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Endothelium-dependent relaxation followed by contraction mediated by NK_1 receptors in precontracted rabbit intrapulmonary arteries

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1 In the present study, we examined whether substance P (SP) and SP methyl ester (SPME), a selective NK_1 agonist, cause biphasic responses consisting of endothelium-dependent relaxation (EDR) and contraction (EDC) in precontracted rabbit intrapulmonary arteries.

2 In arteries contracted with $PGF_{2\alpha}$ (2×10^{-6} M), SP as well as SPME caused only EDR at low concentration (10^{-9} M) and EDR followed by EDC at higher concentrations, indicating the involvement of NK_1 receptors. The SP (10^{-8} M)-induced EDR was abolished in arteries moderately contracted by $PGF_{2\alpha}$ (5×10^{-7} M) and the EDC in arteries maximally contracted by $PGF_{2\alpha}$ (10^{-5} M), indicating that EDR and EDC are inversely dependent on preexisting tone.

3 Indomethacin ($10^{-8}-10^{-6}$ M), a cyclo-oxygenase inhibitor, and ozagrel ($10^{-8}-10^{-6}$ M), a TXA_2 synthetase inhibitor attenuated the EDC in the SPME (10^{-7} M)-induced biphasic response and markedly potentiated the EDR. AA-861 ($10^{-8}-10^{-6}$ M), a 5-lipoxygenase inhibitor, did not affect the EDR or EDC. L-N^G-nitro-arginine methyl ester ($10^{-5}-10^{-4}$ M), a nitric oxide synthase inhibitor, attenuated the EDR and slightly potentiated the EDC.

4 CP-99994 ($10^{-10}-10^{-8}$ M), an NK_1 antagonist, attenuated the EDC and potentiated the EDR in the SPME (10^{-7} M)-induced biphasic response, while the NK_2 antagonist SR-48968 ($10^{-9}-10^{-7}$ M) had no effect. CP-99994 attenuated the SPME (10^{-7} M)-induced EDC under EDR-blockade to a greater extent than the EDR under EDC-blockade, indicating that CP-99994 enhanced the EDR component by preferential inhibition of the EDC component.

5 In conclusion, NK_1 agonists caused a biphasic endothelium-dependent response (EDR and EDC) in submaximally precontracted intrapulmonary arteries. The EDC and EDR mediated by NK_1 receptors may play physiological and/or pathophysiological roles in modulation of vascular tone.
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Keywords: NK_1 receptor; endothelium-dependent contraction; endothelium-dependent relaxation; intrapulmonary artery; nitric oxide; substance P; substance P methyl ester; thromboxane A_2

Abbreviations: EDC, endothelium-dependent contraction; EDR, endothelium-dependent relaxation; EIC, endothelium-independent contraction; L-NAME, L-N^G-nitro-arginine methyl ester; NKA, neurokinin A; NO, nitric oxide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SP, substance P; SPME, substance P methyl ester; TXA_2, thromboxane A_2

