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## Acetylcholine Stimulates the Release of Nitric Oxide from Rat Spinal Cord

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**Background:** Acetylcholine causes synthesis of nitric oxide in vascular endothelium, and presumptive evidence *in vivo* suggests spinally released acetylcholine causes antinociception and increased sympathetic nervous system activity *via* a nitric oxide mechanism. The purpose of this study was to determine, using a recently described bioassay system, whether acetylcholine stimulates nitric oxide release from spinal cord tissue *in vitro*.

**Methods:** Rat thoracolumbar spinal cord slices were incubated in a tissue chamber and perfused with Krebs-Henseleit solution. The perfusate was then passed through endothelium-denuded rat aortic rings and their tension was measured. Vascular rings were precontracted with phenylephrine, then were exposed to spinal cord perfusate with increasing concentrations ( $10^{-12}$ – $10^{-4}$  M) of acetylcholine alone or with various antagonists.

**Results:** Acetylcholine perfusion of spinal tissue caused concentration-dependent relaxations of the aortic rings, an effect blocked by each of the muscarinic antagonists, atropine, pirenzepine, and AFDX-116. Acetylcholine-induced relaxation also was antagonized by an inhibitor of nitric oxide synthase (N-methyl-L-arginine), a nitric oxide scavenger (hemoglobin) and an inhibitor of guanylate cyclase (methylene blue).

**Conclusions:** These results demonstrate release of a vasorelaxant from spinal cord tissue by acetylcholine, which results from an action on muscarinic receptors and exhibits a pharmacology consistent with nitric oxide. Although precise anatomic localization of acetylcholine's action is not possible with this system, these results add to evidence that acetylcholine causes nitric oxide synthesis in the spinal cord. (Key words: Analgesia: postoperative. Gases: nitric oxide. Neuro-

transmitters: acetylcholine. Spinal cord: vasorelaxant release. Sympathetic nervous system: increased activity.)

ACETYLCHOLINE released from spinal cord tissue produces antinociception and increased sympathetic nervous system activity. As such, intrathecal injection of direct muscarinic cholinergic agonists or of cholinesterase inhibitors results in antinociception in animals<sup>1-3</sup> and humans.<sup>4,5</sup> Intrathecal injection of cholinesterase inhibitors increases blood pressure and heart rate by increasing cholinergic stimulation of preganglionic sympathetic neurons.<sup>6,7</sup> These results may prove clinically relevant, because intrathecal neostigmine injection produces dose-dependent, long-lasting analgesia in patients postoperatively<sup>5</sup> and inhibits hypotension from spinal injection of the analgesic clonidine and the local anesthetic bupivacaine in animals.<sup>8,9</sup>

Preliminary studies suggest that both antinociception and increased sympathetic nervous system activity from intrathecal cholinomimetic agents are influenced by local nitric oxide synthesis. Thus, both enhancement of antinociception<sup>10</sup> and blockade of hypotension<sup>7</sup> after intrathecal clonidine by neostigmine are antagonized by intrathecal injection of inhibitors of nitric oxide synthase in sheep. Similarly, antinociception from intrathecal carbamylcholine is antagonized by intrathecal injection of inhibitors of nitric oxide synthase in rats.<sup>11</sup> These results provide presumptive evidence that acetylcholine released from spinal cord tissue stimulates nitric oxide synthesis to cause its effects, similar to the acetylcholine-nitric oxide interaction in vascular endothelium. However, whether acetylcholine stimulates nitric oxide synthesis in the spinal cord has not been directly studied.

The purpose of this study was to determine whether acetylcholine stimulates nitric oxide synthesis in spinal cord tissue. Because nitric oxide is synthesized in small quantities and is rapidly destroyed in the presence of oxygen, we employed a recently developed bioassay technique<sup>12</sup> to measure nitric oxide *via* its vasorelaxant properties. This technique, unlike other methods for

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examining nitric oxide activity, has the advantage of allowing exposure to various concentrations of agents to produce a full concentration-response curve from the same sections of tissue.

