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Contemporary Activation of Different Endothelial Receptors Accounts for a Reserve Mechanism of Nitric Oxide-Mediated Relaxation

S. KYSELÁ, J. TÖRÖK

Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovak Republic

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Summary

The aim of this study was to investigate whether the inhibition of one of the endothelial receptor sites in the rat pulmonary artery (muscarinic, histaminergic, purinergic, α_2-adrenergic) affects the NO-mediated relaxation induced by the activation of the other type of receptors. Acetylcholine (ACh)-, histamine (Hist)-, adenosine (Ade)- , and clonidine (Clon)-induced endothelium-dependent relaxations were reduced by the administration of specific antagonists of muscarinic, H_1-histaminergic, purinergic or α_2-adrenergic receptors, respectively. The inhibition of H_1-histaminergic receptors by chlorphenyramine did not prevent ACh-induced relaxation. Similarly, the inhibition of muscarinic receptors by atropine did not prevent the relaxations to histamine, adenosine and clonidine. On the other hand, the relaxations induced by acetylcholine, histamine, adenosine or clonidine were regularly reduced by NO-synthase inhibitor N^G-nitro-L-arginine methyl ester (10^{-4} mol/l). These results suggest that the inhibition of NO-synthase abolished arterial relaxations induced by all agonists. After inhibition of one type of the endothelial receptors, the NO-dependent relaxation could still be evoked by activation of one of the others.

Key words

Pulmonary artery • Relaxation • Acetylcholine • Histamine • Adenosine • Clonidine • Nitric oxide

Introduction

Acetylcholine, histamine, adenosine, and clonidine have been shown to elicit endothelium-dependent vasorelaxation (Van de Voorde and Leusen 1983, Olanrewaju *et al.* 1995, Ito *et al.* 1995). These relaxations are mediated by the stimulation of specific endothelial receptors leading to the release of relaxing mediator(s) from endothelial cells. It has been demonstrated that a variety of agents (acetylcholine, histamine, adenosine etc.) promote the release of an endothelium-derived relaxing factor – nitric oxide (NO) from endothelial cells as a mediator of vasorelaxation (Török *et al.* 1995, Olanrewaju *et al.* 1995).

The sites of action of receptor-mediated dilators should correlate to the vascular segments containing corresponding receptors for these agonists. The activation of different receptor sites would then lead to the activation of the same mechanism – the production and release of NO. NO is synthesized by the activation of

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Fax +420 2 293 894
E-mail: physres@biomed.cas.cz
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NO-synthase (Palmer *et al.* 1988). Therefore, the inhibition of NO-synthase by analogues of L-arginine, such as N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al.* 1990) could prevent relaxation induced by receptor-mediated dilators. On the other hand, it has been reported that the inactivation of H_1-histaminergic receptors does not prevent the appearance of NO-mediated relaxation to acetylcholine in the rat pulmonary artery (Kyselá and Török 1996). This means that, after inhibition of specific endothelial receptors for one agent, the capability of endothelium to produce NO would be ensured by the activation of other types of receptors present.

The purpose of this study was to determine whether the inhibition of one of endothelial receptor sites in the rat pulmonary artery (muscarinic, histaminergic, purinergic, α_2-adrenergic) affects the NO-mediated relaxation induced by the activation of an other type of receptor.

Materials and Methods

Male Wistar rats weighing 230-400 g were sacrificed by bleeding from the carotid arteries. Pulmonary arteries were isolated, cleaned of adherent connective tissue and cut into rings (3-4 mm in length). The rings were vertically fixed between two stainless steel wires in an incubation bath with oxygenated Krebs solution (95 % O_2/5 % CO_2) and kept at 37 °C. The composition of the Krebs solution was (mmol/l): NaCl 118; KCl 5; $NaHCO_3$ 25; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11; ascorbic acid 1.1; $CaNa_2EDTA$ 0.032.

The arterial rings were connected to a force transducer (Sanborn FTA10) for recording of changes in isometric tension as described earlier (Török *et al.* 1993). Resting tension was adjusted to 10 mN. The preparations were allowed to equilibrate for 60-90 min before use.

