**N⁴-G-Nitro L-Arginine Methyl Ester and Other Alkyl Esters of Arginine are Muscarinic Receptor Antagonists**

Iain L. O. Buxton, Dennis J. Cheek, Delrae Eckman, David P. Westfall, Kenton M. Sanders, and Kathleen D. Keef

Analogues of L-arginine with modifications at the terminal guanidino nitrogen and/or the carboxyl terminus of the molecule have been widely used for their ability to inhibit the production of nitric oxide and are thought to be competitive antagonists of nitric oxide synthase. The present studies were designed to test the possibility that these agents are also muscarinic receptor antagonists. Acetylcholine produced concentration-dependent contraction of endothelium-denuded rabbit coronary artery as well as isolated strips of canine colonic smooth muscle. The arginine analogue N⁴-g-nitro L-arginine methyl ester (L-NAME, 100 μM) but not N⁴-monomethyl L-arginine (L-NMMA, 100 μM) significantly shifted these contractile relations to the right, an effect that was not reversed by addition of 1 mM L-arginine. In radioligand binding studies using the muscarinic radioligand [³H]quinuclidinyl benzilate and tissues known to contain differing contributions of M1, M2, and M3 muscarinic receptors, addition of increasing concentrations of L-NAME resulted in a monophasic competition of binding with affinities (Kᵯ) ranging from 68 μM in endothelium to 317 μM in whole aorta. Addition of the hydrolysis-resistant guanosine 5'-triphosphate analogue GTPγS (100 μM) had no effect on L-NAME competition of [³H]quinuclidinyl benzilate binding. Addition of L-NAME in radioligand binding competition studies using the agonist carbachol did not result in an alteration of the receptor’s affinity for agonist, confirming the competitive nature of the interaction of L-NAME with the muscarinic receptor. Several L-arginine analogues with alkyl ester modifications at the carboxyl end of the molecule as well as those without this modification were evaluated as muscarinic antagonists in radioligand binding experiments. Only those arginine compounds with a modified carboxyl group were able to compete for radioligand binding to the muscarinic receptor. Our results indicate that alkyl esters of L-arginine are muscarinic antagonists and suggest that these compounds are poor choices as nitric oxide synthase inhibitors in studies in which muscarinic receptors are not blocked. (Circulation Research 1993;72:387–395)

**KEY WORDS** • nitric oxide • nitric oxide synthase inhibitors • N⁴-g-nitro L-arginine methyl ester • muscarinic receptors • smooth muscle • radioligand binding

In 1988, Palmer et al¹ demonstrated that L-arginine was the precursor for nitric oxide (NO) synthesis in endothelial cells. Since that time, significant interest has been focused on the study of the synthesis, release, and subsequent actions of NO (for review see Moncada et al²). The effects of NO that have been studied to date include relaxation of smooth muscles. In blood vessels, NO is known to dilate vascular smooth muscle³ and may contribute to the moment-to-moment maintenance of blood vessel tone. In the gastrointestinal tract, NO is thought to be a nonadrenergic noncholinergic neurotransmitter responsible for inhibition of colonic motility.⁴ NO is also thought to be released by macrophages and may play a role in signaling in the immune system.⁵

Numerous congeners of arginine that block the action of nitric oxide synthetase (NOS) in several tissues including endothelium, nerve, and smooth muscle have been described.⁶ The simplest of these compounds, N⁴-g-nitro L-arginine (L-NA), is an inhibitor of NOS both in vivo and in vitro⁷ and is widely used as its methyl ester analogue N⁴-g-nitro L-arginine methyl ester (L-NAME), which is more lipophilic and therefore useful for experiments in vivo.⁸ In studies designed to elucidate the role of NO in the gastrointestinal tract, an inhibitory effect of L-NAME on cholinergic neural responses was sometimes observed. This inhibitory effect would be consistent with an action of L-NAME at the muscarinic receptor. Indeed, although differences in the actions of NOS inhibitors such as L-NAME in vivo have been attributed to differences in metabolism and tissue distribution,⁹,¹⁰ we have hypothesized that some of these effects could be due to muscarinic receptor blockade. Thus, we report here for the first time the ability of L-NAME, L-arginine methyl ester (L-AME), and other L-arginine alkyl esters to act as muscarinic antagonists.