### Materials and Methods

After approval by the Animal Care and Use Committee of our institution, adult male Sprague-Dawley rats were deeply anesthetized with 50 mg/kg intraperitoneal sodium pentobarbital, decapitated, and the aorta removed. The aorta was cut into 3–4-mm long rings and endothelium denuded by rubbing with stainless steel wire, then rings were mounted on transducers and tension was measured continuously with a Grass #7 polygraph (Quincy, MA). The rings were stretched to their optimum length-tension relationship by repeated exposures to 180 mM potassium chloride. Removal of endothelium was confirmed by precontraction with phenylephrine  $10^{-6}$  M and lack of relaxation to  $10^{-7}$  M to  $10^{-6}$  M acetylcholine. Two rats were then killed as stated earlier and spinal cords were removed. Each spinal cord was divided into two parts, then chopped in 0.5-mm thick slices. Tissue sections from each hemispinal cord were put into an incubation chamber surrounded by a temperature-controlled water bath maintained at 26°C. Tissue slices were perfused continuously with a multichannel pump (Manostat, NY) at 4 ml/min with oxygenated modified Krebs-Henseleit solution containing indomethacin  $10^{-5}$  M (composition in mM, 118.3 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 0.027 EDTA, and 11 glucose), gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub> at 26°C. Previous studies demonstrated this tissue preparation and temperature yielded consistent responses to stimulators of nitric oxide synthase.<sup>12</sup> The effluent of spinal cord tissue chambers dripped onto the rings directly. Time from spinal cord tissue exposure to contact with aortic rings was <2 s. Experiments were started after spinal cord slices had been incubated in the chamber for 60 min.

The aortic rings were precontracted by addition of  $10^{-6}$  M phenylephrine into the spinal cord perfusion solution. This resulted in an increase in tension in the aortic rings of 600–900 mg (approximately 40–50% of maximum constriction possible with exposure to higher concentrations of phenylephrine), which remained stable for 60 min in control experiments. Acetylcholine was then added to the perfusion solution, beginning at  $10^{-12}$  M, and increased in log units to  $10^{-4}$

M (n = 11 rings). These data from acetylcholine alone were used as a comparison group to all other experimental treatments. To determine the receptors acted on by acetylcholine, the nonsubtype-selective muscarinic antagonist, atropine (n = 7), or the M1 subtype-selective antagonist, pirenzepine (n = 7) or M2 subtype-selective antagonist, AFDX-116 (n = 6, all  $10^{-5}$  M), were added to the perfusion solution beginning 20 min before acetylcholine. To ascertain whether the vasodilation caused by spinal cord tissue perfusion with acetylcholine was from nitric oxide, the following inhibitors of nitric oxide synthase or nitric oxide action were added to the perfusion solution: N-methyl-L-arginine (n = 7;  $10^{-5}$  M), 7-nitroindazole (7-NI;  $10^{-5}$  M), methylene blue (n = 7;  $10^{-5}$  M), and hemoglobin (n = 8;  $10^{-5}$  M). Methemoglobin was removed by incubation of hemoglobin solution overnight through a semipermeable membrane with sodium nitrite. At the end of experiments, sodium nitroprusside,  $10^{-6}$  M, was added to perfusate to yield complete relaxation.

### Drugs and Solutions

All compounds were obtained from Sigma Chemical (St. Louis, MO) except AFDX-116, which was donated by Boehringer-Ingelheim (Ridgefield, CT). Drugs were dissolved in Krebs solution freshly prepared for each experiment, except the stock solutions of acetylcholine and phenylephrine, which were stored at -20°C. Indomethacin was dissolved in  $3 \times 10^{-5}$  M NaHCO<sub>3</sub> solution and diluted to  $10^{-5}$  M final concentration in the Krebs-Henseleit solution.

### Statistics

All results are expressed as means  $\pm$  SEM. Statistical differences were determined by using two-way analysis of variance for repeated measures.  $P < 0.05$  was considered significant.

### Results

Endothelium was successfully removed from the aortic rings, as evidenced by lack of relaxation response to two doses of acetylcholine (data not shown). Addition of acetylcholine to spinal cord perfusion caused concentration-dependent relaxation of the detector rings, with maximum effect at  $10^{-8}$  M (fig. 1). Pretreatment with the nonsubtype-selective muscarinic receptor antagonist, atropine, or the M1-selective antagonist, pirenzepine, or the M2 selective antagonist, AFDX-116,

