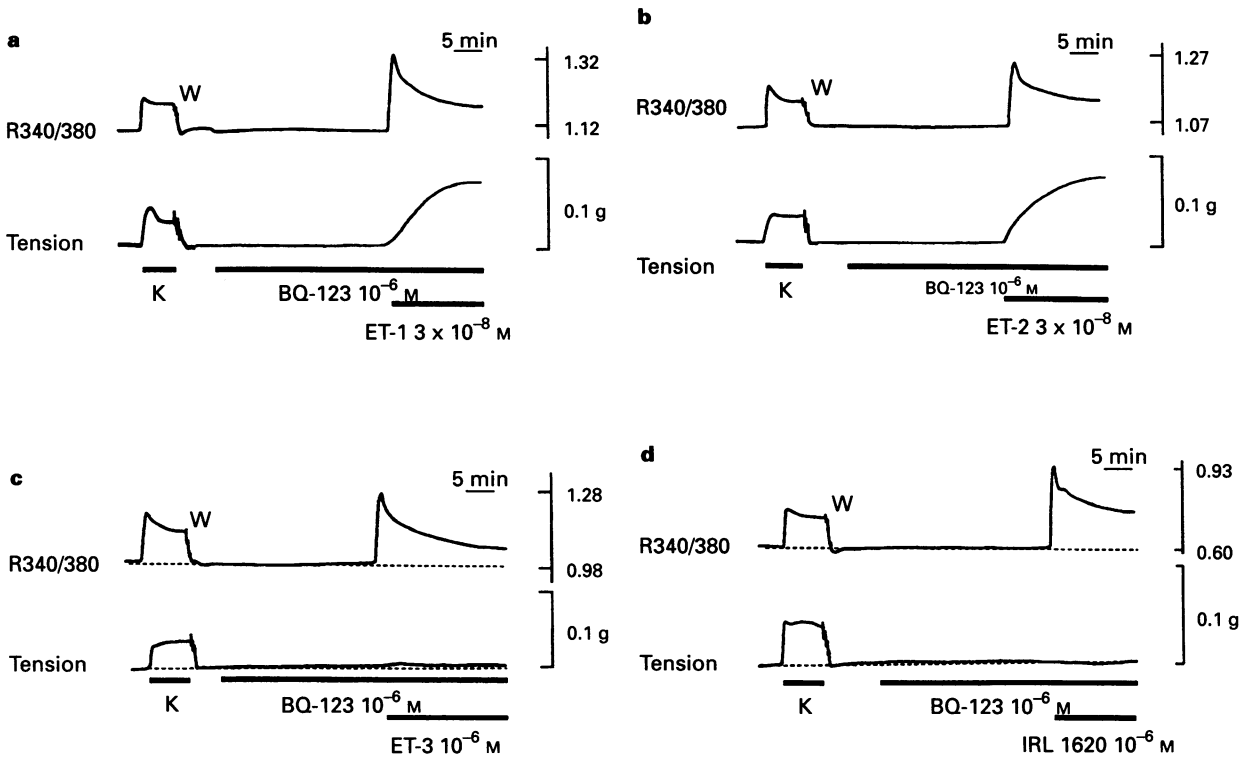

Figure 5 Effects of BQ-123 on cytosolic Ca^{2+} level (upper trace, expressed as $R_{340/380}$) and muscle tension (lower trace) induced by ET-1 (a), ET-2 (b), ET-3 (c) and IRL1620 (d). Each agonist was applied after 30 min treatment with BQ-123, (a) 1 μM BQ-123 against 30 nM ET-1, (b) 1 μM BQ-123 against 30 nM ET-2, (c) 100 nM BQ-123 against 100 nM ET-3 and (d) 1 μM BQ-123 against 1 μM IRL1620. Each trace shown is typical of 4–6 experiments.

