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Effect of N^G-nitro-L-arginine methyl ester on functionally characterized muscarinic receptors in anesthetized cats

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Abstract

This study was undertaken to determine if the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), is a competitive antagonist of muscarinic receptors in vivo. Cats were anesthetized with pentobarbital (36 mg/kg, i.p.). Five peripheral muscarinic responses were characterized based on their sensitivity to intravenous administration of atropine (1–100 μg/kg), pirenzepine (1–100 μg/kg) or gallamine (30–3000 μg/kg) as follows: (1) muscarinic ganglionic transmission through the superior cervical ganglion to the nictitating membrane (M_1), (2) electrically elicited vagal bradycardia (M_2), (3) neurally evoked sudomotor responses (M_3; non-endothelial), (4) basal pupil tone in sympathectomized cats (M_3; non-endothelial) and (5) methacholine-induced depression of arterial blood pressure (M_3; endothelial). Additional groups of animals were administered L-NAME (50 mg/kg, i.v.) to determine if this agent would alter activation of these muscarinic systems. L-NAME was devoid of effect on responses elicited by stimulation of muscarinic M_1, M_2 and M_3 (non-endothelial) receptors. In contrast, L-NAME significantly reduced the depressor responses to i.v. methacholine (M_3; endothelial), as did its non-alkyl ester congener, L-NA (N^G-nitro-L-arginine; 25 mg/kg, i.v.). These results support the conclusion that although L-NAME inhibits synthesis of nitric oxide in vascular endothelial cells, it is not a generalized muscarinic receptor antagonist in vivo. © 1997 Elsevier Science B.V.

Keywords: Muscarinic M_1 receptor; Muscarinic M_2 receptor; Muscarinic M_3 receptor; N^G-nitro-L-arginine methyl ester (L-NAME); N^G-nitro-L-arginine; Atropine; Gallamine; Pirenzepine

1. Introduction

Identification of L-arginine analogs that act as competitive inhibitors of nitric oxide synthase has been instrumental in the rapid expansion of knowledge concerning nitric oxide (NO) mechanisms in general (Moncada et al., 1991; Iadecola et al., 1994). L-NAME (N^G-nitro-L-arginine methyl ester) is one of the most widely used of the family of nitric oxide synthase inhibitors due both to its high potency and to the fact that the alkyl esterification at the carboxy terminus aids in solubility of the compound (Rees et al., 1990; Moncada et al., 1991; Nathan, 1992). The use of L-NAME to study the involvement of NO on effects mediated by acetylcholine and other muscarinic receptor agonists has been complicated by reports that L-NAME lacks specificity and, due to the alkyl ester substitution, is a non-selective muscarinic receptor antagonist (Buxton et al., 1993; Chang et al., 1997). In contrast, other investigators have reported that L-NAME does not block muscarinic mechanisms in vivo (Bellan et al., 1993; Cheng et al., 1994; Santiago et al., 1994).

The present investigation was undertaken to systematically determine if a high dose of L-NAME would antagonize evoked muscarinic receptors in anesthetized cats. Five peripheral muscarinic systems were utilized (e.g., muscarinic ganglionic transmission (M_1); vagal bradycardia (M_2); sudomotor responses (M_3, non-endothelial); pupillary constriction (M_3, non-endothelial); methacholine hypotension (M_3, endothelial)).

Following characterization of the peripheral muscarinic systems as fitting into the muscarinic M_1, M_2 or M_3 receptor subclass, each system was tested for inhibition following intravenous administration of L-NAME (50 mg/kg). L-NAME antagonized methacholine-induced vasodilation but was without significant effect on non-endothelial muscarinic receptor mediated responses regardless of the muscarinic receptor subtype involved. Taken together, these results suggest that L-NAME does not exhibit generalized muscarinic receptor antagonism in vivo.

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2. Materials and methods

2.1. General

Adult cats were anesthetized with pentobarbital (36 mg/kg, i.p.) which was supplemented by 10 mg i.v. bolus injections, as needed, during the experimental period. The trachea, right femoral vein and right femoral artery were cannulated. The blood pressure was recorded from the femoral artery using a Statham P23 pressure transducer and the femoral vein was used for i.v. drug administration. Body temperature was controlled at approximately 37°C with a heating pad. In all experiments, the vago-sympathetic nerve trunks were sectioned at the midcervical level and physiological responses were continuously recorded on a Grass polygraph (7D). Animals receiving gallamine were initially placed on positive pressure ventilation using a Harvard respirator. The end expiratory CO_2 was maintained in the 3–4% range with use of a capnometer (Traverse Medical).