### ACETYLCHOLINE STIMULATION

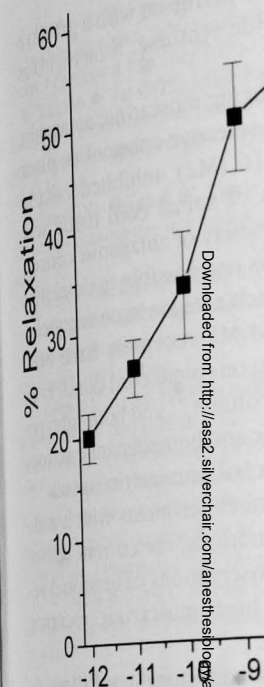


Fig. 1. Concentration-dependent relaxation of aortic rings from spinal cord perfusion. Increasing concentrations of acetylcholine caused a concentration-dependent relaxation of the rings. The maximum relaxation was achieved by exposure of the rings to  $10^{-8}$  M acetylcholine. The relaxation was significantly blocked by the muscarinic antagonist, atropine, pirenzepine, or AFDX-116. Sodium nitroprusside, added at the conclusion of the experiment, yielded complete relaxation.

significantly blocked the aortic ring relaxation (fig. 1). To determine the relaxation caused by acetylcholine, the spinal cord slices were precontracted with phenylephrine. Nitric oxide synthase inhibitors, including N-methyl-L-arginine, methylene blue, and hemoglobin, were used to inhibit nitric oxide scavenger, guanylate cyclase and nitric oxide synthase. The muscarinic antagonist, atropine, pirenzepine, and AFDX-116, and methylene blue, and hemoglobin, were used to precontracted aortic rings without perfusion over 60 min (data not shown). Sodium nitroprusside, added at the conclusion of experiments, relaxed the rings by  $\geq 95\%$  compared to baseline.

### Discussion

The role of spinal cholinergic transmission in spinal anesthesia and fentanyl analgesia is not clear.



## ACETYLCHOLINE STIMULATES SPINAL NITRIC OXIDE RELEASE

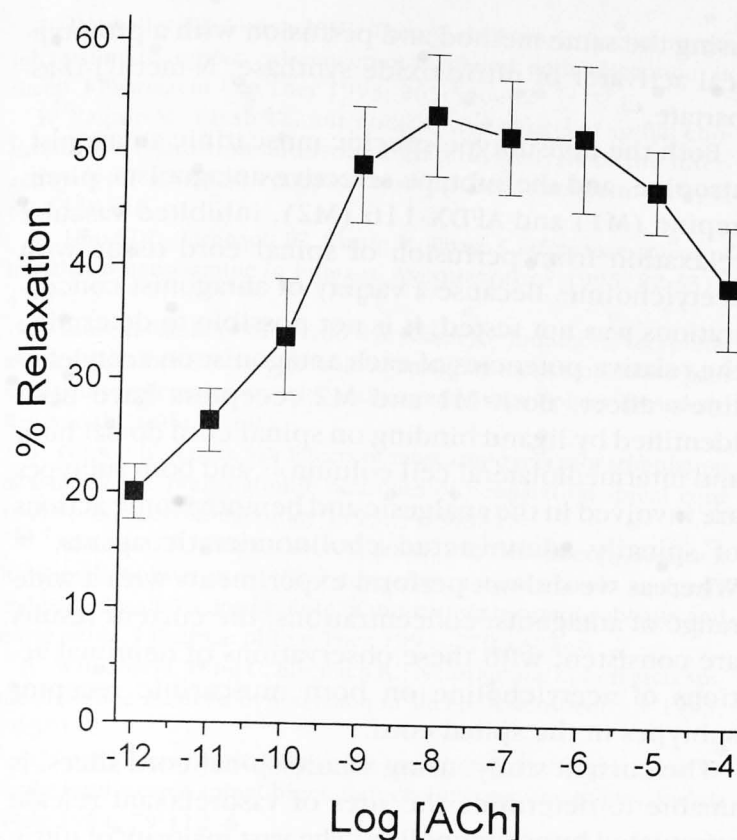


Fig. 1. Concentration-dependent relaxation of detector aortic vascular rings from perfusion of spinal cord slices with increasing concentrations of acetylcholine. Values expressed as mean  $\pm$  SE percent maximum relaxation of 11 rings, determined by exposure of rings to sodium nitroprusside at the conclusion of the experiment.

significantly blocked the acetylcholine-induced aortic ring relaxation (fig. 2). To further determine whether the relaxation caused by acetylcholine perfusion with spinal cord slices was produced by nitric oxide, the nitric oxide synthase inhibitor, N-methyl-L-arginine, the nitric oxide scavenger, hemoglobin, and the soluble guanylate cyclase and nitric oxide synthase inhibitor, methylene blue were used. Each of these agents antagonized acetylcholine perfusion-induced relaxation (fig. 3). The muscarinic antagonists, N-methyl-L-arginine, hemoglobin, and methylene blue had no effects on precontracted aortic ring tension when administered without perfusion over spinal cord slices (data not shown). Sodium nitroprusside, added at the completion of experiments, relaxed precontracted vascular rings by  $\geq 95\%$  compared to baseline tension.

