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Inhibitory Effect of Fentanyl on Acetylcholine-induced Relaxation in Rat Aorta

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Background: **Previous study has shown that fentanyl attenuates acetylcholine-induced vasorelaxation. The goal of the current *in vitro* study was to identify the muscarinic receptor subtype that is mainly involved in the fentanyl-induced attenuation of endothelium-dependent relaxation elicited by acetylcholine.**

Methods: **The effects of fentanyl and muscarinic receptor antagonists on the acetylcholine concentration–response curve were assessed in aortic vascular smooth muscle ring preparations precontracted with phenylephrine. In the rings pretreated independently with pirenzepine, 4-diphenylacetoxyl-*N*-methylpiperidine methiodide, and naloxone, acetylcholine concentration–response curves were generated in the presence and absence of fentanyl. The effect of fentanyl on the concentration–response curve for calcium ionophore A23187 was assessed.**

Results: **Fentanyl (0.297×10^{-6}, 0.785×10^{-6} M) attenuated acetylcholine-induced vasorelaxation in ring preparations with or without 10^{-6} M naloxone. Pirenzepine (10^{-7} to 10^{-6} M) and 4-diphenylacetoxyl-*N*-methylpiperidine methiodide (10^{-9} to 10^{-8} M) produced a parallel rightward shift in the acetylcholine concentration–response curve. The concentrations (− log M) of pirenzepine and 4-diphenylacetoxyl-*N*-methylpiperidine methiodide necessary to displace the concentration–response curve of an acetylcholine by twofold were estimated to be 6.886 ± 0.070 and 9.256 ± 0.087, respectively. Methoctramine, 10^{-7} M, did not alter the acetylcholine concentration–response curve. Fentanyl, 0.785×10^{-6} M, attenuated acetylcholine-induced vasorelaxation in the rings pretreated with 10^{-7} M pirenzepine but had no effect on vasorelaxation in the rings pretreated with 10^{-8} M 4-diphenylacetoxyl-*N*-methylpiperidine methiodide. Fentanyl, 0.785×10^{-6} M, did not significantly alter calcium ionophore A23187–induced vasorelaxation.**

Conclusions: **These results indicate that fentanyl attenuates acetylcholine-induced vasorelaxation *via* an inhibitory effect at a level proximal to nitric oxide synthase activation on the pathway involving endothelial M_3 muscarinic receptor activation in rat aorta.**

ENDOTHELIAL cells contribute to the local regulation of vasomotor tone by releasing dilator and constrictor substances. The vascular endothelium releases endothelium-derived relaxing factor (EDRF),[1] which relaxes vascular smooth muscle by activation of 3',5'-cyclic guanosine monophosphate.[2] In blood vessels, release of EDRF evoked by acetylcholine is mediated by the activation of different muscarinic receptor subtypes. Muscarinic receptors of cultured bovine aortic endothelial cells are likely to be of M_3 or M_1 subtype.[3] Activation of the M_3 receptors mediates endothelium-dependent relaxation evoked by acetylcholine in the rabbit aorta.[4] The endothelial M_3 muscarinic receptor is mainly involved in mediating the acetylcholine-induced endothelium dependent relaxation in rat aorta.[5]

Anesthetics including inhalation anesthetics,[6,7] local anesthetics,[8] thiopental,[9] and propofol[10] attenuate endothelium-dependent relaxation evoked by acetylcholine. Fentanyl is an excellent opioid anesthetic, especially for patients who have poor cardiac reserve. Fentanyl also inhibits endothelium-dependent relaxation elicited by acetylcholine in isolated rat aortic strips.[11] However, from the best information available to the authors, the endothelial muscarinic receptor subtype that is involved in fentanyl-induced attenuation of vasorelaxant response induced by acetylcholine in rat aorta has not been identified previously. The goals of the current *in vitro* study were to characterize the endothelial muscarinic receptor subtypes that mediate acetylcholine-induced relaxation in rat aorta, to identify the muscarinic receptor subtype that is mainly involved in fentanyl-induced attenuation of endothelium-dependent relaxation elicited by acetylcholine, and to investigate the associated cellular mechanism.

Materials and Methods

All the experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Gyeongsang National University Hospital (Jinju, Gyeongnam, Republic of Korea).

Preparation of Aortic Rings for Tension Measurement

Male Sprague-Dawley rats weighing 250–350 g were anesthetized with intraperitoneal administration of pentobarbital sodium (50 mg/kg). The descending thoracic aorta was dissected free, and surrounding connective tissue and fat were removed under a microscope while the vessel was bathed in Krebs solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 25 mM $NaHCO_3$, 11 mM glucose, 0.03 mM EDTA. The aorta was then cut into 2.5-

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or 3-mm rings, which were suspended on Grass isometric transducers (FT-03; Grass Instrument, Quincy, MA) at 2.0 g resting tension in 10-ml temperature-controlled baths (37°C) containing Krebs solution continuously gassed with 95% O_2 and 5% CO_2. The rings were equilibrated at 2.0 g resting tension for 120 min, during which the bathing solution was changed every 15 min. Care was taken not to damage the endothelium. Only one concentration–response curve elicited by endothelium-dependent relaxing agents (acetylcholine or calcium ionophore A23187) was made from each ring in all the experiments.