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The affinity of muscarinic receptors for these compounds suggests that experiments using them may yield both muscarinic receptor antagonism and inhibition of NOS activity in cells, tissues, or whole animals.

Materials and Methods

Tissue Preparation

Mongrel dogs or guinea pigs of either sex were anesthetized with pentobarbital sodium (30 mg/kg). In dogs, a segment of proximal colon (6–14 cm from the ileocecal sphincter), the submandibular glands, right and left atria, coronary vessels, and aorta (15–20 cm including the aortic arch) were removed and kept in oxygenated (97% O₂, 3% CO₂) Krebs-Ringer bicarbonate solution (KR) containing (mM) Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5, Cl⁻ 134, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, and dextrose 11.5. In guinea pigs, the brain was removed and kept on ice in the same solution. Atria, atria, blood vessels, and submandibular gland were trimmed to remove adherent fat or connective tissue and minced in KR to remove blood cells. In aorta from three dogs, the vessels were opened longitudinally, and the endothelium was removed by scraping and collected by centrifugation. After washing out fecal material, the colon was cut into 2×3-cm pieces and pinned out in a dissecting dish bathed with fresh KR. The longitudinal smooth muscle was carefully removed, and the circular smooth muscle was separated from the submucosa by the method of Smith et al.⁷

Contractile Experiments

Male rabbits (2–3 kg) were anesthetized with an overdose of pentobarbital sodium (30 g/kg) injected into the ear vein. The heart was removed and placed in cold (10°C) oxygenated KR. A 1–1.5-cm segment of the left ventricular coronary artery was carefully dissected, cut into ring segments (3 mm long), and denuded of endothelium by gentle rubbing with a stainless-steel pin. Denuded segments were mounted on two triangular tungsten supports (89-μm diameter). The upper triangle was attached to a Gould strain gauge, and the lower was attached to a stable mount. A resting force of 0.6 g was applied to vessel segments in a tissue bath maintained at 37°C filled with oxygenated KR (pH 7.4). Tissues were equilibrated for at least 1 hour and contracted at 10–15-minute intervals with 60 mM KCl before beginning the experiments. The absence of endothelium was assessed during the experiment by measuring the response to acetylcholine (ACh, 0.1–10 μM) in segments contracted with the histamine H₁-receptor agonist 2-(2-aminoethyl)pyridine.⁸ In endothelium-intact vessel, ACh (10 μM) produced near maximal relaxation, whereas in denuded segments, ACh produced contraction (see Keef et al.⁹)

Colonic smooth muscle strips (10 mm×1 mm) were made by cutting across the entire muscularis parallel to the circular muscle fibers. Muscles were then attached with suture to a stable mount and to a Gould strain gauge and immersed in a tissue bath containing KR maintained at 37°C. A resting tension of 1 g was applied, and tissues were equilibrated for 1 hour, during which time the CR was exchanged every 15 minutes. Contractile experiments in canine colon were performed in the presence of 1 μM tetrodotoxin to reduce interference from enteric nerves. Before the start of the experiments, tissues were repeatedly contracted with 1 μM ACh, followed by 10-minute rest periods until a stable amplitude of contraction was obtained. Peak contractile tension was then determined in the presence of 100 μM ACh. Tissues were then reequilibrated with repeated applications of 1 μM ACh as before. The response to cumulative additions of ACh was then measured in the absence or presence of a 100-μM concentration of the l-arginine analogues L-NAME or N⁶0-monomethyl l-arginine (L-NMA). Responses from each tissue strip were normalized to the peak ACh-induced contraction obtained with the first addition of 100 μM ACh.

Membranes Preparation

Membranes from all tissues were prepared in a similar fashion; tissues were washed three times with ice-cold hypotonic buffer A containing (mM) Tris base 50, MgCl₂ 10, and EGTA 1, pH 7.4, and homogenized in 10 vol of the same ice-cold buffer using a Polytron (three times for 15 seconds at setting 8). The homogenate was filtered through a nylon cloth (200 μm) and centrifuged at 1,000g for 10 minutes (4°C) to remove unwanted material. The resulting pellet was discarded, and the supernatant was centrifuged at 80,000g for 60 minutes at 4°C. The resulting pellet containing plasma membrane was quickly frozen in liquid nitrogen and stored as a frozen powder at −80°C for 1–2 weeks until used in radioligand binding studies. Membrane pellets from three animals were pooled.

