Department of Biochemistry and Molecular Biology, School of Life Science, Lanzhou University, Lanzhou, P.R. China

Vasorelaxant responses to endomorphin1[ψ] and endomorphin2[ψ], analogues of endomorphins, in rat aorta rings

YUN FENG, QIAN-YU ZHAO, QIANG CHEN, RUI WANG

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Prof. Rui Wang, PhD, Department of Biochemistry and Molecular Biology, School of Life Science, Lanzhou University, Lanzhou, 730000, People's Republic of China
wangrui@lzu.edu.cn

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Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM2), the endogenous selective μ-opioid receptor agonists, can inhibit phenylephrine (PE) induced contraction which is related to the release of nitric oxide from vascular endothelium in aorta rings of rats and rabbits. The reduced (CH_2NH) amide bond is a useful peptide bond surrogate in the design of opioid mimetics because it could enhance conformational flexibility and metabolic stability. The present work was designed to investigate the vascular activities of interrelated endomorphin analogues: endomorphin-1[ψ] (Tyr[ψ(CH_2NH)]Pro-Trp-Phe-NH_2, EM1[ψ]) and endomorphin-2[ψ] (Tyr[ψ(CH_2NH)]Pro-Phe-Phe-NH_2, EM2[ψ]). The effect of EM1[ψ] (1, 2, 3, 4, 5 μM) and EM2[ψ] (0.001, 0.01, 0.1, 1, 5 μM) were evaluated on rat thoracic aortic rings pre-contracted with PE (0.1 μM). EM1[ψ] and EM2[ψ] both caused a concentration-dependent relaxation. The IC_{50} of EM1[ψ] and EM2[ψ] was 3.332 μM and 1.226 μM, respectively. The vasorelaxant effect of EMs[ψ] is 216.9 and 237.1 fold more potent than EMs. Moreover, the vasorelaxant effect of EMs[ψ] was blocked by naloxone (NaCl, 1 μM) and was reduced by N^{ω}-nitro-L-arginine (L-NNA, 1 μM) and removal of endothelium. The present study demonstrated that EMs[ψ] had more potent vasorelaxant effects and their activities were naloxone-sensitive and endothelium-dependent and partially NO-dependent, similar to the mechanism of parent EMs.

1. Introduction

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM2) are endogenous opioid peptides isolated from the bovine frontal cortex (Zadina et al. 1997) and human brain (Hackler et al. 1997). These tetrapeptides are most potent and selective μ-opioid receptor agonists and differ in one amino acid at the third position (Trp vs Phe).

The endogenous opioid system participates in the regulation of vascular smooth muscle tone, regional blood flow and blood pressure in normal and hypertensive states (Olson et al. 1998). The endogenous μ-opioid receptor agonist endomorphins (EMs) have potent cardiovascular effect. EMs can decrease systemic arterial pressure in rabbit (Champion et al. 1997a), rat (Czapla et al. 1998), cat (Champion et al. 1998a) and mouse (Champion et al. 1998b), and have significant vasodilator activity in the hindquarters vascular bed of the rat (Champion et al. 1997b). It has been reported that EMs inhibit phenylephrine (PE) induced contraction which is related to the release of nitric oxide (NO) from vascular endothelium in aorta rings of rat (Hugghins et al. 2000; Qi et al. 2002) and rabbit (Wu et al. 2001). Most of these activities arise from the high affinity and selectivity of EMs for opioid receptors (Champion et al. 1997a, 1997b, 1998b; Czapla et al. 1998). In EMs, the conformation of N-terminal dipeptide (Tyr-Pro) is important to their receptor profile and biological activities. Also, it has been reported that the receptor affinity of EMs greatly depend on the Phe residue at the fourth position whereas the selectivity of EMs depend on the Pro residue at the second position (Podlogar et al. 1998). However, EMs as well as peptides in general, have a limited *in vivo* efficacy, since they are easily degraded by different proteases (Péter et al. 1999; Shane et al. 1999; Szatmari et al. 2001; Tömböly et al. 2002). To resolve these problems, structure-activity studies of EMs along with the search for new peptidomimetics or more stable peptide analogues are of particular interest (Cardillo et al. 2002; Keller et al. 2001; Leitgeb et al. 2003; Okada et al. 2000; Paterlini et al. 2000). It has been reported that replacement of Pro^2 with other amino acids produced a vastly change of receptor profile, such as TIPP (Tyr-Tic-Phe-Phe-OH) (Martin et al. 2002) and TAPP (Tyr-D-Ala-Phe-Phe-NH_2) (Spetea et al. 1998). Stereochemical inversion of Pro resulted in drastic loss of activity in [D-Pro^2]EM1 (Paterlini et al. 2000) and [D-Pro^2]EM2 (Huo et al. 2001). Moreover, further research indicated that the substitution of D-isomer amino acid is an effective strategy in prolonging a durations in vasodilator activity. It has been demonstrated that the D-Ala^2-endomorphin-2 analogue produced similar dose-dependent decreases in systemic arterial pressure as endomorphin-2, and the duration of this effect persisted longer following analogue administration (Champion and Kadowitz 1998, 1999). Some studies suggested that the reduced (CH_2NH) amide bond is a

