Arch Pharm Res Vol 28, No 6, 709-715, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Induces Vasodilatation of Rat Mesenteric Artery *in vitro* Mainly by Inhibiting Receptor-Mediated Ca^{2+}-Influx and Ca^{2+}-Release

Yong-Xiao Cao[1,2], Jian-Pu Zheng[1], Jian-Yu He[1], Jie Li[1], Cang-Bao Xu[1,2], and Lars Edvinsson[1,2]

[1]Department of Pharmacology, Medical School of Xi'an Jiaotong University, and Key Laboratory of Environment and Genes Related to Diseases, Xi'an Jiaotong University, Ministry of Education, Xi'an, Shaanxi 710061, China and [2]Division of Experimental Vascular Research, Department of Medicine, Lund University, Lund S-221 85, Sweden

(Received December 29, 2004)

The purpose of this study was to investigate the effect of atropine on peripheral vasodilation and the mechanisms involved. The isometric tension of rat mesenteric artery rings was recorded *in vitro* on a myograph. The results showed that atropine, at concentrations greater than 1 μM, relaxed the noradrenalin (NA)-precontracted rat mesenteric artery in a concentration-dependent manner. Atropine-induced vasodilatation was mediated, in part, by an endothelium-dependent mechanism, to which endothelium-derived hyperpolarizing factor may contribute. Atropine was able to shift the NA-induced concentration-response curve to the right, in a non-parallel manner, suggesting the mechanism of atropine was not mediated via the α_1-adrenoreceptor. The β-adrenoreceptor and ATP sensitive potassium channel, a voltage dependent calcium channel, were not involved in the vasodilatation. However, atropine inhibited the contraction derived from NA and $CaCl_2$ in Ca^{2+}-free medium, in a concentration dependent manner, indicating the vasodilatation was related to the inhibition of extracellular Ca^{2+} influx through the receptor-operated calcium channels and intracellular Ca^{2+} release from the Ca^{2+} store. Atropine had no effect on the caffeine-induced contraction in the artery segments, indicating the inhibition of intracellular Ca^{2+} release as a result of atropine most likely occurs via the IP_3 pathway rather than the ryanodine receptors. Our results suggest that atropine-induced vasodilatation is mainly from artery smooth muscle cells due to inhibition of the receptor-mediated Ca^{2+}-influx and Ca^{2+}-release, and partly from the endothelium mediated by EDHF.

Key words: Atropine, Vasodilatation, Rat mesenteric artery, Ca^{2+}, EDHF

INTRODUCTION

Atropine, a henbane alkaloid found predominantly in solanaceous plants, is the most important muscarinic antagonist to have been used in the clinic for decades. Its main effects include the inhibition of secretions, tachycardia, and pupillary dilatation, paralysis of accommodation and relaxation of visceral smooth muscle. Its mechanism of action is mostly dependent on competitive antagonism to muscarinic cholinergic (M) receptors. In China, atropine as well as other henbanes, such as scopolamine, anisodamine and anisodine, in large doses, have been utilized to prevent patient deaths from bacteremic or hemorrhagic shock (Liu *et al.*, 2004; Jin and Zhou, 2004; Xu 1997; Chen, 1983). For example, atropine was used to treat 13 patients suffering from serious infected shock, of which 11 were cured (Qian, 1999). Also, henbane drugs were used to treat 26 children suffering from serious infected shock, of which 24 were cured (Xu, 1997). The mechanism of atropine in preventing bacteric shock is believed to be by improving the circulation due to a peripheral vasodilator effect. However, atropine, as an M receptor blocker, barely explains the vasodilator effect.

The vasodilator effect of high doses of henbane is due to its peripheral action (Brown and Taylor, 2001). Hall *et al.* (1992) claimed that a high concentration of atropine enhanced the secretion of endothelium derived relaxing factor (EDRF) from the vascular endothelium. Atropine has both contraction and relaxation effects on cavernosal smooth muscle of rabbits at lower and higher concentrations, respectively (Choi *et al.*, 1999). However, effluent flow tests on isolated rabbit ears showed that scopolamine has no direct vasodilator action, and that the vasodilatation of

Correspondence to: Yong-Xiao Cao, Department of Pharmacology, Medical School of Xi'an Jiaotong University, No. 76, Yanta West Road, Xi'an, Shaanxi Province 710061, P. R. China
Tel: 86-29-82655140, Fax: 86-29-82657764
E-mail: yxy@xjtu.edu.cn

scopolamine is not derived from blockade of the muscarinic cholinergic receptor in the vascular wall (Liu *et al.,* 2004). In addition, the endothelium may play a partial role in the vasodilatation of atropine on pulmonary artery rings (Hanghighi *et al.,* 2002). Choi *et al.* (1999), however, found that a high concentration of atropine leads to vasodilation mediated *via* an increase of intracellular calcium sequestration, not by hyperpolarization or secretion of EDRF.

