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Spinal muscarinic cholinergic and nitric oxide systems in cardiovascular regulation

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Abstract

Pharmacological activation of muscarinic receptors located in the thoracic spinal cord evokes a marked increase in blood pressure and heart rate. We have previously demonstrated that the cardiovascular response to stimulation of spinal cord muscarinic cholinergic receptors is dependent upon a pharmacologically described ascending spino-bulbar pathway. The purpose of the study was to determine whether the blood pressure and heart rate responses to intrathecal (i.t.) injection of the muscarinic cholinergic receptor agonist carbachol are mediated by a local nitric oxide (NO)-generating system. Freely moving rats were previously prepared with chronic indwelling i.t. and intra-arterial catheters. Both the pressor and tachycardic responses produced by i.t. injection of carbachol were inhibited in a dose-dependent manner by i.t. pre-treatment with the NO synthase inhibitor *N*-ω-Nitro-L-arginine methylester (L-NAME). To confirm the site of action of the drugs employed in conscious rats, a separate group of rats was anesthetized, and using surgical procedures previously developed in this laboratory, drug distribution was limited specifically to the lower thoracic spinal cord. When carbachol was administered by i.t. injection and localized to the lower thoracic area, muscarinic cholinergic receptor stimulation again produced a marked pressor response, but without the accompanying tachycardia. The ability of *N*-ω-Nitro-L-arginine methylester (L-NAME) to inhibit the pressor response to carbachol in conscious rats was confirmed in anesthetized rats, although higher doses of L-NAME than those employed in conscious rats were required. L-NAME-induced inhibition of the carbachol-evoked pressor response was reversed by the L-, but not the D-isomer, of arginine. Moreover, i.t. pre-treatment with Methylene blue, that interferes with NO production and function, effectively inhibited the expression of the pressor response to i.t. injection of carbachol. The 'anti-muscarinic' action of L-NAME was not due to a direct interaction with spinal muscarinic receptors, as L-NAME did not significantly displace [^{3}H]methyl-scopolamine from spinal cord membranes in vitro. The results of this study support the hypothesis that spinal muscarinic cholinergic receptors participate in a sympathoexcitatory pathway that interacts either directly or indirectly with a local NO-generating system involved in the regulation of blood pressure.

Keywords: Nitric oxide (NO); Blood pressure; Spinal cord; Hypertension; Muscarinic receptor; N^{ω}-Nitro-L-arginine methyl ester (L-NAME)

1. Introduction

The spinal cord and brain stem have a significant role in the maintenance of systemic blood pressure and heart rate. Our previous studies have focused on the pharmacological characterization of a spinal cholinergic (muscarinic) pathway that mediates a hypertensive response (Marshall and Buccafusco, 1987; Magri' and Buccafusco, 1988; Buccafusco and Magri', 1990; Takahashi and Buccafusco, 1991a,b). In rats, intrathecal (i.t.) injection of cholinergic muscarinic receptor agonists evokes an atropine-reversible hypertensive response (Marshall and Buccafusco, 1987). In fact, the hypertensive response to i.t. injection of carbachol and neostigmine in hypertensive rats (SHR) is even more intense than that in normotensive animals (Buccafusco and Magri', 1990). This pressor response to muscarinic receptor stimulation does not result from direct activation of spinal pre-ganglionic neurons. Rather, spinal cholinergic receptors mediate an increase in blood pressure through interactions with other neurotransmitter pathways within

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the medulla and spinal cord (Marshall and Buccafusco, 1987; Takahashi and Buccafusco, 1992; Feldman and Buccafusco, 1993b).

One interaction of this spinal cholinergic pressor system is through an ascending pathway that links to other cholinergic neurons located in the lower medulla, possibly within the rostral ventrolateral medulla (Feldman and Buccafusco, 1993a). This link between spinal and medullary cholinergic systems reflects the most rostral component of the ascending pathway. Cholinergic neurons within the rostral ventrolateral medulla provide tonic sympathoexcitatory output via a descending glutamatergic pathway (Bazil and Gordon, 1993; Gordon and McCann, 1988; Morrison et al., 1989). In addition to the ascending cholinergic pressor system, a component of the pressor response to spinal muscarinic receptor stimulation is mediated locally through facilitation of descending sympathoexcitatory vasomotor tone (Takahashi and Buccafusco, 1992). Moreover, preliminary studies in this laboratory have suggested that the pressor response to stimulation of spinal muscarinic receptors is mediated through the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Feldman and Buccafusco, 1992, 1993b). It is possible that spinal cholinergic neurons facilitate descending sympathetic activity that is mediated, at least in part, by a descending glutamatergic (NMDA) pathway.

Nitric oxide (NO) is a vasoactive substance produced by a reaction catalyzed by NO synthase. One form of NO synthase present in vascular endothelial cells generates NO that produces relaxation of smooth muscle tone and vasodilation (Furchgott and Zawadzki, 1980; Palmer et al., 1987; Vanhoutte, 1989). Although NO was originally linked to the vasodilator action of acetylcholine in the peripheral vasculature, in the central nervous system NO was first associated with glutamatergic rather than cholinergic synapses (Garthwaite et al., 1989; Knowles et al., 1989). The voltage-dependent, post-synaptic release of NO modulates the neuronal activity of surrounding neurons regardless of specific neuronal connections (Gally et al., 1990). NO modulates neuronal activity principally by activating soluble guanylate cyclase and increasing cGMP levels in other neurons. Three isozymes of NO synthase, including a predominantly brain form (bNO synthase), exist in the brain, where generation of NO has been suggested as an important neural mediator in a variety of physiological and behavioral functions (Garthwaite et al., 1989; Knowles et al., 1989; Snyder, 1992; Bredt and Snyder, 1994).