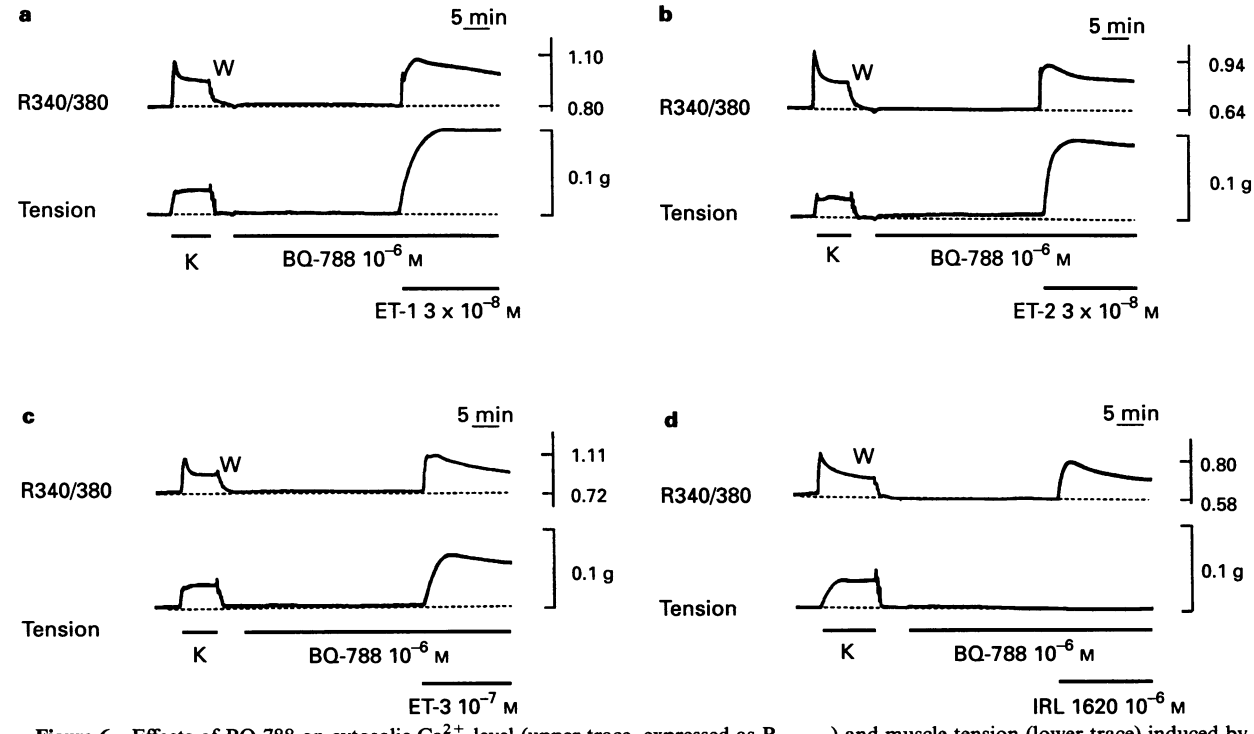

Figure 6 Effects of BQ-788 on cytosolic Ca^{2+} level (upper trace, expressed as $R_{340/380}$) and muscle tension (lower trace) induced by ET-1 (a), ET-2 (b), ET-3 (c) and IRL1620 (d). ET-1 (30 nM), ET-2 (30 nM), ET-3 (100 nM) and IRL1620 (1 μM) were applied after 30 min treatment with 1 μM BQ-788. Each trace shown is typical of 4–6 experiments.

iris dilator (Kokubu *et al.*, 1993). In endothelial cells and vascular smooth muscle, Ca^{2+}-channel blockers also have no effect on the ET-1-induced elevation of $[Ca^{2+}]_i$, indicating that the L-type (voltage-dependent) Ca^{2+} channels are not involved in the ET-1-activated Ca^{2+} influx (Masaki *et al.*, 1991; Aoki *et al.*, 1994). Abdel-Latif & Zhang (1991) also made a similar