The vasodilator mechanism of atropine remains unclear. It is well known that atropine dilates arterioles rather than large arteries. The rat mesenteric artery is a representative peripheral resistance vessel (Alm *et al.,* 2002); therefore, it was used in this study in order to investigate the effects of atropine on vasodilatation and the mechanisms involved.

MATERIALS AND METHODS

Animals and reagents

Sprague-Dawley rats (weighing 200~300 g) were obtained from the Animal Center of Xi'an Jiaotong University. Atropine, noradrenaline (NA), acetylcholine (ACh), NG-nitro-L-arginine methyl ester (L-NAME), Triton X-100, glibenclamide (Glib), indomethacin (Indo), and propranolol (Prop) were obtained from Sigma CO. All substances were dissolved in distilled water, with the exception of indomethacin, which was dissolved in 1% Na_2CO_3.

In vitro pharmacology

The rats were anesthetized and sacrificed. The superior mesenteric artery was gently removed, immersed in cold oxygenated Kreb's solution and dissected free of adhering tissue under a microscope. For the endothelium-denuded experiments, the artery endothelium was denuded by perfusion of the vessel for 10 seconds with 0.1% Triton X-100 followed by another 10 seconds with Kreb's solution (Cao *et al.,* 2004). The vessels were then cut into 1 mm long cylindrical segments and mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer for continuous recording of the isometric tension, and the other to a displacement device. The mounted artery segments were immersed in tissue baths containing 1 mL of Kreb's solution, which was continuously aerated with a 95% O_2 and 5% CO_2 gas mixture, and maintained at 37°C. The artery segments were equilibrated for 1.5 h, with a resting tension of 2 mN, prior to the experiments. The contractile capacity of each vessel segment was examined by exposure to a K^+-rich (60 mM) buffer solution, where NaCl was exchanged for an equimolar concentration of KCl. When two reproducible contractions had been achieved the vessels were used for further experiments. After equilibration, a vasoconstrictor was added to the bath. After obtaining a sustained tension, atropine (10 nM~100 μM) was added cumulatively to the baths and concentration-response curves to atropine constructed.

In the experiment involving endothelium, the completeness of endothelium denudation was tested with acetylcholine (ACh) (10 μM) following pre-contraction with NA. The lack of relaxation in response to ACh in the denuded preparation indicated an effective functional removal of the endothelium. The rings with endothelium producing less than a 30% relaxation in response to ACh were discarded.

Statistical analysis

The effects of atropine were expressed as the percentage of relaxation compared to the agonist-precontraction. Statistical analysis was performed with unpaired Student's *t*-tests or one-way ANOVA. A *P*-value of less than 0.05 was regarded as significant.

RESULTS

Vasodilatation effect of atropine on rat mesenteric artery precontracted by NA

Atropine, from 1 to 100 μM, concentration-dependently relaxed the artery segments, both with and without endothelium pre-contracted by NA (Figs.1 A and B). In the artery ring segments with endothelium the maximum relaxation effect (R_{max}) of atropine was 90.1±2.2%. In rings denuded of endothelium, the effect of atropine was attenuated slightly, with an R_{max} of atropine of 67.1±2.7%. In a comparison of both the R_{max} values, the *P* value was less than 0.01. The effect of atropine on the artery segments without endothelium was reduced by 25.7% (Fig. 1C).

Vasodilatation effect of atropine on rat mesenteric artery precontracted by 5-HT

Atropine, from 0.1 to 300 μM, showed a concentration-dependent relaxation of the artery rings both with and without endothelium pre-contracted by 5-HT, a general agonist of 5-HT receptors (Fig. 2). In the artery ring segments with endothelium, the R_{max} of atropine was 91.9 ±1.5%. In rings denuded of endothelium, the effect of atropine was attenuated slightly, with an R_{max} of atropine of 71.3±1.5%. In a comparison of both the R_{max} values, the *P* value was less than 0.01. Atropine, from 10 nM to 100 μM, concentration-dependently relaxed the artery segments with endothelium precontracted by 5-CT, a specific agonist of 5-HT_1 receptor (Fig. 3).