Like the brain, the spinal cord contains a discrete distribution of NO synthase-containing neurons (Valtschanoff et al., 1992, 1993). The purpose of the present study was to determine whether the hypertensive response to spinal muscarinic receptor stimulation is mediated by a local NO-generating system. Experiments were designed to determine whether i.t. pre-treatment with the competitive NO synthase inhibitor L-NAME could block the pressor response to i.t. injection of the muscarinic agonist carbachol. The selectivity of action of L-NAME was assessed by determining whether the effects of L-NAME could be reversed by pre-treatment with L-arginine and by determining whether L-NAME interacted directly with spinal cord muscarinic receptors.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN, USA), weighing 280–380 g, were housed in an environmentally controlled room on a 12-h/12-h day-night cycle; and were maintained on Wayne Rodent blox and tap water. All animal protocols were previously approved by the institutional Committee on Animal Use for Research and Education.

2.2. Implantation of a chronic i.t. catheter

Rats were anesthetized with methohexital (65 mg/kg) and placed in a stereotaxic frame. Catheterization of the spinal subarachnoid space was performed under aseptic conditions by inserting a sterile, saline-filled polyethylene (PE_{10}) catheter caudal to a micro-incision in the atlanto-occipital membrane. The catheter was advanced to the (thoracic) T_{12} level of the spinal cord (see Section 2.4). The distal end of the catheter was plugged with a 30-gauge stainless steel wire and anchored to the skull with acrylic cement after being passed through a burr hole in the occipital bone. Each rat was allowed 3–5 days to recover. Only normally moving, healthy animals were employed in subsequent experiments. Upon the completion of an experiment, catheter placement was confirmed by dorsal laminectomy and dye injection.

2.3. Measurement of blood pressure from freely moving rats

After 2 days of recovery from i.t. surgery, rats were again anesthetized with methohexital and a midsagittal incision was made in the abdomen. The left iliac artery was then exposed and a polyethylene catheter (PE_{50}) filled with heparinized saline (20 U/ml) was inserted so that the tip rested in the abdominal aorta below the origin of the renal arteries. The distal end of the catheter was plugged with 22-gauge wire and directed around the pelvis before emerging in the subcutaneous nuchal adipose. The catheter was passed through a spring support and connected to a water tight swivel mounted 300 mm above the cage floor. The abdominal incision was closed with 3.0 silk using a three layer closure. This surgical procedure allowed the chronically catheterized rat unrestricted movement to all areas of his cage for the duration of the experiment while

receiving a constant infusion of heparinized saline (8 ml/day). Each rat was allowed a 48-h period of recovery prior to the experiment. Only healthy animals having positive weight gain were employed in experiments.

2.4. Procedure for localization of i.t. administered drugs to the lower thoracic spinal cord and measurement of blood pressure in anesthetized rats

Anesthesia was achieved with urethane 0.86 g/kg i.p. that was supplemented with inhalation halothane. A polyethylene catheter (PE_{50}) filled with heparinized saline (2 U/ml) was inserted into the right femoral artery. Blood pressure was monitored by connecting the arterial line to a pressure transducer (Abbott Critical Care Systems, North Chicago, IL, USA) that was coupled to a Beckman polygraph recorder. Heart rate was obtained from the pressure pulses and displayed as an integrated signal by using a biotachometer (Beckman 9857B, Schiller Park, IL, USA). Mean arterial pressure was calculated as diastolic + (pulse pressure/3). I.v. access was accomplished by inserting a polyethylene (PE_{50}) catheter into the left femoral vein or left subclavian vein.

Halothane administration was terminated and an additional i.v. injection of urethane (0.30 g/kg) administered to maintain complete anesthesia for the duration of the experiment. The following i.t. cannulation procedure was developed to insure that subsequent drug distribution was relegated to the thoraco-lumbar spinal cord (Feldman and Buccafusco, 1993a). Rats were placed in a stereotaxic frame with the head positioned such that the interaural line was 50 mm above the operating table. A midsagittal incision was performed and the musculature between C_7 and T_3 was reflected. A dorsal laminectomy was performed at the level of T_1-T_2 and the dura was longitudinally transected. The incision was extended to the lateral borders of the exposed spinal cord. A saline filled catheter (PE_{10}) was then inserted 50–53 mm from the most caudal portion of the exposed cord. The length of the catheter was determined empirically based upon the weight of the animal. Cerebrospinal fluid was collected with micro-sponges (Alcon Surgical) placed on and around the exposed spinal cord to prevent redistribution of drug solution from the local spinal site to rostral brain regions. Artificial cerebrospinal fluid was given to replace the loss of spinal fluid. A rectal temperature probe and thermostatically controlled heating pad sustained body temperature at 37°C throughout the experiment. I.v. infusion of 5% dextrose in water (D_5W) at a rate of 5 ml/kg/h was used to maintain the animal's water balance and to help replace insensible losses. Tracheal intubation was performed to preserve normal respiratory dynamics. The trachea was cannulated with PE_{240} tubing and then sutured into the surrounding fascia. Arterial blood gases were measured to insure proper pO_2, pCO_2, HCO_3 and oxygen saturation. Respiratory rate, tidal volume and inflow gas mixture were individually adjusted. Animals were ventilated with 2 l/min of room air supplemented with 100% O_2. Any break in the vascular integrity of the spinal cord or surrounding vessels terminated the experiment. Proper placement of the catheter was confirmed at necropsy.