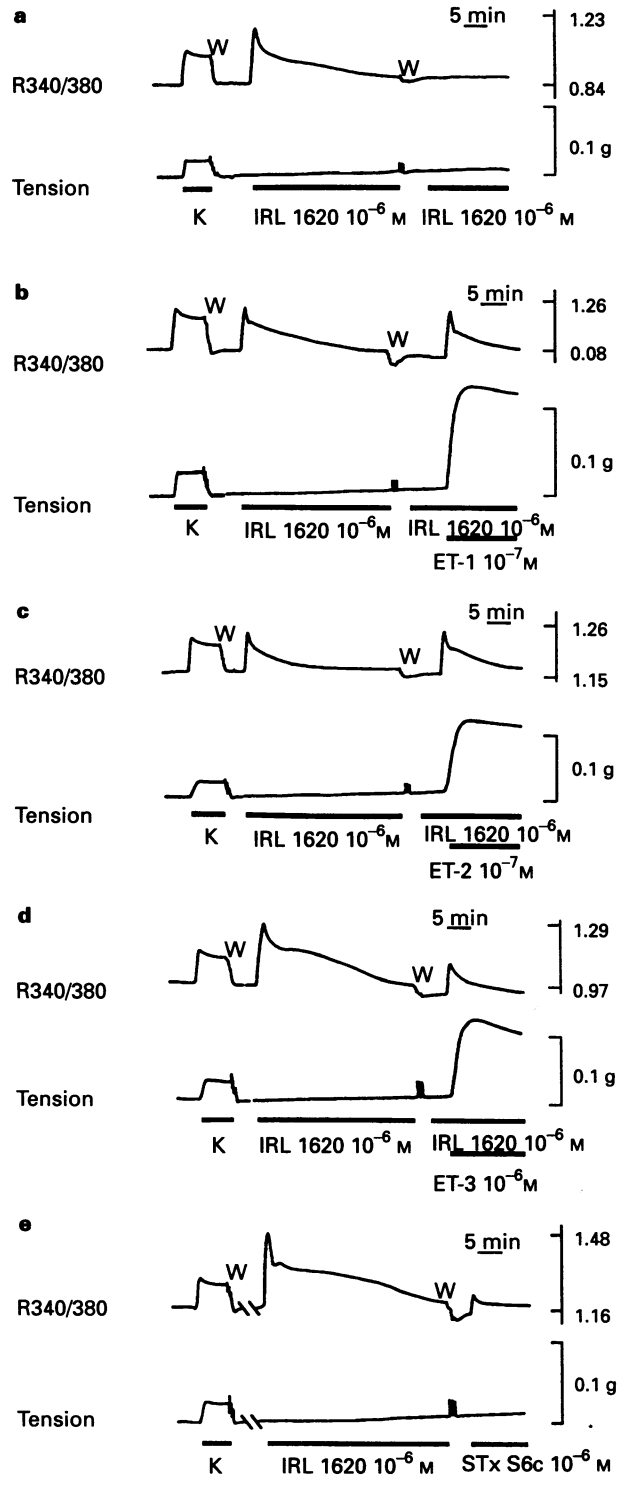

Figure 7 The effect of 1 μM IRL1620 treatment on cytosolic Ca^{2+} level (upper trace, expressed as $R_{340/380}$) and muscle tension (lower trace) induced by 1 μM IRL1620 (a), 100 nM ET-1 (b), 100 nM ET-2 (c), 1 μM ET-3 (d) and 1 μM sarafotoxin S6c (STx S6c) (e). Each trace shown is typical of 4–6 experiments.

observation in rabbit iris sphincter, finding that neither nifedipine nor nicardipine had any effect on ET-1-induced concentration-response curves and IP_3 accumulation. They concluded that ET-1 acts via a mechanism other than activation of dihydropyridine-sensitive channels in this tissue. These findings suggest that in the muscle of rabbit iris dilator, functional L-type (voltage-dependent) Ca^{2+} channels may not exist or that Ca^{2+} may enter the muscles through non-selective cation channels.