useful peptide bond surrogate in the design of opioid mimetics because it could enhance conformational flexibility and metabolic stability (Chen et al. 2002; Schiller et al. 1999). In order to enhance endomorphins' metabolic stabilities and their biological activities, interrelated analogues EM1[ψ] and EM2[ψ] have been synthesized (EM1[ψ], Tyr[ψ(CHNH)]Pro-Trp-Phe-NH; EM2[ψ], Tyr[ψ(CHNH)]Pro-Phe-Phe-NH_2).

Isolated rat aorta has a sparse or absent innervation and therefore permits the direct effects of opioid receptor agonists on vascular smooth muscle to be tested. The present study was done to investigate the role of EMs[ψ], two analogues of EMs, on thoracic aorta isolated from rat and to compare their responses with EMs. Furthermore, we investigated the vasorelaxant mechanisms of EMs[ψ].

2. Investigations and results

2.1. Effects of EM1, EM2, EM1[ψ], EM2[ψ] on contractile responses of rat thoracic aorta rings to PE

In endothelium-intact aortic rings, EM1 (0.001, 0.01, 0.1, 1, 5 μM), EM2 (0.001, 0.01, 0.1, 1, 5μM), EM1[ψ] (1, 2, 3, 4, 5 μM), EM2[ψ] (0.001, 0.01, 0.1, 1, 5 μM) could inhibit the contractile responses of rings to PE (0.1 μM) and the effects were concentration-dependent. The values of IC_{50} were calculated and obtained from concentration-response curves. The IC_{50} values of EM1, EM2, EM1[ψ], EM2[ψ] were 722.8, 290.7, 3.332, 1.226 μM, respectively. The vasorelaxant effect of EM1[ψ] is 216.9 fold more prononnced than that of EM1, and similar to EM1[ψ], the vasorelaxant effect of EM2[ψ] is 237.1 fold stronger than that of EM2. Vascular tension changes to opioid peptides are expressed as % decrease rate of the tension, the maximal response of aortic rings to PE was taken as 100%. The results are summarized in Fig. 1.

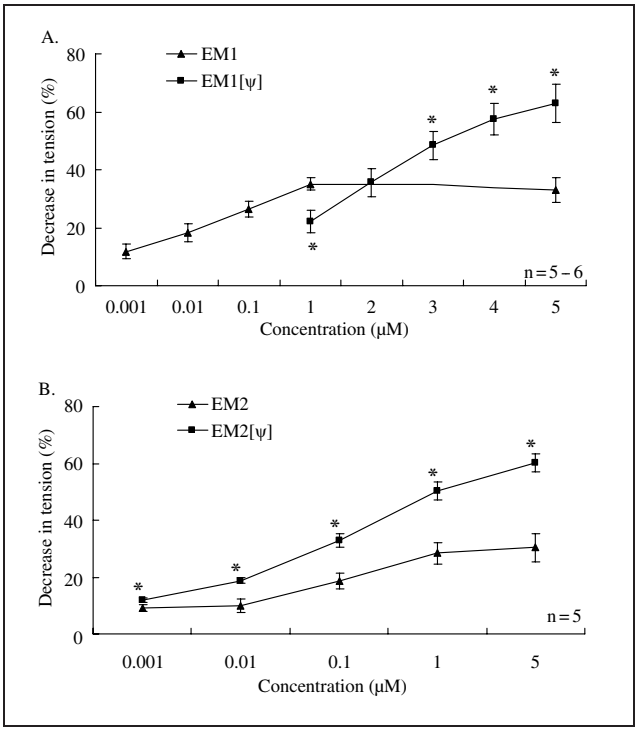

Fig. 1: Dose-respose curve of the vasorelaxation caused by EMs and EMs[ψ] on the PE (0.1 μM) precontracted rat aorta. The values are presented as the means ±S.E.M., n = 5−6 each group. n indicates number of experiments. Asterisk (*) represent significant results ($P < 0.05$) in comparison EMs[ψ] with their parent peptides

