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Dopaminergic and Cholinergic Muscarinic Receptor Effects of L-Alphacetylmethadol (LAAM) and Its Metabolites[1] (41882)

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Abstract. In isolated rat hearts L-alphacetylmethadol (LAAM) produced a concentration-dependent decrease in the spontaneous beating rate. This effect was completely prevented by 1.0 μM atropine. Chronic treatment of rats with LAAM increased the number of striatal dopamine receptors measured by [^{3}H]spiroperidol binding. The affinity of these binding sites for [^{3}H]spiroperidol was unchanged by LAAM treatment. There were no significant changes in the number or affinity of binding sites for the labeled muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) with chronic LAAM treatment. The ability of LAAM, nor-LAAM, or dinor-LAAM to antagonize the binding of [^{3}H]spiroperidol (40 pM) or [^{3}H]QNB (125 pM) to striatal membrane fragments was tested. The measured affinity constants for LAAM and metabolites were 100–3000 times higher than the affinity constants of unlabeled spiroperidol at [^{3}H]spiroperidol binding sites. The affinity constants of LAAM and metabolites at muscarinic binding sites were 10–20 times higher than pilocarpine and 5000–8000 times higher than atropine. These results suggest that LAAM can produce some of its effects by acting as a weak agonist at muscarinic receptor sites.

Stickney (1) reported that L-alphacetylmethadol (LAAM) produced an atropine-sensitive slowing of guinea pig isolated atria. We confirmed this bradycardic effect in isolated perfused guinea pig hearts (2). Atropine blocked LAAM-induced negative chronotropy during the period prior to nerve stimulation. Atropine also prevented the LAAM-induced depression of peak heart rate and the inhibition of norepinephrine release in response to nerve stimulation. We have concluded from these data that LAAM can activate both postsynaptic and presynaptic muscarinic receptors in guinea pig hearts. We attempted to confirm the muscarinic receptor mediated cardiac slowing effect of LAAM in rat hearts.

We previously reported that chronic treatment of mice and guinea pigs with LAAM resulted in changes in the striatal dopamine system (3). These changes consisted of augmented apomorphine induced stereotypy scores and increased numbers of [^{3}H]spiroperidol binding sites in striatal slices from LAAM-treated mice and guinea pigs.

Using diverse experimental approaches, the present study was designed to test the possibility that muscarinic receptors were involved in the striatal dopaminergic and peripheral cardiac actions of LAAM in rats.

Methods. *Treatment of rats with LAAM.* Male Wistar rats (250–350 g) were treated on alternate days for 40 days with 20 mg/kg po LAAM. The dose was determined to be effective in suppressing withdrawal signs for 24 hr in morphine-dependent rats (4).

Dosing was discontinued 4 days prior to sacrifice in order to allow sufficient time to completely eliminate residual LAAM and its active metabolites from the body (5).

Isolation and preparation of rat hearts. Hearts from rats (250–350 g) were isolated and perfused as previously described for guinea pigs (2, 6). The animals were sacrificed by decapitation and a flap of the thorax around the sternum was removed. A cannula was inserted into the ascending aorta and the heart perfused *in situ* at 34°C with a Krebs–Henseleit solution containing 0.2% bovine serum albumin and aerated with 95% O_2, 5% CO_2. A small incision was made in the pulmonary artery to allow the perfusate from the coronaries to escape and the inferior vena cava was severed near the diaphragm. The heart was removed from the thorax and suspended

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from a stand via the aortic cannula and allowed to stabilize for 20 min. A PE-200 catheter inserted into the right ventricle via the pulmonary artery and connected to a Statham P-23D pressure transducer detected the heart rate, which was recorded on a Brush model 2400 electronic recorder. Perfusion was maintained at a constant rate of 7 ml/min. The effect of *in vitro* perfusion with LAAM (1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, and 1×10^{-4} *M*) on heart rate was tested.

The ability of a single concentration of atropine (1×10^{-6} *M*) to antagonize the effects of LAAM was tested in separate experiments. Perfusion with atropine was initiated 1 min prior to the start of LAAM infusion and continued through the end of the experiment.

[^{3}H]Spiroperidol binding. Changes in the binding of [^{3}H]spiroperidol to dopamine receptors were assessed 4 days following termination of LAAM dosing. The striata were homogenized in 100 vol (w/v) of cold 0.5 *M* Tris buffer, pH 7.7, with a Brinkman polytron. The tissue homogenates were centrifuged twice at 25,000 rpm (Sorvall OTD-2) for 15 min. The final pellet was homogenized in 150 vol of cold 0.05 *M* Tris buffer containing 0.1% ascorbic acid, 120 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, pH 7.1, at 37°C for 10 min and returned to ice (7).