Experimental Protocols

The rings were precontracted with 10^{-7} M phenylephrine. The first series of this *in vitro* experiment was conducted to assess the effect of fentanyl on endothelium-dependent relaxation evoked by acetylcholine. When the contractile response to phenylephrine was stabilized, incremental concentration of acetylcholine (10^{-9} to 10^{-5} M) was added to the organ bath to generate a concentration–response curve in the endothelium-intact rings. The effect of fentanyl (9.52×10^{-8}, 0.297×10^{-6}, 0.785×10^{-6} M) on the concentration–response curve for acetylcholine was assessed by comparing the vasorelaxant response in the presence and absence of fentanyl. The fentanyl was added directly to the organ bath 20 min before phenylephrine-induced contraction.

The second series of experiments was designed to determine which subtype of endothelial muscarinic receptor is functionally important in mediating endothelium-dependent relaxation evoked by acetylcholine in rat aorta. The effect of preferential muscarinic receptor subtype antagonists (M_1: 10^{-7}, 3×10^{-7}, 10^{-6} M pirenzepine, M_2: 10^{-7} M methoctramine, M_3: 10^{-9}, 3×10^{-9}, 10^{-8} M 4-diphenylacetoxyl-*N*-methylpiperidine methiodide [4-DAMP]) on the concentration–response curve for acetylcholine was assessed by comparing each vasorelaxant response in the presence and absence of each preferential muscarinic receptor subtype antagonist. The incubation period for each different preferential muscarinic receptor subtype antagonist was 20 min before phenylephrine-induced contraction.

In the third series of experiments, the endothelial muscarinic receptor subtype dependence of fentanyl-induced attenuation of endothelium-dependent relaxation evoked by acetylcholine was examined. In the rings pretreated with 10^{-7} M pirenzepine or 10^{-8} M 4-DAMP, the effect of 0.785×10^{-6} M fentanyl on the concentration–response curve for acetylcholine was assessed by comparing the vasorelaxant response in the presence and absence of 0.785×10^{-6} M fentanyl. The incubation period for the muscarinic receptor subtype antagonist (10^{-7} M pirenzepine or 10^{-8} M 4-DAMP) plus 0.785×10^{-6} M fentanyl or muscarinic receptor subtype antagonist alone was 30 min before phenylephrine-induced contraction.

In the fourth series of experiments, the effect of 0.785×10^{-6} M fentanyl on the concentration–response curve for calcium ionophore A23187 (10^{-9} to 10^{-6} M) was assessed by comparing the vasorelaxant response in the presence and absence of 0.785×10^{-6} M fentanyl. The fentanyl was added directly to the organ bath 20 min before phenylephrine-induced contraction.

Finally, to investigate the participation of opioid receptors in the fentanyl-induced attenuation of endothelium-dependent relaxation induced by acetylcholine, the acetylcholine concentration–response curve was assessed 30 min after the nonspecific opioid antagonist naloxone, 10^{-6} M, was added to the bath, either alone or after combined pretreatment with fentanyl (0.297×10^{-6}, 0.785×10^{-6} M).

Drug and Solutions

All drugs were of the highest purity commercially available: phenylephrine HCl, acetylcholine, calcium ionophore A23187, pirenzepine, methoctramine, 4-DAMP, naloxone (Sigma Chemical, St. Louis, MO), fentanyl citrate (Hana Pharmaceutical Co., Ltd., Seoul, Republic of Korea). All concentrations are expressed as the final molar concentration in the organ bath. Calcium ionophore A23187 was initially dissolved in dimethyl sulfoxide (0.05% volume/volume)[9] and subsequently diluted in distilled water. All other drugs were dissolved and diluted in distilled water.

Data Analysis

Values are expressed as mean ± SD. Vasorelaxant responses to acetylcholine and calcium ionophore A23187 are expressed as the percentage relaxation of the precontraction induced by 10^{-7} M phenylephrine. The logarithm of drug concentration (ED_{50}) eliciting 50% of the maximal relaxation response was calculated by nonlinear regression analysis by fitting the dose–response relation for each vasorelaxant to a sigmoidal curve using commercially available software (Prism version 3.02; Graph Pad Software, San Diego, CA). The maximum relaxant response (R_{max}) was measured as the maximal response to each vasorelaxant, with R_{max} = 100% indicating complete reversal of phenylephrine-induced contraction. The concentration ratio (CR) is defined as the concentration of agonist required to induce 50% maximal response in the presence of antagonist divided by the agonist concentration that elicits the same degree of response in the absence of antagonist. The pA_2 value represents the concentration of antagonists necessary to displace the concentration–response curve of an agonist by twofold. Preferential muscarinic receptor subtype antagonist pA_2 values (−log M) were calculated from Arunlakshana and Schild plots[12] and were obtained from the x-intercept of the plot of log (CR-1) against log