We made another interesting observation in this study: with fura-2-loaded dilator muscle viz BQ-123 (1 μM) did not affect the ET-1- or ET-2-induced increase in $[Ca^{2+}]_i$ and muscle tension, or IRL1620-induced increase in $[Ca^{2+}]_i$, whereas low concentrations of BQ-123 (100 nM) significantly reduced the sustained contraction induced by ET-3 without affecting the transient and sustained increase in $[Ca^{2+}]_i$. Further, BQ-788 (1 μM) reduced the transient increase in $[Ca^{2+}]_i$ induced by ETs and IRL1620, but did not affect the sustained elevation of $[Ca^{2+}]_i$ and contraction. Thus, the activation of ET_A receptors is important for the muscle contraction, and the muscle contraction is caused by only a small increase in $[Ca^{2+}]_i$. In addition to our findings, many investigators have also demonstrated that ETs mobilized Ca^{2+} from both intracellular and extracellular sources in various cell types, such as aorta, brain capillary endothelial cells, mesangial cells, glial cells and PC12 phaeochromacytoma cells (Miashiro *et al.*, 1988; Takuwa *et al.*, 1990; Vigne *et al.*, 1990; Martin *et al.*, 1990; Thomas *et al.*, 1991). ETs activate phospholipase C, which catalyzes hydrolysis of phosphatidyl inositol biphosphate into inositol trisphosphate (IP_3) and diacylglycerol (Vigne *et al.*, 1990; Martin *et al.*, 1990; Thomas *et al.*, 1991; Abdel-Latif & Zhang, 1991; EL-Mowafy & Abdel-Latif, 1991; Osborne & Barnett, 1992). IP_3 induces the release of Ca^{2+} from its intracellular storage site in cells, presumably the endoplasmic reticulum (Newby & Henderson, 1990). In the rabbit iris sphincter muscle, ET-1 induced the accumulation of IP_3 within 5 min (Abdel-Latif & Zhang, 1991; Osborne & Barnett, 1992; EL-Mowafy & Abdel-Latif, 1994), which may have caused the ETs-induced transient increase in $[Ca^{2+}]_i$ in our experiments. As shown in Figure 6, the selective ET_B receptor antagonist, BQ-788 (Fukuroda *et al.*, 1994) almost completely eliminated the transient increase in $[Ca^{2+}]_i$ induced by ETs and IRL1620. These findings suggest that the ET_B receptor mediating increase in $[Ca^{2+}]_i$ induced by ET-1, ET-2, ET-3 and IRL1620 is related to the transient increase in $[Ca^{2+}]_i$ mediated by the accumulation of IP_3. Added to these findings, the results shown in Figure 7 indicate that the accumulation of IP_3 may be produced by activation of not only ET_A receptors but also ET_B receptors. EL-Mowafy & Abdel-Latif (1994) demonstrated another observation that ETs induce both IP_3 and cyclic AMP formation, two second messengers with apparently opposite physiological functions to contraction of smooth muscle. In the present experiment, ET related peptide-induced increase in $[Ca^{2+}]_i$, which is not accompanied by muscle contraction, may be related to the production of intracellular second messengers including cyclic AMP. We cannot show for certain whether the transient increase in $[Ca^{2+}]_i$ through ET_B receptors activates the cyclic AMP-protein kinase A pathway and influences the ETs-induced muscle contraction. But as shown in Figure 7a, we have provided evidence that treatment with IRL1620 decreases the transient increase in $[Ca^{2+}]_i$, and completely eliminates it with two applications. This observation makes it clear that the increase in $[Ca^{2+}]_i$ evoked by activation of ET_B receptors is reduced by exposure to and repeated applications of ET_B agonists (tachphylaxis). And further the application of ET-1, ET-2 and ET-3 after the treatment with IRL1620 produces smaller increases in $[Ca^{2+}]_i$ and the sustained contraction, whose amplitude is not significantly different from those produced without IRL1620, in spite of no-response for sarafotoxin S6c. The increase in $[Ca^{2+}]_i$ and contraction induced by the activation of ET_A receptors were not changed by tachphylaxis (down-regulation) of ET_B receptors. Furthermore the ET_B selective antagonist, BQ-788, reduced ET-1-, ET-2- and ET-3-induced transient elevation of $[Ca^{2+}]_i$, but did not affect their sustained contraction (Figure 6). These results suggest that the transient increase in $[Ca^{2+}]_i$ is mainly produced by ET_B receptor subtypes, and the contractile response is produced by activation of ET_A receptors; the elimination of elevation of $[Ca^{2+}]_i$ produced by exposure to the ET_B agonist, IRL1620, is not due to exhaustion or

reduction of Ca^{2+} in Ca^{2+} stores but to the decrease of the products of the intracellular second messengers stimulated by activation of ET_B receptors.