Introduction

The pulmonary circulation is regulated by various neuronal, hormonal and humoral factors including neuropeptides (Barnes & Liu, 1995). The pulmonary arteries are innervated by sensory nerve containing substance P (SP), neurokinin A (NKA) and CGRP (Allen *et al*., 1989). Treatment with the C-fibre activator capsaicin and electrical stimulation of vagal nerves results in release of SP and NKA in the perfused guinea-pig lung (Saria *et al*., 1988). SP is also localized in endothelial cells and is released by hypoxia and changes in perfusion flow (Milner *et al*., 1989; Ralevic *et al*., 1990). Tachykinin NK_1 receptors have been shown to locate on the vascular endothelium (Greeno *et al*., 1993; Bowden *et al*., 1996). SP causes only endothelium-dependent relaxation (EDR) *via* nitric oxide (NO) production in precontracted preparations of guinea-pig and rabbit pulmonary arteries *via* activation of NK_1 receptors (D'Orleans-Just *et al*., 1986; Emonds-Alt *et al*., 1993; Floch *et al*., 1994). In non-contracted preparations of rabbit intrapulmonary arteries, SP evokes endothelium-dependent contraction (EDC) *via* activation of NK_1 receptors and TXA_2 production at low concentrations (Shirahase *et al*., 1995) and endothelium-independent contraction (EIC) *via* NK_2 receptors at higher concentrations (D'Orleans-Just *et al*., 1986; Shirahase *et al*., 1995). Neuronal and/or endothelial SP may modulate vascular tone by causing EDR and EDC. However, there have been few reports on the simultaneous occurrence of EDC and EDR under active tone in pulmonary arteries or on their pharmacological nature. In the present study, we demonstrated that SP and SP methyl ester (SPME), a selective NK_1 agonist, induce only EDR at low concentrations and a biphasic endothelium-dependent response (EDR followed by EDC) at higher concentrations in isolated submaximally precontracted rabbit intrapulmonary arteries. We also pharmacologically characterized the EDR and EDC components in the biphasic endothelium-dependent response.

Methods

Male Japanese white rabbits (2–3 kg) (Oriental Bio Service, Kyoto, Japan) were fed regular chow (CR-3, Clea Japan, Osaka, Japan) and allowed access to tap water *ad libitum*. Animals were anaesthetized with sodium pentobarbital (25 mg kg^{-1}, i.v.) and exsanguinated from the common carotid artery. The thoracic cavity was opened and the lungs were excised and placed in aerated Krebs-Henseleit solution of

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the following composition (mM): NaCl, 120; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 10. The peripheral portions of the intrapulmonary arteries (diameter 0.3–1.0 mm) were isolated from the lungs and carefully cleaned of lung parenchymal, fat and connective tissue. The arteries were helically cut and the strips were fixed vertically between hooks in a 10 ml organ bath containing a nutrient solution maintained at 37 ± 0.5°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The pH of the solution was 7.4. The end of each strip was attached to the lever of a force-displacement transducer (NEC San-Ei Instrument Co. Ltd, Tokyo, Japan) connected to an ink-writing oscillograph (NEC San-Ei Instrument Co. Ltd and isometric changes in tension were recorded. The applied tension was adjusted to 0.5 g. Each strip was allowed to equilibrate for 1 h, during which time the nutrient solution was changed every 10 min and the applied tension was readjusted. In several experiments, the intact and endothelium-removed strips were prepared from the same artery. The functional integrity of the endothelium in the intact preparations was checked with acetylcholine (ACh), which causes EDR in the presence of active tone (Altiere *et al*., 1986). The endothelium was removed by intimal rubbing. The rubbed preparations showed no ACh-induced relaxation. The elimination of endothelium was verified morphologically by scanning electron microscopy as described previously (Shirahase *et al*., 1987).

In the first set of experiments, SP (10^{-10}–10^{-7} M) and SPME (10^{-10}–10^{-6} M) were applied non-cumulatively to the arteries with and without endothelium contracted by $PGF_{2\alpha}$ at 2×10^{-6} M. Relaxation and contraction were expressed as changes in tension from the plateau level of $PGF_{2\alpha}$-induced contraction. SPME (10^{-10}–10^{-7} M) was also applied under EDC- and EDR-blockade. To eliminate EDC and EIC, ozagrel (10^{-5} M), a TXA_2 synthetase inhibitor (Iizuka *et al*., 1981), and SR-48968 (10^{-7} M), an NK_2 antagonist (Emonds-Alt *et al*., 1992) were pretreated before application of SPME. Ozagrel (10^{-5} M) and SR-48968 (10^{-7} M) abolishes the substance P-induced EDC and EIC, respectively, in the isolated rabbit intrapulmonary artery (Shirahase *et al*., 1995). To eliminate EDR and EIC, L-NAME (10^{-4} M), a NO synthase inhibitor, and SR-48968 (10^{-7} M) were pretreated before application of SPME. L-NAME (10^{-4} M) abolishes the substance P-induced EDR in the isolated rabbit intrapulmonary artery (Shirahase *et al*., 1997). Then, concentration-response curves of SPME for EDR and EDC were constructed. To examine the effects of magnitude of preexisting tonus, SP (10^{-8} M) was applied to the intrapulmonary arteries with endothelium contracted by $PGF_{2\alpha}$ at 5×10^{-7}, 2×10^{-6} and 10^{-5} M.