Radioligand Binding Studies

Binding of the nonselective muscarinic receptor antagonist [³H]quinuclidinyl benzilate ([³H]QNB) was measured by a rapid filtration method similar to that described previously.¹⁰ Membrane pellets were resuspended in buffer A to yield approximately 0.4 mg/ml protein as determined by the method of Bradford.¹¹ Radioligand binding assays were carried out in a volume of 500 μL consisting of 440 μL membrane suspension, 50 μL radioligand, and 10 μL buffer or drug. Saturation binding experiments used concentrations of [³H]QNB from 20 to 5,000 pM, and nonspecific binding was determined by the addition of 1 μM atropine. Equilibrium binding, carried out at 30°C for 75 minutes, was stable for 120 minutes and completely reversible. Bound and free radioligand were separated by rapid vacuum filtration over GF/C filters (Whatman Inc., Clifton, N.J.) that had been pretreated with 0.3% polyethyleneimine as described by Bruns et al.¹² The filters were washed with two 5-ml aliquots of ice-cold buffer containing (mM) Tris-HCl 5, MgCl₂ 1, and EGTA 0.1 and counted at 48% efficiency in a liquid scintillation counter (model LS 6000IC, Beckman Instruments, Inc., Fullerton, Calif.). All determinations were made in triplicate, and specific binding of [³H]QNB at 0.3 nM was examined in competition studies using increasing concentrations of unlabeled agonists and antagonists.

Data Analysis

Saturation and competition binding curves were analyzed using the computer program GRAPHPAD INSLIT (version 3.0, GraphPad Software, San Diego, Calif.). This program uses nonlinear least-squares regression to fit binding data to equations that adhere to the laws of
mass action. For saturation binding data, nonlinear curve fitting was used to generate both the $K_d$ and $B_{max}$ values in preference to the linear transform. Both methods yield similar results. For competition binding data, the nonlinear least-squares approach fits the data to either one or two classes of binding sites and assists in determining if the two-site model is significantly better than the one-site fit of the data ($F$ test). The affinity of receptors for $l$-arginine analogues in competition for radioligand binding ($K_d$) was determined by the method of Cheng and Prusoff. Data are expressed as the mean±SEM. Statistical significance was determined by Student’s $t$ test, where a value of $p<0.05$ was considered significant.

**Drugs**

$ACH$, the stable muscarinic agonist carbachol, and the muscarinic antagonist atropine, as well as GTP analogue GTP-γS, EGTA, Tris base, and the putative NOS inhibitors L-NAME, L-NMMA, Na-benzoyl $l$-arginine ethyl ester (BAEE), L-AME, $l$-arginine ethyl ester (L-AEE), and L-NA were purchased from Sigma Chemical Co., St. Louis, Mo. [3H]QNB (45.7 Ci/mmol) was obtained from Dupont-NEN, Boston. All other materials were obtained from standard sources. Arginine analogues (except L-NA) were prepared fresh immediately before use by dissolution in buffer containing (mM) Tris-HCl 5, MgCl$_2$ 1, and EGTA 0.1, pH 7.4, with sonication. L-NA was dissolved at a stock concentration of 100 mM in 0.05N NaOH in water and immediately diluted to the desired concentration using the same Tris/MgCl$_2$/EGTA buffer shown above. Solutions of L-NAME, L-NMMA, L-AME, and L-AEE were significantly less effective or ineffective as muscarinic antagonists if stored frozen at $-20^\circ$C and reused.

**Results**

Concentration–response relations for $ACH$-induced contraction in both rabbit coronary artery and canine colonic smooth muscle were determined by cumulative addition of $ACH$ (0.01–100 $\mu$M). The concentration of $ACH$ that produced half-maximal contraction in coronary rings (Figure 1B) was 1.5 $\mu$M (log $ED_{50}=-5.81±0.14$), whereas in colonic strips 2.1 $\mu$M $ACH$ (log $ED_{50}=-5.67±0.10$) was required to produce half-maximal contraction (Figure 1D).