Table 1. Effect of Fentanyl on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
No drug	8	1.302 ± 0.202	−7.49 ± 0.20	96.96 ± 3.54
Fentanyl				
9.52×10^{-8} M	6	1.126 ± 0.197	−7.34 ± 0.27	96.74 ± 3.74
0.297×10^{-6} M	6	1.223 ± 0.311	−7.05 ± 0.15*†	91.52 ± 6.02
0.785×10^{-6} M	6	1.343 ± 0.347	−6.68 ± 0.21*†‡	86.41 ± 7.38*†

* $P < 0.05$ *vs.* no drug. † $P < 0.05$ *vs.* 9.52×10^{-8} M fentanyl. ‡ $P < 0.05$ *vs.* 0.297×10^{-6} M fentanyl.

molar antagonist concentration, where slope was not different from unity. The slopes and pA_2 values calculated from Arunlakshana and Schild plots[12] are expressed as mean ± SE. Statistical analysis was performed using the Student *t* test for paired samples or one-way analysis of variance followed by the Scheffé F test. Differences were considered statistically significant at $P < 0.05$. N refers to the number of rats whose descending thoracic aortic rings were used in each protocol.

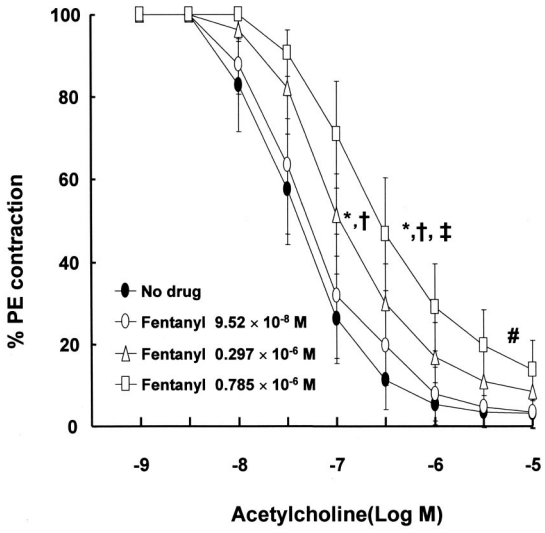

Fig. 1. Effect of fentanyl on acetylcholine dose–response curve. Fentanyl (0.297×10^{-6}, 0.785×10^{-6} M) produced a significant rightward shift (* $P < 0.05$ *vs.* no drug, † $P < 0.05$ *vs.* 9.52×10^{-8} M fentanyl, ‡ $P < 0.05$ *vs.* 0.297×10^{-6} M fentanyl) in the acetylcholine dose–response curve in a concentration-dependent manner. Fentanyl (0.785×10^{-6} M) also attenuated (# $P < 0.05$) the maximal relaxation induced by acetylcholine compared with rings with no drug or 9.52×10^{-8} M fentanyl. PE = phenylephrine.

Results

Effect of Fentanyl on Acetylcholine-induced Relaxation

Fentanyl, 9.52×10^{-8} M, did not significantly alter the acetylcholine concentration–response curve. However, fentanyl (0.297×10^{-6}, 0.785×10^{-6} M) significantly attenuated ($P < 0.05$) the endothelium-dependent relaxation evoked by acetylcholine in a concentration-dependent manner (table 1 and fig. 1). Fentanyl, 0.785×10^{-6} M, significantly attenuated ($P < 0.05$) maximal relaxation evoked by acetylcholine compared with the rings with no drug or 9.52×10^{-8} M fentanyl (table 1 and fig. 1).

Effect of Preferential Muscarinic Receptor Subtype Antagonists on Acetylcholine-induced Relaxation

Pirenzepine (10^{-7}, 3×10^{-7}, 10^{-6} M) caused a parallel rightward shift ($P < 0.05$) in the acetylcholine concentration–response curve in a concentration-dependent manner (table 2 and fig. 2A). Analysis of the data by Arunlakshana and Schild plot for antagonism of acetylcholine-induced relaxation by pirenzepine yielded a slope (0.949 ± 0.126) that was not significantly different from unity and a pA_2 value of 6.886 ± 0.070 (fig. 2B). Methoctramine, 10^{-7} M, did not alter the acetylcholine concentration–response curve (table 3). 4-DAMP (10^{-9}, 3×10^{-9}, 10^{-8} M) produced a parallel rightward shift ($P < 0.05$) in the acetylcholine concentration–response curve (table 4 and fig. 3A) in a concentration-dependent manner. Analysis of the data by Arunlakshana and Schild plot for antagonism of acetylcholine-induced relaxation by 4-DAMP yielded a slope (0.942 ± 0.096) that was not significantly different from unity and a pA_2 value of 9.256 ± 0.087 (fig. 3B).