In the second set of experiments, the effects of various enzyme inhibitors and receptor antagonists were examined in arteries contracted with $PGF_{2\alpha}$ at 2×10^{-6} M. Enzyme inhibitors and receptor antagonists were applied 5 min prior to the administration of $PGF_{2\alpha}$ and 20 min prior to the administration of SPME (10^{-7} M). The effects of CP-99994 (10^{-10}–10^{-8} M), an NK_1 receptor antagonist (Desai *et al*., 1992), on the SPME-induced EDR in the presence of ozagrel and SR-48968, and on the EDC in the presence of L-NAME and SR-48968 were also examined.

SP (Peptide Institute, Osaka, Japan), SPME (Peptide Institute), indomethacin (Wako Pure Chemical Industries, Ltd, Osaka, Japan), L-NAME (Wako Pure Chemical Industries, Ltd, Osaka, Japan), $PGF_{2\alpha}$ (Cayman Chemical Company, Ann Arbor, MI, U.S.A.), sodium pentobarbitone (Tokyo Kasei Kogyo, Co., Ltd, Tokyo, Japan) and ACh (Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan) were purchased from the sources indicated. Ozagrel hydrochloride (Ono Pharmaceutical Co. Ltd, Osaka, Japan), (+)−(2s,3s)-3-(2-methoxybenzylamino)-2-phenylpiperidine (CP-99994) (Pfizer Inc., Groton, CT, U.S.A.), (s)-N-methyl-N-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide (SR-48968) (Sanofi Recherche, Cedex, France) and 2-(12-hydroxy-5,10-dodecadiynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861) (Takeda Chemical Industries, Ltd, Osaka, Japan) were gifts from the sources indicated. SP, SPME, ozagrel hydrochloride, CP-99994, ACh, sodium pentobarbital and L-NAME were dissolved in distilled water, $PGF_{2\alpha}$ in ethanol, and AA-861 and SR-48968 in dimethylsulphoxide.

Data are expressed as means ± s.e.mean. The statistical significance of differences was analysed by Student's *t*-test for paired data. A P value less than 0.05 was considered significant.

Results

Responses to SP and SPME in endothelium-intact and removed intrapulmonary artery

SP (10^{-10}–10^{-7} M) and SPME (10^{-10}–10^{-6} M) were non-cumulatively applied to the endothelium-intact and -removed strips contracted by $PGF_{2\alpha}$ (2×10^{-6} M). SP and SPME caused only relaxation at 10^{-9} M and biphasic responses consisting of relaxation followed by contraction at concentrations of 10^{-8} M and higher in the endothelium-intact strips (Figure 1). These responses were abolished in endothelium-removed strips with the exception of SP (10^{-7} M), in which partial contraction remained (EIC). Mean values of EDR and EDC induced by SP and SPME are shown in Figure 2.

EDR and EDC may counteract each other in the biphasic response. To observe the concentration-response relationship for EDR and EDC without this counteraction, SPME (10^{-10}–10^{-7} M) was applied to strips pretreated with ozagrel (10^{-5} M) and SR-48968 (10^{-7} M), or with L-NAME (10^{-4} M) and SR-48968 (10^{-7} M), respectively. SPME-induced EDR reached the maximal level at 10^{-8} M, while EDC did not reach this level even at 10^{-7} M (Figure 3).