Effect of atropine on rat mesenteric artery precontracted by KCl

After removal of the endothelium, artery segments were

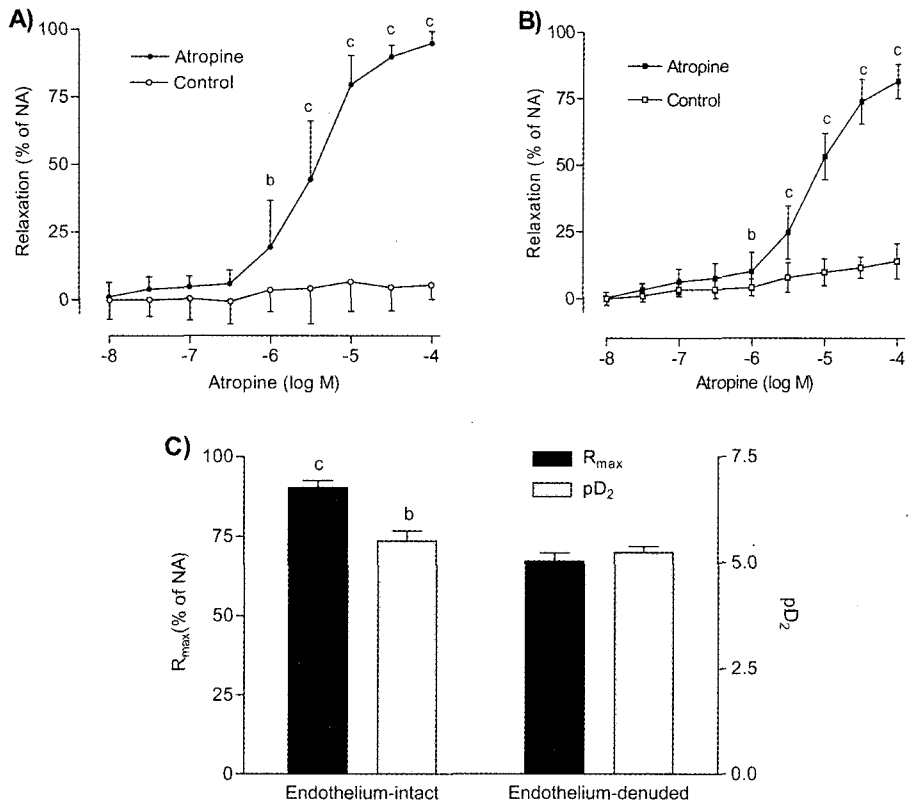

Fig. 1. The concentration-response curves of atropine on the vasodilatation effect of the rat mesenteric artery pre-contracted by NA with intact endothelium (A) and denuded endothelium (B). The relaxation was expressed as the percentage of the precontraction by NA. (C): Maximal relaxation and pD_2 of atropine on the rat mesenteric artery precontracted by NA. R_{max} refers to the maximal relaxation, calculated as the percentage of the corresponding precontraction with NA. pD_2: the negative logarithm of the drug concentration that elicited 50 % relaxation. n = 8. $^bP < 0.05$ and $^cP < 0.01$ *vs.* control (A and B) or *vs.* endothelium-denuded group (C).

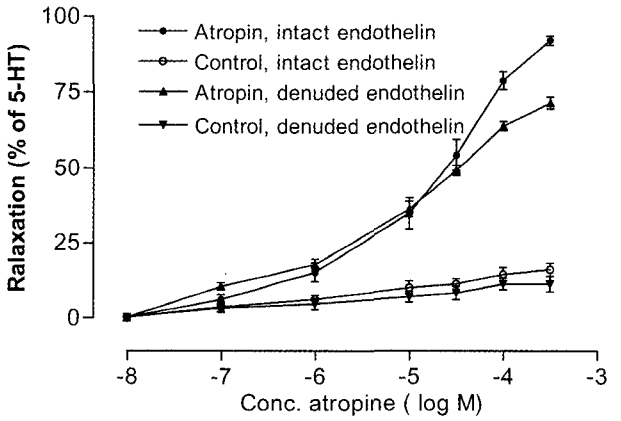

Fig. 2. The concentration-response curves of atropine on the vasodilatation effect of the rat mesenteric artery of intact and denuded endothelium precontracted by 5-HT, a general agonist of 5-HT_1 and 5-HT_2 receptors. n = 8.