2.5. [^{3}H]Methylscopolamine binding to spinal cord membranes

Rats were sacrificed by decapitation and the spinal cord was rapidly removed by high pressure injection of isotonic saline applied to the caudal end of the sacral vertebral column. The tissue was washed and immediately placed in 4 ml of ice-cold incubation buffer (50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$) and, subsequently, homogenized using a Bellco glass homogenizer with a Teflon pestle. The homogenate was then centrifuged at $37\,000 \times g$ for 20 min. After centrifugation, the supernatant was decanted, the pellet resuspended in 4 ml of incubation buffer and the protein content was assayed (Bio-Rad Protein Assay-Bio-Rad laboratories, Hercules, CA, USA). Binding reactions were carried out in a reaction volume of 1 ml containing the following: 100 μg of receptor protein, 1 nM [^{3}H]methylscopolamine, increasing concentrations of atropine or L-NAME (in duplicate) and incubation buffer. Non-specific binding was determined in the presence of 10 μM atropine. After a 90-min incubation at room temperature, the reaction mixture was filtered through Schleicher and Schuell No. 32 glass filters and washed 3 times with 3 ml of ice-cold buffer using a Brandel Cell Harvester (Gaithersburg, MD, USA). The filters were then dried, placed in scintillation fluid for at least 4 h and the radioactivity determined in a liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

2.6. Drugs

Drugs were administered in 10 μl of sterile saline via PE_{10} tubing that connected a constant speed infusion pump to the i.t. cannula. Drug solutions were infused over a 1-min period, including an additional 6 μl of saline to clear the contents of the catheter. All reagents and drugs were purchased from Sigma Chemical and the [^{3}H]methylscopolamine was purchased from New England Nuclear.

2.7. Statistical Analysis

Data are presented as the mean ± S.E.M. and the differences between or among experimental groups were determined by analysis of variance (ANOVA) or repeated measures ANOVA employed when experimental groups involved multiple comparisons. A Fisher's protected least squares difference test was employed for post-hoc analyses of significant ANOVA data. Differences between 2 groups of data were determined using a modified t-test with a Bonferroni correction for multiple comparisons utilizing

the error mean squared term from the ANOVA. The area under the curve (AUC) for carbachol time course data, including both positive and negative areas in relationship to baseline, was determined between 0.1 and 30 min. The criterion for statistical significance was $P < 0.05$ for all statistics. Data derived from the fractional specific binding of [^{3}H]methylscopolamine to spinal cord membranes were fit by non-linear (least squares) regression using the mass action expression for single non-interacting sites (Sigma Plot Jandel Scientific, Corte Madra, CA, USA).

3. Results

3.1. The effects of pre-treatment with L-NAME on the increase in mean arterial pressure and heart rate to i.t. injection of carbachol in unanesthetized rats

In our previous studies, i.t. injection of carbachol was shown to evoke an atropine-reversible, dose-dependent increase in blood pressure and heart rate (Marshall and Buccafusco, 1987; Magri' and Buccafusco, 1988; Buccafusco and Magri', 1990). In rats pre-treated with saline, subsequent i.t. administration of carbachol (5 μg, 27 nmol) elicited a reproducible peak increase in mean arterial pressure of 30–45 mm Hg and a duration of response of 30–45 min. The increase in mean arterial pressure peaked at 2 min and was within 10 mm Hg of the pre-drug baseline by 30 min after injection (Fig. 1). The pressor response to carbachol was accompanied by an immediate increase in heart rate that peaked simultaneously (at about 100 beats/min) with the pressor response (Fig. 2). Immediately after peaking, heart rate values decreased slightly at the 5-min time point and then returned to the previous peak level. Because the animals were unanesthetized and baroreceptor reflexes were intact, this biphasic nature to the heart rate response to i.t. carbachol most likely represents a transient reflex slowing of heart rate due to the pressor response. Thereafter, heart rate slowly returned to baseline levels within 45–60 min.

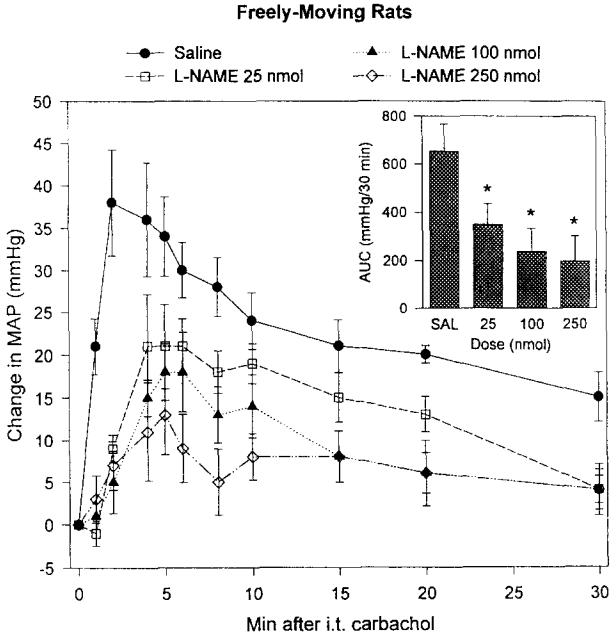

Fig. 1. The increase in mean arterial pressure (MAP) after i.t. injection of carbachol in conscious rats pre-treated with saline or a given dose of L-NAME. Saline pre-treatment, closed circles; i.t. pre-treatment with L-NAME, open squares (25 nmol), closed triangles (100 nmol) and opened diamonds (250 nmol). The abscissa displays time (min) subsequent to i.t. administration of carbachol. L-NAME significantly inhibited the pressor response to i.t. injection of carbachol in a dose-dependent manner ($P < 0.05$ vs. control). Results are expressed as mean ± S.E.M. Inset: The data for each curve expressed as area under the curve (AUC). * Significantly different from saline (SAL) control mean ($P < 0.05$, $n = 6$).