To examine the effect of magnitude of preexisting tonus, SP (10^{-8} M) was applied to the endothelium-intact strips

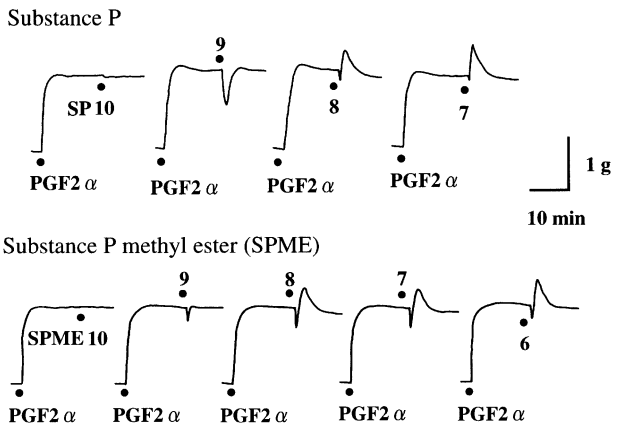

Figure 1 Representative tracings of responses induced by substance P (SP) and substance P methyl ester (SPME) in endothelium-intact rabbit intrapulmonary arteries precontracted with $PGF_{2\alpha}$ (2×10^{-6} M). Figures with dots show concentrations of peptides (−log M).

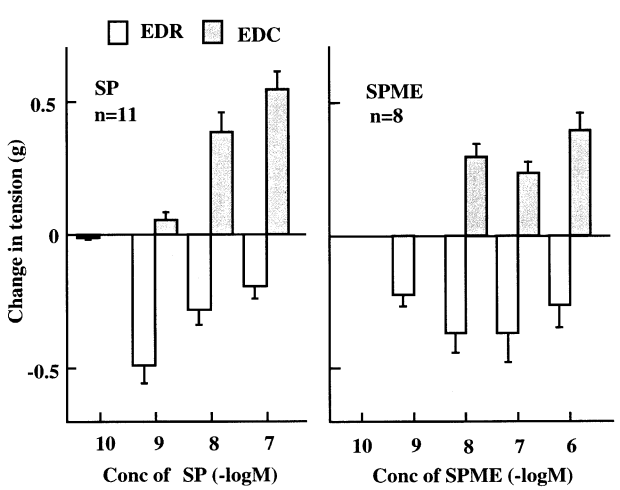

Figure 2 Endothelium-dependent relaxation (EDR) and contraction (EDC) induced by SP and substance P methyl ester (SPME) in endothelium-intact rabbit intrapulmonary arteries precontracted with $PGF_{2\alpha}$ (2×10^{-6} M). Data are means ± s.e.mean.

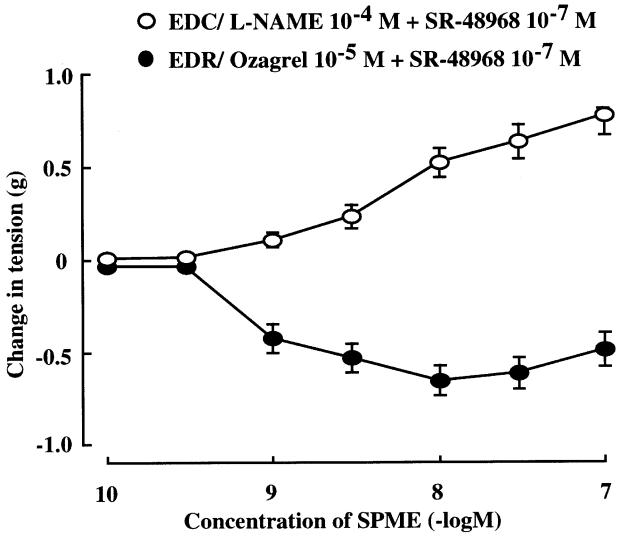

Figure 3 Concentration-response curves of SPME (10^{-10}–10^{-7} M) for EDR under EDC-blockade and for EDC under EDR-blockade in endothelium-intact rabbit intrapulmonary arteries precontracted with $PGF_{2\alpha}$ (2×10^{-6} M). Data are means ± s.e.mean ($n=9$) EDC: SPME was applied in the presence of L-NAME (10^{-4} M) and SR-48968 (10^{-7} M), which eliminate EDR and EIC, respectively. EDR: SPME was applied in the presence of ozagrel (10^{-5} M) and SR-48968 (10^{-7} M), which eliminate EDC and EIC, respectively.