Table 2. Effect of Pirenzepine on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
No drug	11	1.253 ± 0.220	−7.44 ± 0.20	96.84 ± 6.36
Pirenzepine				
10^{-7} M	8	1.258 ± 0.337	−7.19 ± 0.19*	94.99 ± 7.14
3×10^{-7} M	7	1.254 ± 0.296	−6.86 ± 0.12*†	92.35 ± 6.52
10^{-6} M	6	1.129 ± 0.139	−6.48 ± 0.24*†‡	94.92 ± 4.69

* $P < 0.05$ *vs.* no drug. † $P < 0.05$ *vs.* 10^{-7} M pirenzepine. ‡ $P < 0.05$ *vs.* 3×10^{-7} M pirenzepine.

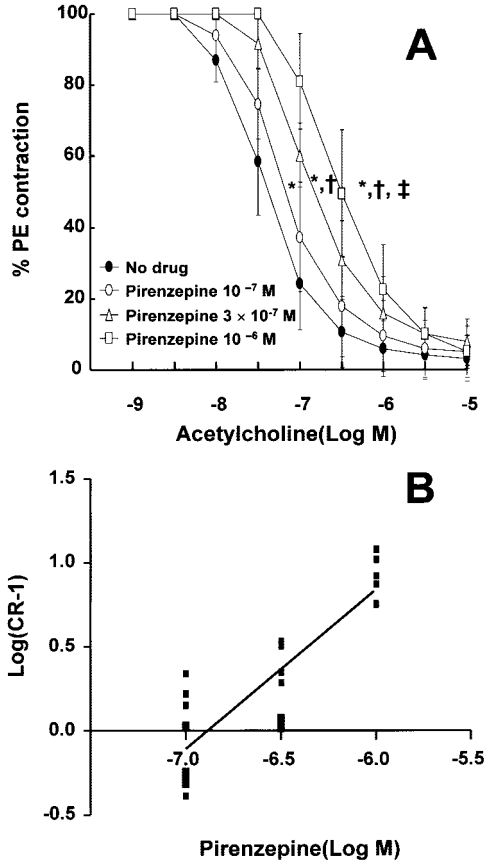

Fig. 2. (*A*) Effect of pirenzepine on acetylcholine dose–response curve. Pirenzepine (10^{-7}, 3×10^{-7}, 10^{-6} M) produced a parallel rightward shift (* $P < 0.05$ *vs.* no drug, † $P < 0.05$ *vs.* 10^{-7} M pirenzepine, ‡ $P < 0.05$ *vs.* 3×10^{-7} M pirenzepine) in the acetylcholine dose–response curve in a concentration-dependent manner. (*B*) A Schild plot was constructed with the concentration ratio (CR: ED_{50} in the presence and absence of pirenzepine) for individual experiments. The slope of the Schild plot for pirenzepine was 0.949 ± 0.126 (r^2 = 0.7505), and the concentration (− log M) of pirenzepine necessary to displace the concentration–response curve of an acetylcholine by twofold was 6.886 ± 0.070. PE = phenylephrine.

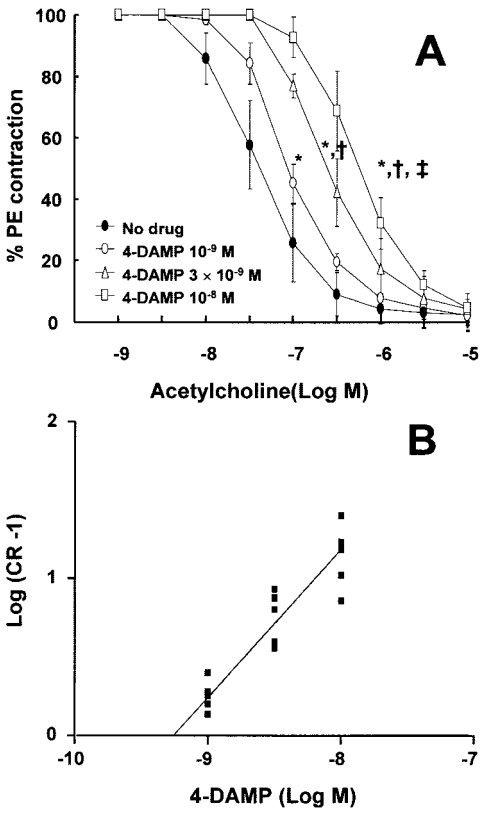

Fig. 3. (*A*) Effect of 4-diphenylacetoxyl-*N*-methylpiperidine methiodide (4-DAMP) on acetylcholine dose–response curve. 4-DAMP (10^{-9}, 3×10^{-9}, 10^{-8} M) produced a parallel rightward shift (* $P < 0.05$ *vs.* no drug, † $P < 0.05$ *vs.* 10^{-9} M 4-DAMP, ‡ $P < 0.05$ *vs.* 3×10^{-9} M 4-DAMP) in the acetylcholine dose–response curve in a concentration-dependent manner. (*B*) A Schild plot was constructed with the concentration ratio (CR: ED_{50} in the presence and absence of 4-DAMP) for individual experiments. The slope of the Schild plot for 4-DAMP was 0.942 ± 0.096 (r^2 = 0.856), and the concentration (− log M) of 4-DAMP necessary to displace the concentration–response curve of an acetylcholine by twofold was 9.256 ± 0.087. PE = phenylephrine.