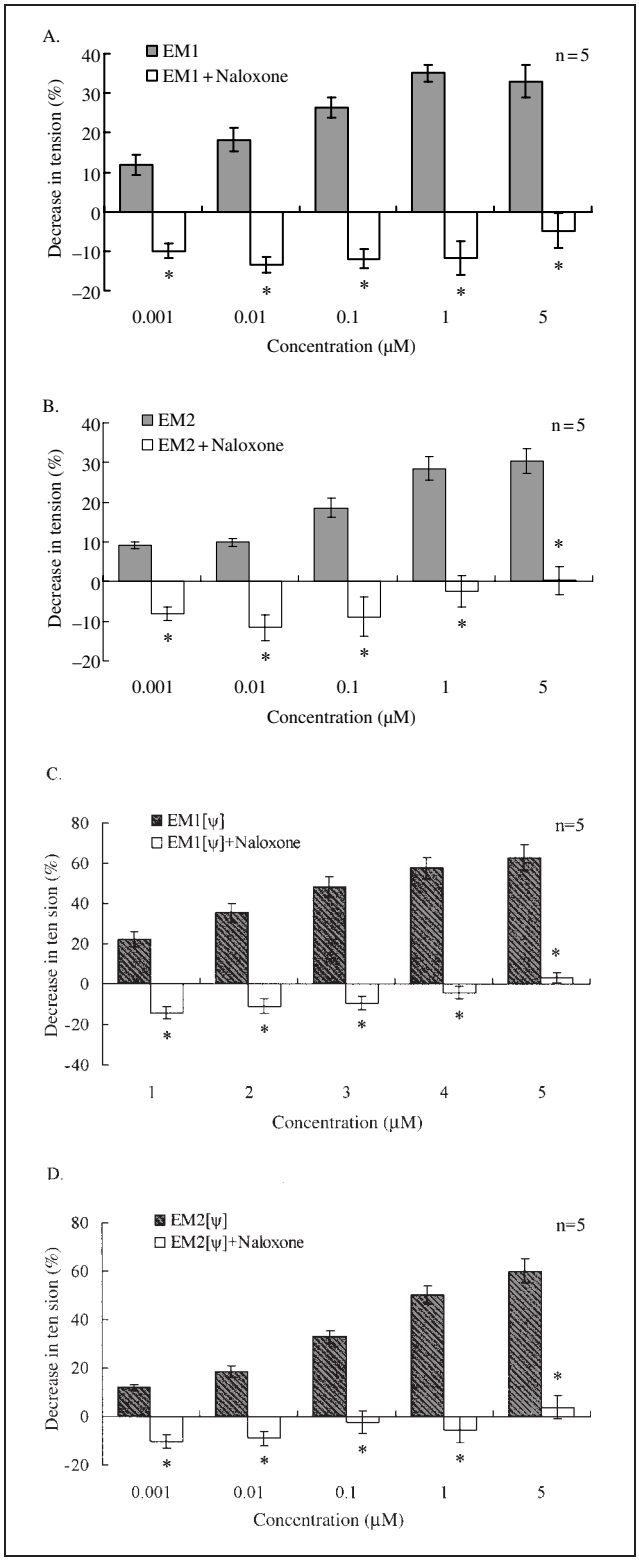

Fig. 2: Vascular tension changes to opioid peptides are expressed as % decrease rate, the maximal response of aortic rings to PE was taken as 100%. Influence of naloxone (1 μM) on vasorelaxation responses to EMs, EMs[ψ] in aortic rings precontracted with PE (100 nM). The values are presented as the means ±S.E.M., n = 5 each group. n indicates number of experiments. Asterisk (*) represent significant results ($P < 0.05$) in comparison with the control

2.2. Effects of naloxone on EM1, EM2, EM1[ψ], EM2[ψ] inhibits PE-induced contraction

EMs are endogenous opioid peptides which are most potent and selective μ-opioid receptor agonist. Naloxone is a