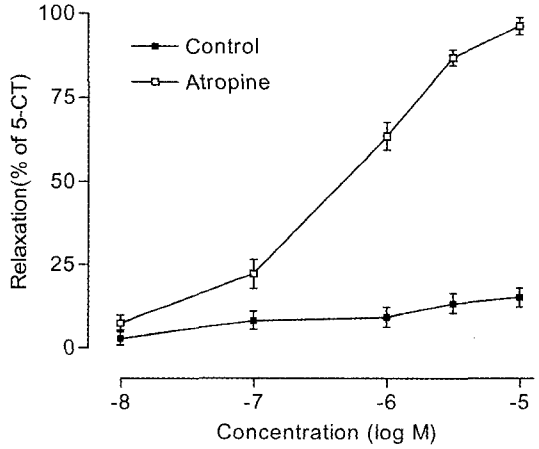

Fig. 3. The concentration-response curves of atropine on the vasodilatation of the rat mesenteric artery of intact endothelium precontracted by 5-CT, a specific 5-HT_1 agonist. n = 8.

equilibrated for 1.5 h KCl (10, 20, 40, and 80 mM) was cumulatively added, and concentration-response curves constructed. After washout, the arteries were incubated with 30 μM atropine for 10 minutes. Then, concentration-response curves of KCl were again constructed, as above. The concentration-response curve of KCl in the presence of atropine was not obviously shifted compared with the control (without atropine) (Fig. 4).

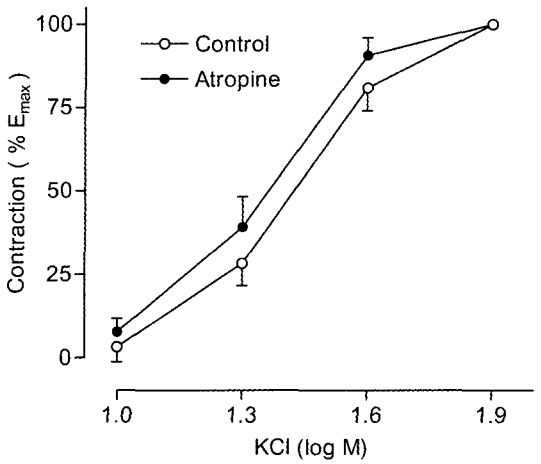

Fig. 4. Effect of atropine on the concentration-response curve of KCl on the rat mesenteric artery segments without endothelium. The curve induced by KCl in the presence of atropine was not obviously shifted compared to that without atropine (n = 8).

Effect of atropine on the NA-induced concentration-contraction curve

NA induced a potent and sustained constriction of mesenteric artery segments, in a concentration-dependent manner. Atropine, at 1~100 μM, potently inhibited the NA-induced vasoconstriction, and concentration-dependently shifted the concentration-contractile curves toward the right, in a non-parallel manner, with a decreased E_{max} (Fig. 5). Prazosine, an α_1 receptor antagonist, at 10 pM~10 nM, unlike atropine, shifted the concentration-contractile curves toward the right, in a parallel manner, with an unremarkable difference in the E_{max}.

Vasodilatation effect of atropine in the presence of different blockers

The endothelium-intact artery rings were incubated with L-NAME (100 μM) and indomethacin (10 μM); and endothelium-denuded artery rings were incubated in the