2.2. Physiological activation of muscarinic responses

Vasodilator responses were elicited by bolus injections of methacholine (3 μg/kg) at 15 min intervals. In preliminary experiments it was determined that 3 μg/kg of methacholine produced a depressor response of 50–75% of the maximal response seen at 30 μg/kg. Vagal bradycardia was evoked by electrical stimulation of the right vagosympathetic nerve trunk (2–4 Hz) with a silver bipolar electrode using a Grass Stimulator (S88) and isolation unit (SIU5). Stimulation parameters were 10 V, 2 ms duration and 10 s trains. In preliminary experiments, a frequency of 2 or 4 Hz produced 50–70% of the maximal slowing. The stimuli were presented at approximately 2 min intervals. Dose–response relationships were determined based on the area under the response curve (AUC) for both blood pressure and heart rate responses using a computer based digitizer (Jandel Scientific).

Electrodermal (sudomotor) responses were measured from the left forepaw footpad using Beckman miniature biopotential skin electrodes (11 mm diameter) in reference to a shaven inactive region on the same leg (Koss and Davison, 1976). These skin potential responses were amplified with a Grass 7P low level preamplifier using a 0.8 s time constant. For preganglionic nerve stimulation the chest was opened by section of ribs 2–4 with artificial respiration provided with a Harvard respirator. The sympathetic nerve trunk was crushed proximally, covered with mineral oil and electrically stimulated using a pair of silver bipolar electrodes. Square wave pulses of submaximal voltage and duration (6–8 V; 2 ms) were obtained from a Grass S88 stimulator and isolation unit (SIU5). One or two pulses were presented at 30 s intervals. These parameters produced skin potential responses of approximately 60–90% of maximal.

Muscarinic ganglionic transmission was elicited by stimulation of the preganglionic cervical sympathetic nerve. Nictitating membrane contractions were recorded using a Grass FT.03 force displacement transducer with the basal tension set at 10 g and the animals positioned in a Kopf stereotaxic unit (Koss and Rieger, 1976). A bipolar silver electrode was placed beneath the sectioned vagosympathetic nerve trunk at the mid-cervical level and the nerve covered with mineral oil. Electrical stimuli were derived from a Grass S88 stimulator and isolation unit (SIU5). Stimulation parameters were maximal (8–10 V; 2 ms duration; 10 s trains) and presented at approximately 2 min intervals. After consistent control responses were established, hexamethonium was continuously infused at 1 mg/kg in order to block nicotinic transmission. The residual transmission (muscarinic) was about 20–25% of the maximal responses prior to hexamethonium infusion.

Tonic muscarinic activation of the pupil was measured under the general ambient lighting conditions of the laboratory using a clear mm ruler (estimated to the nearest $\frac{1}{4}$ mm). As the cervical sympathetic nerve trunk was sectioned, the basal pupil size was inversely correlated to muscarinic parasympathetic neuronal input to the iris sphincter muscle. Under these conditions, the basal pupil diameter ranged from 0.5–2.0 mm.

2.3. Experimental protocols

In the first series of experiments, an attempt was made to characterize the primary muscarinic receptor subtypes involved in the physiological activation of the five effector systems. Responses were elicited as detailed above with subsequent observation of effects of i.v. administration of saline or cumulative doses of one of the three muscarinic antagonists given at 15 min intervals. When possible, several effector responses were measured in a single animal. In some experiments, atropine was administered if there was no significant response to gallamine or pirenzepine, however, no more than two such preparations were included in any experimental group.

The second series of experiments were directed towards determination of the effect of inhibition of nitric oxide synthesis on each of the five characterized muscarinic responses. Following a 15 min control period, a single i.v. injection of L-NAME (50 mg/kg) was made over a 5 min period and the evoked responses followed for an additional 60 min. In one additional group of animals, concerning methacholine-induced vasodilation, L-NA (N^G-nitro-L-arginine; 25 mg/kg) was administered instead of L-NAME. All responses were compared with matched saline controls.