The preparations were precontracted with phenylephrine (PE, 10^{-5} mol/l) before relaxations to dilator agonists (acetylcholine, histamine, adenosine, clonidine) were recorded. The extent of relaxation was expressed as a percentage of the PE-induced contraction. The relaxations were also obtained in the presence of specific antagonists of muscarinic (atropine; 10^{-5} mol/l), H_1-histaminergic (chlorphenyramine; 10^{-6} mol/l), purinergic (8-phenyltheophylline; 10^{-5} mol/l) and α_2-adrenergic (yohimbine; 10^{-4} mol/l) as well as during the inhibition of NO-synthase by N^G-nitro-L-arginine methyl ester (L-NAME; 10^{-4} mol/l). These inhibitors were added to the organ bath 30 min before PE.

The following drugs were used: phenylephrine, acetylcholine, histamine, adenosine, clonidine, atropine, chlorphenyramine, 8-phenyltheophylline, yohimbine, L-NAME (all from Sigma).

Experimental values shown in the text and figures are means ± S.E.M. For the statistical evaluation of differences between groups, one-way analysis of variance (ANOVA) was used. The differences of means were considered to be significant at $p<0.05$.

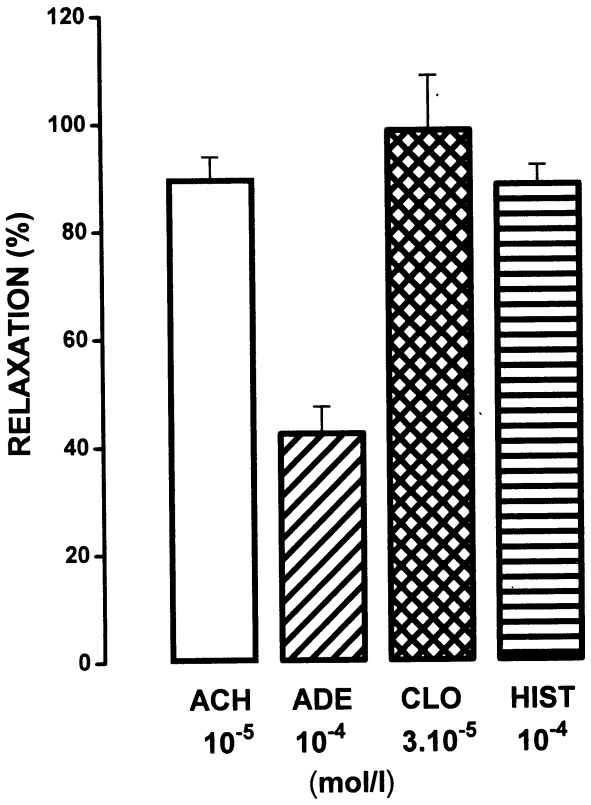

Fig. 1. *Relaxations induced by acetylcholine (ACH; 10^{-5} mol/l; n=12); adenosine (ADE; 10^{-5} mol/l; n=6); clonidine (CLO; 3.10^{-5} mol/l; n=8) and histamine (HIST; 10^{-4} mol/l; n=12) in rat pulmonary artery precontracted with phenylephrine (10^{-5} mol/l). The data were calculated as percentage relaxation of phenylephrine-induced tension. Experimental values are means ± S.E.M.*

Results

Acetylcholine (10^{-5} mol/l), adenosine (10^{-4} mol/l), histamine (10^{-4} mol/l), and clonidine (3×10^{-5} mol/l) caused a relaxation of rat pulmonary artery rings precontracted with phenylephrine (10^{-5} mol/l;

Fig. 1). The relaxation to acetylcholine reached 90.0±3.9 % (n=12) and it was not significantly different from the relaxation induced by histamine (89.1±3.1 %; n=12) or clonidine (99.2±9.7 %; n=8). The relaxation to adenosine was 43.1±4.5 % (n=6).