contracted by 5×10^{-7}, 2×10^{-6} and 10^{-5} M $PGF_{2\alpha}$, for which the precontraction levels were 0.9 ± 0.1, 1.9 ± 0.1 and 2.5 ± 0.2 g (mean ± s.e.mean, $n=5$), respectively. SP induced biphasic response consisting of EDR (0.5 ± 0.1 g) followed by EDC (0.4 ± 0.1 g) in the arteries contracted by $PGF_{2\alpha}$ at 2×10^{-6} M. The EDC decreased to 0.1 ± 0.02 g and the EDR increased to 0.7 ± 0.1 g in the arteries contracted by $PGF_{2\alpha}$ at 10^{-5} M, while the EDC increased to 1.0 ± 0.2 g and the EDR decreased to 0.2 ± 0.1 g in the arteries contracted at 5×10^{-7} M, indicating that EDR increased and EDC decreased depending on the magnitude of precontraction.

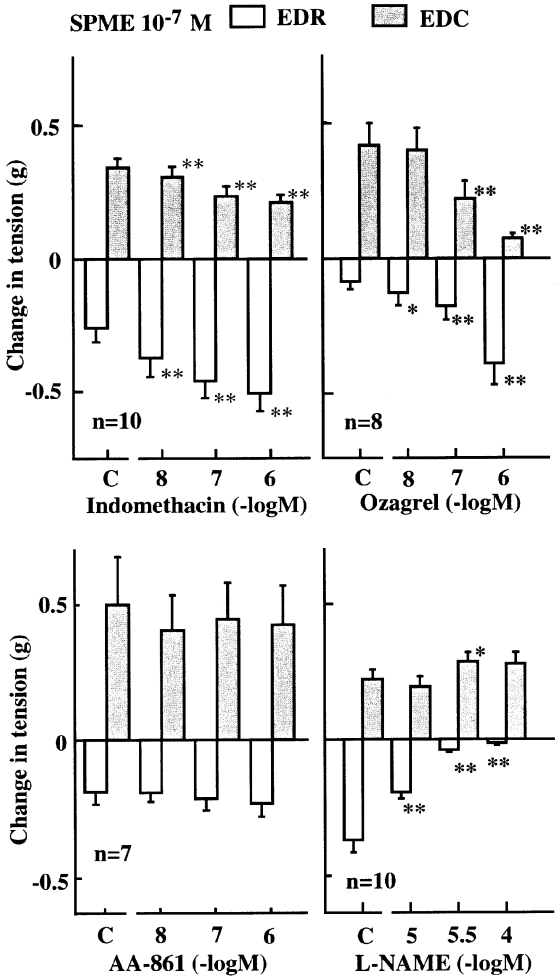

Figure 4 Effects of indomethacin (cyclo-oxygenase inhibitor), ozagrel (TXA_2 synthetase inhibitor), AA-861 (5-lipoxygenase inhibitor) and L-NAME (nitric oxide synthase inhibitor) on the EDC and EDR component in the biphasic endothelium-dependent response induced by SPME (10^{-7} M) in endothelium-intact rabbit intrapulmonary arteries precontracted with $PGF_{2\alpha}$ (2×10^{-6} M). C = control. Data are means ± s.e.mean. $^*P<0.05$, $^{**}P<0.01$, Student's t-test for paired data.

