Prejunctional Regulation by Endogenous and Exogenous Acetylcholine of Adrenergic Nerve Function in Isolated Canine Mesenteric Arteries

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Transmural electrical stimulation (5–30 Hz) produced a frequency-dependent increase in the perfusion pressure of isolated, perfused dog mesenteric artery segments without the endothelium, which was abolished by prazosin or tetrodotoxin. Physostigmine inhibited the pressor response to transmural electrical stimulation, whereas atropine potentiated the response. Treatment with acetylcholine (10^{-6} and 10^{-5} M) dose-dependently inhibited the response to electrical nerve stimulation. The effect was reversed by the addition of atropine and AF-DX 116 at a concentration (10^{-7} M) that selectively blocked the M_2 receptor subtype, but not by pirenzepine or 4-DAMP. Acetylcholine did not alter the pressure raised by norepinephrine in perfused arterial segments nor the contraction caused by exogenous norepinephrine in the artery strips. ^3H -overflow evoked by transmural electrical stimulation from tissues prelabeled with [^3H] norepinephrine was decreased by acetylcholine (10^{-6} M) in the superfused dog mesenteric arterial strips. It is concluded that acetylcholine inhibits adrenergic neurogenic contractions by interfering with the release of norepinephrine, which possibly results from activation of the prejunctional M_2 receptor subtype. (*Hypertens Res* 1997; 20: 119–125)

Key Words: acetylcholine, muscarine M_2 receptor, adrenergic nerve stimulation, prejunctional inhibition, mesenteric artery

Although vasomotor nerves are mainly adrenergic, there are some arteries innervated by cholinergic vasodilator nerves (1). Previous studies have shown that stimulation of muscarinic receptors located on noradrenergic nerve terminals by exogenously applied acetylcholine (ACh) inhibits the release of transmitter, as determined by measuring the release of incorporated ^3H -norepinephrine during transmural nerve stimulation (2–4). At least three different subtypes of the muscarinic receptor have been pharmacologically characterized. Muscarinic receptors that show high affinity for the antagonist pirenzepine are referred to as M_1 subtypes (5–7), whereas those with low affinity for this agent are classified as M_2 and M_3 subtypes (6, 7). M_2 receptor subtypes, as compared with M_3 subtypes, display a high affinity for AF-DX 116 (11-[(2-[(diethyl-amino) methyl]-1-piperidiny] acetyl)-5, 11-dihydro-6H-pyrido-[2, 3-b] [1, 4]-benzodiazepine-6-one) (6). The M_3 receptors are blocked by lower concentrations of 4-DAMP (4-diphenylacetoxy-*N*-methyl-piperidine methiodide) (8), as compared with M_2 receptors. Modulatory prejunctional muscarinic receptors are present on neurons liberating various neurotransmitters, and there is evidence that the subtypes of muscarinic receptors involved at these sites are not identical for all neurons. The prejunctional musca-

rinic inhibitory heteroreceptors present on sympathetic nerves in different tissues also vary as to their subtype; those in the heart (9–11) and rabbit iris (12) appear to be muscarine M_2 receptors, whereas those in the rabbit vas deferens are of the M_1 subtype (13). Nevertheless, little is known about the subtype of these receptors present on adrenergic nerve endings modulating norepinephrine release in dog mesenteric arteries.

Aims of the present work were to characterize the subtype of muscarinic receptors involved in the inhibitory effect of acetylcholine on the pressor response to electrical nerve stimulation in perfused dog mesenteric arterial segments and to determine whether endogenous ACh from cholinergic nerve interferes with the response to adrenergic nerve stimulation. For this purpose, antagonists such as atropine (non-specific), pirenzepine (M_1 antagonist) (5), AF-DX 116 (M_2 antagonist) (6), and 4-DAMP (M_3 antagonist) (13) and the agonist ACh were used.