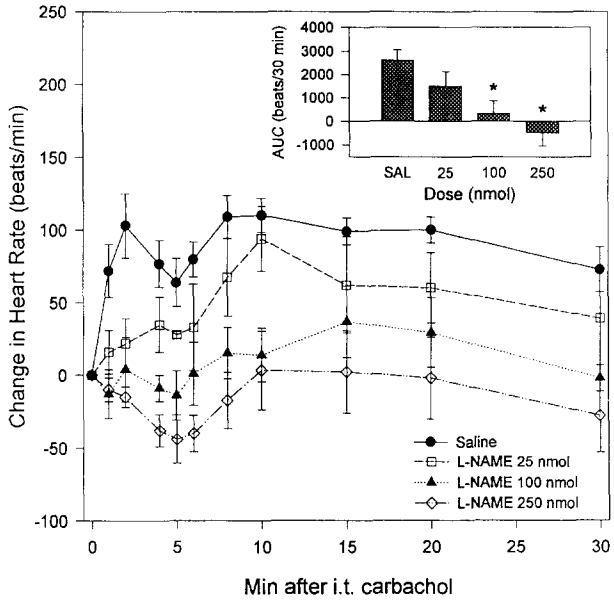

Fig. 2. The change in heart rate after i.t. injection of carbachol in conscious rats pre-treated with saline or a given dose of L-NAME. Saline pre-treatment, closed circles; i.t. pre-treatment with L-NAME, open squares (25 nmol), closed triangles (100 nmol) and opened diamonds (250 nmol). L-NAME significantly inhibited the increase in heart rate to i.t. injection of carbachol in a dose-dependent manner ($P < 0.05$ vs. control). Inset: The data for each curve expressed as area under the curve (AUC). * Significantly different from saline (SAL) control mean ($P < 0.05$, $n = 6$).

Rats were randomly assigned to 1 of 4 i.t. pre-treatment groups: saline (control), 25 nmol L-NAME, 100 nmol L-NAME and 250 nmol L-NAME. These doses of L-NAME were selected based upon the results of a preliminary dose-finding study (data not shown). After the first carbachol experiment, rats were allowed to recover for at least 2 days. They were then reassigned to receive a second regimen on a random basis. No rat received more than two regimens. L-NAME was administered by i.t. injection 20 min prior to i.t. injection of carbachol (Table 1). During the 20-min pre-treatment time, L-NAME produced no change in resting mean arterial pressure except at the 250-nmol dose that produced a transient, but significant

Table 1
Mean arterial pressure and heart rate in freely moving rats: baseline, 10 min after saline or L-NAME pre-treatment and 30 s prior to i.t. carbachol injection

Drug	Baseline	10 min after saline/L-NAME	Immediately prior to carbachol	*n*
Blood pressure (mm Hg)				
Saline	106.5 ± 2.8	107.5 ± 3.1	110.0 ± 3.2	8
L-NAME 25	108.0 ± 4.2	121.7 ± 7.6	117.0 ± 4.3	6
L-NAME 100	96.8 ± 5.2	102 ± 6.6	106.9 ± 5.5	5
L-NAME 250	109.3 ± 2.4	121 ± 5.2 [a,b]	118 ± 3.5	6
Heart rate (beats / min)				
Saline	348.7 ± 8.0	363.6 ± 9.5	359.1 ± 9.3	8
L-NAME 25	399.8 ± 18.7	393.6 ± 22.9	369.8 ± 24	6
L-NAME 100	342.2 ± 27.6	386.0 ± 21.2	378.0 ± 26.0	5
L-NAME 250	373.3 ± 23.7	406 ± 26.0	417.3 ± 25.1	6

L-NAME, *N*-ω-Nitro-L-arginine. Drug doses (25, 100 and 250) are expressed in nmol. Baseline values were measured immediately before pre-treatment with i.t. L-NAME or isotonic saline. 10 min after saline/L-NAME, the midpoint between saline or L-NAME pre-treatment and i.t. carbachol. Immediately prior to carbachol, time point 30 s before i.t. injection of carbachol (approximately 20 min after pre-treatment). Each value represents the mean ± S.E.M. [a] Significantly different from respective saline mean; [b] significantly different from the respective baseline mean; $P < 0.05$ (two-tailed).

12-mm Hg increase. There was no significant effect of L-NAME on resting heart rate (Table 1).

I.t. pre-treatment with L-NAME produced a dose-dependent inhibition of the peak pressor response to subsequent i.t. injection of carbachol. In L-NAME-pre-treated rats, there was a prolonged time to peak and reduced duration of action of carbachol compared with saline controls (Fig. 1). Since onset and duration of the pressor response to carbachol were altered in the pre-treated animals, a better estimate of the scope of inhibition produced by pre-treatment regimens was the area under the carbachol pressor-time curve (AUC). The AUCs for the groups pre-treated with 25-, 100- and 250-nmol doses of L-NAME were reduced by 47, 64 and 70%, relative to the respective saline control mean (Fig. 1, inset). As with the pressor response, a dose-dependent reduction in the tachycardic response to carbachol was produced by pre-treatment with L-NAME. In fact, pre-treatment with the highest dose of L-NAME transiently lowered heart rate to below pre-carbachol levels (Fig. 2). L-NAME-induced inhibition of the carbachol tachycardic response was also reflected by a dose-dependent decrease in the AUC such that pre-treatment with the 250-nmol dose completely abolished the response (Fig. 2, inset).