2.4. Drugs used and statistics

The drug doses refer to the respective salts which were dissolved in sterile saline. The agents used and their sources were as follows: hexamethonium bromide and methacholine chloride were purchased from Sigma (St. Louis, MO, USA), atropine sulfate, pirenzepine hydrochlo-

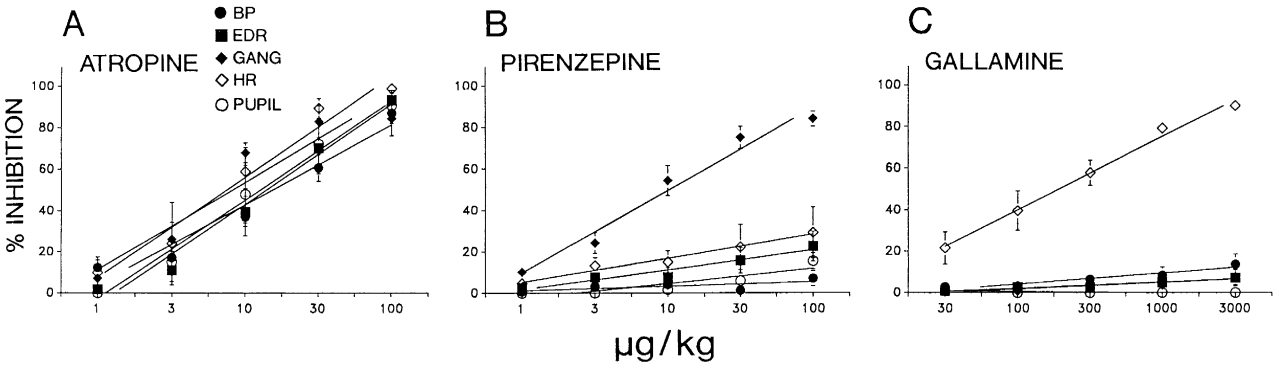

Fig. 1. Characterization of muscarinic receptor subtypes on five peripheral effectors in anesthetized cats. BP represents vascular hypotensive effect of i.v. methacholine (3 μg/kg); EDR indicates preganglionic activation of electrodermal (sudomotor) responses; GANG represents muscarinic transmission to nictitating membrane via superior cervical ganglion; HR indicates vagal-evoked bradycardia; PUPIL represents basal pupil size. Note that atropine is non-selective, pirenzepine significantly antagonized only ganglionic transmission (M_1) and that gallamine produced significant blocking action only against the vagal bradycardia (M_2). Values represent means ± S.E.M.

ride and gallamine triethiodide were purchased from Research Biochemicals International (Natick, MA, USA) as were L-NAME (N^G-nitro-L-arginine methyl ester) and L-NA (N^G-nitro-L-arginine).

Statistical evaluation of the muscarinic antagonist dose–response curves was by analysis of variance (ANOVA) with repeated measures with $P < 0.05$ testing for significance. Statistical differences ($P < 0.05$) between L-NAME (or L-NA) and saline treated animals were determined using one-way ANOVA and Dunnett's *t*-test.

3. Results

3.1. Muscarinic receptor subtype characterization

As shown in Fig. 1A, intravenous administration of atropine produced an equivalent antagonism of all muscarinic receptor mediated responses in the relatively narrow dose range of 3–30 μg/kg. There were no significant differences between the five effector organs studied with the ED_{50}s for atropine ranging between 6.0 ± 2.4 μg/kg