Effects of inhibitors of arachidonic acid metabolism and NO synthesis on the biphasic endothelium-dependent response

The effects of various inhibitors on biphasic endothelium-dependent responses induced by SPME (10^{-7} M) were examined. The EDC component was attenuated in a concentration-dependent manner by indomethacin (10^{-8}–10^{-6} M), a cyclo-oxygenase inhibitor (Vane, 1971) and ozagrel (10^{-8}–10^{-6} M), a TXA_2 synthetase inhibitor, resulting in concentration-dependent enhancement of the EDR component (Figure 4). AA-861 (10^{-8}–10^{-6} M), a specific 5-lipoxygenase inhibitor (Yoshimoto *et al.*, 1982), affected neither EDC nor EDR (Figure 4). The EDR component was attenuated in a concentration-dependent manner by L-NAME (10^{-5}–10^{-4} M), an inhibitor of NO synthase, resulting in slight enhancement of the EDC component (Figure 4).

Effects of tachykinin NK_1 and NK_2 receptor antagonists on the biphasic endothelium-dependent response

The effects of NK_1 and NK_2 antagonists on the biphasic endothelium-dependent response induced by SPME (10^{-7} M)

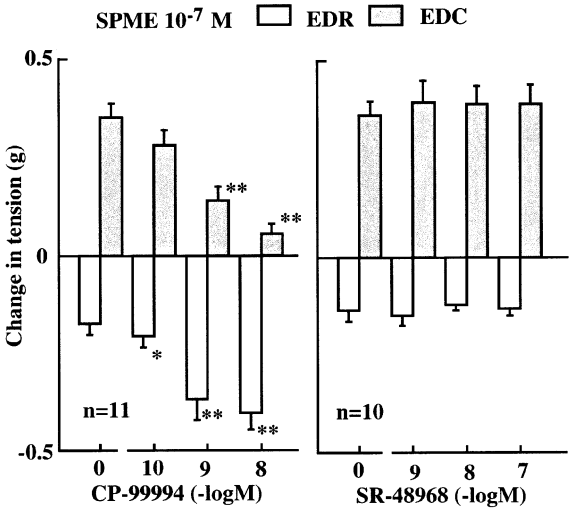

Figure 5 Effects of CP-99994 (tachykinin NK_1 receptor antagonist) and SR-48968 (NK_2 receptor antagonist) on the EDC and EDR component in the biphasic endothelium-dependent response induced by SPME (10^{-7} M) in endothelium-intact rabbit intrapulmonary arteries precontracted with $PGF_{2\alpha}$ (2×10^{-6} M). C = control. Data are means ± s.e.mean. $^*P<0.05$, $^{**}P<0.01$, student's *t*-test for paired data.

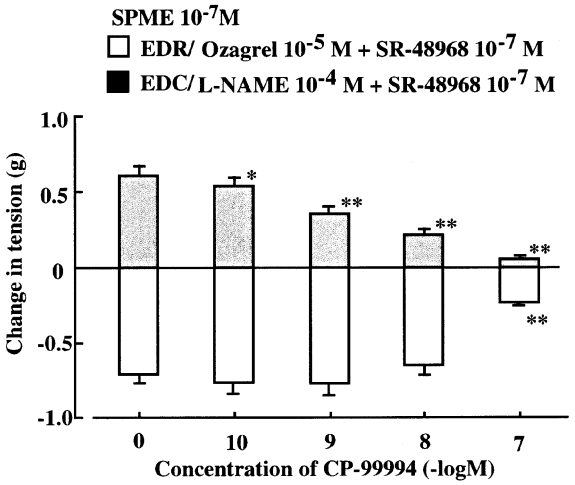

Figure 6 Effects of CP-99994 (tachykinin NK_1 receptor antagonist) on the EDR component induced by SPME (10^{-7} M) under EDC blockade and the EDC component under EDR blockade in endothelium-intact rabbit intrapulmonary arteries precontracted with $PGF_{2\alpha}$ (2×10^{-6} M). EDR: ozagrel (10^{-5} M) and SR-48968 (10^{-7} M) were applied to eliminate EDC and EIC, respectively. EDC: L-NAME (10^{-4} M) and SR-48968 (10^{-7} M) were applied to eliminate EDR and EIC, respectively. C = control. Data are mean ± s.e.mean ($n=11$). $^*P<0.05$, $^{**}P<0.01$, Student's *t*-test for paired data.

