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OPIATE-LIKE EFFECTS OF NORLAUDANOSOLINECARBOXYLIC ACIDS ON THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS*

JOHN M. LASALA,† THEODORE J. CICERO‡ and CARMINE J. COSCIA†

†E. A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, MO 63104, U.S.A. and ‡Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

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Abstract—Norlaudanosolinecarboxylic acid (NLCA), a condensation product of dopamine and 3, 4-dihydroxyphenylpyruvic acid, has been found to exhibit opiate-like effects both *in vitro* and *in vivo*. The ability of NLCA to displace [^{3}H] naloxone was measured in the presence and absence of 100 mM NaCl (IC_{50} values = 2×10^{-5} M and 7.8×10^{-7} M respectively). This large "Na^+ shift" suggested that NLCA was a relatively pure opiate agonist. Two analogs of NLCA, 3′-*O*-methyl NLCA (MNLCA) and 3′, 4′-deoxy-NLCA (DNLCA), that have been shown to accumulate during L-dopa chemotherapy of Parkinsonism and phenylketonuria, respectively, also behaved as opiate agonists, but the concentrations required were higher than for NLCA. In addition, NLCA, like many opiates, decreased serum luteinizing hormone (LH) levels by approximately 50 per cent in both castrated and normal rats, 1–2 hr after its subcutaneous administration. Similarly, in normal males, serum testosterone levels were markedly depressed (60 per cent) after treatment with NLCA. The NLCA-induced depression in serum LH was naloxone reversible.

The norlaudanosolinecarboxylic acids (NLCAs, Fig. 1) are a group of tetrahydroisoquinoline alkaloids (TIQs) which can accumulate in phenylketonuric (PKU) humans and in Parkinsonian patients maintained on L-dopa chemotherapy [1, 2]. In an attempt to assess the biological significance of these compounds, it seemed reasonable to test NLCAs for their possible opiate properties. NLCA, a condensation product of dopamine (DA) and 3, 4-dihydroxyphenylpyruvic acid, has been implicated in the biosynthesis of morphine in plants [3–5]. Moreover, liver homogenates can catalyze phytomimetic transformations of related TIQs into compounds with the morphinandienone skeleton *in vitro* [6]. More importantly, an inspection of Dreiding atomic models of NLCAs suggests that these TIQs can assume conformations with a juxtaposition of key groups similar to that required for the opiate activity of morphine and methionine-enkephalin, as determined by computer generated models [7].

The possibility that TIQs may have opiate-like properties has also been suggested by other lines of evidence. First, a TIQ derived from L-dopa and acetaldehyde, 3-carboxysalsolinol, elicited analgesia alone and potentiated morphine-induced analgesia in the rat [8]. Second, relatively high concentrations of aldehyde-derived TIQs, and related tetrahydroprotoberberine alkaloids, competed with naloxone for binding sites in whole brain homogenates [9].

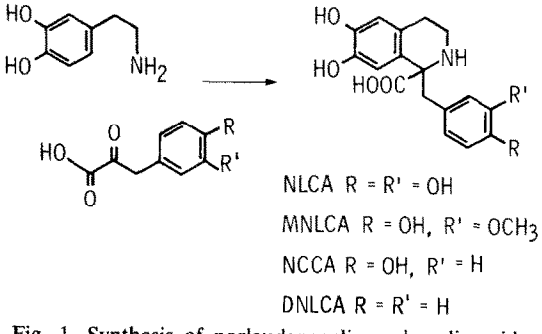

Fig. 1. Synthesis of norlaudanosolinecarboxylic acids.

Finally, intracranially administered salsolinol, the acetaldehyde–dopamaine condensation product, enhances the analgesia produced by subcutaneously injected morphine [10].

In the studies described in this paper, the possibility that the NLCAs have opiate-like properties was evaluated in an *in vitro* preparation and *in vivo*. For the *in vitro* studies, the ability of NLCAs to displace [^{3}H] naloxone from binding sites in rat brain membranes was assessed [11–13]. This assay has provided a sensitive means of characterizing opiate-like compounds. In addition, since 100 mM sodium favors the binding of opiate antagonists, relative to agonists, this so-called "sodium shift" can be used to predict whether a given compound will behave as an opiate agonist or antagonist *in vivo*. As an *in vivo* assay, we have employed the opiate-induced changes in serum luteinizing hormone (LH) and testosterone [14, 15]. It has been found that these two hormones are markedly sensitive to opiate agonists and antagonists (decreasing and increasing, respectively, after

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treatment) and this technique has been used to assess structure–activity relationships [16]. Using these *in vivo* and *in vitro* techniques, we have established that the NLCAs behave like opiate agonists.

MATERIALS AND METHODS

Materials. Catecholamines, α-ketoacids, and most other biochemicals (except where noted) were purchased from the Sigma Chemical Co., St. Louis, MO. (±)-NLCAs were prepared as described previously [17]. 3,4-Dihydroxyphenylpyruvate was synthesized from (±)-3, 4-dihydroxyphenylalanine [18].