### Discussion

The role of spinal cholinomimetic agents as adjuncts to spinal anesthesia and for postoperative analgesia is

being determined in ongoing clinical trials. The current study provides evidence that cholinomimetic agents may stimulate nitric oxide synthesis in the spinal cord, and previous *in vivo* experiments suggest that this nitric oxide synthesis is necessary for the expression of analgesic<sup>10</sup> and hemodynamic<sup>7</sup> effects of spinally administered cholinomimetic agents.

A variety of methods has been used to measure nitric oxide synthesis in central nervous system tissue, each with its own advantages and drawbacks.<sup>13</sup> Direct measurement of nitric oxide is limited in most cases by the instability of the molecule itself and by sensitivity of the assay. As such, several indirect measures of nitric oxide synthesis or action have been devised. We chose not to measure nitric oxide synthesis by enzymatic analysis (such as conversion of <sup>14</sup>C-arginine to <sup>14</sup>C-citrulline) because we wanted to maintain some anatomic integrity rather than using homogenized tissue.

We recently modified a bioassay system for nitric oxide synthesis from vascular endothelium to investi-

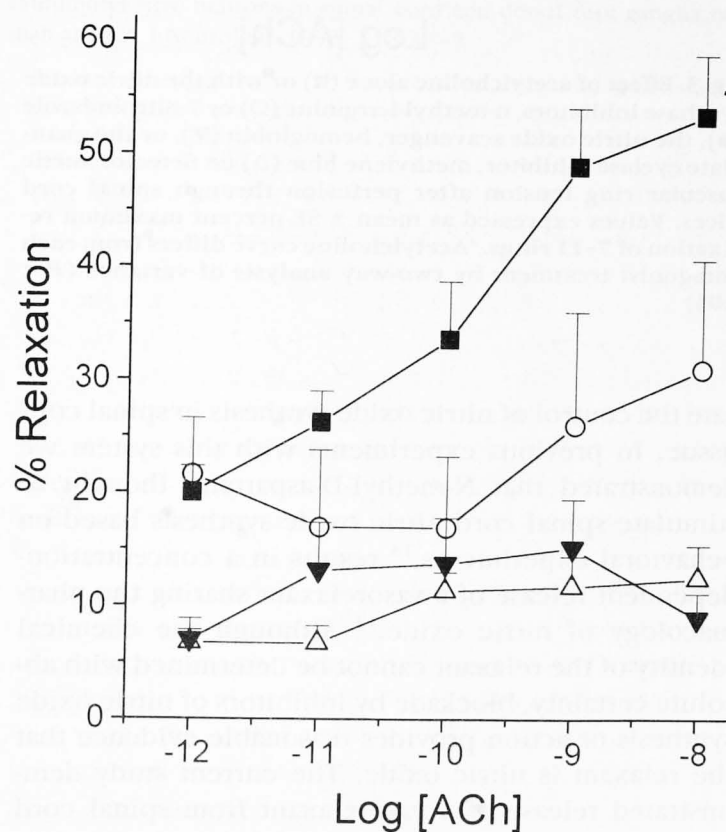


Fig. 2. Effect of acetylcholine alone (■) or with the muscarinic antagonists atropine (○), pirenzepine (▼), or AFDX-116 (△) on detector aortic vascular ring tension after perfusion through spinal cord slices. Values expressed as mean  $\pm$  SE percent maximum relaxation of 6-11 rings. \*Acetylcholine curve differs from each antagonist treatment by two-way analysis of variance ( $P < 0.05$ )