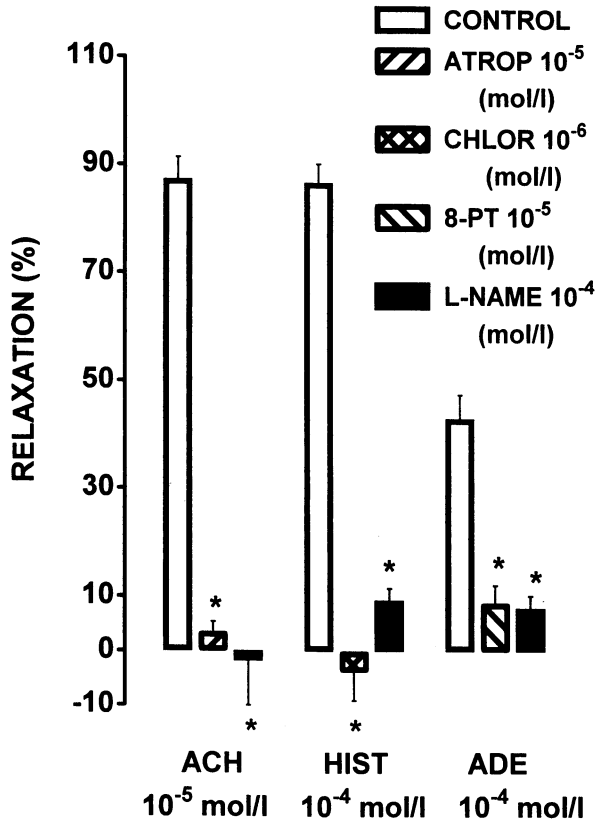

Fig. 2. *The effect of L-NAME (10^{-4} mol/l) and specific antagonists (atropine 10^{-5} mol/l ATROP; chlorphenyramine 10^{-6} mol/l CHLOR; 8-phenyltheophylline 10^{-5} mol/l 8-PT) on relaxations induced in rat pulmonary artery (precontracted by 10^{-5} mol/l phenylephrine) by acetylcholine (ACH; n=6 for L-NAME-treated group and n=9 for atropine-treated group); histamine (HIST; n=6 for L-NAME-treated group and n=11 for chlorphenyramine-treated group) and adenosine (ADE; n=4 for L-NAME-treated group and n=8 for 8-phenyltheophylline-treated group). For other legend see Figure 1.*

The administration of the potent and selective muscarinic antagonist atropine (10^{-5} mol/l) abolished acetylcholine-induced relaxation (Fig. 2). The relaxation to acetylcholine in the control group was 87.3±4.1 % (n=9), whereas in the atropine-treated group it was completely abolished (3.53±1.8; n=9; $p<0.05$; Fig. 2). The administration of chlorphenyramine (10^{-6} mol/l), H_1-histaminergic antagonist, inhibited histamine-induced relaxation. The relaxation in the control group reached 86.7±3.5 % (n=11). After the administration of chlorphenyramine the relaxation to histamine was almost reversed to a slight contraction (–3.8±5.3 %; n=11; $p<0.05$; Fig. 2). Similarly, when the purinergic antagonist 8-phenyltheophylline (8-PT; 10^{-5} mol/l) was present in the incubation bath, the adenosine-induced relaxation was reduced. The relaxation in the 8-PT-treated group reached only 8.9±3.2 % (n=8; $p<0.05$; Fig. 2).

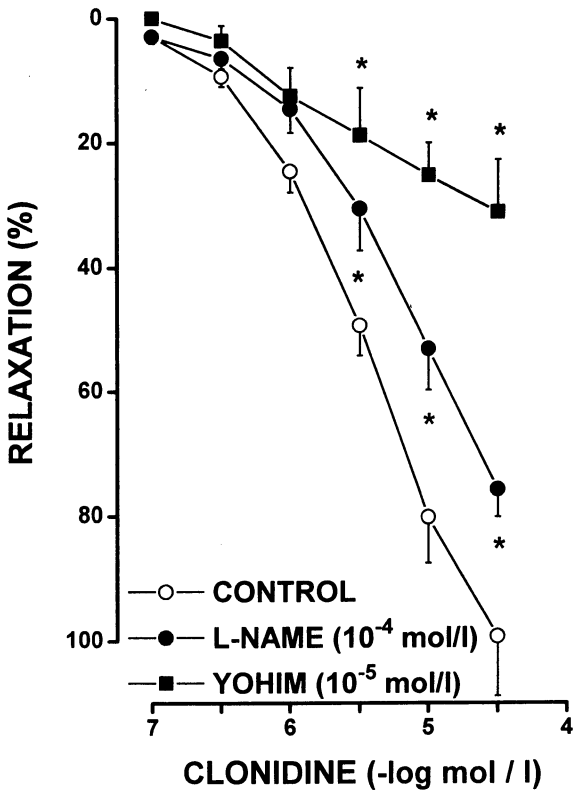

Fig. 3. *Concentration-response curve for clonidine-induced relaxation in rat pulmonary artery precontracted by 10^{-5} mol/l phenylephrine. Effects of L-NAME (10^{-4} mol/l; n=9; full dots) and yohimbine (YOHIM; 10^{-4} mol/l; n=4; full squares) on clonidine-induced relaxation of rat pulmonary artery. For other legend see Figure 1.*

Yohimbine (10^{-5} mol/l), a potent and selective α_2-adrenergic antagonist, reduced the dose-dependent clonidine-induced relaxation (Fig. 3). The maximal relaxation was attained at 3 x 10^{-5} mol/l and represented only 31.1±8.4 % (n=4; $p<0.05$; Fig. 3) in the yohimbine-treated group.