MacCumber *et al.* (1991) demonstrated the presence of immunoreactive ET-1 and ET-3 in the rabbit eye and showed that the iris-ciliary body contains the highest concentrations of ET among the various tissues of the eye. Characterization of the receptors as in binding studies and genetic experiments and the physiological role of the transient increase in $[Ca^{2+}]_i$, which did not produce contraction of smooth muscle may need further study, as is also true of the intracellular signal transduction in the ET receptors mediating contractile response. In the present studies, we have shown that ET_A and ET_B receptor subtypes exist in the rabbit iris dilator muscle, and the ET_A receptors are divided into BQ-123-sensitive ET_A subtypes activated by ET-1, ET-2 and ET-3, and BQ-123-insensitive ET_A subtypes activated by ET-1 and ET-2, which cause the increase in $[Ca^{2+}]_i$ and sustained contraction. ET_B receptor subtypes activated by ET-1, ET-2, ET-3, IRL1620 and sarafotoxin S6c cause the transient and sustained increase in $[Ca^{2+}]_i$, which is not able to contract the smooth muscle.

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References

ABDEL-LATIF, A.A. (1991). Biochemical and functional interactions between the inositol 1,4,5-trisphosphate-Ca^{2+} and cyclic AMP signalling systems in smooth muscle. *Cell. Signal.*, **3,** 371–385.

ABDEL-LATIF, A.A. & ZHANG, Y. (1991). Species differences in the effects of endothelin-1 on myo-inositol triphosphate accumulation, cyclic AMP formation and contraction of isolated iris sphincter of rabbit and other species. *Invest. Ophthalmol. Vis. Sci.*, **32,** 2432–2438,

ABDEL-LATIF, A.A., ZHANG, Y. & YOUSUFZAI, S.Y.K. (1991). Endotheline-1 stimulates the release of arachidonic acid and prostaglandins in rabbit iris sphincter smooth muscle: activation of phospholipase A_2. *Current Eye Res.*, **10,** 259–265.

AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1994). Sensitivity of G-protein involved in endothelin-1-induced Ca^{2+} influx to pertussis toxin in porcine endothelial cells in situ. *Br. J. Pharmacol.*, **111,** 989–996.

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348,** 730–732.

BORGES, R., VON GRAFENSTEIN, R. & KNIGHT, D.E. (1989). Tissue selectivity of endothelin. *Eur. J. Pharmacol.*, **165,** 223–230.

EL-MOWAFY, A.M. & ABDEL-LATIF, A.A. (1994). Characterization of iris sphincter smooth muscle endothelin receptor subtypes which are coupled to cyclic AMP formation and polyphosphoinositide hydrolysis. *J. Pharmacol. Exp. Ther.*, **268,** 1343–1351.

FUKURODA, T., OZAKI, S., IHARA, M., ISHIKAWA, K., YANO, M. & NISHIKIBE, M. (1994). Synergistic inhibition by BQ-123 and BQ-788 of endothelin-1-induced contraction of rabbit pulmonary artery. *Br. J. Pharmacol.*, **113,** 336–338.

GEPPETTI, P., PATACCHINI, R., MEINI, S. & MANZINI, S. (1989). Contractile effect of endothelin on isolated iris sphincter muscle of the pig. *Eur. J. Pharmacol.*, **168,** 119–121.

GRANSTAM, E., WANG, L. & BILL, A. (1992). Ocular effects of endothelin-1 in the cat. *Current Eye Res.*, **11,** 325–332.

HAY, D.W.P. (1990). Mechanism of endothelin-induced contraction in guinea-pig trachea: comparison with rat aorta. *Br. J. Pharmacol.*, **100,** 383–392.