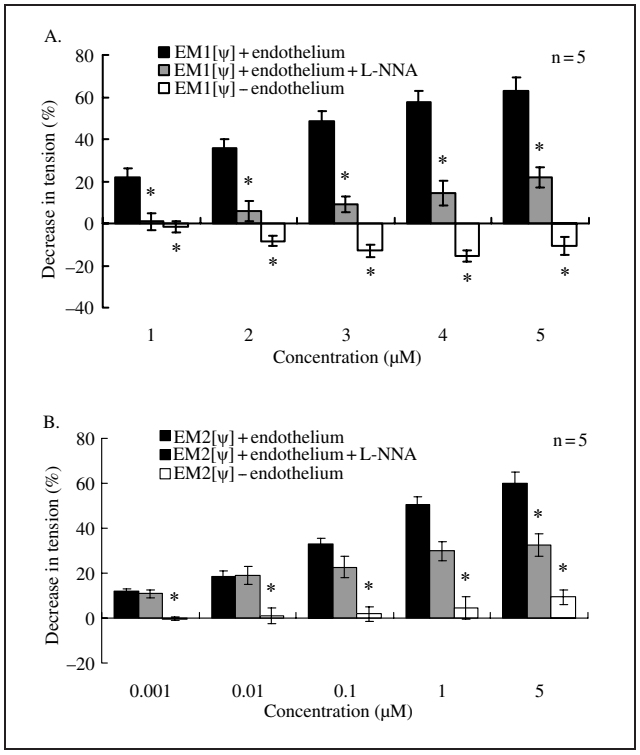

Fig. 3: Vasorelaxation effect of EMs[ψ] in rat aortic rings pretreated with L-NNA (1 μM) or endothelium denuded. The values are presented as the means ±S.E.M., n = 5 each group. n indicates number of experiments. Asterisk (*) represent significant results ($P < 0.05$) in comparison with the control (endothelium intact group)

selective opioid receptor antagonist. Naloxone (1 μM) can completely antagonize the vasorelaxant effects of EMs, EMs[ψ] ($P < 0.05$). In consistence with the results with EMs, it is indicated that the vasorelaxant effect of EMs[ψ] in opioid-receptor mediated and not a non-specific action. The results are summarized in Fig. 2.

2.3. Effects of EM1[ψ] and EM2[ψ] on contractile responses of rat endothelium-denuded aorta rings to PE

In contrast to endothelium-intact aortic rings, EMs[ψ] could not inhibit PE-induced contraction in endothelium-denuded rat aorta rings. As can be seen from Fig. 3, even the vasorelaxant effect of EM2[ψ] (5 μM) to the endothelium-denuded aortic rings was lower than 10%. In conclusion, the vasorelaxant responses of EMs[ψ] were endothelium-dependent. The results are summarized in Fig. 3.

2.4. Effects of L-NNA on EM1[ψ] and EM2[ψ] inhibits PE-induced contraction to rat endothelium-intact aorta rings

The effect of nitric oxide synthase is inhibited by L-NNA. In PE pre-contraction rings with intact endothelium, pretreatment with L-NNA (1 μM) caused significant ($P < 0.05$) decreases in the relaxant responses and downward shift in the concentration-response relationships of EM1[ψ]. However, L-NNA could only partially antagonize the vasorelaxant effects of EM2[ψ]. The results are summarized in Fig. 3.

3. Discussion

Previous studies of our group showed that the introduction of a reduced (CH_2NH) bond between Tyr^1 and Pro^2 in EMs caused a significant decrease of μ-opioid receptor agonist potency in GPI assay compared with parent EMs, and at the same time, EM1[ψ] and EM2[ψ] showed 3.2 and 2.1 fold reduced δ-opioid receptor agonist potency in MVD assay, respectively (data for agonist potency of EMs[ψ] in GPI and MVD assay were not shown). The selectivity of agonist potency of EMs[ψ] turned from the μ- to the δ-opioid receptor.

The present work was performed in order to investigate possible effects of EMs[ψ] in the isolated rat thoracic aorta. The results showed that EMs[ψ] concentration-dependently relaxed the PE pre-contracted rat thoracic aorta in agreement with the previous results of EMs (Hackler et al. 1997; Champion et al. 1998b, 1997b; Hugghins et al. 2000; Qi et al. 2002). At the same time, the vasorelaxant effect of EM1[ψ] is 216.9 fold stronger than that of EM1, and similar to EM1[ψ], EM2[ψ] is 237.1 fold more potent than EM2.