The incubation tubes contained varying concentrations of [^{3}H]spiroperidol in a volume of 0.1 ml, 0.1 ml of 0.1% ascorbic acid with or without 1.0 μM apomorphine, and 1.8 ml of tissue homogenate. Tubes were incubated for 20 min at 37°C and filtered through Whatman GF/B glass microfilters under vacuum, using the rapid filtration method of Creese *et al.* (7). The tubes were rinsed and the filters were washed with 3 × 5-ml aliquots of Tris buffer. The filters were placed into liquid scintillation vials containing Aquasure and counted on a Packard Prias Tri-Carb scintillation counter with counting efficiencies ranging from 53 to 63%. The specific binding to dopaminergic receptors was estimated as the difference of binding in the absence and presence of 1 μM apomorphine. The kinetics of [^{3}H]spiroperidol binding was determined using Scatchard analysis and subjected to a Student's *t* test for significance. Protein was measured by the Lowry method.

[^{3}H]QNB binding. Saturation binding of [^{3}H]QNB was carried out according to the method of Hulme *et al.* (8). Striata from three rats were homogenized on ice in 10 vol of 0.32 *M* sucrose. The homogenate was centrifuged at 1000*g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 10,000*g* for 20 min. The resulting pellet was resuspended in Krebs–Henseleit buffer (pH 7.4) to a final protein concentration of 1.0 mg/ml. The membrane suspension was preincubated at 30°C for 15 min. Eight-hundred-microliter aliquots were pipetted into 7-ml plastic scintillation vials which contained 50 μl of [^{3}H]QNB and 50 μl of either Krebs–Henseleit (K–H) buffer or atropine in K–H buffer. The reaction was incubated for 15 min at 30°C and terminated by centrifugation at 14,000*g* for 15 min. The supernatant was discarded and the pellets were gently washed three times with 1.5 ml of Krebs–Henseleit buffer. The vials were allowed to air dry. Five milliliters of liquid scintillation cocktail was added to each vial, the vials were shaken by hand, and counted by liquid scintillation spectrometry.

Binding assays were carried out which tested the ability of different drugs to displace a single concentration of [^{3}H]spiroperidol (40 p*M*) from dopamine binding sites or a single concentration of [^{3}H]QNB (125 p*M*) from muscarinic binding sites. The assay conditions were the same as described in saturation binding experiments. Cold spiroperidol, LAAM, nor-LAAM, and dinor-LAAM were used to antagonize [^{3}H]QNB. Probit analysis was used to determine the concentration of drug necessary to displace 50% of the ^{3}H-labeled ligand (EC_{50}).

Results. *In vitro effect of LAAM on isolated rat hearts.* Perfusion of isolated rat hearts with LAAM produced a decrease in heart rate (Fig. 1). This cardiac slowing effect of LAAM was statistically significant at all tested concentrations of LAAM. The highest concentration, 100 μM LAAM, decreased heart rate by 61% (190 to 74 bpm). In seven hearts atropine (1.0 μM) was infused beginning 1 min prior to the infusion of the first concentration of LAAM and continued to the end of the experiment. The negative chronotropic effect of LAAM was completely inhibited in these experiments.

[^{3}H]Spiroperidol and [^{3}H]QNB binding. Scatchard analysis of [^{3}H]spiroperidol binding

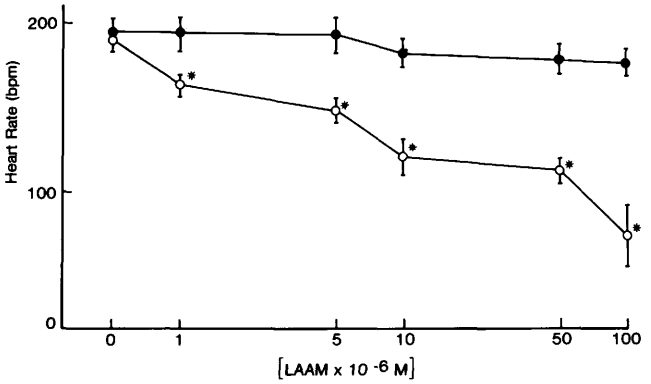

FIG. 1. Effect of LAAM alone (O, $N = 8$) or LAAM in the presence of 1×10^{-6} M atropine (●, $N = 7$) on the beating rate of isolated rat hearts. *LAAM significantly less than atropine + LAAM $P < 0.05$ (Student's t test).

showed no change in the apparent affinity constant with chronic LAAM treatment (control K_D = 40 ± 5.1 pM vs LAAM K_D = 37 ± 8.8 pM) (Fig. 2). The maximum number of binding sites was significantly greater in striata from LAAM treated rats (control B_{max} = 75.1 ± 3.2 fmole/mg protein vs LAAM B_{max} = 94.2 ± 6.1 fmole/mg protein).