To determine whether the contractile responses to $ACH$ in coronary artery and colon were modified by arginine analogues, cumulative additions of $ACH$ were also obtained after preexposure of tissues to either L-NAME or L-NMMA. L-NAME (100 $\mu$M), but not L-NMMA (100 $\mu$M), produced a significant shift to the right of the concentration–response relation for $ACH$ in both coronary artery (log $EC_{50}=-4.42±0.31$, Figure 1B) and colon (log $EC_{50}=-5.15±0.12$, Figure 1D). Examples of the effect of 100 $\mu$M L-NAME on contractions produced by 1 $\mu$M $ACH$ in coronary artery and colon are shown in Figures 1A and 1C, respectively.

**FIGURE 1. Effects of $N^\text{G}$-nitro $l$-arginine methyl ester (L-NAME) and $N^\text{G}$-monomethyl $l$-arginine (L-NMMA) on acetylcholine (ACH)–induced contractions of the rabbit coronary artery and the canine proximal colon.** Panels A and B show the effects of arginine analogues in the rabbit coronary artery denuded of endothelium. Panel A: Tracings showing concentration–response relation for $ACH$ in the absence (●) of arginine analogue or in the presence of 100 $\mu$M L-NAME (○) or 100 $\mu$M L-NMMA (□). Values are mean±SEM of experiments performed in five to 13 animals. Panels C and D show the effects of arginine analogues in the canine colon. Panel C: Tracings showing examples of the contractile response obtained on addition of 1 $\mu$M $ACH$ in the presence and absence of 100 $\mu$M L-NAME. Panel D: Graph showing concentration–response relation for $ACH$ in the absence (●) of arginine analogue or in the presence of 100 $\mu$M L-NAME (○) or 100 $\mu$M L-NMMA (□). Values are mean±SEM of experiments performed in five to eight animals. *Significantly different from control ($p<0.05$).
several experiments cumulative additions of ACh were repeated once more in the presence of both L-NNAME and L-arginine (1 μM). There was no significant difference in the response to ACh plus L-NNAME in the presence or absence of L-arginine in either tissue type (n = 3; data not shown).

The effect of L-NNAME to lower the potency of ACh in contractile studies taken together with the general structural similarity of ACh and the arginine analogues that inhibit NOS (Figure 2) suggested to us that these nitroarginine analogues might bind to muscarinic receptors. Since the results in both rabbit coronary artery and colon suggested that L-NNAME was interacting with a muscarinic receptor of the M2 or M3 subtype, it was unclear whether the effect of L-NNAME might be receptor-subtype specific. To test this possibility, we prepared membranes from different tissues representative of each of the muscarinic receptor subtypes (M1, M2, and M3) well known to be expressed in mammalian tissues. Saturation isotherms were performed as we have described elsewhere to determine the affinity and density of muscarinic receptors in several tissues for the muscarinic antagonist radioligand [3H]QNB (Table 1). As demonstrated by the work of Doods et al., canine atria were used as a source of M2 receptors, submandibular gland was used as a source of M3 receptors, and whole brain was used as a source of all three receptor types. Our own data confirm the presence of M2 and M3 receptors in colonic smooth muscle. Choices of tissues representing each of the muscarinic receptor subtypes thought to exist in the cardiovascular system and elsewhere were made to ensure the general applicability of our data. Direct assessment of the effects of L-NNAME was determined both in blood vessels and endothelial cells.