EM1 and EM2, endogenous opioid peptides, interact potently and selectively with the μ-opioid receptor. Some studies showed that EMs decrease mean arterial pressure in the anesthetized rat, rabbit and mouse by a naloxone-sensitive mechanism (Champion et al. 1997a, 1997b, 1998b; Czapla et al. 1998). It has been reported that the affinity of EMs greatly depends on the Phe residue at the fourth position and Pro at the second position confers high selectivity to the μ-opioid receptor (Podlogar et al. 1998). A selective opioid receptor antagonist naloxone (1 μM) completely suppressed the vasorelaxant responses induced by both EMs and EMs[ψ], which provided evidence like EMs, the vasorelaxant activity of EMs[ψ] is mediated via the opioid receptor system.

We investigated the vasorelaxant effects of EMs[ψ] in endothelium-denuded rat aorta rings. The findings of the present study were that the vasorelaxant effects of EMs[ψ] could be completely reversed by endothelium removal, in agreement with the previous studies of vasorelaxant responses of EMs in isolated aorta (Hugghins et al. 2000; Qi et al. 2002; Wu et al. 2001), so the vasorelaxant effects of EMs[ψ] were mediated through the mechanism of the endothelium system. That is to say, the vasorelaxation induced by EMs[ψ] was dependent on the presence of functional endothelium.

Furthermore, the results from this study show that the vasorelaxant responses to EM1[ψ] in isolated aortic rings of rats could be significantly prevented by pretreatment with L-NNA. Two primary vasodilatory stimuli produced by the endothelium are NO and prostaglandins. It has been reported that the cyclo-oxygenase inhibitor or the K_{ATP}^+-channel antagonist does not alter the vasodilator responses to EMs, whereas the NO synthase inhibitor does attenuate it (Champion et al. 1998a; Champion and Kadowitz 1998, 1999). Accordingly, the release of vasodilator prostaglandins or the opening of K_{ATP}^+-channels cannot be involved in the mediation of the responses to the EMs (Champion et al. 2002). The vasodilator responses to EMs are mediated, in large part, by the release of NO (Champion et al. 2002). The mechanism underlying the vasorelaxant effects of EM1[ψ] was in agreement with EMs. It is well known that the vascular endothelium synthesizes nitric oxide (NO) from L-arginine via the enzyme nitric oxide synthase (NOS), nitric oxide synthase inhibitor L-NNA suppresses production of NO by inhibiting the activity of NOS and NO modulates vascular tone as endothelium-derived relaxing factor (EDRF) (Moncada et al. 1991).

However, it was interesting that EM2[ψ] could only partially be inhibited by L-NNA and it was shown that

EM2[ψ] exerted vasorelaxant effects by both NO-dependent and NO-independent mechanisms. It is generally accepted that EDRF is NO or an NO derivative (Bellan et al. 1991; Broten et al. 1992; Ignarro et al. 1987, 1988a, 1988b). More recent evidence, however, suggests that NO may be only one of several EDRFs mediating the vasodilator response to ACh (Huang et al. 2000; Ross et al. 1991; Waldron et al. 1999). An additional endothelium-dependent vasodilatory mechanism is characterized as the hyperpolarization-mediated relaxation that remains after the inhibition of the synthesis of NO and prostaglandins. This mechanism is due to the action of a so-called endothelium-derived hyperpolarizing factor (EDHF) and is dependent on either the release of diffusible factor(s) and/or to a direct contact-mediated mechanism (Sandow 2004). It has been suggested that in the absence of NO production, EDHF may play an important role in regulating vascular tone in the mouse (Scotland et al. 2001). It was implied that EM2[ψ], different from EM2, could probably have vasorelaxant effects not only through a NO-dependent pathway but also through a hyperpolarization-mediated pathway such as EDHF. The mechanism underlying the vasorelaxant effects of EMs[ψ] needs to be studied further.

In summary, the present study demonstrated that these two newly synthesized analogues (EMs[ψ]) have vasorelaxant effects more potent than those of EMs. The activities of EMs[ψ] were naloxone-sensitive and endothelium-dependent and partially NO-dependent.