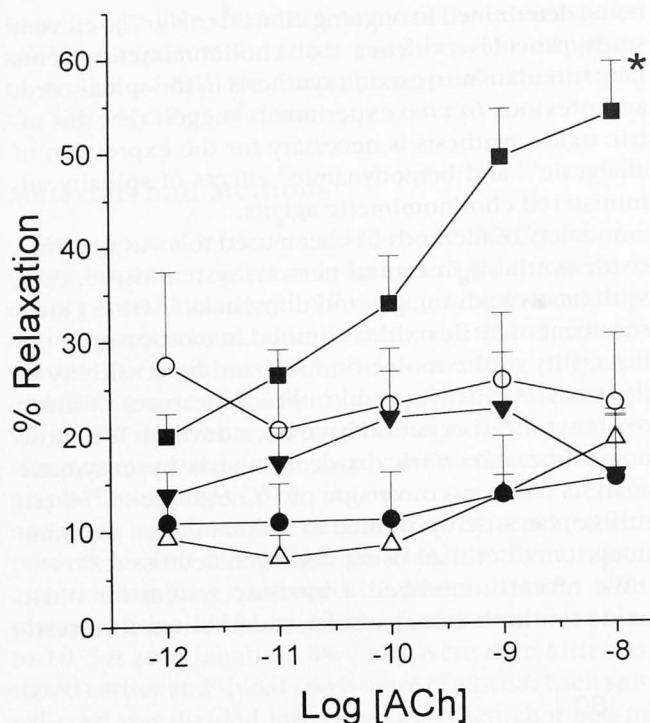


Fig. 3. Effect of acetylcholine alone (■) or with the nitric oxide synthase inhibitors, n-methyl-L-arginine (○) or 7-nitroindazole (●), the nitric oxide scavenger, hemoglobin (▼), or the guanylate cyclase inhibitor, methylene blue (△) on detector aortic vascular ring tension after perfusion through spinal cord slices. Values expressed as mean  $\pm$  SE percent maximum relaxation of 7–11 rings. \*Acetylcholine curve differs from each antagonist treatment by two-way analysis of variance ( $P < 0.05$ )

gate the control of nitric oxide synthesis in spinal cord tissue. In previous experiments with this system we demonstrated that N-methyl-D-aspartate, thought to stimulate spinal cord nitric oxide synthesis based on behavioral experiments,<sup>14</sup> results in a concentration-dependent release of a vasorelaxant sharing the pharmacology of nitric oxide.<sup>12</sup> Although the chemical identity of the relaxant cannot be determined with absolute certainty, blockade by inhibitors of nitric oxide synthesis or action provides reasonable evidence that the relaxant is nitric oxide. The current study demonstrated release of a vasorelaxant from spinal cord slices by acetylcholine, which was inhibited by a nitric oxide synthase blocker, a nitric oxide scavenger, and an inhibitor of guanylate cyclase, consistent with its identity as nitric oxide. The maximum degree of vascular relaxation from acetylcholine perfused through spinal tissue in these studies is similar to that produced

using the same method and perfusion with a prototypical activator of nitric oxide synthase, N-methyl-D-aspartate.<sup>12</sup>

Both the nonsubtype-specific muscarinic antagonist, atropine, and the subtype selective antagonists, pirenzepine (M1) and AFDX-116 (M2), inhibited vascular relaxation from perfusion of spinal cord tissue with acetylcholine. Because a variety of antagonist concentrations was not tested, it is not possible to determine the relative potencies of each antagonist on acetylcholine's effect. Both M1 and M2 receptors have been identified by ligand binding on spinal cord dorsal horn and intermediolateral cell column,<sup>15</sup> and both subtypes are involved in the analgesic and hemodynamic actions of spinally administered cholinomimetic agents.<sup>7,16</sup> Whereas we did not perform experiments with a wide range of antagonist concentrations, the current results are consistent with these observations of neuronal actions of acetylcholine on both muscarinic receptor subtypes in the spinal cord.

The current study, using whole spinal cord slices, is unable to determine the sites of vasorelaxant release stimulated by acetylcholine. The vast majority of nitric oxide synthase in the spinal cord is of the neuronal isoform, with  $<5\%$  consisting of the membrane-bound, endothelial form.<sup>12</sup> Similarly, it has been suggested that 7-NI is a specific inhibitor of neuronal nitric oxide synthase,<sup>17</sup> and inhibition of acetylcholine-induced relaxation by this agent is consistent with an acetylcholine action on neuronal nitric oxide synthase. Nitric oxide synthase is localized to the superficial dorsal horn and the intermediolateral cell column regions of the spinal cord,<sup>18</sup> but the current study used tissue including both areas and was therefore unable to distinguish separate release from each area.

In summary, acetylcholine perfusion of spinal cord tissue *in vitro* causes concentration-dependent release of a vasorelaxant with pharmacologic properties consistent with nitric oxide. Blockade by muscarinic antagonists confirms *in vivo* experiments and supports the concept of nitric oxide mediation of analgesic and hemodynamic actions of spinally administered cholinomimetic agents.

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