Table 3. Effect of Methoctramine on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
No drug	6	1.199 ± 0.205	−7.41 ± 0.22	98.88 ± 1.33
Methoctramine				
10^{-7} M	6	1.113 ± 0.212	−7.51 ± 0.23	99.04 ± 2.35

Table 4. Effect of 4-DAMP on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
No drug	10	1.132 ± 0.218	−7.44 ± 0.22	97.60 ± 5.08
4-DAMP				
10^{-9} M	6	1.329 ± 0.207	−7.06 ± 0.10*	97.97 ± 1.77
3×10^{-9} M	6	1.210 ± 0.204	−6.59 ± 0.09*†	95.82 ± 5.02
10^{-8} M	6	1.163 ± 0.226	−6.21 ± 0.18*†‡	95.39 ± 4.68

* $P < 0.05$ *vs.* no drug. † $P < 0.05$ *vs.* 10^{-9} M 4-diphenylacetoxyl-*N*-methylpiperidine methiodide (4-DAMP). ‡ $P < 0.05$ *vs.* 3×10^{-9} M 4-DAMP.

Table 5. Effect of Fentanyl on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings Pretreated with 10^{-7} M Pirenzepine

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
10^{-7} M pirenzepine	6	1.390 ± 0.294	−7.12 ± 0.21	96.68 ± 3.54
10^{-7} M pirenzepine + 0.785 × 10^{-6} M fentanyl	6	1.181 ± 0.294	−6.71 ± 0.11*	95.22 ± 5.37

* $P < 0.05$ *vs.* 10^{-7} M pirenzepine.

Effect of Fentanyl on Acetylcholine-induced Relaxation in the Rings Pretreated with Muscarinic Receptor Subtype Antagonists (10^{-7} M Pirenzepine or 10^{-8} M 4-DAMP)

In the rings pretreated with 10^{-7} M pirenzepine, 0.785 × 10^{-6} M fentanyl significantly attenuated ($P < 0.05$) acetylcholine-induced relaxation (table 5 and fig. 4). In the rings pretreated with 10^{-8} M 4-DAMP, 0.785 × 10^{-6} M fentanyl had no effect on acetylcholine-induced relaxation (table 6 and fig. 5).

Effect of Fentanyl on Calcium Ionophore A23187–induced Relaxation

Fentanyl, 0.785 × 10^{-6} M, did not significantly alter calcium ionophore A23187–induced relaxation (table 7 and fig. 6).

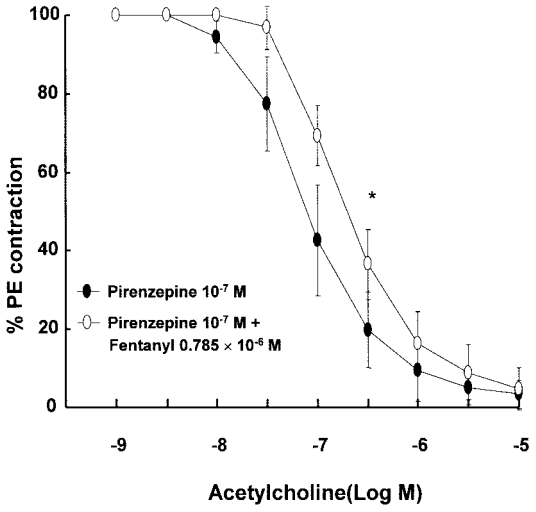

Fig. 4. Effect of fentanyl on acetylcholine dose–response curve in rings pretreated with 10^{-7} M pirenzepine. Fentanyl, 0.785 × 10^{-6} M, attenuated (* $P < 0.05$ *vs.* 10^{-7} M pirenzepine) acetylcholine-induced vasorelaxation compared with rings pretreated with 10^{-7} M pirenzepine alone. PE = phenylephrine.

Effect of Fentanyl on Acetylcholine-induced Relaxation in Rings Pretreated with 10^{-6} M Naloxone

Fentanyl (0.297 × 10^{-6}, 0.785 × 10^{-6} M) significantly attenuated ($P < 0.05$) the endothelium-dependent relaxation evoked by acetylcholine in rings pretreated with 10^{-6} M naloxone in a concentration-dependent manner (table 8 and fig. 7).

Discussion

Despite widespread use of fentanyl for open heart surgery, we believe that this is the first study to determine the muscarinic receptor subtype that is primarily involved in fentanyl-induced attenuation of endothelium-dependent relaxation elicited by acetylcholine in rat aorta. Our results indicate that fentanyl attenuates acetylcholine-induced relaxation *via* an inhibitory effect on the pathway involving endothelial M_3 muscarinic receptor activation. This is independent of opioid receptor activation. Endothelial M_3 muscarinic receptor is functionally important in mediating acetylcholine-induced relaxation in rat aorta.