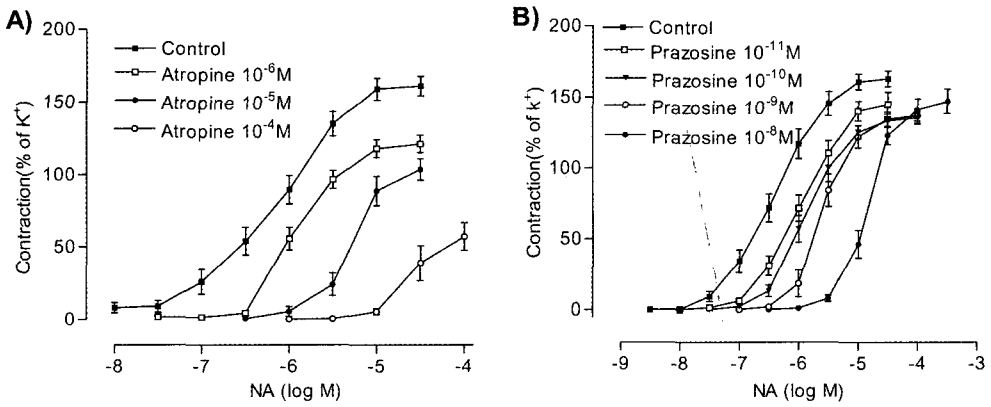

Fig. 5. Comparison of the inhibitory effects of atropine 10^{-6}~10^{-4} M (A, n=12) or prazosine, a α_1 receptor antagonist 10^{-11}~10^{-8} M (B, n=8) on the NA-induced concentration-contractile curves on the rat mesenteric artery denuded of endothelium. Atropine shifted the concentration-contractile curves toward the right, in a non parallell manners, and the pD_2 of atropine was 4.87±0.11, with 95% confidence intervals, from 3.66 to 6.08. Prazosine shifted the curves toward the right, in a parallel manner, and the pA_2 value of prazosine was 10.60 ± 0.23, with 95% confidence intervals, from 9.30 to 11.78. Each point represents the mean±SEM.

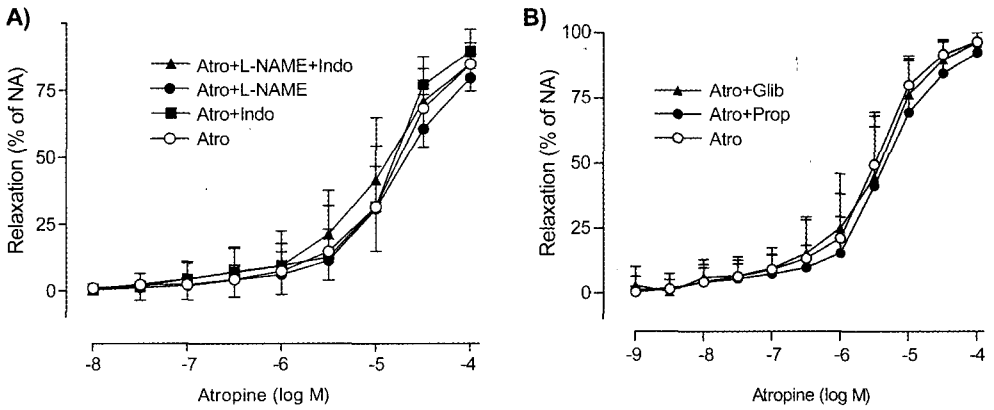

Fig. 6. The vasodilatation effect of atropine on endothelium-intact artery rings (A) in the presence of L-NAME and indomethacin, and on endothelium-denuded artery rings (B) in the presence of propranolol and glibenclamide. n = 8. L-NAME, indomethacin, propranolol, and glibenclamide did not significantly affect the atropine-induced relaxation.

presence of propranolol (5 μM) or glibenclamide (10 μM) for 30 minutes (Tijen *et al* 2003). The vasodilatation effect of atropine on NA-pre-contraction artery rings was recorded in order to test the effects of nitric oxide (NO), prostaglandins (PGs), β-adrenoceptors and the ATP sensitive potassium channels that may contribute to atropine-induced vasodilatation. The results (Fig. 6) showed that endothelium-intact artery rings, with L-NAME or indomethacin, and endothelium-denuded artery rings, with propranolol or glibenclamide, had no significant effect on the atropine-induced relaxation response

Effect of atropine on the contraction of artery segments in Ca^{2+}-free solution

After testing the contraction activity with K^+ (60 mM), the endothelium-denuded artery segments were exposed to Ca^{2+}-free solution, containing EGTA (100 μM), for 5 minutes, followed by the addition of 10 μM NA. After obtaining a sustained NA-induced contraction, 1 mM $CaCl_2$ was used to contract the segments again (Jiang *et al.*, 1998). The segments were then washed with normal Kreb's solution (45 min contact for Ca^{2+} refilling of the intracellular stores) and twice with Ca^{2+}-free solution (15 min contact times). The second contractile responses to NA and $CaCl_2$ were tested in the presence of atropine (3, 10, and 30 μM) for 5 min. Then, the effect of atropine on the contraction induced by NA and $CaCl_2$ in the Ca^{2+}-free Kreb's solution was tested in endothelium-denuded preparations. Atropine, at the three different concentrations, significantly inhibited the contraction induced by NA and $CaCl_2$ in a concentration-dependent manner. The inhibitory ratios were 36.0 ±16.9, 57.4±22.5, and 74.8±10.6% to NA; and 36.0±16.9, 57.4±22.5, and 74.8±10.6% to $CaCl_2$, respectively (Fig. 7, Table I).