The binding of the muscarinic antagonist [^{3}H]QNB to striatal membranes was unchanged by chronic LAAM treatment (Fig. 3). The maximum number of binding sites was identical in striatal membrane fragments from both control and LAAM treated rats (control B_{max} = 750 ± 37 fmole/mg protein vs LAAM B_{max} = 752 ± 60 fmole/mg protein). There was no significant difference in the affinity of [^{3}H]QNB for its binding sites (control K_D = 0.15 ± 0.03 nM vs LAAM K_D = 0.11 ± 0.04 nM).

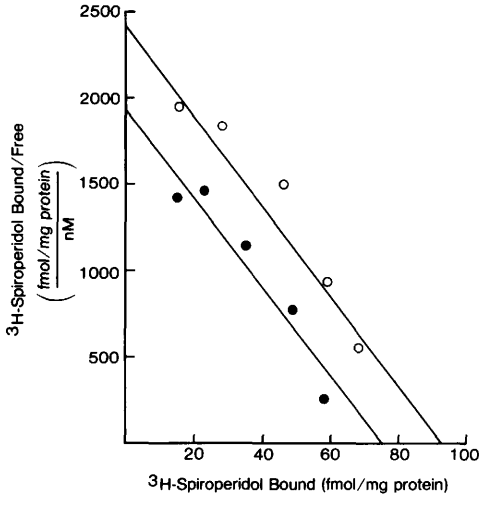

FIG. 2. Scatchard plots of [^{3}H]Spiroperidol binding to rat striatal membrane fragments. The number of binding sites was significantly increased by chronic LAAM treatment ($P < 0.05$ Student's t test). Control (●, $N = 8$); LAAM treated (O, $N = 9$). Linear regression analysis was used to determine the line of best fit.

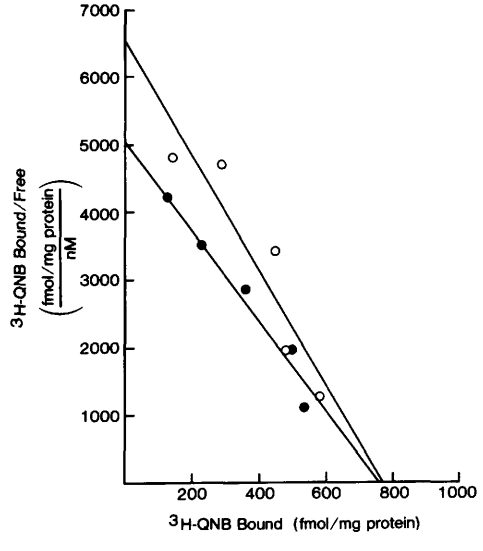

FIG. 3. Scatchard plots of [^{3}H]QNB binding to rat striatal membrane fragments. There were no significant differences in the apparent affinity constant or number of binding sites ($P > 0.05$ Student's t test). Control (●, $N = 8$); LAAM-treated (O, $N = 8$).

Antagonism of [^{3}H]spiroperidol and [^{3}H]-QNB binding. LAAM, nor-LAAM, and dinor-LAAM were tested for their ability to antagonize the binding of a single concentration of either [^{3}H]spiroperidol (40 p*M*) or [^{3}H]QNB (125 p*M*) to rat striatal membranes. The metabolites of LAAM show a higher affinity than the parent compound for [^{3}H]spiroperidol binding sites, but all three were significantly less potent than cold spiroperidol at displacing [^{3}H]spiroperidol from striatal binding sites (Table I).

The potencies of LAAM, nor-LAAM, and dinor-LAAM were similar vs the binding of [^{3}H]QNB to striatal membranes but slightly less potent than pilocarpine and much less potent than atropine. Pilocarpine had an affinity 10–20 times greater and atropine had an affinity 5000–8000 times greater than LAAM and metabolites for [^{3}H]QNB binding sites (Table I).

Discussion. An earlier report from our laboratory demonstrated that chronic treatment with LAAM altered dopaminergic mechanisms in the striata of mice and guinea pigs (3). Other narcotic agonists like morphine or methadone have been shown to produce changes in the striatal dopamine system similar to those produced by chronic treatment with neuroleptics (9–12). These changes were thought to be due to long-term blockade of dopamine receptors by the narcotics (13). In the present study, we attempted to determine if the increased number of [^{3}H]spiroperidol binding sites following chronic LAAM treatment was due to the ability of LAAM to block striatal dopamine receptors or resulted from some other section of LAAM.