Addition of increasing concentrations of L-NNAME in equilibrium binding–competition experiments using membranes from each of the five tissue sources and the nonspecific radioligand [3H]QNB (0.3 nM) resulted in a complete monophasic competition of binding in each case, with affinities of the receptor for L-NNAME that varied from 65 μM in colonic smooth muscle to 235 μM in canine submandibular gland (Figure 3, Table 2). In whole canine aorta, competition of [3H]QNB binding by L-NNAME (Figure 3) resulted in a mean inhibitory affinity (Ki) of 317±68 μM (n = 6), whereas in endothelium scraped from aorta, L-NNAME competition of [3H]QNB binding yielded a Ki of 86±51 μM (n = 3). Similar experiments undertaken in canine coronary arteries (n = 17) also demonstrated that L-NNAME competed for specific [3H]QNB binding, although the binding in the absence of L-NNAME was unimpressive (11.4 ± 1.0 fmol/mg at 450 pM [3H]QNB). The affinity of muscarinic receptors for L-NNAME determined in several tissues agrees with the ability of L-NNAME to interfere with agonist-induced contraction when added to the contractile experiments at a concentration of 100 μM (Table 2). Addition of the hydrolysis-resistant guanine nucleotide GTPγS (100 μM) did not shift the competition curve for L-NNAME, suggesting that L-NNAME

![Figure 2. Structures of acetylcholine (ACh), arginine (ARG), and ARG analogues used as inhibitors of nitric oxide synthase. L-NMMA, N6-monomethyl L-arginine; L-NA, N⁴-nitro L-arginine; L-AME, L-arginine methyl ester; L-AEE, L-arginine ethyl ester; L-NAME, N⁴-nitro L-arginine methyl ester; BAEE, Nα-benzoyl L-arginine ethyl ester.](http://circres.ahajournals.org/)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>K\textsubscript{s} (pM)</th>
<th>B\textsubscript{max} (fmol/mg)</th>
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</thead>
<tbody>
<tr>
<td>Heart (atria)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>189±22</td>
<td>156±45</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2+M3</td>
<td>123±18</td>
<td>134±24</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>263±39</td>
<td>277±33</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1+M2+M3</td>
<td>205±56</td>
<td>190±41</td>
</tr>
</tbody>
</table>

Values are mean±SEM of three to five experiments.

Specific binding of [3H]quinuclidinyl benzilate (5–5,000 pM) to membranes prepared from each of the tissues shown was determined by filtration as described in the text. K\textsubscript{s} and B\textsubscript{max} values were calculated from nonlinear fit of the saturation binding data.
acted in a fashion consistent with a muscarinic antagonist (Table 3).

To examine the prediction that L-NAME interacted with muscarinic receptors in a manner consistent with competition for the agonist binding site, we measured the ability of the muscarinic agonist carbachol to compete for [3H]QNB binding in the presence and absence of L-NAME in colonic smooth muscle (Figure 4, left

**FIGURE 3.** Graphs showing Nω-nitro-L-arginine methyl ester (L-NAME) competition of [3H]quinuclidinyl benzilate binding in tissues containing different subtypes of muscarinic receptors (M1, M2, and M3). Membranes were prepared from different tissues as described in “Materials and Methods.” Binding of [3H]quinuclidinyl benzilate (=0.3 nM) was measured in the absence (100%) and in the presence of increasing concentrations of L-NAME. Nonspecific binding was calculated by assessing binding in the presence of 1 μM atropine. Values for L-NAME competition were determined in triplicate and normalized to binding in the presence of atropine (0%) and absence of competitor (100%). Curves are computer-generated best fit assuming a single class of binding sites determined in a single representative experiment performed in triplicate. Values for $K_I$ were calculated by the method of Cheng and Prusoff (as described in the text). Data for several experiments are presented in Table 2.

**TABLE 2. Effects of L-Arginine Analogues in Radioligand Binding Competition Studies With Muscarinic Receptors**

<table>
<thead>
<tr>
<th>L-ARG analogue</th>
<th>Heart (atria) (M2)</th>
<th>Colon (M2+M3)</th>
<th>Submandibular gland (M3)</th>
<th>Brain (M1+M2+M3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NAME</td>
<td>123±22</td>
<td>68±18</td>
<td>204±57</td>
<td>108±19</td>
</tr>
<tr>
<td>L-AME</td>
<td>95±34</td>
<td>105±42</td>
<td>185±50</td>
<td>82±38</td>
</tr>
<tr>
<td>L-AEE</td>
<td>189±43</td>
<td>204±56</td>
<td>167±55</td>
<td>320±87</td>
</tr>
<tr>
<td>BAEE</td>
<td>255±62</td>
<td>305±89</td>
<td>313±41</td>
<td>430±120</td>
</tr>
<tr>
<td>Nonesterified L-ARGs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-ARG</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>L-NA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