[^{3}H (G)] Naloxone (23 Ci/mmole) and [1,2,6,7-^{3}H (N)] testosterone (105 Ci/mmole) were purchased from New England Nuclear, Boston, MA. Levorphanol and naloxone were gifts from the Roche Laboratories, Nutley, NJ and Endo Laboratories, Garden City, NY, respectively.

Opiate binding assay Brains (without cerebella) were obtained from 150 to 200 g male Sprague–Dawley rats and were sectioned into four parts for convenient handling during homogenization. Each portion was homogenized in 40 ml of 0.05 M Tris, pH 7.5, before centrifuging at 10,000 *g* for 10 min at 3°. After decanting the supernatant fraction, the pellet was resuspended in 50 additional ml of 0.05 M Tris and recentrifuged at 10,000 *g*. The resulting pellets were combined, resuspended in a total of 10 ml of 0.05 M Tris, and stored at −20° until needed. These membrane preparations were rehomogenized, using 4–5 strokes in a glass homogenizer, prior to use to assure uniform consistency.

Incubations were performed in an ice bath for 2.5 hr. Each tube contained 200 μl of 0.05 M Tris, 50 μl of 10^{-5} M bacitracin (to inhibit peptidase activities), 50 μl of drug solution (dissolved in 0.05 M Tris), 50 μl of [^{3}H]naloxone (10^{-7} M), and 100 μl of membranes representing 18 mg wet weight of tissue. Fifty μl of 1 M NaCl were added when required, or 50 μl of 0.05 M Tris, to make a final incubation volume of 500 μl. Following incubation, the tubes were centrifuged at 1600 *g* at 3° for 30 min. The supernatant fraction was aspirated, and the pellet was washed twice with 1 ml of 0.05 M Tris (centrifuging after each wash). The resulting pellet was solubilized with 200 μl of NCS (Amersham Searle, Chicago, IL) and transferred to scintillation vials with 3 × 1 ml washes of Scintiverse (Fisher Scientific, St. Louis, MO). The final volume of the scintillation mixture was 10 ml. All points represent specific binding defined as total number of counts ([^{3}H]naloxone) minus the number of counts of [^{3}H]naloxone bound in the presence of 10^{-6} M levorphanol [11].

LH assay. Rats were injected subcutaneously with either drug or vehicle and were decapitated at selected intervals. Blood was collected from the carcasses and allowed to stand for 1–3 hr before centrifuging at 1000 *g* for 30 min. All sera were stored at −20° until assayed.

LH was assayed by the double-antibody radioimmunoassay (RIA) originally described by Niswender *et al.* [19]. Reagents for the radioimmunoassay were provided by the Rat Pituitary Hormone Distribution Program of the NIAMDD. The anti-LH sera were provided by Dr. Gordon Niswender (Colorado State University) and the LH used for iodination (LER-1056C2) was supplied by Dr. Leo Reichert (Emory University). Castration and sham operations were performed under nembutal anesthesia, and the animals were injected with NLCA 24 hr later.

Serum testosterone assay. Plasma testosterone levels were determined by a highly specific radioimmunoassay utilizing an antibody generated in sheep to an 11-α-succinyl testosterone-BSA hapten supplied by Dr. W. Weist, Department of Obstetrics and Gynecology, Washington University. Serum was extracted with a mixture of benzene–petroleum ether (2:5). After drying the organic phase under N_2 at 45°, samples were taken up in 300 μl of 0.05 M Tris buffer (pH 8.0), and heated at 60° to dissolve them; they were then transferred to 10 × 76 mm culture tubes in which the RIA took place. Unbound steroid was removed by adsorption to dextran coated charcoal. The standard curve was linear upon plotting the logit transform of bound–unbound radioactivity against the log of testosterone concentration.

RESULTS

Effects of NLCAs on [^{3}H]naloxone binding in brain. The effect of (±)-NLCA in displacing stereospecific binding of [^{3}H]naloxone to rat brain membranes is shown in Fig. 2. The IC_{50} was determined graphically by plotting the concentration of NLCA versus the percent inhibition of [^{3}H]naloxone binding in a log-probit analysis. The IC_{50} of NLCA in the presence of 100 mM NaCl was higher than that in the absence of salt. This marked shift in binding affinity is characteristic of opiate agonists. In fact, the IC_{50} (+NaCl)/IC_{50} (-NaCl) ratio of 25.6 is in the same range as that of similar morphine-like compounds [20].

Table 1 shows the effects of two NLCA analogs, DNLCA and MNLCA, on [^{3}H]naloxone binding.