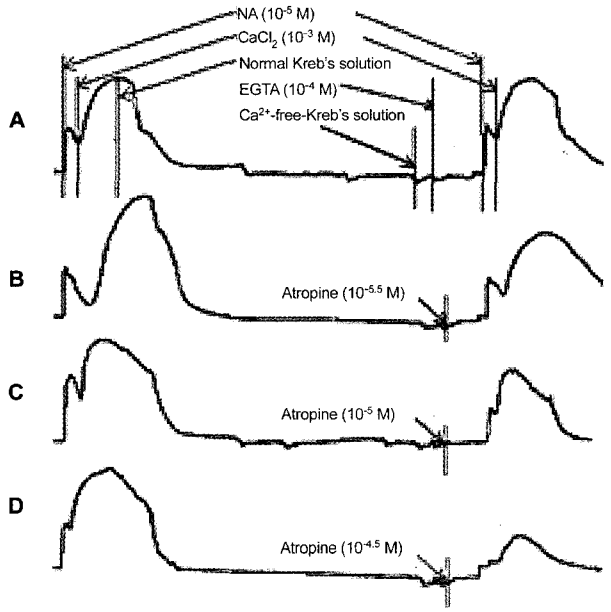

Fig. 7. Effect of atropine on the contraction induced by NA and $CaCl_2$ in Ca^{2+}-free Kreb's solution. The endothelium-denuded artery segments were contracted with K^+ (60 mmol·L^{-1}), and then washed with normal Kreb's solution until the basal tension returned. The segments were exposed to Ca^{2+}-free solution, containing EGTA (100 μM), for 5 min and 10 μM NA then added. After obtaining a sustained contraction, $CaCl_2$ (1 mM) was used to contract the segments again. The segments were then washed four times with normal Kreb's solution (45 min contact time for Ca^{2+} refilling of the intracellular stores) and twice with Ca^{2+}-free solution. The second contractile responses to NA and $CaCl_2$ were tested in the presence of atropine (3, 10 or 30 μM) for 5 min. The effects of atropine on the contraction induced by NA and $CaCl_2$ in the Ca^{2+} free Kreb's were then tested in endothelium-denuded preparations. A: control group; B: atropine (3 μM) group; C: atropine (10 μM) group; D: atropine (30 μM) group. Atropine, at the three concentrations, *significantly inhibited the contraction derived from NA and $CaCl_2$ in a* concentration dependent manner.

Table I. The effects of atropine on the vasoconstriction effect of 10^{-5} M NA or 1 mM $CaCl_2$ on the rat mesenteric artery in Ca^{2+}-free-Kreb's solution.

	Concentration (M)	n	Inhibitory ratios	
			NA (10^{-5} M)	Ca^{2+}(1 mM)
Control	-	8	5.9 ± 5.7	2.5 ± 7.1
Atropine	$10^{-5.5}$	8	36.0 ± 5.4**	19.0 ± 4.0*
Atropine	10^{-5}	8	57.4 ± 7.1**	40.1 ± 6.6**
Atropine	$10^{-4.5}$	8	74.8 ± 3.3**	63.7 ± 5.9**

*p<0.05 and **p<0.01 *vs.* control. p <0.05 *vs.* atropine 10^{-5}M group

Effect of atropine on the contraction induced by caffeine in Ca^{2+}-free solution

Sustained contractions of endothelium-denuded arteries to caffeine (30 mM) were obtained in Ca^{2+}-free solution. After washout and reloading, atropine (30 μM) was added to the tissue baths. The effect of atropine on the vasoconstriction induced by caffeine in rat mesenteric artery segments in Ca^{2+}-free solution was also tested in endothelium-denuded preparations. Caffeine induced a transient contraction in Ca^{2+}-free solution containing EGTA, with an inhibitory rate of 13.9±9.7% (n = 8). After incubation with atropine for 5 minutes, the caffeine-induced inhibitory rate was 14.4±17.7% (n = 8), showing that atropine did not affect the caffeine-induced contraction.