3.2. The effect of pre-treatment with L-NAME on the pressor response to i.t. injection of carbachol in anesthetized rats

In rats pre-treated with i.t. injection of saline, i.t. injection of 5 μg of carbachol produced an increase in mean arterial pressure that was qualitatively similar to that produced in unanesthetized rats (Figs. 1 and 3). Although the magnitude and duration of the pressor response was similar in both unanesthetized and anesthetized animals, the time to peak was slightly slower (4 min after injection) in the anesthetized group. The delay in the initiation of the pressor response to carbachol allowed the appearance of a transient depressor response of 5–15 mm Hg and less than 1 min in duration (see Fig. 5). In control rats, this response was complete prior to the first data sampling point presented in Fig. 3. Unlike the response in unanesthetized rats, the pressor response to carbachol was not accompanied by a significant change in heart rate (data not shown).

Because of the excellent reproducibility of the pressor response to carbachol, rather than employing a new set of

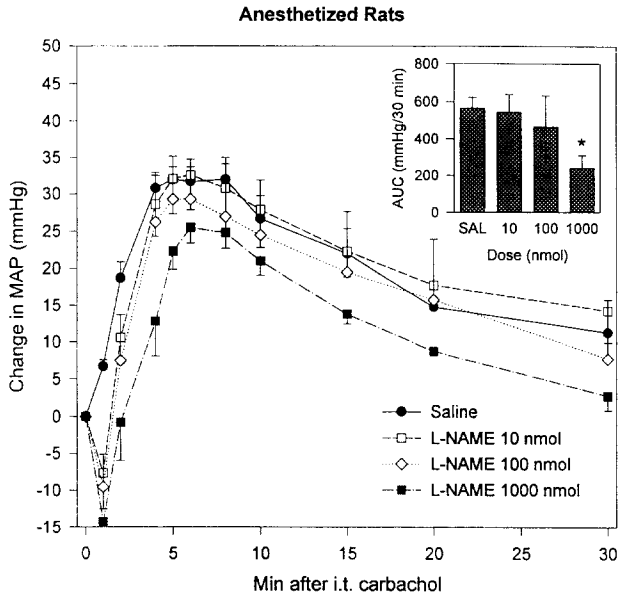

Fig. 3. The increase in mean arterial pressure (MAP) after i.t. injection of carbachol in anesthetized rats pre-treated with saline or a given dose of L-NAME. Saline pre-treatment, closed circles; i.t. pre-treatment with L-NAME, open squares (10 nmol), open diamonds (100 nmol) and closed squares (1000 nmol). The abscissa displays time (min) subsequent to i.t. administration of carbachol. Only the 1000 dose of L-NAME significantly inhibited the pressor response to carbachol ($P < 0.05$ vs. control). Note: The initial depressor phase in saline-pre-treated rats preceded the first time point of measurement. Inset: The data for each curve expressed as area under the curve (AUC). * Significantly different from saline (SAL) control mean ($P < 0.05$, $n = 5–8$).

control (saline pre-treatment) animals for each experimental series, control animals were randomly interspersed among the 3 primary experimental groups. Thus, 2–3 rats were employed for each of the groups. This design served to both limit the numbers of control animals for the study and to provide confirmation that the typical control responses to i.t. injection of carbachol were obtained longitudinally throughout the study. As the control responses to i.t. carbachol were very similar (see below), the 8 experiments were combined to provide control means for the 3 study groups. Each animal was employed only one time.

After i.t. injection of L-NAME (10 or 100 nmol), mean arterial pressure remained within 5% of saline control means (Table 2). The highest dose of L-NAME (1000 nmol) produced a gradual 17-mm Hg increase in mean arterial pressure such that blood pressure was significantly higher than pre-treatment levels just prior to carbachol treatment. When L-arginine (but not D-arginine) was administered 10 min after L-NAME (see below), the L-NAME-induced increase in resting mean arterial pressure was not observed. In contrast to the effect of L-NAME, i.t. injection of Methylene blue resulted in a decrease in resting mean arterial pressure by about 20 mm Hg that remained at that level just prior to i.t. carbachol injection.

Administration of the various pre-treatment regimens (Table 2) produced no significant heart rate change from saline controls. Also, for each drug regimen employed below, over the course of the experiment, heart rate did not change by more than 10% compared with resting or saline-pretreatment values. Therefore, only data for the carbachol-induced pressor response will be presented below.

I.t. pre-treatment with 10 or 100 nmol of L-NAME did not significantly alter the peak pressor response to i.t. injection of carbachol (Fig. 3). However, the highest dose of L-NAME (1000 nmol) significantly reduced the magnitude and delayed the time to peak of the pressor response to carbachol (Fig. 3). As in the control situation, in rats pre-treated with L-NAME, a transient depressor response preceded the pressor response (Figs. 3 and 5). However, L-NAME pre-treatment did not appear to inhibit the expression of the depressor response. Rather the highest dose of L-NAME appeared to be associated with the greatest fall in mean arterial pressure.

Based upon measurement of AUC, there was a trend towards a dose-dependent inhibition of the overall expression of the carbachol-evoked response of 3.6, 17.6 and 57.5%, respectively, for the 3 doses of L-NAME (Fig. 3, inset).