The nitric oxide formation by nitric oxide synthase (NOS) from L-arginine in mammalian cells activates guanylate cyclase, resulting in an increase in smooth muscle 3′,5′-cyclic guanosine monophosphate production, which correlates with its relaxing effect.[13] The NOSs for release of nitric oxide in endothelial cells bind calmodulin in a calcium (Ca^{2+})–dependent manner.[13] Calcium ionophore A23187 increases intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in the endothelium by increasing the Ca^{2+} permeability of the cell membrane, as well as intracellular organelles containing Ca^{2+}, and therefore can activate NOS. Calcium ionophore A23187 at low concentrations induces an increase of intracellular free Ca^{2+} in endothelial cells but not vascular smooth muscle, causing endothelium-dependent relaxation.[14] However, a high concentration of calcium ionophore A23187 induces an increase of free Ca^{2+} in vascular smooth muscle cells,

Table 6. Effect of Fentanyl on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings Pretreated with 10^{-8} M 4-DAMP

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
10^{-8} M 4-DAMP	6	1.293 ± 0.172	−6.22 ± 0.31	88.96 ± 10.45
10^{-8} M 4-DAMP + 0.785 × 10^{-6} M fentanyl	6	1.133 ± 0.194	−6.24 ± 0.24	90.73 ± 4.34

4-DAMP = 4-diphenylacetoxyl-*N*-methylpiperidine methiodide.

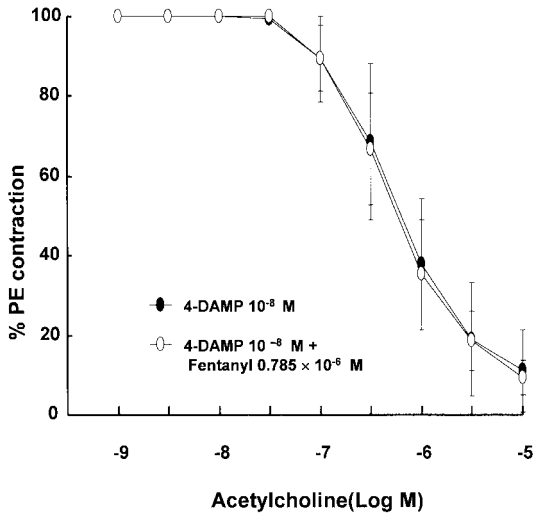

Fig. 5. Effect of 0.785×10^{-6} M fentanyl on acetylcholine dose–response curve in rings pretreated with 10^{-8} M 4-diphenylacetoxyl-*N*-methylpiperidine methiodide (4-DAMP). Fentanyl, 0.785×10^{-6} M, had no effect on the acetylcholine dose–response curve. PE = phenylephrine.

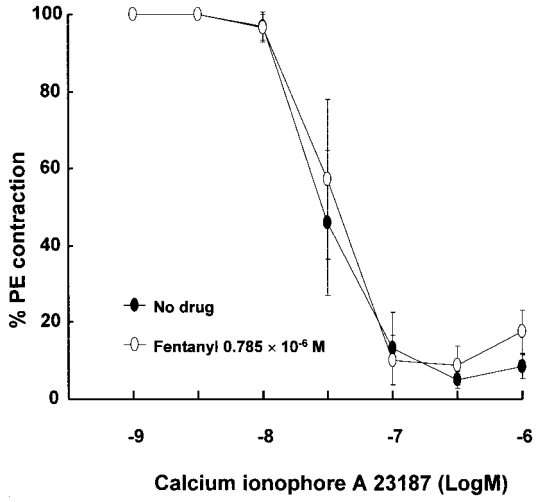

Fig. 6. Effect of fentanyl on calcium ionophore A23187 dose–response curve. Fentanyl, 0.785×10^{-6} M, did not significantly alter the calcium ionophore A23187 dose–response curve. PE = phenylephrine.

causing endothelium-independent contraction.[15] Although unstimulated endothelial cells continuously produce nitric oxide, NOS activity can be enhanced by both receptor-dependent agonists (acetylcholine, adenosine triphosphate, bradykinin) and a receptor-independent agonist (calcium ionophore A23187).[13] Similar to the results reported by Lee *et al.*,[11] 0.785×10^{-6} M fentanyl attenuated endothelium-dependent relaxation evoked by acetylcholine but not by calcium ionophore A23187. Taken together, these results suggest that the possible site for fentanyl to interfere with acetylcholine-induced relaxation seems to be on the level proximal to the biochemical pathway converting L-arginine to EDRF.