DISCUSSION

This study showed that atropine, at concentrations greater than 1 μM, relaxed the mesenteric artery precontracted by NA, a strong α-adrenoceptor agonist, in a concentration- dependent manner. Removal of the functional endothelium decreased 22.2~25.5% of the relaxant effect of atropine, indicating that atropine-induced vasodilatation

is mediated, at least in part, by an endothelium-dependent mechanism.

ACh is commonly used to assess endothelium-dependent vasodilatation (Furchgott *et al.,* 1980). The dilatory mediators released by ACh have mainly been characterized as NO, PGs and EDHF. NO is produced by nitric oxide synthase in endothelial cells, which dilates vascular smooth muscle by activating guanylate cyclase (Moncada *et al.*, 1991). NO production can be inhibited with L-NAME. Dilatory PGs are produced by cyclo-oxygenase from arachidonic acid in endothelial cells, which relax smooth muscle cells by activating adenylate cyclase. The formation of PGs can be inhibited by indomethacin, a cyclo-oxygenase inhibitor. EDHF is an endothelium-derived mediator, distinct from NO and PGs, which hyperpolarizes and relaxes smooth muscle cells. Both its dilatory and hyperpolarizing effects on the rat mesenteric artery can be inhibited by a combination of the potassium channel inhibitors, charybdotoxin and apamin (Doughty *et al.,* 1999). To further examine the dilatory mediators that contribute to the vasodilatation effect of endothelium derived atropine, L-NAME and indomethacin were used to block NO production and PGs formation, respectively. The results showed that NO and PGs did not contribute to the vasodilatation of endothelium derived atropine, but that EDHF may account for the effect.

Vasodilatation due to atropine mainly derives from vascular smooth muscle cells. The next step is to determine the factors that could be involved in the vasodilatation. Atropine was able to shift the NA-induced concentration-response curve toward the right, in a non-parallel manner, with a decreased E_{max}; prazosine, a specific α_1-receptor antagonist, shifted the NA-induced concentration-response curve toward the right, in a parallel manner. These results suggest the mechanism of atropine is not mediated *via* the α_1-adrenoreceptor. Propranolol (a general β-adrenoceptor antagonist) and glibenclamide (an ATP sensitive potassium channel inhibitor) did not affect the vasodilation of atropine, indicating that β-adrenoceptors and ATP sensitive potassium channels are not involved in the vasodilation.

The mechanism of vascular smooth muscle contraction involves different signal transduction pathways, all of which converge to increase intracellular calcium (Broekaert *et al.,* 1979). Both extracellular Ca^{2+} influx, through VDCC or receptor operated calcium channels (ROCC), and the release of intracellular Ca^{2+} can result in an increase of intracellular calcium. The release of intracellularly stored Ca^{2+} is mainly regulated by an inositol 1,4,5-triphosphate (IP_3) receptor system and a ryanodine receptor system. The former directly induces Ca^{2+} release when the receptors are bound to IP_3. The later may function through a Ca^{2+} induced Ca^{2+} release mechanism when the ryanodine receptors are activated by using drugs, such as caffeine (Leijten *et al.,* 1984). In our experiments, atropine did not affect the KCl concentration-response curve of mesenteric artery segments without endothelium, indicating that VDCC do not account for the vasodilator action of atropine. However, atropine inhibited the contraction derived with NA and $CaCl_2$ in Ca^{2+} free medium, in a concentration dependent manner, which suggests a mechanism of the vasodilatation related to the inhibition of extracellular Ca^{2+} influx through ROCC and intracellular Ca^{2+} release from Ca^{2+} stores. Besides, atropine did not affect the caffeine-induced contraction of artery segments, which rules out the possible involvement of ryanodine receptors in the release of intracellularly stored Ca^{2+}. Therefore, it is likely that the IP_3 receptor contributes to the Ca^{2+} release.

In conclusion, atropine possesses a significant vasodilatation effect, which derives partly from the endothelium mediated by EDHF, but mainly from artery smooth muscle cells by inhibiting the receptor-mediated Ca^{2+}-influx and Ca^{2+}-release.

ACKNOWLEDGEMENT

We would like to thank Dr. Shemin Lu for his critical reading and linguistic correction of the manuscript.

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