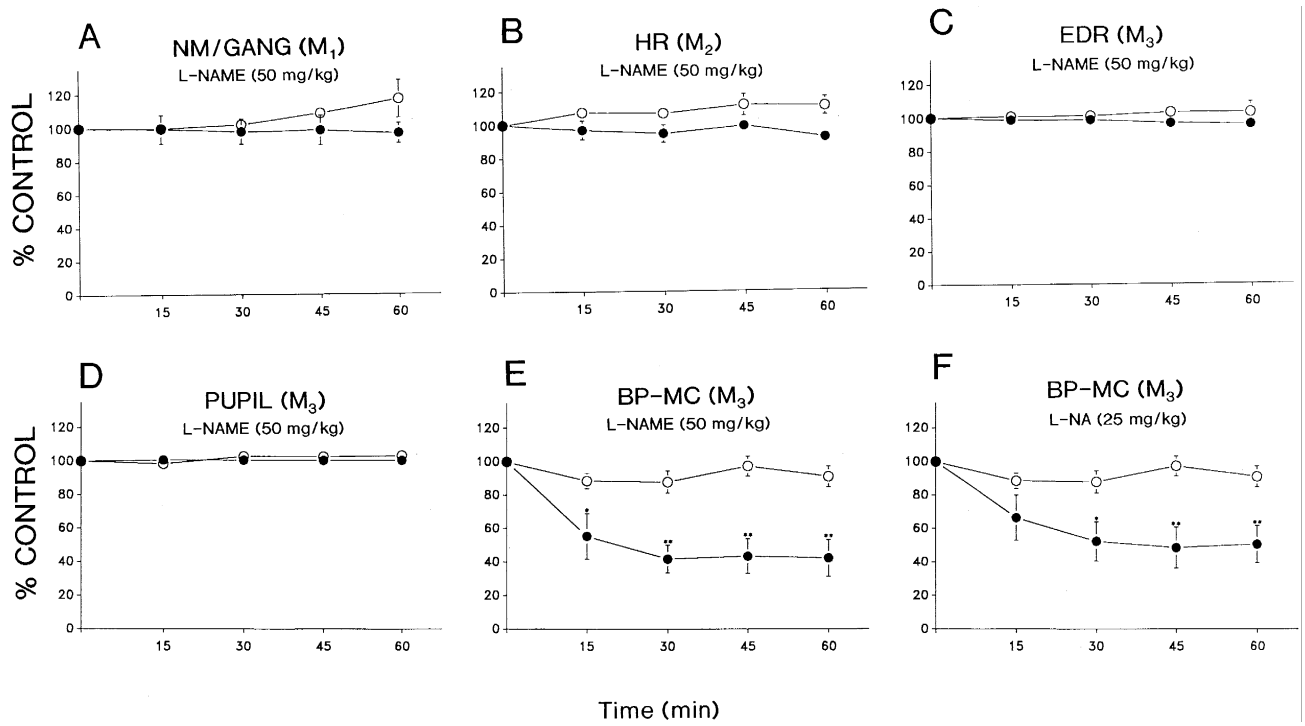

Fig. 2. Effect of saline or nitric oxide synthase inhibition with L-NAME on muscarinic responses as in Fig. 1. Open circles represent responses to saline; solid circles represent responses to either L-NAME (50 mg/kg, i.v.; panels A–E) or to L-NA (25 mg/kg, i.v.; panel F). Saline or inhibitors were administered at time zero with responses followed for 60 min. Note that L-NAME had no effect on non-endothelial muscarinic activation (A–D). In contrast, L-NAME prevented methacholine-induced vasodilation (BP-MC) as did L-NA. Values represent means ± S.E.M. for 5–6 cats per group. * $P < 0.05$ ** $P < 0.01$.

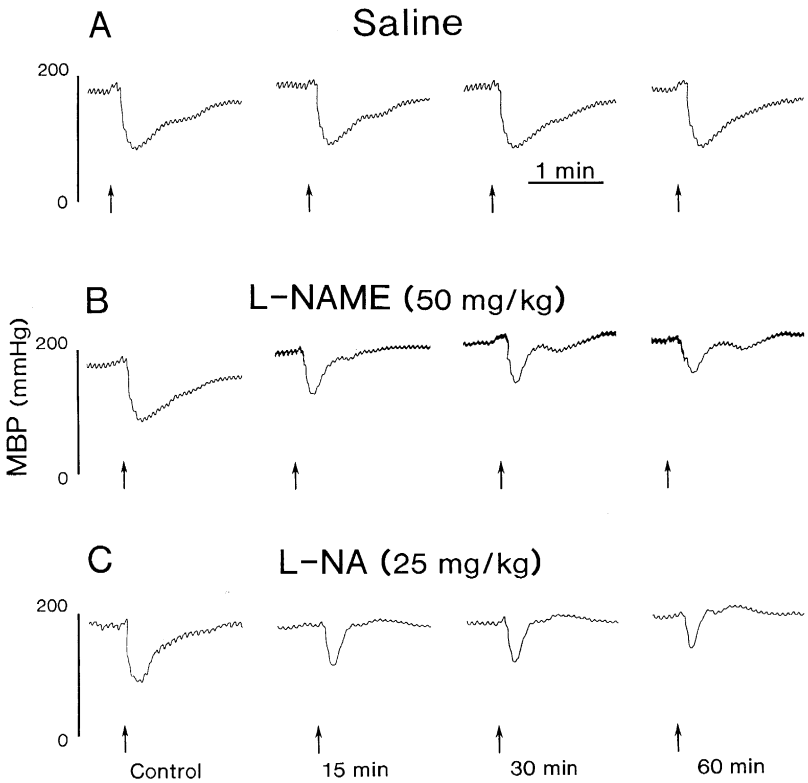

Fig. 3. Effect of intravenous administration of saline, L-NAME (50 mg/kg), or L-NA (25 mg/kg) on methacholine-induced systemic hypotension in three anesthetized cats. Methacholine (3 μg/kg, i.v.) was administered at 15 min intervals (arrows). Panel A represents control saline responses. Panels B and C show effects of L-NAME and L-NA, respectively (given immediately after the first methacholine-induced hypotensive response in each example). Note inhibition of methacholine-induced hypotensive responses after inhibition of nitric oxide synthase.