were examined. CP-99994 (10^{-10}–10^{-8} M), a tachykinin NK_1 receptor antagonist, concentration-dependently attenuated the EDC component but enhanced the EDR component in the biphasic response induced by SPME (10^{-7} M) (Figure 5). SR-48968 (10^{-9}–10^{-7} M), an NK_2 antagonist, had no effect on the EDC or EDR component (Figure 5). SPME at 10^{-9} M caused only EDR as shown in Figure 2, which was attenuated by CP-99994 (10^{-9}–10^{-7} M) in a concentration-dependent manner (data not shown).

In strips pretreated with ozagrel (10^{-5} M) and SR-48968 (10^{-7} M) to eliminate EDC and EIC components, SPME (10^{-7} M) evoked only EDR, which was attenuated by CP-99994 (10^{-7} M) (Figure 6). In strips pretreated with L-NAME (10^{-4} M) and SR-48968 (10^{-7} M) to eliminate EDR and EIC components, SPME (10^{-7} M) evoked only EDC, which was concentration-dependently attenuated by CP-99994 (10^{-10}–10^{-7} M) (Figure 6). These results showed that the EDC component was more sensitive to NK_1 antagonist than the EDR component in the biphasic response induced by SPME (10^{-7} M).

Discussion

SP causes EDR in various peripheral arteries including rabbit pulmonary arteries *via* NO production in the presence of active tone (Emonds-Alt *et al*., 1993). We reported previously that SP causes EDC *via* production of TXA_2 in the non-contracted rabbit pulmonary artery (Shirahase *et al*., 1995). However, there have been few reports on SP-induced EDR and EDC in the same pulmonary arterial preparations. In the present study, we found that SP and SPME, a selective NK_1 agonist, caused only EDR at low concentrations and biphasic endothelium-dependent responses (EDR followed by EDC) at concentrations of 10^{-8} M and higher in the precontracted rabbit intrapulmonary arteries, and that SP (10^{-8} M)-induced EDC decreased and EDR increased depending on the magnitude of precontraction.

EDR appeared at lower concentrations of SP and SPME in comparison with EDC (Figure 2). EDR did not increase in a concentration-dependent manner since the following EDC counteracted EDR at higher concentrations of SP and SPME. In separate experiments (Figure 3), concentration-response curves of SPME for EDC and EDR were independently constructed using ozagrel to eliminate EDC and L-NAME to eliminate EDR, respectively. The EDR was about 10 fold more sensitive to SPME than the EDC. We speculated that when endothelial cells are exposed to endogenous NK_1 agonists, the EDR pathway is first activated at low concentrations and then the EDC pathway is driven at higher concentrations to counteract the EDR as an auto-regulatory mechanism. Although the precise mechanism by which EDR was more sensitive to NK_1 activation than EDC is not clear, the nature of endothelial NK_1 receptors and/or their signalling process involved in EDC and EDR are considered to be different. The guinea-pig bronchi have been reported to contain unusual septide-selective NK_1 receptors (Zeng & Burcher, 1994). Alternatively, sensitivity to second messengers after activation of NK_1 receptors may be different between EDC and EDR pathways. NO is produced from arginine by Ca^{2+}-dependent eNOS and TXA_2 from arachidonic acid liberated by Ca^{2+}-dependent phospholipase A_2. Stimulation of NK_1 receptors leads to activation of phospholipase C and to accumulation of IP_3, resulting in an increase in intracellular Ca^{2+} level. eNOS may be activated by lower concentrations of intracellular Ca^{2+} than phospholipase A_2. Similarly to NK_1 agonists, Ca^{2+} ionophores such as A-23187 and ionomycin caused only EDR at low concentrations and a biphasic endothelium-dependent response (EDR followed by EDC) at higher concentrations (unpublished data).