In the next series of experiments, arginine was employed to confirm that L-NAME was acting as a competitive inhibitor of spinal NO synthase. In this protocol, rats first were pre-treated with 1000 nmol of L-NAME by i.t. injection. 10 min later, 1000 nmol of either L- or D-arginine also was administered by i.t. injection. Finally, i.t. carbachol was administered 10 min later. The protocol for control animals consisted of i.t. saline pre-treatment, followed 10 min later by an additional i.t. saline injection, that was followed 10 min later by i.t. carbachol. In a few preliminary experiments, we determined that i.t. administration of D- or L-arginine alone did not produce any statistically significant changes in baseline mean arterial pressure or heart rate. L-Arginine pre-treatment antagonized the L-NAME-induced inhibition of the carbachol pressor response to within 1% of control values (Figs. 4 and 5). The AUC for the L-NAME-inhibited carbachol

Table 2
Mean arterial pressure and heart rate in anesthetized rats: baseline, 10 min after saline or L-NAME pre-treatment and 30 s prior to i.t. carbachol injection

Drug	Baseline	10 min after saline/L-NAME	Immediately prior to carbachol	*n*
Blood pressure (mm Hg)				
Saline	90.5 ± 3.8	88.9 ± 5.1	87.5 ± 4.1	8
L-NAME 10	92.3 ± 7.4	86.7 ± 6.6	88.4 ± 7.2	5
L-NAME 100	86.6 ± 6.5	79.7 ± 8.0	79.5 ± 8.3	5
L-NAME 1000	88.8 ± 4.3	100 ± 3.9	105.8 ± 3.0 [a,b]	5
Methyl blue	91.0 ± 1.5	71.5 ± 2.6 [a,b]	67.6 ± 2.8 [a,b]	5
L-NAME/L-ARG	81.1 ± 3.5	88.4 ± 3.5	89 ± 4.4	5
L-NAME/D-ARG	86.1 ± 2.2	89.9 ± 1.7	98.1 ± 1.8 [a]	4
Heart rate (beats / min)				
Saline	451.3 ± 9.9	440.6 ± 12.1	444.4 ± 9.3	8
L-NAME 10	424 ± 8.9	420 ± 8.9	421 ± 5.5	5
L-NAME 100	464 ± 11.1	467 ± 9.6	468 ± 8.1	5
L-NAME 1000	454 ± 8.7	442 ± 6.8	442 ± 10.7	5
Methyl blue	459 ± 5.0	454 ± 13.6	454 ± 12.5	5
L-NAME/L-ARG	438 ± 11.1	439 ± 10.7	438 ± 9.1	5
L-NAME/D-ARG	452 ± 11.8	448 ± 10.0	436 ± 8.0	4

L-NAME, *N*-ω-Nitro-L-arginine. Drug doses (10, 100 and 1000) are expressed in nmol; Methyl blue, 1000 nmol Methylene blue; L-ARG, L-arginine; D-ARG, D-arginine. Saline, L-NAME or Methylene blue was administered 20 min prior to carbachol; arginine was administered 10 min prior to carbachol.
[a] Significantly different from respective saline mean; [b] significantly different from the baseline mean; $P < 0.05$ (two-tailed).

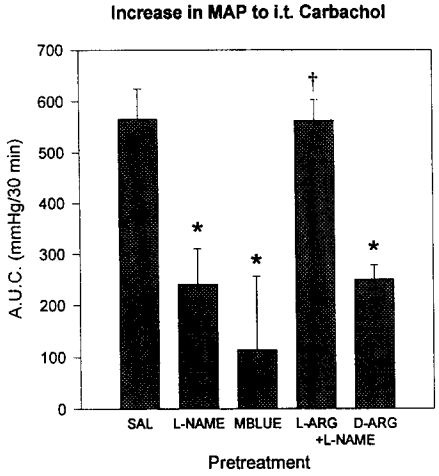

Fig. 4. Effect of inhibitors of NO function on the pressor response to i.t. injection of carbachol in anesthetized rats. Data is displayed as the area under the pressure-time curve. The abscissa displays i.t. pre-treatments prior to the administration of carbachol as follows: saline (SAL) control, L-NAME (1000 nmol), Methylene blue (1000 nmol) and L-NAME (1000 nmol), followed by L- or D-arginine (1000 nmol). Saline, L-NAME or Methylene blue was administered 20 min prior to carbachol; arginine was administered 10 min prior to carbachol. * Significantly different from saline (SAL) control mean, $P < 0.05$; † significantly different from L-NAME and significantly different from D-ARG + L-NAME group means ($P < 0.05$, $n = 5–8$).

response was 240.2 ± 69.8 mm Hg/30 min. L-Arginine totally reversed this L-NAME-induced inhibition to 562.5 ± 40.7 mm Hg/30 min (that was similar to the saline pre-treatment mean 565.8 ± 58.0 mm Hg/30 min). In contrast, D-arginine was completely without effect in reversing the L-NAME-induced inhibition of the carbachol response (Figs. 4 and 5).

As further confirmation of the role of spinal NO synthase in the expression of the pressor response to i.t.

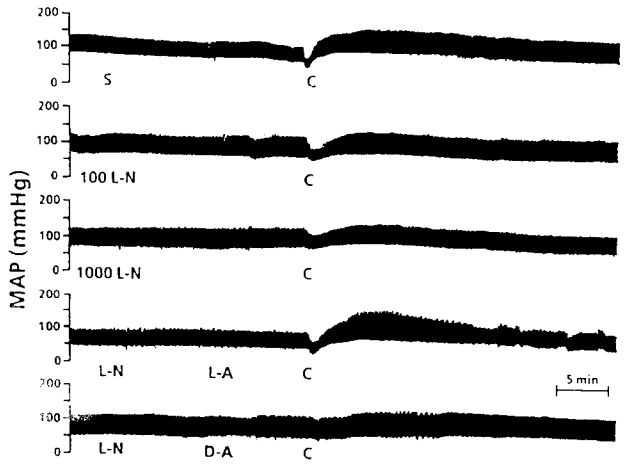

Fig. 5. Blood pressure tracings from representative experiments in anesthetized rats treated with i.t. injection of carbachol (at point C). S, saline; 100 L-N, 100 nmol L-NAME; 1000 L-N, 1000 nmol L-NAME; L-N, 1000 nmol L-NAME; L-A, 1000 nmol L-arginine; D-A, 1000 nmol D-arginine. Saline or L-NAME was administered 20 min prior to carbachol; arginine was administered 10 min prior to carbachol.