Materials and Methods

Studies on Mechanical Response

The Animal Care and Use Committee at Shiga University of Medical Science approved the use of dog

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blood vessels in this study. Mongrel dogs of either sex, weighing 7 to 13 kg, were anesthetized with intravenous injections of sodium thiopental (30 mg/kg) and killed by bleeding from the carotid arteries. Proximal portions of the superior mesenteric artery of 0.9 to 5.0 mm outside diameter were isolated. The artery segments were placed in a bath of 40-ml capacity and perfused luminally with modified Ringer-Locke solution maintained at $37 \pm 0.3^\circ\text{C}$ and aerated with a mixture of 95% O_2 and 5% CO_2 at a constant rate of 1 ml/min with a pressure of 40 to 50 mmHg (4). Constituents of the solution were as follows (mM): NaCl 120, KCl 5.5, CaCl_2 2.2, MgCl_2 1.0, NaHCO_3 25.0, and dextrose 5.6. The pH of the solution was 7.36 to 7.43. The perfusion pressure was measured *via* a pressure transducer (Nihon-Kohden Kogyo Co., Tokyo, Japan) placed upstream of the artery segment. Perfused segments were placed between a pair of stimulating electrodes made of platinum plate. The gap between the segment and the electrodes was wide enough to allow undisturbed contractions and yet sufficiently narrow enough to permit stimulation of intramural nerve terminals. Under resting conditions, electrical square pulses of supramaximal intensity were applied transmurally at frequencies of 5, 10, 20, and 30 Hz for 40, 20, 10, and 7 s, respectively (total number of pulses, 200–210), every 10 to 15 min to stimulate perivascular nerves innervating the arterial wall. Transmural electrical stimulation was applied repeatedly until steady responses were obtained, and then the agents, such as acetylcholine (ACh), atropine, pirenzepine, AF-DX 116, and 4-DAMP, were directly applied to the bathing media. At the end of the experiment, tetrodotoxin was applied to determine whether the induced response was due to stimulation of perivascular nerves. In preliminary experiments, the stimulation-induced increase in perfusion pressure was observed to be steady for over 2 h. The endothelium was removed by rubbing with cotton twine along with a polyethylene catheter inserted into the lumen of the segment. Successful removal of the endothelium was determined by abolishment of the depressor response induced by 10^{-6} M ACh applied intraluminally.

Some of the mesenteric arteries (outside diameter 0.5 to 0.8 mm) were cut into helical strips of approximately 20 mm long to evaluate the effect of ACh on responses to norepinephrine. The endothelium was removed by gently rubbing the intimal surface of the strip with a cotton ball. Removal of the endothelium was verified by abolishment of relaxation caused by 10^{-6} M ACh. The strips were fixed vertically between hooks in a muscle bath of 20-ml capacity containing the modified Ringer-Locke solution, which was maintained at $37 \pm 0.3^\circ\text{C}$ and aerated with a mixture of 95% O_2 and 5% CO_2 . Isometric mechanical responses were recorded on an ink-writing oscillograph. The resting tension was adjusted to 1.5 g, which is optimal for inducing maximum contraction (14). Norepinephrine (10^{-7} to 10^{-5} M) was applied cumulatively to the bathing media to obtain the concentration-response curve.

³H-Overflow Study

Isotope experiments were carried out on helical strips of dog mesenteric arteries as described previously (15). The tissue was preincubated for 60 min at 37°C with 5×10^{-7} M [^3H] norepinephrine (specific activity, 56.9 Ci/mmol). The tissue was then superfused with the modified Ringer-Locke solution containing cocaine (3×10^{-5} M) and corticosterone (4×10^{-5} M) at a rate of 1 ml/min. The preincubated strips were stimulated electrically 5 times for 3 min at a frequency of 5 Hz. Stimulation was applied after 126 (S_1), 144 (S_2), 162 (S_3), 180 (S_4), and 198 min (S_5) of superfusion. At the end of the superfusion, tritium counts in the superfusate collected each 3 min and in the tissues solubilized by toluene were measured. The stimulation-evoked ³H-overflow of total tritium was calculated as percent of the tissue tritium content at the time of stimulation. ACh was added 12 min before S_4 . Effects of the drug on stimulation-evoked ³H-overflow were expressed as the ratio between the overflow evoked by S_4 or S_5 and that evoked by S_3 . The ratios were compared with those obtained in the absence of treatment with the drug. ACh did not affect the spontaneous overflow of tritium.

PERFUSED DOG MESENTERIC ARTERY (E-)

Fig. 1. Modification by physostigmine (eserine; 10^{-7} M) and atropine (10^{-6} M) of the pressor response to transmural electrical stimulation (TES, 20 Hz for 10 s) in perfused dog mesenteric artery segments without the endothelium (E-), $n=10$. Stimulation-induced increases in the perfusion pressure before application of physostigmine are expressed as zero; the mean absolute value was 40.4 ± 8.6 mmHg. Significantly different from zero, $^a p < 0.05$; Significantly different from eserine, $^b p < 0.01$. Vertical bars represent SEM.