L-ARG, L-arginine; L-NAME, Nω-nitro-L-arginine methyl ester; L-AME, L-arginine methyl ester; L-AEE, L-arginine ethyl ester; BAEE, Nω-benzoyl-L-arginine ethyl ester; L-NA, Nω-nitro-L-arginine; L-NMMA, Nω-monomethyl L-arginine; NC, no competition. Values for alkyl esters of L-ARG are mean±SEM in three to five experiments performed in triplicate. Experiments using nonesterified arginines (repeated twice) did not yield any significant competition (NC) of [3H]quinuclidinyl benzilate binding at any concentration tested (see Figure 5, right panel).

The affinity of muscarinic receptors for the arginine analogues tested ($K_I$) in each of the four tissues was determined in radioligand binding–competition studies as described in “Materials and Methods.” All data were best fit assuming a single class of binding sites, and affinities entered for calculation of $K_I$ were those determined independently, as described in Table 1.
TABLE 3. Agonist Competition of \(^{3}H\)Quinuclidinyl Benzilate Binding to Muscarinic Receptors in Heart and Colon: Effects of GTP Analogue GTP\(_y\)S in the Absence and Presence of 100 \(\mu M\) \(N\)-Nitro-\(L\)-Arginine Methyl Ester

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-NAME (100 (\mu M))</th>
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<tbody>
<tr>
<td></td>
<td>(K_h) high (nM)</td>
<td>R high (%)</td>
</tr>
<tr>
<td>Atria</td>
<td>37.3±3.6</td>
<td>36.6</td>
</tr>
<tr>
<td>+GTP(_y)S</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Colon</td>
<td>0.82±0.13</td>
<td>45.5</td>
</tr>
<tr>
<td>+GTP(_y)S</td>
<td>1.85±0.42</td>
<td>25.2</td>
</tr>
</tbody>
</table>

L-NAME, \(N\)-nitro \(L\)-arginine methyl ester; GTP\(_y\)S, hydrolysis-resistant guanine nucleotide; NA, not applicable. Values are mean±SEM of three experiments performed in triplicate and were determined by computer-generated best fit assuming one or two affinity states. Data from colon were best fit assuming two states of the receptor in the absence and in the presence of GTP\(_y\)S, reflecting distinct affinities of M2 and M3 receptors for the agonist (see Reference 10).

The affinity of atrial and colonic muscarinic receptors for carbachol and proportions of high and low affinity sites (K and R) in competition for \(^{3}H\)quinuclidinyl benzilate binding, in membranes prepared as described in the “Materials and Methods,” were determined in the absence or presence of 100 \(\mu M\) GTP\(_y\)S with (+) and without (−) 100 \(\mu M\) L-NAME present during the incubation.

In the absence of L-NAME, carbachol interacted with both high- and low-affinity sites as expected.\(^{10}\) In the presence of 100 \(\mu M\) L-NAME, however, 30% of sites were occupied by L-NAME, and carbachol was still able to compete for binding in a biphasic manner to a decreased number of binding sites (Figure 4, right panel; Table 3). Addition of 100 \(\mu M\) GTP\(_y\)S shifted the agonist competition curve to the right, suggesting that L-NAME did not interfere with receptor:GTP-binding protein interaction.

To determine the structural component responsible for the ability of L-NAME to compete for binding to muscarinic receptors, we tested other arginine-derived NOS inhibitors in heart, submandibular gland, colon, and brain (Figure 5, Table 2). Nitroarginine compounds such as L-NAME, L-NAME, and \(N\)-\(L\)-arginine (L-NAAA; data not shown) did not compete for \(^{3}H\)QNB binding in any tissue tested. Compounds with an alkyl or aryl ester such as L-NAME, L-NAME, L-NAME, and BAEE, on the other hand, were all able to compete for \(^{3}H\)QNB binding in all tissues (Table 2). Arginine itself was not able to compete for binding of radioligand to muscarinic receptors in any of the tissues tested.