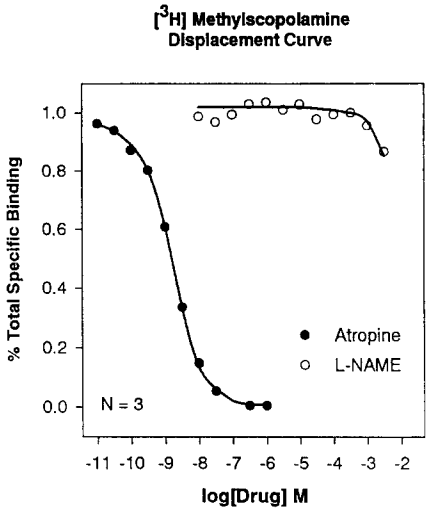

Fig. 6. Displacement of [^{3}H]methylscopolamine (muscarinic receptor) binding to rat spinal cord membranes by atropine or L-NAME. Each binding assay (tissue derived from 3 rats) was performed in duplicate.

muscarinic receptor stimulation, in the next series Methylene blue was employed for its ability to interfere with the function of NO in vivo. I.t. pre-treatment with 1000 nmol of Methylene blue, like L-NAME, produced an effective blockade of the pressor response to subsequent i.t. injection of carbachol (Fig. 4). As indicated by measurement of the AUC, after Methylene blue administration, the pressor response to carbachol was reduced by 80% vs. control.

3.3. Effect of L-NAME on [^{3}H]methylscopolamine binding to rat spinal cord membranes

As indicated in Fig. 6, L-NAME displaced [^{3}H]methylscopolamine binding to spinal cord membranes only at concentrations above 1 mM. In comparison, atropine, a classical muscarinic receptor antagonist displaced [^{3}H]-methylscopolamine binding with an IC_{50} of 1.5 nM.

4. Discussion

The results of this study support the hypothesis that NO has a role in the modulation of central sympathetic outflow and cardiovascular hemodynamics. Thus, pre-treatment with L-NAME produced a small but significant increase in mean arterial pressure (for the highest doses employed) in both anesthetized and freely moving rats. This increase in resting mean arterial pressure to L-NAME was reversed by L-arginine, whereas the dextro-rotatory arginine analog was inert in this regard. The ability of L-NAME to increase baseline blood pressure suggests that spinal cord-generated NO may provide some tonic inhibition of ongoing sympathetic nerve activity, a finding consistent with the results of an earlier study (Togshi et al., 1992). Alternatively, the L-NAME-induced increase in mean arterial pressure may be explained by redistribution of L-NAME to the systemic

circulation. However, doses of L-NAME as employed in this study are not sufficient to increase blood pressure when administered systemically (Rees et al., 1990).

In contrast to L-NAME, i.t. injection of Methylene blue decreased baseline mean arterial pressure. This apparent discrepancy may be explained by the fact that L-NAME is selective in its action as an inhibitor of NO synthase, whereas Methylene blue may inhibit the function of NO by more than one mechanism, one of which could be responsible for the fall in resting mean arterial pressure. For example, Methylene blue has been shown to inhibit guanylyl cyclase, to scavenge free radicals, and may even inhibit NO synthase (Marshall et al., 1988; Mayer et al., 1993). Nevertheless, both Methylene blue and L-NAME had similar inhibitory actions regarding the pressor response to i.t. injection of carbachol (see below).

I.t. pre-treatment with L-NAME attenuated the pressor and tachycardic responses to i.t. injection of carbachol in a dose-dependent manner. This finding suggests that the sympathoexcitatory response to spinal muscarinic receptor stimulation is mediated, at least in part, by a NO-generating system. The experiments performed in conscious rats provided a clear profile of the effects of carbachol and L-NAME without the potential confounding effects of general anesthesia. In this situation, drug distribution could not be limited to one area of the spinal cord. I.t. administration of carbachol in anesthetized rats allowed us to localize the drug solution to the lower thoracic spinal cord. Similar profiles (magnitude and duration) of carbachol-induced pressor responses were produced under control (i.t. saline pre-treatment) conditions in both conscious and anesthetized animals. One difference between the carbachol responses in the two groups was the presence of a small transient depressor effect prior to the onset of the hypertensive response. This biphasic response to carbachol is most often observed in anesthetized animals and most likely reflects the presence of inhibitory cardiovascular muscarinic receptors that are revealed in the anesthetized state (Marshall and Buccafusco, 1987; Brezenoff and Jenden, 1969, 1970). The inability of carbachol to elicit a significant tachycardic response in anesthetized rats was expected, since muscarinic cholinoceptive sites are somatotopically organized such that tachycardic responses to carbachol are elicited predominantly at cervical and upper thoracic levels (Marshall and Buccafusco, 1987; Takahashi and Buccafusco, 1991a,b). The selectivity of response observed to carbachol in anesthetized rats further supports the contention that drug localization did indeed occur in this preparation.

The ability of carbachol to elicit a tachycardic response in conscious rats suggests that stimulation of spinal muscarinic receptors results in enhanced cardiac sympathetic activity and/or withdrawal of vagal tone. This latter effect is sufficient to override the effects of vascular baroreceptor stimulation resulting from the accompanying pressor response. The influence of the baroreceptor reflex in the heart rate response to i.t. injection of carbachol is suggested by the observation that immediately after peaking, heart rate values decreased slightly and then returned to the previous peak level. This biphasic nature to the heart rate response to carbachol most likely represents a transient reflex slowing of heart rate caused by the pressor response. However, the independence of spinal cardiac pathway from the vasomotor pathway is suggested both by the difference in anatomical origin of each response (as discussed in the preceding paragraph) and by the observation that the heart rate response usually outlasted the pressor response.