The addition of L-NAME (10^{-4} mol/l, inhibitor of NO-synthase) to the incubation medium abolished the acetylcholine-, histamine-, and adenosine-induced relaxations (Fig. 2). The relaxation to acetylcholine in

L-NAME-treated group was reversed to a slight contraction (–1.8±8.2 %; n=6; p<0.05; Fig. 2). The residual relaxations induced by histamine and adenosine represented only 9.3±2.3 % (n=6; p<0.05; Fig. 2) and 7.9±2.4 % (n=4; p<0.05; Fig. 2), respectively. Clonidine-induced relaxation of the L-NAME-treated arterial rings was inhibited (dose-response curve was shifted to the right) but the value of maximal relaxation reached almost 80 % of the control response (n=9; p<0.05; Fig. 3).

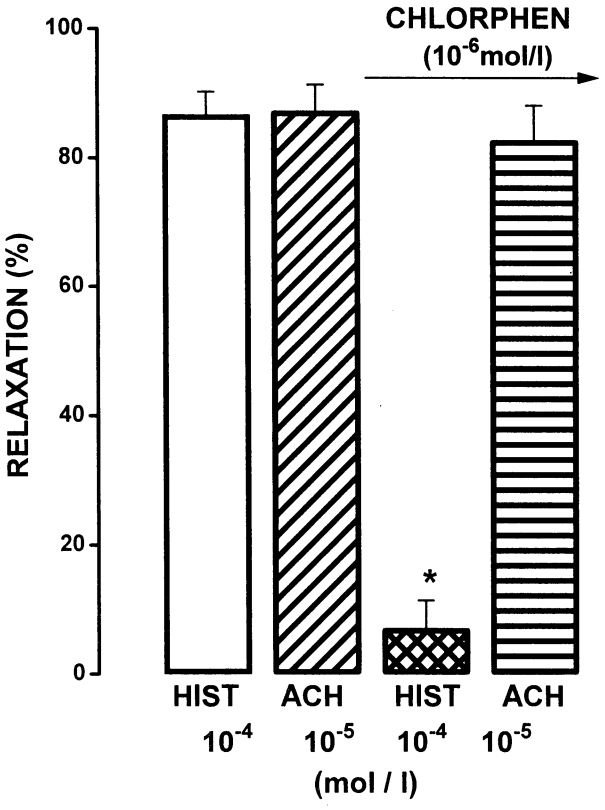

Fig. 4. *The effect of chlorphenyramine (CHLORPHEN; 10^{-6} mol/l) on relaxant responses induced in rat pulmonary artery (precontracted by 10^{-5} mol/l phenylephrine) by histamine (HIST; 10^{-4} mol/l; n=12) and acetylcholine (ACH; 10^{-5} mol/l; n=12). For other legend see Figure 1.*

Figure 4 shows the response to histamine (10^{-4} mol/l) and acetylcholine (10^{-5} mol/l) before and after treatment with chlorphenyramine (10^{-6} mol/l). The relaxation to histamine was completely inhibited in the presence of chlorphenyramine (7.1±4.3 %; n=12; p<0.05; Fig. 4). Following the addition of acetylcholine (without washing chlorphenyramine out from the bath) the pulmonary artery was relaxed similarly as under control conditions. The relaxation (87.3±4.1 %; n=12) was not significantly different from that before chlorphenyramine treatment (82.8±5.3 %; n=12; Fig. 4).