Discussion

The results presented in this article demonstrate for the first time that the widely used amino acid analogues...
of arginine with alkyl or aryl esterification of the carboxyl group are antagonists of muscarinic receptors. This conclusion is based on observations in intact segments of vascular and visceral smooth muscle as well as the results of radioligand binding studies with each of the muscarinic receptors commonly expressed in mammalian cardiovascular and other tissues.

The results of experiments with L-NAME and L-NMMA in the rabbit coronary artery revealed that L-NAME significantly reduced the amplitude of ACh-induced contractions but L-NMMA did not. The inhibition of contraction by L-NAME was not reversed with L-arginine. These observations support the hypothesis that L-NAME antagonizes muscarinic receptors.

NO may be spontaneously generated from several different sources in isolated segments of canine colon. These sources include enteric neurons, vascular endothelial cells, and possibly an inducible form of NOS in smooth muscle cells.\(^4,18\) Arginine analogues would therefore be predicted to have an excitatory effect on the response to ACh in the canine colon because of the removal of an inhibitory substance (i.e., NO). In contrast to this predicted effect of arginine analogues, we observed instead that the contractile response to ACh was reduced in the presence of 100 \(\mu M\) L-NAME and was unchanged in the presence of 100 \(\mu M\) L-NMMA. The inhibition of ACh-induced contractions in the presence of L-NAME is compatible with the hypothesis that this analogue antagonizes muscarinic receptors, whereas the lack of an effect of L-NMMA could be interpreted in a number of different ways. To provide direct evidence for the effect of these analogues on muscarinic receptors, we therefore undertook a series of radioligand binding studies.

Our radioligand binding studies demonstrate that each of the alkyl esters of L-arginine tested, L-NAME, L-AME, and L-BAEE, were effective antagonists of muscarinic receptors regardless of the complement of receptor subtypes present in the tissue tested. In contrast, the nitroarginine compounds without altered carboxyl groups, L-NA, L-NMMA, and L-NAA, did not compete for \(^3\)H]QNB binding in any tissue tested. These results correlate well with the L-NAME-induced reduction in ACh-induced contractions observed in both the rabbit coronary artery and the canine colon, an effect not produced by L-NMMA.

The notion that the alkyl esters of L-arginine compete for muscarinic receptor binding as antagonists acting at the known binding site for agonist is supported by our data showing that 1) guanine nucleotide does not alter the ability of alkyl esters of L-arginine to compete for \(^3\)H]QNB binding and 2) these esters compete for agonist binding directly and reversibly, without interfering with GTP-dependent agonist binding. The data of Table 3 demonstrate that in atria, where all M2 muscarinic receptors are shifted to low affinity in the presence of GTP\(_7\)S, addition of L-NAME does not alter this effect. Furthermore, L-NAME does not alter the agonist competition in canine colon, in which it is known\(^10\) that addition of GTP\(_7\)S shifts the agonist binding com-

**Figure 5.** Graphs showing the effects of nitric oxide synthase inhibitors on competition of \(^3\)H]quinoxulidyl benzilate (\(^3\)H]QNB) binding in smooth muscle membranes. L-NAME, \(N^\ominus\)-nitro L-arginine methyl ester; L-AME, L-arginine methyl ester; L-AEE, L-arginine ethyl ester; BAEE, Na-benzoyl L-arginine ethyl ester; L-ARG, L-arginine; L-NA, \(N^\ominus\)-nitro L-arginine; L-NMMA, \(N^\ominus\)-monomethyl L-arginine. Left panel: Arginine analogues possessing an alkyl ester modification at the carboxyl end of the amino acid were used in competition for \(^3\)H]QNB binding in membranes prepared from colonic smooth muscle. Data, normalized to binding in the absence of competitor (100%) and binding occurring in the presence of 1 \(\mu M\) atropine (0%), are expressed as the percentage of maximal \(^3\)H]QNB binding (0.3 nM). Curves are computer-generated best fit assuming the presence of a single class of binding sites. Data are the mean of triplicate observations from three representative experiments. Right panel: L-ARG and L-ARG analogues without an alkyl ester modification at the carboxyl end of the amino acid were used in competition for \(^3\)H]QNB binding in colonic smooth muscle membranes and found to be without effect. The results of additional experiments are presented in Table 2.
petition curve to a low-affinity curve best fit assuming two agonist:receptor complexes (M2 and M3). The assignment of L-NAME and the other alkyl esters of l-arginine as nonselective muscarinic antagonists also follows from our data. In competition studies using tissues known to possess different forms of the muscarinic receptor, the competition curve is monophasic. If the alkyl esters of l-arginine were selective for one form of the muscarinic receptor, one might expect that curves would be shallow and might be best fit assuming the presence of more than one receptor:antagonist complex. This was not the case. The simplest conclusion from these studies then is that the alkyl esters of l-arginine compete at the agonist binding site of several forms of the muscarinic receptor nonselectively and with moderate affinity, a proposal that is supported by the similarity of the structure of these compounds when compared against ACh (Figure 2).