As in the conscious rats, i.t. pre-treatment with L-NAME attenuated the carbachol-induced pressor response in anesthetized rats, although higher doses of L-NAME were required. The requirement for higher doses of L-NAME in anesthetized rats may be attributed to the effects of the general anesthetic. Nevertheless, the similarity of drug responses in terms of mean arterial pressure for conscious and anesthetized rats will allow for future studies in which the use of conscious rats is not possible. Although we did not quantify the initial depressor response to carbachol, as indicated in Fig. 5, L-NAME pre-treatment delayed the onset, but did not appear to inhibit the expression of this transient response.

The ability of both L-NAME and Methylene blue pretreatment to inhibit the pressor response to i.t. injection of carbachol provides strong evidence that the local production of NO is a requirement for the complete expression of this response. It is difficult to explain the apparently disparate findings that L-NAME both increased resting blood pressure and decreased the evoked (carbachol-induced) blood pressure response. Local micro-injection of NO donors, or inhibitors of NO synthase into specific brain regions, such as the paraventricular nucleus (Horn et al., 1994), the nucleus tractus solitarius (Harada et al., 1993) or via the lateral cerebral ventricle (Cabrera and Bohr, 1995), have been reported to produce, respectively, decreases and increases in blood pressure. Indeed, some degree of inhibition of sympathetic tone appears to be maintained by tonic NO formation within several brain and spinal regions. Since NO formation mediates or enhances the pressor action of carbachol within the spinal cord, it is likely that the NO-generating system linked to the carbachol-evoked pressor response is separate from that inhibiting sympathetic tone. To suggest this possibility is the observation that cells within the intermediolateral nucleus are not activated by muscarinic cholinergic receptor agonists (Gibson and Logan, 1995). Also, micro-injection of carbachol into specific spinal sites of the lower thoracic region evoked pressor responses predominantly from the intermediomedial, rather than the intermediolateral nucleus, region (Takahashi and Buccafusco, 1992). The spinal muscarinic pressor system appears to be an indirect, possibly ascending system that facilitates descending sympathetic activity (Takahashi and Buccafusco, 1991a,b; and

see Buccafusco, 1996). Therefore, spinal NO-generating systems may play dual, potentially opposite, roles in modifying spinal sympathetic neurotransmission.

The potential of L-NAME to bind to spinal muscarinic receptors was addressed in the concluding experiments of this study. The specificity of L-NAME as an inhibitor of NO synthase was supported both by in vitro and in vivo experimentation. The results of the muscarinic receptor binding experiments demonstrated that greater than 1.0 mM concentrations of L-NAME were required to displace [^{3}H]methylscopolamine from spinal cord muscarinic receptors in vitro. This low degree of potency at muscarinic receptors was in contrast to the nM affinity of the classical muscarinic antagonist atropine. Specificity of L-NAME action was also indicated in vivo in the anesthetized rat, since the ability of L-NAME to attenuate the pressor response to i.t. carbachol was reversed by L-arginine, but not by D-arginine. Our results are in disagreement with those from an earlier study that indicated that L-NAME exhibited μM affinity for guinea-pig brain muscarinic receptors (Buxton et al., 1993). Factors underlying this discrepancy may include species differences and tissue differences (spinal cord vs. whole brain). We have demonstrated that the spinal cord exhibits a different profile of expression of muscarinic receptor subtypes than that of higher brain regions (Wei et al., 1994). Another difference between the two studies was our use of [^{3}H]methylscopolamine and the prior group's use of [^{3}H]quinuclidinyl benzylate as the muscarinic receptor probe. [^{3}H]Quinuclidinyl benzylate, in contrast to [^{3}H]methylscopolamine, is an irreversible receptor antagonist and is quite lipid soluble. Hence, quinuclidinyl benzylate would have access to intracellular binding sites that may not necessarily reflect cell surface binding of functional significance (Aronstam et al., 1987).

Finally, the results of this study are in agreement with our recent findings regarding the role of a spinal NO-generating system that mediates the cardiovascular and behavioral responses to naloxone-precipitated withdrawal in the morphine-dependent rat. As with the pressor response to i.t. injection of carbachol, we have reported that the pressor response to i.t. injection of naloxone in conscious morphine-dependent rats is mediated through a spinal muscarinic receptor pressor pathway (Holland et al., 1993). More recently, we demonstrated that the pressor response to i.t. injection of naloxone in dependent rats was blocked by i.t. pre-treatment with L-NAME (Buccafusco et al., 1995). Therefore, this spinal cholinergic-NO link appears to play a role in several aspects of cardiovascular regulation, including the regulation of tonic sympathetic activity (Togshi et al., 1992), the autonomic responses associated with precipitated morphine withdrawal (Holland et al., 1993; Buccafusco et al., 1995) and, possibly, the maintenance of elevated blood pressure in the SHR (Magri' and Buccafusco, 1988).

In conclusion, the present study supports the concept that a spinal NO-generating system plays a role in both the maintenance of resting blood pressure and in the expression of the evoked pressor to spinal muscarinic receptor stimulation. Since this muscarinic pressor system does not directly interact with pre-ganglionic sympathetic neurons, it is possible that the release of NO may provide part of the link between the cholinoceptive system and pre-ganglionic cell bodies. This link may be limited to the release of NO from cholinoceptive neurons or it may include other spinal systems. One site of interaction may include the descending glutamatergic pathway that is the purported primary substrate for mediating sympathetic tone to preganglionic neurons (see Buccafusco, 1996).

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