Figure 5 illustrates the relaxations of rat pulmonary artery to adenosine (10^{-4} mol/l), histamine (10^{-4} mol/l) and clonidine (3.10^{-5} mol/l) before and after treatment with atropine (10^{-5} mol/l). Adenosine-induced relaxation (30.6±6.9 %; n=4) reached almost half of relaxation to acetylcholine (74.1±3.4 %; n=4). Atropine completely inhibited relaxant response to acetylcholine (0.9±0.9 %; n=6; p<0.05), but did not affect the following relaxation to adenosine (24.2±4.1 %; n=6; Fig. 5A). There was no significantly difference in the relaxation to acetylcholine (69.6±10.2 %; n=7) and histamine (62.5±11.0 %; n=10; Fig. 5B). Atropine significant inhibited the relaxation to acetylcholine (3.5±1.8 %; n=9; p<0.05), but did not change the relaxation to histamine (53.2±8.0 % ; n=9; Fig. 5B). Similarly, the addition of atropine to the incubation bath abolished the acetylcholine-induced the relaxation (3.5±2.1 %; n=4; p<0.05; Fig. 5C). On the other hand, the relaxation to clonidine was unchanged after treatment with atropine (87.8±5.8 %; n=4) compared to the control response (99.2±9.7 %; n=4; Fig. 5C).

Discussion

Acetylcholine, histamine, adenosine and clonidine activate specific receptors of endothelial cells. The sites of action of these vasodilators should correlate to the vascular segments containing specific receptors for these agents. Our results indicate that acetylcholine, histamine, adenosine and clonidine were able to elicit relaxation in the rat pulmonary artery. The acetylcholine at concentration 10^{-5} mol/l reached a 90 % decrease in tension of the contraction induced by phenylephrine. Relaxations to histamine and clonidine were of the same magnitude as that obtained with acetylcholine. Relaxation to clonidine reached almost 100 % and was markedly higher compared to the clonidine-induced relaxations (60 %) in the rat mesenteric artery (Bockman *et al.* 1996). Adenosine-induced maximum relaxation presented only 40 %, but it was comparable with those obtained in the rat pulmonary artery (Sheridan *et al.* 1997) and in other arteries and species (De Mey and Vanhoutte 1982, McCormack *et al.* 1989).

Relaxations induced by acetylcholine and histamine were blocked by appurtenant receptor antagonists, atropine and chlorphenyramine. These results are in agreement with our previous findings and the results of other authors (Chen and Suzuki 1989, Szarek *et al.* 1992, Kyselá and Török 1996).