Using functional studies, muscarinic receptor subtypes have been identified on endothelial cells: the M_1 and M_2 muscarinic subtypes in rabbit saphenous artery,[16] the M_2 subtype in rabbit ear artery,[17] and the M_3 subtype in rabbit aorta.[4] Acetylcholine-induced vasorelaxation in canine pulmonary artery is mediated primarily by endothelial M_3 muscarinic receptors and to a lesser extent by M_1 muscarinic receptors.[18] Pirenzepine is a selective M_1 muscarinic receptor subtype antagonist (pA_2: 7.8–8.5) whose selectivity for the M_1 muscarinic receptor subtype is approximately 10 times higher than for the M_3 muscarinic receptor subtype (pA_2: 6.7–7.1).[19] Pirenzepine, 10^{-7} M, which is approximately 10 times higher than the reported affinity (pA_2: 7.8–8.5)[19] of pirenzepine for the M_1 muscarinic receptor subtype, produced a rightward shift in the acetylcholine concentration–response curve. The estimated affinity (pA_2: 6.886 ± 0.070) of pirenzepine in the current *in vitro* study is higher than the reported affinity (pA_2: 7.8–8.5)[19] of pirenzepine for the M_1 muscarinic receptor subtype but close to the reported affinity (pA_2: 6.7–7.1)[19] of pirenzepine for the M_3 muscarinic receptor subtype. The M_2 muscarinic receptor subtype antagonist methoctramine, 10^{-7} M, which is 10 times higher than the pA_2 (approximately 7.9)[20,21] reported for a homologous population of M_2 muscarinic receptors, had no effect on acetylcholine-induced relaxation. The M_3 muscarinic receptor antagonist 4-DAMP (10^{-9}, 3×10^{-9}, 10^{-8} M) caused a parallel rightward shift in the acetylcholine concentration–response curve in a concentration-dependent manner. The estimated affinity (pA_2: 9.256 ± 0.087) of 4-DAMP in this *in vitro* study is close to the pA_2 values (M_3: 8.4–9.4, M_1: 8.6–9.2)[19] of 4-DAMP for the M_3 and M_1 muscarinic receptor subtypes, respectively. Although pA_2 values (M_3: 8.9–9.3, M_1: 8.6–9.2) for M_3 and M_1 receptors of 4-DAMP[19] show an overlapping affinity, the conclusion that 4-DAMP (10^{-9}, 3×10^{-9}, 10^{-8} M) attenuates acetylcholine-induced relaxation by acting on M_3 muscarinic receptors rather than M_1 muscarinic receptors is prompted by the fact that the estimated affinity (pA_2: 6.886 ± 0.070) of pirenzepine in this *in vitro* study is close to pA_2 (6.7–7.1)[19] of pirenzepine reported for a

Table 7. Effect of Fentanyl on Calcium Ionophore A23187–induced Relaxation in Isolated Rat Aortic Rings

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
No drug	6	1.304 ± 0.095	−7.55 ± 0.16	94.97 ± 2.08
Fentanyl 0.785×10^{-6} M	6	1.231 ± 0.224	−7.52 ± 0.20	91.10 ± 5.04

Table 8. Effect of Fentanyl on Acetylcholine-induced Relaxation in Isolated Rat Aortic Rings Pretreated with 10^{-6} M Naloxone

	No. of Rats	Precontraction by Phenylephrine, g	Log ED_{50}	Maximal Relaxation, %
No drug	6	1.717 ± 0.230	−7.57 ± 0.19	98.75 ± 1.68
Fentanyl				
0.297×10^{-6} M	6	1.860 ± 0.221	−7.24 ± 0.05*	97.05 ± 3.83
0.785×10^{-6} M	6	1.946 ± 0.325	−6.89 ± 0.19*†	98.83 ± 1.93

* $P < 0.05$ *vs.* no drug. † $P < 0.05$ *vs.* 0.297×10^{-6} M fentanyl.

homologous population of M_3 muscarinic receptor subtype. The previous study by Chen and Liao[5] showed that acetylcholine-induced relaxation is mediated by endothelial M_3 muscarinic receptors in the thoracic aorta of Wistar rats. Pirenzepine and 4-DAMP displace the binding of tritiated acetylcholine to the endothelial membrane in Wistar Kyoto rats.[22] In accord with previous studies,[5,22] all these results suggest that M_1 and M_2 muscarinic receptors had little antagonistic effect on acetylcholine-induced relaxation, indicating that the acetylcholine-induced relaxation in rat aorta is mediated primarily by endothelial M_3 muscarinic receptors. Fentanyl, 0.785×10^{-6} M, attenuated acetylcholine-induced relaxation in the rings pretreated with 10^{-7} M pirenzepine but had no effect on in the rings pretreated with 10^{-8} M 4-DAMP. Reinforced with our results from previous *in vitro* protocols, these results indicate that 0.785×10^{-6} M fentanyl attenuates acetylcholine-induced relaxation *via* an inhibitory effect on the pathway involving endothelial M_3 muscarinic receptor activation.