The effects of the alkyl esters L-NAME, L-AME, and L-BAEE on muscarinic receptors have important implications in the interpretation of a variety of experiments. ACh was the first agonist shown to release a substance from blood vessels that was later identified as NO,19 and it is still frequently used as a method for releasing NO from endothelial cells (for review see Reference 20). Our results clearly suggest that a portion of the inhibitory effects of L-NAME and related analogues on ACh-induced relaxation could be due to antagonism at the muscarinic receptor. We provide functional and radioligand binding evidence in blood vessels, suggesting that the addition of L-NAME and related compounds will block both smooth muscle and endothelial cell receptors. Direct measurement of muscarinic receptors in blood vessels and their subtype assignment is difficult because of the small numbers of receptors present.21–23 Indeed, muscarinic receptors on endothelial cells were determined by Tracey and Peach24 to be lost during isolation and culture, but although these authors were able to determine that freshly prepared endothelial cells possess mRNAs for each of the commonly expressed muscarinic receptor subtypes (m1, m2, and m3), they could not determine which were translated. Thus, conclusions as to the muscarinic receptor subtype(s) present on the endothelial cell must be made with caution. Functional studies have suggested that endothelial cells probably possess both M2 and M3 receptors.25,26 Although the muscarinic receptor component on endothelium is not clear, we provide evidence that L-NAME is an antagonist at these as well as other muscarinic receptor subtypes.

In studies in which cholinergic excitatory nerves are stimulated to release ACh, alkyl esters of arginine may also produce complicated effects on the response, involving both the excitatory effect of removing NO that is due to NOS inhibition and the inhibitory effect of antagonizing muscarinic receptors. The overall change in contractile amplitude obtained on addition of L-NAME to a tissue is therefore likely to differ depending on how the two opposing effects sum together in a particular tissue. Indeed, it was our initial observation that the nerve-stimulated cholinergic contraction in canine colon was sometimes reduced on addition of L-NAME, and it was this observation that initially led us to explore the hypothesis that arginine analogues might also antagonize muscarinic receptors.

The ability of alkyl esters of arginine to antagonize muscarinic receptors also has important implications in interpreting in vivo studies. For example, in these studies various physiological parameters have been monitored during exposure to L-NAME.27,28 If the introduced analogue not only antagonizes NOS but also inhibits muscarinic receptors, then the physiological effects observed are likely to be the sum of these two actions. A key observation in determining whether the effects of L-NAME are due to antagonism of NOS or to antagonism of muscarinic receptors is the ability of l-arginine to reverse the effects of L-NAME. We have found that the antagonism of muscarinic receptors by L-NAME cannot be overcome by l-arginine, whereas reversal of NOS antagonism by l-arginine has been widely reported and recently reviewed.2

We suggest that the results presented here, that alkyl and aryl esters of l-arginine are muscarinic antagonists, can be extended from the specific tissues in which they were obtained (rabbit coronary artery, canine heart, coronary artery, aorta, endothelium, colon, submandibular gland, and guinea pig brain) to any tissue containing M1, M2, or M3 receptors. Furthermore, studies using these amino acid analogues should take into account the possibility that their actions are, at least in part, due to antagonism of muscarinic receptors.

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