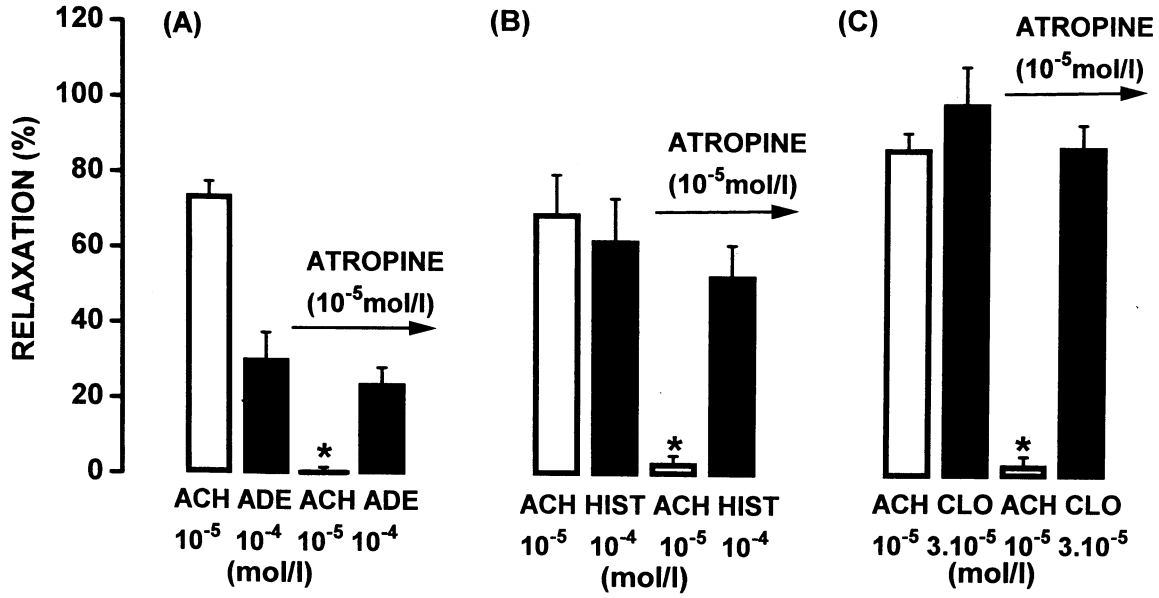

Fig. 5. ***[A]*** *The effect of atropine (ATROPINE; 10^{-5} mol/l) on relaxations induced by acetylcholine (ACH; 10^{-5} mol/l; n=6) and adenosine (ADE; 10^{-4} mol/l; n=6) in rat pulmonary artery precontracted by 10^{-5} mol/l phenylephrine.* ***[B]*** *Relaxant responses induced in rat pulmonary artery (precontracted by 10^{-5} mol/l phenylephrine) by acetylcholine (ACH; 10^{-5} mol/l; n=9) and histamine (HIST; 10^{-4} mol/l; n=9) before and after treatment with atropine (ATROPINE; 10^{-5} mol/l).* ***[C]*** *Relaxations of rat pulmonary artery (precontracted by 10^{-5} mol/l phenylephrine) to acetylcholine (ACH; 10^{-5} mol/l; n=4) and clonidine (CLO; 3×10^{-5} mol/l; n=4) under control conditions and following the addition of atropine (ATROPINE; 10^{-5} mol/l). For other legend see Figure 1.*

Clonidine has been shown to produce contraction (mainly by activation of α_1-adrenergic receptors) in many vessels (rabbit aorta and femoral artery, rat mesenteric artery) at basal tone (Satake and Shibata 1987, Kojima *et al.* 1989). In contrast, clonidine induced relaxation in the precontracted pulmonary artery with increased tone. Our results demonstrate that yohimbine, the antagonist of α_2-adrenergic receptors, inhibited clonidine-induced relaxation in phenylephrine-precontracted rat pulmonary artery. It means that clonidine-induced relaxation is mediated by α_2-adrenergic receptors in the rat pulmonary artery. Similar findings have been reported in the study of Bockman *et al.* (1996) who demonstrated that rauwolscine – another α_2-adrenergic antagonist – reduced relaxations in rat and pig mesenteric arteries. The presence of α_2-adrenergic receptors on the endothelial cells has been demonstrated in systemic and pulmonary vessels of the dog (Miller and Vanhoutte 1985). These authors showed that catecholamines activate endothelial α_2-adrenoceptors resulting in the release of an endothelium-derived relaxing factor which counters contractions induced by adrenergic agonists.

8-phenyltheophylline, a selective and potent antagonist of purinergic receptors, inhibited the relaxation to adenosine in the rat pulmonary artery. This suggests that adenosine-induced relaxation is mediated by purinergic receptors in the pulmonary circulation of the rat. Similar results have been observed in human pulmonary arteries in which adenosine-induced relaxation was mediated by P_2-purinergic receptors located on smooth muscle cells (McCormack *et al.* 1989).

Endothelial cells play an important role in mediating the relaxation of arteries elicited by a number of endogenous agents. This mediation involves the production and release of a relaxing substance from endothelial cells when the respective receptors are activated. It represents one of the principle mechanisms responsible for the vasodilator effect of these substances in the intact organism. A key enzyme in the production of nitric oxide is NO-synthase (Palmer *et al.* 1988). Inhibition of NO-synthase by analogues of L-arginine such as L-NAME (Rees *et al.* 1990) could prevent the relaxation induced by endothelium-dependent receptor-mediated vasodilator compounds. In accordance with our previous results (Kyselá and Török 1996, Török *et al.*