PERFUSED DOG MESENTERIC ARTERY (E-)

-Transmural stimulation, 20Hz

Fig. 2. Typical recording of the response to transmural electrical stimulation (20 Hz for 10 s) of a perfused mesenteric artery segment without the endothelium (E-) before and after treatment with ACh (10^{-6} and 10^{-5} M) and atropine (10^{-6} M). Drugs were cumulatively applied directly to the organ bath. Each dot under the tracing represents the application of electrical stimulation. TTX = 3×10^{-7} M tetrodotoxin.

PERFUSED DOG MESENTERIC ARTERY (E-)

-Transmural stimulation, 20Hz

Fig. 3. Effects of ACh (10^{-6} and 10^{-5} M) on the pressor response to transmural electrical stimulation (TES, 20 Hz for 10 s) and modification by atropine (10^{-6} M) of the inhibition due to ACh in perfused dog mesenteric artery segments without the endothelium (E-), $n = 16$. Stimulation-induced increases in the perfusion pressure before application of ACh are expressed as control. Significantly different from control, $^a p < 0.01$, $^b p < 0.001$; significantly different from acetylcholine (10^{-6} M), $^c p < 0.01$; significantly different from atropine, $^d p < 0.01$. Vertical bars represent SEM.

Statistical Analysis and Drugs Used

The results shown in the text, table, and figures are expressed as mean values $\pm$ SEM. Statistical analyses were made with Student's paired and unpaired *t*-test or Tukey's method after one-way analysis of variance. The drugs used were acetylcholine chloride (ACh, Daiichi Pharmaceutical Co., Tokyo, Japan), atropine sulphate (Tanabe Pharmaceutical Co., Osaka, Japan), physostigmine sulphate (Sigma, St.

PERFUSED DOG MESENTERIC ARTERY (E-)

-Transmural stimulation, 20Hz

Fig. 4. Modification by pirenzepine (10^{-8} , 10^{-7} and 10^{-6} M) and atropine (10^{-6} M) of the inhibitory effect of ACh (10^{-5} M) on the pressor response induced by transmural stimulation at 20 Hz of perfused dog mesenteric artery segments without the endothelium (E-), $n = 5$. Stimulation-induced increases in the perfusion pressure after application of atropine (10^{-6} M) are expressed as 100%; the mean absolute value was 37.5 ± 5.8 mmHg. Significantly different from zero, $^a p < 0.05$, $^b p < 0.01$; significantly different from pirenzepine (10^{-8} M), $^c p < 0.05$, $^d p < 0.01$; significantly different from atropine, $^e p < 0.01$, $^f p < 0.001$. Vertical bars represent SEM.

Louis, MO, USA), pirenzepine, AF-DX 116, 4-DAMP, [3 H]norepinephrine (NEN Research Products, DE, USA), prazosin hydrochloride (Wako Pure Chemical Industries Ltd., Osaka, Japan), tetrodotoxin, and norepinephrine hydrochloride (Sankyo Co., Tokyo).

Fig. 5. Typical recording of the response to transmural electrical stimulation (20 Hz for 10 s) of a perfused mesenteric artery segment without the endothelium (E-) before and after treatment with ACh (10^{-6} and 10^{-5} M) and AF-DX 116 (10^{-7} , 10^{-6} and 10^{-5} M), and atropine. Drugs were cumulatively added directly to the organ bath. Each dot under the tracing represents the application of electrical stimulation. TTX= 3×10^{-7} M tetrodotoxin.

Fig. 6. Modification by AF-DX 116 (10^{-7} , 10^{-6} and 10^{-5} M) and atropine (10^{-6} M) of the inhibitory effect of ACh (10^{-5} M) on the pressor response induced by transmural stimulation at 20 Hz of perfused dog mesenteric artery segments without the endothelium (E-), $n = 5$. Stimulation-induced increases in the perfusion pressure after application of atropine (10^{-6} M) are expressed as 100%; the mean absolute value was 36.9 ± 4.7 mmHg. Significantly different from zero, $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$; significantly different from AF-DX 116 (10^{-7} M), $^d p < 0.01$, $^e p < 0.001$; significantly different from atropine, $^f p < 0.01$, $^g p < 0.001$. Vertical bars represent SEM.