The mechanism by which EDR preceded EDC in the biphasic response also remains to be clarified. Production and/or action of NO are considered to be more rapid than those of TXA_2 after stimulation by SP or SPME. The EDC component in the biphasic endothelium-dependent response was decreased and the EDR component increased depending on the magnitude of active tone, suggesting a role of EDC or EDR in auto-regulation of vascular tone.

We reported previously that SP-induced EDC is mediated by production of TXA_2 in non-contracted pulmonary arteries (Shirahase *et al*., 1995). Indeed, cyclo-oxygenase and TXA_2 synthetase inhibitors showed concentration-dependent attenuation of the EDC component accompanied by enhancement of the EDR component, indicating that EDR was partially masked by the following EDC in the biphasic endothelium-dependent response. We have also shown that EDC induced by various agonists is mediated by TXA_2 in canine cerebral arteries (Shirahase *et al*., 1987; 1988a,b; 1991; Kurahashi *et al*., 1994). L-NAME concentration-dependently inhibited EDR and slightly enhanced EDC, indicating that NO had little effect on the maximal level of the following EDC. AA-861 affected neither EDR nor EDC, indicating that 5-lipoxygenase metabolites were not involved in the biphasic endothelium-dependent response.

There are three types of tachykinin receptors, NK_1, NK_2 and NK_3. In the pulmonary artery, EDC and EIC are mediated by NK_1 and NK_2 receptors, respectively (Shirahase *et al*., 1995). EDR induced by SP is mediated by NK_1 receptors in guinea-pig and rabbit pulmonary arteries (Emonds-Alt *et al*., 1993; Floch *et al*., 1994). We demonstrated that SPME, a selective NK_1 agonist induced only EDR at a low concentration (10^{-9} M), which was effectively blocked by the selective NK_1 antagonist CP-99994. However, CP-99994 showed concentration-dependent attenuation of the EDC component accompanied by enhancement of the EDR component in the biphasic response induced by SPME at 10^{-7} M. This concentration of SPME was shown to be submaximal for EDC and supramaximal for EDR in the concentration-response curves (Figure 3). Therefore, CP-99994 is considered to inhibit the EDC more effectively than the EDR component, resulting in apparent enhancement of the EDR which had been partially masked by the EDC. Indeed, EDR under EDC-blockade was not enhanced and was attenuated by CP-99994 (Figure 6). The EDC under EDR-blockade was shown to be much more effectively inhibited by the NK_1 antagonist than the EDR under EDC-blockade.

The balance between EDC and EDR is important for homeostasis of vascular tone and is impaired in various diseases showing circulatory failure. Indeed, SP-induced EDR is impaired in pulmonary hypertension (Uren *et al*., 1992; Brett *et al*., 1996). Thus, inhibitors of the cyclo-oxygenase-TXA_2 synthetase pathway are considered to dilate blood vessels by inhibition of EDC and enhancement of EDR in pulmonary hypertension. NK_1 antagonists also dilate pulmonary arteries exposed to high concentrations of tachykinins by preferential inhibiton of NK_1-mediated EDC accompanied by apparent enhancement of EDR. On the other hand, tachykinins are involved in pulmonary inflammation such as asthma, and cause vasodilation and increases in vascular permeability through activation of NK_1 receptors and NO production (Kageyama *et al*., 1997). TXA_2 may be simultaneously produced by tachykinins to counteract NO in an auto-regulatory mechanism. Recently, a number of NK_1 antagonists have been synthesized, but none of these have been successfully developed as anti-inflammatory drugs. Under high concentrations of tachykinins, NK_1 antagonists may potentiate the proinflammatory effect of NO by preferential inhibition of the EDC (TXA_2) pathway. Further studies are needed to clarify the pathophysiological role of tachykinin-induced EDR and EDC and therapeutic significance of their pharmacological modulation.

In conclusion, tachykinin NK_1 receptor stimulation causes biphasic endothelium-dependent response (EDR followed by EDC) in submaximally precontracted intrapulmonary arteries. The EDR component in the biphasic endothelium-dependent response is more resistant to NK_1 antagonists than the EDC component.

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