4. Experimental

4.1. Chemicals and drugs

Peptides were synthesized by the solid-phase method on a *p*-methylbenzhydrylamine resin (MBHA) using *tert*-butyloxycarbonyl (Boc)-protected amino acids (Sigma Chemical Co.) and dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) as coupling agent. Deprotection of Boc-amino protecting groups was accomplished with 33% trifluoroacetic acid (TFA) in dichloromethane (DCM). The free N-terminal amino group of the resin-bound peptide was reductively alkylated by the requisite Boc-proteced α-aminoaldehyde in the presence of sodium cyanoborohydride ($NaBH_3CN$) in DMF containing 1% AcOH. The aldehydes Boc-Tyr-H were prepared by $LiAlH_4$ reduction of their corresponding *N*,*O*-dimethyl hydroxamates at 0–10 °C in dried THF (Sasaki and Coy 1987). Peptides were cleaved from the resin by HF/anisole treatment in the usual manner. After evaporation of HF, the resin was washed three times with ether and subsequently three times with 10% acetic acid. The crude peptides were obtained in solid form by reverse-phase HPLC on a DELTAPAK C_{18} column (Waters, 5 μ 300 Å 7.8 × 300 mm) with a linear gradient of 10–100% B in 60 min at flow rate of 1 ml/min (A = 0.05% trifluroacetic acid in water and B = acetonitrile). Each peptide was >95% pure as determined by analytical reverse-phase HPLC on a DELTAPAK C_{18} column (Waters, 5 μ 300 Å 3.9 × 150 mm) using a linear gradient. Purified peptides were verified by mass spectrometry (Mariner 5074). Phenylephrine hydrochloride (PE) and acetylcholine (ACh) were the products of Shengyang First Pharmaceutical Factory in China. Naloxone hydrochloride (Nx) and N^{ω}-nitro-L-arginine (L-NNA) were purchased from Sigma Chemical Co.

4.2. Animal preparation

Wistar rats weighing 200 to 250 g were purchased from the Medical Laboratory Animal Center of Lanzhou Medical College. Before use, rats were housed in individual cages and maintained on a 12-hour light/dark cycle with unlimited access to food and water. A minimum of 5 animals was used for each experiment. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

4.3. Vascular reactivity experiments

Rats were killed by a bit to the head; the thoracic aorta was removed carefully; and then fat and tissue were dissected away in normal Krebs' solution of the following composition: NaCl 118.0 mM, KCl 1.7 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1.2 mM, KH_2PO_4 1.0 mM, $NaHCO_3$ 25.0 mM, and glucose 11.0 mM, pH 7.40. The aorta was cut into rings about 5 mm in length in a 10 mL bath and constantly gassed with 95% O_2 + 5% CO_2 at 37.0 ± 0.5 °C. Two stainless steel hooks were inserted into the aortic lumen; one was fixed in the bottom bath and the other was connected to a force transducer. The aorta rings were equilibrated in the Krebs' solution and maintained under an optimal tension of 1 g for 90 min. Contractions were recorded isometrically via a force-displacement transducer (JZ 101 muscle force transducer) connected to a computer with BL-420E system (TME, Chengdu, China). In denuded aorta, the endothelium was removed by rubbing with a cotton swab in the rings. Before each experiment, contractile responses to PE 0.1 μM were used to assess vascular smooth muscle function, whereas relaxation responses to ACh (1 μM) in rings precontracted with PE (0.1 μM) were used to test endothelial integrity. Following these, different concentrations of EM1 (0.001, 0.01, 0.1, 1, 5 μM), EM2 (0.001, 0.01, 0.1, 1, 5 μM), EM1[ψ] (1, 2, 3, 4, 5 μM), EM2[ψ] (0.001, 0.01, 0.1, 1, 5 μM) were added 15 min after the rings were contracted by PE (0.1 μM) in turn. In other test groups, Nal (1 μM) or L-NNA (1 μM) was added to the bath 10 min prior to EM1[ψ], EM2[ψ].

4.4. Statistical analyses

All responses were expressed as a % decrease of the tension induced by PE. The data are presented as the means ±S.E.M. for the number of experiments indicated. Statistical analysis was performed using the Student's t-test, and $P < 0.05$ was regarded as significantly different. The values of IC_{50} were calculated and obtained from concentration-response curves. The values of inhibition of these points ranged from 10% to 70%.

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