for methacholine-induced hypotension to 12.4 ± 2.8 μg/kg for sudomotor activation. In contrast (at doses up to 100 μg/kg) the muscarinic M_1 receptor antagonist, pirenzepine, produced significant antagonism only of the sympathetic ganglionic evoked responses (Fig. 1B); the muscarinic M_2 receptor antagonist, gallamine, demonstrated a high degree of selectivity for the vagal bradycardia (Fig. 1C).

3.2. Effects of L-NAME and L-NA

Following receptor characterization, we utilized separate groups of cats to determine the extent to which L-NAME might antagonize muscarinic receptors in addition to inhibition of nitric oxide synthesis. There was no significant alteration of response amplitude of the five effector organs studied over the 60 min control period in response to i.v. saline (Fig. 2). Fig. 2A–D illustrate that i.v. administration of L-NAME (50 mg/kg) did not diminish the amplitude of evoked muscarinic responses including those involved with sympathetic ganglionic transmission (M_1), vagal bradycardia (M_2), sudomotor responses (M_3) or basal pupillary constriction (M_3). In contrast, both L-NAME (50 mg/kg) and L-NA (25 mg/kg) selectively inhibited methacholine-induced systemic vasodilator responses (Fig. 2E and F). Fig. 3 illustrates the effects of i.v. saline, L-NAME and L-NA on methacholine-induced systemic hypotension in three different preparations.

Both L-NAME (50 mg/kg) and L-NA (25 mg/kg) caused a sustained elevation of systemic arterial blood pressure. The basal mean systemic blood pressure was 134 ± 7 mm Hg before and 170 ± 28 mm Hg 60 min after i.v. administration of L-NAME ($P < 0.01$; $n = 22$). In the five animals given L-NA, the mean blood pressure rose from 151 ± 6 mm Hg to 168 ± 7 mm Hg after 60 min ($P < 0.05$).

4. Discussion

During the last decade there has been greatly expanded understanding of the heterogeneity of muscarinic receptor subtypes. Molecular biological and in vitro binding studies have defined five distinct muscarinic receptor subtypes, three of which (M_1, M_2 or M_3) can be clearly demonstrated using both functional and binding techniques in the peripheral nervous system (Hulme et al., 1990; Caulfield, 1993; Eglen et al., 1994). Although there are many studies utilizing in vitro techniques, there are only a few comprehensive studies using in vivo preparations (Caulfield, 1993). In the present study we used putatively selective muscarinic M_1 and M_2 receptor antagonists (pirenzepine

and gallamine), in comparison with atropine, to define the predominant muscarinic receptors involved with the activation of five muscarinic cholinergic systems in anesthetized cats.

Muscarinic transmission through the cat superior cervical ganglion fits the muscarinic M_1 receptor profile (rank order of potency: atropine = pirenzepine ≫ gallamine) and is consistent with observations by others in cats (Koss and Rieger, 1976; Bachoo and Polosa, 1992). Similarly, vagal bradycardia fits the muscarinic M_2 receptor profile (rank order of potency: atropine > gallamine = pirenzepine) which is consistent with the conclusions of others that vagal slowing of the heart rate is mediated almost exclusively by the activation of muscarinic M_2 receptors (Caulfield, 1993).

The remaining three systems studied appear to involve mainly muscarinic M_3 receptor activation as they were antagonized by atropine but not by pirenzepine or gallamine. Muscarinic M_3 receptor designation for the pupillary sphincter is consistent with previous reports in primates including man (Gabelt and Kaufman, 1992; Woldemussie et al., 1993). Other investigators have shown in rats that neural activation of the footpad sweat glands is mediated primarily by muscarinic M_3 receptors (Schiavone and Brambilla, 1991). Finally, the conclusion that methacholine-induced vasodilation is mainly due to muscarinic M_3 receptor activation is in agreement with previous studies using perfused rat mesenteric and renal vascular beds (Hendriks et al., 1992; Eltze et al., 1993) as well as studies of human forearm blood flow (Bruning et al., 1994).