Fentanyl-induced inhibition of nitric oxide–mediated relaxation induced by acetylcholine does not imply that fentanyl constricts rat aorta. Fentanyl produces hypotension by an inhibition of central sympathetic outflow in intact dogs anesthetized with enflurane.[23,24] *In vivo*, most vessels are continuously exposed to pressure and flow, and the amount of EDRF released has been shown to be directly related to the flow and pressure,[25] and the flow-induced endothelium-dependent regulation of the tones of large arteries may be of physiologic importance on regional hemodynamics. Taking into consideration the above facts,[23–25] the net hemodynamic effects of fentanyl *in vivo* are a composite of effects of fentanyl on blood vessel and central sympathetic activity. In addition, fentanyl decreases tongue mucosal blood flow without change in other hemodynamic variables and has no effect on total peripheral vascular resistance in the rabbit.[26] Any clinical implication of fentanyl on regional hemodynamics must be tempered by the fact that aorta was used in this *in vitro* experiment, whereas organ blood flow is controlled by changes of the diameter of the arteriole with diameter less than 150 μm of the vascular network. Given this limitation, however, because basal release of EDRF is important in the control of blood pressure, fentanyl-induced inhibition of basal EDRF formation may contribute to vasoconstriction observed in previous *in vivo* microcirculation[26] and may contribute to an understanding of endothelial portion of the whole effect of fentanyl.

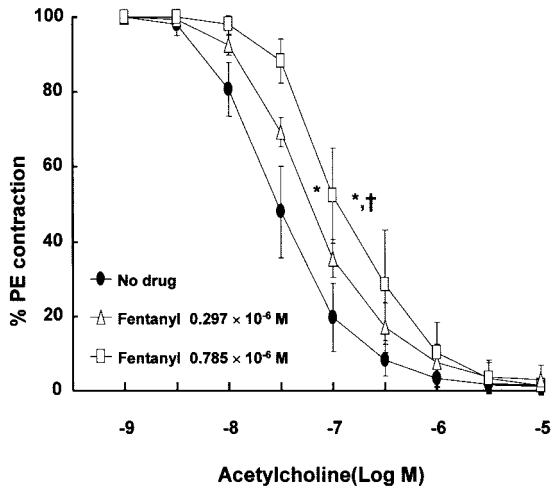

Fig. 7. Effect of fentanyl on acetylcholine dose–response curve in rings pretreated with 10^{-6} M naloxone. Fentanyl (0.297×10^{-6}, 0.785×10^{-6} M) produced a significant rightward shift (* $P < 0.05$ *vs.* no drug, † $P < 0.05$ *vs.* 0.297×10^{-6} M fentanyl) in the acetylcholine dose–response curve in a concentration-dependent manner. PE = phenylephrine.

Fentanyl attenuates acetylcholine-mediated contraction of porcine coronary artery, and this fentanyl-induced attenuation is not opioid receptor mediated.[27] The fact that fentanyl attenuated acetylcholine-induced relaxation in rings pretreated with 10^{-6} M naloxone strongly suggests that this fentanyl-induced attenuation seems to be caused by a direct effect on the pathway for acetylcholine-induced relaxation.

Fentanyl, 9.52×10^{-8} M, which is the concentration encountered in clinical settings because of 80% plasma protein binding,[28] did not significantly alter acetylcholine-induced relaxation. In this *in vitro* study, 0.297×10^{-6} M fentanyl was used in the organ bath because this concentration corresponds to the plasma concentration of 100 ng/ml occurring in patients anesthetized with fentanyl for major surgery,[29] without taking into consideration the protein-bound fraction of fentanyl. Fentanyl (0.297×10^{-6}, 0.785×10^{-6} M), which is above the clinically relevant concentration (9.52×10^{-8} M) encountered in clinical settings, significantly attenuated acetylcholine-induced relaxation. However, rapid redistribution of fentanyl (octanol:water partition coefficient = 813)[30] to

lipid-rich tissue may create a discrepancy between serum concentration and actual tissue concentration. Because cerebrospinal fluid contains little protein compared with plasma,[31] active fentanyl concentration in cerebrospinal fluid averages approximately 46% of the total plasma fentanyl concentration, which is more than twice the free fraction of plasma fentanyl.[32] Small changes in the amount or binding capacity of proteins in certain pathologic conditions (*e.g.*, liver disease, hemodilution, hypoproteinemia) could result in an increase in the free fraction of fentanyl. Because fentanyl is highly lipophilic, we believe the fentanyl concentrations used in this *in vitro* experiment are justified by the fact that 6.9 ng/ml fentanyl in human blood corresponds to the 2.96×10^{-3} M fentanyl calculated in brain lipid.[33] Taking the above four factors into consideration, 0.297×10^{-6} M fentanyl required for an inhibitory effect on acetylcholine-induced relaxation might be the concentration encountered in clinical settings.

In conclusion, these results indicate that fentanyl attenuates acetylcholine-induced relaxation *via* an inhibitory effect at a level proximal to NOS activation on the pathway involving endothelial M_3 muscarinic receptor activation in rat aorta. This phenomenon did not occur through opioid receptor activation. Acetylcholine-induced relaxation in rat aorta is mediated primarily by endothelial M_3 muscarinic receptors.

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