1998), we have repeatedly demonstrated that L-NAME inhibited endothelium-dependent relaxation to both acetylcholine and histamine. Similarly, the addition of L-NAME to the incubation bath reduced adenosine-induced relaxation. With regard to adenosine, the data in the literature are somewhat controversial. Adenosine-mediated relaxation in bovine coronary arteries is positively correlated to activation of the cAMP-dependent protein kinase in arterial smooth muscle (Silver *et al.* 1984). Furthermore, adenosine-induced relaxation did not depend on the presence of functional endothelial cells either in human and dog pulmonary arteries (De Mey and Vanhoutte 1982, McCormack *et al.* 1989). In some vessels of the rat (aorta, pulmonary artery), the endothelium may play a significant role in adenosine-induced relaxation and the endothelium-released mediator could be either nitric oxide or another endothelial factor (White *et al.* 1985, Abiru *et al.* 1995). L-NAME reduced clonidine-induced relaxation, but maximal relaxation reached almost 80 % of control relaxation. This would suggest that nitric oxide is only partially involved in clonidine-induced relaxation of the rat pulmonary artery. It has been reported that activation of α_2-adrenergic receptors leads to the relase of nitric oxide from endothelial cells, which can induce relaxation directly (Bockman *et al.* 1996, Angus *et al.* 1986) or reduce the contractile responses (Miller and Vanhoutte 1985). Ito *et al.* (1995) found that L-NMMA, another NO-synthase inhibitor, inhibited in lower doses (10^{-6} mol/l) clonidine-induced vasodilation, but it did not inhibit clonidine-induced vasodilation of rat mesenteric arteries in higher doses (10^{-5}-10^{-4} mol/l). The authors assumed that clonidine may be involved in the spontaneous release of nitric oxide (inhibition at lower doses), and not in the stimulated release of nitric oxide. Similarly, MacLeod *et al.* (1987) reported that clonidine did not increase the tissue levels of cGMP, which is an effector for nitric oxide, but an inhibitor of cGMP enhanced the contractile response to clonidine in the aorta, suggesting that clonidine may be involved in the spontaneous but not in the stimulated release of nitric oxide. Direct stimulation of smooth muscle cells in the clonidine-induced relaxation of the rat pulmonary artery could also participate. This is in agreement with the observation that endothelium-dependent relaxation appears to be less prominent in vessels chronically exposed to a low oxygen content of the blood (pulmonary artery), when α_2-adrenergic activation of smooth muscle cells is dominant under acidotic conditions (McGrath 1982). Stimulation of α_2-adrenergic receptors by specific agonists and the release of nitric oxide would counteract the vasoconstrictor effect induced by the same agonists, e.g. catecholamines.

A number of agents have been shown to elicit vasodilation through the release of nitric oxide. A variety of other agents promote NO synthesis through the stimulation of specific endothelial cell receptors. In our study, acetylcholine, histamine and adenosine induced relaxations of the rat pulmonary artery by activation of specific endothelial receptors, leading to the release of nitric oxide. Clonidine-induced relaxation was also partially mediated by nitric oxide. The activation of different receptor sites would activate the same mechanism, i.e. the production and release of nitric oxide. On the other hand, after inhibition of one type of endothelial receptors the capability of endothelium to produce nitric oxide would be ensured by activation of the other type of receptors present. Our previous results showed that relaxation to histamine was abolished after treatment with an H_1-histaminergic receptor antagonist chlorphenyramine. Following the addition of cumulative concentrations of acetylcholine without washing chlorphenyramine out from the bath, the pulmonary artery rings were relaxed similarly as under control conditions (Kyselá and Török 1996). This means that the production of nitric oxide by endothelial cells after inhibition of histamine-induced relaxation may be initiated by activation of muscarinic receptors. Similarly, the acetylcholine-induced relaxation was reduced after inhibition of muscarinic receptors, but the relaxation to histamine was unchanged compared to the control response. Similar results were reported by Krstič *et al.* (1989) in the rat renal artery, where atropine did not prevent histamine-induced relaxation. Our results have shown that adenosine and clonidine were also able to induce relaxation after inhibition of muscarinic receptors by atropine. There was no significant difference in the values of maximal relaxation to adenosine and clonidine before and after inhibition of muscarinic receptors.

The endothelium-dependent relaxations of the rat pulmonary artery induced by stimulation of muscarinic, H_1-histaminergic, purinergic and α_2-adrenergic receptors are mediated by nitric oxide. After the inhibition of one type of endothelial receptors the nitric oxide-dependent relaxation could still be mediated by the activation of another type of receptor. Vasodilators, which exert their action due to the direct release of nitric oxide, act uniformly and in a

nonselective manner in whole rat pulmonary vasculature. On the other hand, there is a segmental heterogeneity in the effects exerted by receptor-mediated vasodilators and their action in particular vascular segments correlate with the presence of receptors for these agents. In case of damage of some endothelial receptor type, the vasodilation would be ensured by activation of an other type of receptor present, naturally on the assumption that the given receptor type is also present in the segment.

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Reprint requests

S. Kyselá, Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Sienkiewiczova 1, 813 71 Bratislava, Slovak Republic, e-mail: kysela@unpf.savba.sk.