HENRY, P.J. (1994). Inhibitory effects of nordihydroguaiaretic acid on ET_A-receptor-mediated contractions to endothelin-1 in rat trachea. *Br. J. Pharmacol.*, **111,** 561–569.

HIMPENS, B. & SOMLYO, A.P. (1988). Free-calcium and force transient during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.*, **395,** 507–530.

HISAYAMA, T. & TAKAYANAGI, I. (1988). Ryanodine: its possible mechanism of action in the caffeine-sensitive calcium store of smooth muscle. *Pflügers. Arch. Physiol.*, **412,** 376–382.

HISAYAMA, T., TAKAYANAGI, I. & OKAMOTO, Y. (1990). Ryanodine reveals multiple contractile and relaxant mechanisms in vascular smooth muscle: simultaneous measurements of mechanism activity and of cytoplasmic free Ca^{2+} level with fura-2. *Br. J. Pharmacol.*, **100,** 677–684.

HUANG, X.-N., HISAYAMA, T. & TAKAYANAGI, I. (1990). Endothelin-1 induced contraction of rat aorta: contributions made by Ca^{2+} influx and activation of contractile apparatus associated with no change in cytoplasmic Ca^{2+} level. *Naunyn-Schmied Arch. Pharmacol.*, **341,** 80–87.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, F., HISHIKIBE, M. & YANO, M. (1992). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.*, **50,** 247–255.

ISHIKAWA, H., YOSHITOMI, T., HARADA, Y., KATORI, M. & ISHIKAWA, S. (1993). The presence of two sites of action of endothelins in the isolated rabbit iris sphincter and dilator muscles. *Current Eye Res.*, **12,** 1049–1055.

KARAKI, H., SUDJARWO, S.A., HORI, M., TAKAI, M., URADE, Y. & OKADA, T. (1993). Induction of endothelium-dependent relaxation in the rat aorta by IRL1620, a novel and selective agonist at the endothelin ET_B receptor. *Br. J. Pharmacol.*, **109,** 486–490.

KLOOG, Y., AMBAR, I., SOKOLOVSKY, M., KOCHVA, E., WOLLBERG, Z. & BDOLAH, A. (1988). Sarafotoxin, a novel vasoconstrictor peptide: Phosphoinositide hydrolysis in rat heart and brain. *Science*, **242,** 268–270.

KOKUBU, N., SATOH, M. & TAKAYANAGI, I. (1993). Contractile-responses and calcium movements induced by α_1-adrenoceptor stimulant, norepinephrine, in rabbit iris dilator muscle. *Gen. Pharmacol.*, **24,** 1541–1545.

KURATA, R., TAKAYANAGI, I. & HISAYAMA, T. (1993). Eicosanoid-induced Ca^{2+}-free media are mediated by different signal transduction pathways in rat aorta. *Br. J. Pharmacol.*, **110,** 875–881.

MACCUMBER, M.W., JAMPEL, H.D. & SNYDER, S.H. (1991). Ocular effects of the endothelins. *Arch. Opthalmol.*, **109,** 705–709.

MARTIN, E.R., BRENNER, B.M. & BALLERMANN, B.J. (1990). Heterogeneity of cell surface endothelin receptors. *J. Biol. Chem.*, **265,** 14044–14049.

MASAKI, T., KIMURA, S., YANAGISAWA, M. & GOTO, K. (1991). Molecular and cellular mechanism of endothelin regulation. Implications for vascular function. *Circulation*, **84,** 1457–1468.

MIASHIRO, N., YAMAMOTO, H., KANAIDE, H. & NAKAMURA, M. (1988). Does endothelin mobilize calcium from intracellular store sites in rat aortic vascular smooth muscle cells in primary culture? *Biochem. Biophys. Res. Commun.*, **156,** 312–317.

MORELAND, S., MCMULLEN, D.M., DELANEY, C.L., LEE, V.G. & HUNT, J.T. (1992). Venous smooth muscle contains vasoconstrictor ET_B-like receptors. *Biochem. Biophys. Res. Commun.*, **184,** 100–106.