Treatment of rats with LAAM (20 mg/kg po for 40 days) resulted in a change in [^{3}H]spiroperidol binding to striatal membranes similar to that seen in mice and guinea pigs (3). Scatchard analysis of saturation binding data demonstrated that the maximum number of bindings sites was significantly increased with no change in the apparent affinity constant (Fig. 1). The micromolar concentrations of LAAM and its metabolites which were required to antagonize a 40 p*M* concentration of [^{3}H]spiroperidol indicate that the drugs are probably not acting as antagonist at these sites (Table I). While there appeared to be increased affinity for [^{3}H]spiroperidol binding sites with successive metabolism of LAAM to nor-LAAM and then to dinor-LAAM, the EC_{50} values were 100–3000 times higher than for cold spiroperidol. These data cast doubt on a dopaminergic antagonist action for LAAM or its major metabolites.

TABLE I. CONCENTRATION OF DIFFERENT DRUGS (MICROMOLAR) REQUIRED TO DISPLACE 50% OF 40 p*M* [^{3}H]SPIROPERIDOL OR 125 p*M* [^{3}H]QNB FROM RAT STRIATAL MEMBRANE FRAGMENTS

	EC_{50} (μM) vs [^{3}H]Spiroperidol	EC_{50} (μM) vs [^{3}H]QNB
LAAM	30	42
Nor-LAAM	4.8	35
Dinor-LAAM	1.5	24
Spiroperidol	0.01	—
Pilocarpine	—	2.3
Atropine	—	0.005

We have previously reported that LAAM produced a negative chronotropic effect in isolated guinea pig hearts. In addition, LAAM inhibited the release of norepinephrine from cardiac sympathetic nerves induced by nerve stimulation. These effects were not blocked by 10 μM naltrexone but could be antagonized by 1.0 μM atropine. It was concluded that these actions of LAAM were mediated by atrial (postsynaptic) and nerve terminal (presynaptic) muscarinic receptors, respectively (2). In the present study, LAAM produced a similar negative chronotropic response in isolated rat hearts and this response was completely antagonized by 1.0 μM atropine (Fig. 2). These actions at peripheral synapses and the fact that the striatum contains muscarinic receptors which influence dopamine neurons (20, 1) led us to examine the possibility that the striatal effects of LAAM might be mediated by stimulation of muscarinic receptors.

In order to test this possibility, we measured the binding of [^{3}H]QNB to rat striatal membranes following chronic treatment with LAAM (20 mg/kg po alternate days for 40 days). No significant changes in the binding affinity or the maximum number of binding sites occurred (Fig. 3). It has been reported that prior exposure to agonists can desensitize muscarinic receptors in smooth muscle (14) but not in rat brain (15). Additional experiments were performed on striatal membranes from untreated rats which tested the ability

of LAAM, nor-LAAM, or dinor-LAAM to antagonize the binding of 125 p*M* [^{3}H]QNB (Table I). There were no differences in the affinities of LAAM or its metabolites for the [^{3}H]QNB binding sites. The EC_{50} values for pilocarpine and atropine were similar to those previously reported (16). The concentration of LAAM and its metabolites necessary to displace 50% of 125 p*M* [^{3}H]QNB were 10–20 times higher than pilocarpine and 5000–8000 times higher than atropine. These data indicate that LAAM, nor-LAAM, and dinor-LAAM have low affinities for striatal muscarinic binding sites. In spite of a relatively low affinity, LAAM may produce some of its effects through stimulation of muscarinic receptors in the striatum. Activation of guanylate cyclase in a neuroblastoma clone has been reported to be due to stimulation of a population of low-affinity muscarinic receptors (17). The mechanism by which this muscarinic action of LAAM would produce a supersensitivity of dopamine receptor sites is unclear since most published reports indicate that dopamine neurons synapse on cholinergic neurons in the striatum rather than the reverse (18, 19). It is possible that the muscarinic receptors stimulated by LAAM reside on dopamine nerve terminals. Evidence has been presented supporting the possible existance of presynaptic cholinergic receptors on dopamine nerve endings in the rat striatum (20, 21). Nomura *et al.* (22) reported that destruction of dopaminergic neurons in the striatum and mesolimbic areas of the rat brain by 6-hydroxydopa reduced the number of muscarinic receptor sites measured by [^{3}H]QNB binding without changing the affinity constant indicating a nerve terminal locus for these receptors. Giorguieff *et al.* (23) demonstrated the presence of nicotinic and muscarinic receptors on dopamine nerve terminals in superfused rat striatal slices. Both types of cholinergic receptors enhanced the spontaneous release of dopamine. De Belleroche and Bradford (24) on the other hand have reported presynaptic muscarinic receptors which inhibit the release of dopamine from striatal terminals during nerve stimulation. Such an inhibition of dopamine release would lead to a postsynaptic dopamine receptor supersensitivity (up regulation) like that observed in our experiments. A possible explanation for the lack of measurable change in striatal muscarinic receptors following chronic treatment with LAAM is that the presynaptic receptors represent a small percentage of the total pool of striatal muscarinic receptors.

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