L-NA (N^G-nitro-L-arginine) and its more soluble congener, L-NAME (N^G-nitro-L-arginine methyl ester), are among the most commonly used inhibitors of nitric oxide synthase. The report that L-NAME and other alkyl ester nitric oxide synthase inhibitors are muscarinic antagonists has complicated interpretation of data obtained using these agents. For example, Buxton et al. (1993) showed, using receptor binding techniques, that L-NAME is a competitive antagonist at muscarinic M_1, M_2 or M_3 receptors of peripheral and central nervous system tissues. In addition, acetylcholine constriction of denuded rabbit coronary or isolated canine colonic smooth muscle was blocked by L-NAME but not by the non-alkyl ester substituted N^G-monomethyl-L-arginine. In support of this conclusion, others have shown that although both L-NAME and L-NA inhibited vasodilation produced by acetylcholine in the rat diaphragm, only the effect of L-NA was reversed with L-arginine (Chang et al., 1997). As before, the conclusion from these results is that L-NAME, but not L-NA, is a muscarinic receptor antagonist in addition to having the ability to inhibit nitric oxide synthase.

In contrast, other investigators have presented evidence that L-NAME does not block muscarinic receptors. For example, Buccafusco et al. (1995) found L-NAME to displace [^{3}H] methylscopolamine from spinal cord membranes only at very high concentrations (above 1 mM), whereas atropine exhibited an IC_{50} of 1.5 nM. Cheng et al. (1994) reported that L-NAME and L-NA exhibited a similar ability to inhibit acetylcholine vasodilation in the cat pulmonary circulation. Santiago et al. (1994) also observed similar inhibition by both L-NAME and L-NA of endothelium-dependent vasodilator responses in the cat mesenteric vasculature. This comparability of the magnitude of effect would not be expected if L-NAME also possessed significant muscarinic receptor blocking activity. L-NAME also was lacking in action to block electrically evoked vagal bradycardia (Cheng et al., 1994) and it did not inhibit carbachol induced contractions of the isolated rabbit iris sphincter muscle (Chuman et al., 1996), both muscarinic receptor mediated responses.

The present results showing that L-NAME did not depress responses due to activation of non-endothelial muscarinic M_1, M_2 or M_3 receptors is added support for the conclusion of others that L-NAME is not a muscarinic blocking agent in vivo. The difference between results obtained from in vivo studies when compared to those undertaken in vitro might be due to higher sustained agonist concentrations reached in vitro or to the fact that L-NAME is readily converted to L-NA in vivo (Schwarzacher and Raberger, 1992).

In this study, both L-NAME and L-NA significantly depressed methacholine-induced hypotension, an effect shown to be mediated by the activation of vascular muscarinic M_3 receptors (Hendriks et al., 1992; Eltze et al., 1993). Using isolated arterial rings, methacholine relaxation responses are attenuated by removal of the endothelium as well as by treatment with the nitric oxide synthase inhibitor, L-NA (Monge et al., 1993; Pratt et al., 1996). Taken together, these studies suggest that methacholine-induced hypotension is likely mediated by the release of nitric oxide from endothelial cells as has clearly been demonstrated for acetylcholine (Rees et al., 1990; White et al., 1993; Cheng et al., 1994). In this regard, it is of interest that, in humans, the forearm vasodilator response to acetylcholine, but not that due to methacholine, is selectively attenuated by nitric oxide synthase inhibition with N^G-monomethyl-L-arginine (Chowienczyk et al., 1993; Rongen et al., 1993). These investigators proposed that methacholine-induced forearm vasodilation, in humans, is mediated by mechanisms other than those involving formation of nitric oxide. These results contrast with those of the present study, using systemic arterial blood pressure in cats, where methacholine-induced vasodilation clearly was antagonized by both L-NAME and L-NA.

In conclusion, we utilized pirenzepine, gallamine and atropine to characterize the primary muscarinic receptor subtypes involved in the activation of five peripheral cholinergic systems in vivo in anesthetized cats. The observed lack of depression of any of the non-vascular muscarinic receptor activated systems by L-NAME is strong evidence that L-NAME is not a generalized blocker of

muscarinic receptors as previously suggested from binding and in vitro studies. Thus, we suggest that L-NAME is sufficiently selective as a nitric oxide synthase inhibitor to continue to be a useful tool in experiments concerning nitric oxide synthase inhibition of peripheral cholinergic systems in vivo.

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