Fig. 7. Modification by 4-DAMP (10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M) and atropine (10^{-6} M) of the inhibitory effect of ACh (10^{-5} M) on the pressor response induced by transmural stimulation at 20 Hz of perfused dog mesenteric artery segments without the endothelium (E-), $n = 5$. Stimulation-induced increases in the perfusion pressure after application of atropine (10^{-6} M) are expressed as 100%; the mean absolute value was 38.0 ± 6.1 mmHg. Significantly different from zero, $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$; significantly different from 4-DAMP (10^{-9} M), $^d p < 0.05$, $^e p < 0.01$, $^f p < 0.001$; significantly different from atropine, $^g p < 0.05$, $^h p < 0.01$, $^i p < 0.001$. Vertical bars represent SEM.

Table 1. The Ratio of ^3H -Overflow Evoked by Transmural Electrical Stimulation in the Absence and Presence of ACh

	N	S ₄ /S ₃	S ₅ /S ₃
Control	4	1.00 $\pm$ 0.01	0.98 $\pm$ 0.01
Acetylcholine (10^{-6} M)	4	0.50 $\pm$ 0.03 ^a	0.57 $\pm$ 0.07 ^a

N, number of preparations used. Significantly different from controls, ^a $p < 0.001$.

The strip was treated for 12 min with 10^{-6} M ACh before the application of transmural electrical stimulation (S₄).

Results

Effect of Acetylcholine on the Response to Adrenergic Nerve Stimulation in Perfused Segments

In dog mesenteric artery segments perfused at a rate of 1 ml/min, transmural electrical stimulation (5, 10, 20, and 30 Hz for 40, 20, 10, and 7, respectively) produced a frequency-related increase in the perfusion pressure; the mean values were 3.6 ± 0.6 , 20.4 ± 1.7 , 37.2 ± 3.1 , and 63.8 ± 5.2 mmHg ($n = 5$), respectively. At a frequency of 20 Hz applied at an interval of 10 to 15 min, the pressor response was consistent and reproducible; therefore, the effect of various agents on the response to this frequency of stimulation was evaluated. The pressor response was abolished by treatment with prazosin (10^{-5} M) or tetrodotoxin (3×10^{-7} M), suggesting the involvement of norepinephrine released by activation of perivascular adrenergic nerves.

Physostigmine (10^{-7} M) slightly but significantly inhibited the pressor response to the transmural electrical stimulation. The inhibition was reversed to a potentiation by the addition of atropine (10^{-6} M) (Fig. 1).

ACh (10^{-6} and 10^{-5} M) did not alter the perfusion pressure in endothelium-denuded arterial segments, but significantly inhibited the pressor response to the transmural electrical stimulation dose-dependently (Fig. 2). The inhibition was almost completely reversed by the addition of atropine (10^{-6} M). The quantitative data are summarized in Fig. 3.

Modifications by selective antagonists of M₁, M₂ and M₃ muscarinic receptor subtypes (pirenzepine, AF-DX 116, and 4-DAMP, respectively) of the inhibitory effect of ACh on the pressor response to electrical nerve stimulation were investigated. The inhibition was not significantly affected by 10^{-8} M pirenzepine but was reversed at 10^{-7} and 10^{-6} M dose-dependently (Fig. 4). AF-DX 116 in concentrations from 10^{-7} to 10^{-5} M significantly reversed the inhibition by ACh of the pressor response in a dose-dependent manner (Fig. 5). The quantitative data are summarized in Fig. 6. The inhibitory effect of ACh on the pressor response was not altered by 10^{-9} M 4-DAMP, but was antagonized at 10^{-8} M or higher (Fig. 7).

Effect of ACh on the Response to Norepinephrine in Perfused Artery Segments and Arterial Strips

The addition of norepinephrine (7×10^{-6} to 8×10^{-6} M) produced a sustained increase, averaging 21.3 ± 0.9 mmHg, in the pressure of perfused

mesenteric artery segments denuded of endothelium ($n = 3$). The sustained level of pressure was not influenced by the addition of ACh (10^{-6} and 10^{-5} M), but was lowered by beraprost (10^{-7} M, 16.2 ± 0.7 mmHg, $n = 3$), a stable analog of PGI₂. In arterial strips, contractions elicited by exogenous norepinephrine were not significantly reduced by treatment with ACh; the mean values of contractions induced by 5×10^{-7} M norepinephrine were 15.7 ± 3.2 , 14.8 ± 2.5 , and $14.9 \pm 2.5\%$ ($n = 5$, relative to contractions induced by 30 mM K^+) in control strips and those treated with 10^{-6} and 10^{-5} M ACh, respectively, and the values at 2×10^{-6} M norepinephrine were 90.6 ± 8.8 , 89.7 ± 9.1 , and $92.8 \pm 8.8\%$ ($n = 5$), respectively.