NAKAMICHI, K., IHARA, M., KOBAYASHI, M., SAEKI, T, ISHIKAWA, K. & YANO, M. (1992). Different distribution of endothelin receptor subtypes in pulmonary tissues revealed by the novel selective ligands BQ-123 and [$Ala^{1,3,11,15}$]ET-1. *Biochem. Biophys. Res. Commun.*, **182,** 144–150.

NEWBY, A.C. & HENDERSON, A.H. (1990). Stimulus-secretion coupling in vascular endothelial cells. *Annu. Rev. Physiol.*, **52,** 661–674.

OSBORNE, N.N. & BARNETT, N.L. (1992). Endotheline-1 stimulates phosphatidylinositol hydrolysis in the iris/ciliary complex and is a potent constrictor of the sphincter muscle. *Exp. Eye Res.*, **54,** 285–290.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348,** 732–735.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Changes in cytosolic calcium level in vascular smooth muscle strips measured simultaneously with contraction using fluorescent calcium indicator fura 2. *J. Pharmacol. Exp. Ther.*, **246,** 294–302.

SHETTY, S.S., OKADA, T., WEBB, R.L., DELGRANDE, D. & LAPPE, R.W. (1993). Functionally distinct endothelin B receptors in vascular endothelium and smooth muscle. *Biochem. Biophys. Res. Commun.*, **191,** 459–464.

SHINKAI, M., TSURUOKA, H., WAKABAYASHI, S., YAMAMOTO, Y. & TAKAYANAGI, I. (1994). Pre- and postjunctional actions of endothelin in the rat iris sphincter preparation. *Naunyn-Schmied Arch. Pharmacol.*, **350,** 63–67.

TAKAI, M., UMEMURA, I., YAMASAKI, K., WATANABE, T., FUJITANI, Y., ODA, K., URADE, Y., INUI, Y., YAMAMURA, T. & OKADA, T. (1992). A potent and specific agonist, Suc-[Glu^{9}, $Ala^{11,15}$]-endothelin-1 (8-21), IRL1620, for the ET_B receptor. *Biochem. Biophys. Res. Commun.*, **184,** 953–959.

TAKAYANAGI, I. & KANEKO, M. (1982). Contractile response of rabbit dilator pupillae muscle to isotonic high K^+ or norepinephrine, and Ca^{2+} movement. *Eur. J. Pharmacol.*, **79,** 159–165.

TAKUWA, Y., KASUYA, Y., TAKUWA, N., KUDO, M., YANAGISAWA, M., GOTO, K., MASAKI, T. & YAMASHITA, K. (1990). Endothelin receptor is coupled to phospholipase C via a pertussis toxin insensitive guanine nucleotide binding regulatory protein in vascular smooth muscle cells. *J. Clin. Invest.*, **85,** 653–658.

THOMAS, C.P., KESTER, M. & DUNN, M.J. (1991). A pertussis toxin-sensitive GTP-binding protein couples endothelin to phospholipase C in rat mesangial cells. *Am. J. Physiol.*, **260,** F347–F352.

UYAMA, Y., IMAIZUMI, Y. & WATANABE, M. (1992). Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor, on contractile response in skinned ileal smooth muscle. *Br. J. Pharmacol.*, **106,** 208–214.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.*, **143,** 299–330.

VIGNE, P., MARSAULT, R., BREITTMAYER, J.P. & FRELIN, C. (1990). Endothelin stimulates phosphatidylinositol hydrolysis and DNA synthesis in brain capillary endothelial cells. *Biochem. J.*, **266,** 415–420.

XUAN, Y.-T., WANG, O.-L. & WHORTON, A.R. (1994). Regulation of endothelin-induced Ca^{2+} mobilization in smooth muscle cells by protein kinase C. *Am. J. Physiol.*, **266,** C1560–C1567.

XUAN, Y.-T., WHORTON, A.R. & WATKINS, W.D. (1989). Inhibition by nicardipine on endothelin-mediated inositol phosphate formation and Ca^{2+} mobilization in smooth muscle cell. *Biochem. Biophys. Res. Commun.*, **160,** 758–764.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332,** 411–415.

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