Effect of ACh on ^3H -Overflow Elicited by Transmural Electrical Stimulation

In order to determine the prejunctional action of ACh, the modulation by ACh of the release of [^3H] norepinephrine evoked by perivascular nerve stimulation was studied. Transmural electrical stimulation at a frequency of 5 Hz for 3 min at intervals of 18 min increased ^3H -overflow from superfused mesenteric arterial strips, which had been incubated for 60 min in [^3H] norepinephrine and stabilized for 126 min during superfusion with normal bathing media. ACh was applied just after the third stimulation (S₃). The ratios of the overflow, S₄/S₃ and S₅/S₃, in the control series were 1.00 ± 0.01 and 0.98 ± 0.01 ($n = 4$), respectively, and were significantly decreased by treatment with 10^{-6} M ACh (Table 1).

Discussion

Transmural electrical stimulation (5–30 Hz) produced a frequency-related pressor response in isolated perfused dog mesenteric artery segments that was abolished by treatment with prazosin and tetrodotoxin. Therefore, the observed response is expected to derive mainly from norepinephrine released from electrically stimulated adrenergic nerve endings. The present study showed that physostigmine, an acetylcholinesterase inhibitor, slightly but significantly inhibited the pressor response to the electrical nerve stimulation, and atropine potentiated the response, suggesting the involvement of endogenous ACh in inhibition of the neurally induced vasoconstriction. The neurogenic pressor response was also attenuated by exogenous ACh, the inhibition being reversed by atropine. ACh did not decrease the perfusion pressure raised by norepinephrine in endothelium-denuded arterial seg-

ments nor the contractile response to norepinephrine in arterial strips denuded of the endothelium. In addition, ACh decreased the ^3H -overflow evoked by nerve stimulation in superfused mesenteric arterial strips previously soaked in ^3H -norepinephrine-containing media. Therefore, it is concluded that the inhibitory effect of ACh on the pressor response to nerve stimulation is associated with an inhibition of the release of norepinephrine from nerve endings, possibly by activation of prejunctional muscarinic receptors. Prejunctional modulation by exogenous ACh of the response to adrenergic nerve stimulation has also been reported in canine (16) and human (17) saphenous veins and guinea-pig carotid arteries (18), whereas only limited information is available concerning the inhibitory effect of endogenous ACh on the response to efferent nerve stimulation (19). Under the experimental conditions used in the present study, the inhibition by neurogenic ACh was appreciably less than that elicited by exogenous ACh, possibly because the amount released from the nerve by the stimulation frequency applied is not sufficient to elicit evident attenuation.

There are three different muscarinic receptor subtypes, M_1 , M_2 , and M_3 (5, 7). The present study revealed that the ACh-induced inhibition of the pressor response to adrenergic nerve stimulation was not altered by the addition of pirenzepine at 10^{-8} M, a concentration sufficient to selectively inhibit M_1 receptor subtype (16, 20) or by 10^{-9} M 4-DAMP, which is enough to selectively block M_3 subtype (21-23). It has been reported that pA_2 values for AF-DX 116 at M_2 receptors range from 6.8 to 7.4 (6, 11, 12). The present study demonstrated that the inhibition by ACh of the pressor response to adrenergic nerve stimulation was significantly reversed by AF-DX 116 in concentrations of 10^{-7} M or higher, suggesting that the prejunctional muscarinic receptor subtype involved is M_2 . Similar findings have also been reported in cat (24) and bovine (19) cerebral and guinea-pig carotid arteries (18) and canine saphenous veins (16). On the other hand, involvement of prejunctional M_3 receptor subtype in the inhibition (19) and of M_1 receptor subtype in the facilitation of norepinephrine release (18) has also been reported.

In conclusion, evidence obtained from the present study indicates that the inhibitory effect of endogenous and exogenous ACh on the pressor response to nerve stimulation is exerted *via* an inhibition of norepinephrine release from nerve endings. The prejunctional muscarinic receptor responsible for inhibiting the transmitter release is supposed to be of the M_2 subtype.

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