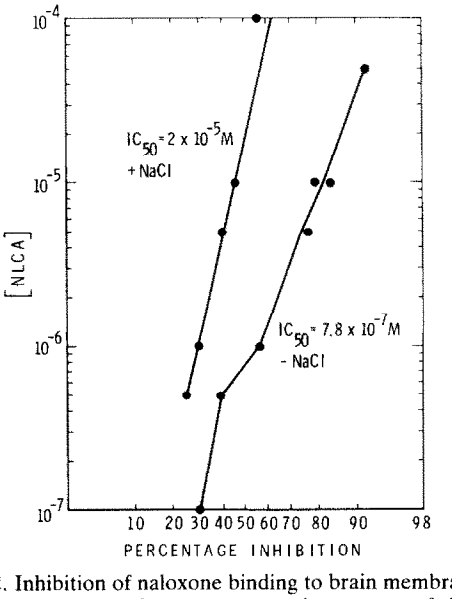

Fig. 2. Inhibition of naloxone binding to brain membranes by NLCA. Values shown represent the average of three trials.

Table 1. Effects of DNLCA and MNLCA on stereospecific naloxone binding to brain membranes

	Concentration (MC)	% Inhibition With 100 mM NaCl	% Inhibition Without NaCl
DNLCA	5×10^{-8}		11
	10^{-7}		19
	5×10^{-7}		34
	10^{-6}	12	41
	5×10^{-6}	24	68
	10^{-5}	31	70
	5×10^{-5}	50	84
	10^{-4}	58	
MNLCA	10^{-7}		5
	5×10^{-7}		26
	10^{-6}	12	35
	5×10^{-6}	23	44
	10^{-5}	31	54
	5×10^{-5}	40	68
	10^{-4}	45	

DNLCA and MNLCA binding above 10^{-4} M could not be determined because of the limited solubility of these compounds in the Tris buffer used as vehicle, and, hence, accurate IC_{50} values could not be estimated in the presence of NaCl. Methanol–HCl is a considerably better solvent for the NLCAs, but will, by itself, reduce binding. Estimations of IC_{50} values in the absence of NaCl, however, indicated that the relative potency of MNLCA was an order of magnitude lower than NLCA, while DNLCA was somewhere between the two. DNLCA and MNLCA did, however, exhibit comparable sodium shifts (26.8 and 25 respectively).

Effect of (±)-NLCA on serum LH and testosterone levels. Figure 3A shows the time course of the effect of NLCA (10 mg/kg) on serum LH levels. The vehicle in this case was acidified methanolic-saline. NLCA produced a 40–50 percent decrease in serum LH 1–2 hr after its injection. This effect was mirrored closely by a decrease in serum testosterone levels which occurred 1–2 hr after the initial LH depression (Fig. 4). The rate of decline in LH was similar to that reported for morphine [16], but the effects of morphine were much more prolonged (4–5 hr); in addition, this opiate virtually reduced LH to non-detectable levels. The shorter duration of the NLCA

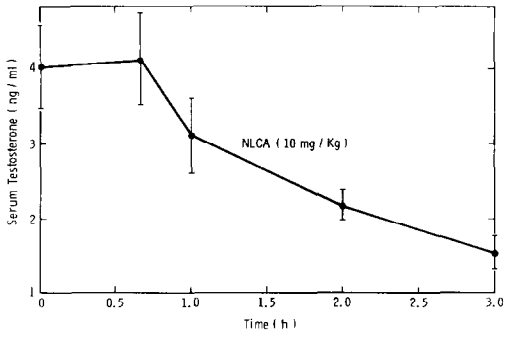

Fig. 4. Effect of NLCA on serum testosterone levels of male rats. Values shown are the means ± S.E.M.; N = 6.

effect is consistent with turnover studies of a 3′-deoxy-NLCA, NCCA (Fig. 1) (B. Roth and C. J. Coscia, unpublished observations). NCCA is converted rapidly to its 6-*O*-methyl ether in liver and kidney. Four hr after intraperitoneal injection of NCCA into mice, over 90 per cent of the label that was initially detected in liver and kidney had been cleared from these organs.

Figure 3B shows that, regardless of the dose administered, maximal inhibition of serum LH with NLCA did not exceed 50 per cent. The dose–response curve shown in this figure indicates that doses up to 20 mg/kg produced reductions in serum LH, but that with doses in the 30–50 mg/kg range, LH levels inexplicably rose to control levels or above (Fig. 3B).

Reversal of NLCA-induced LH depressions (Fig. 5) was observed after simultaneous injection of naloxone (2 mg/kg) and NLCA (7 mg/kg). The concentrations of both drugs used were the lowest dosages necessary to produce a maximum effect. Naloxone itself elevates LH levels in a dose-dependent manner [21].

Effect of NLCA on castrated rats. In an attempt to show more pronounced depressions in serum LH, rats were castrated to remove the negative feedback control of testosterone, and were injected with various amounts of NLCA 24 hr later. Figure 6 shows that, after castration, there was a large increase in serum LH over shams. NLCA was able to decrease this response in a dose-dependent fashion, with a 53 per cent inhibition achieved at 50 mg/kg, the largest

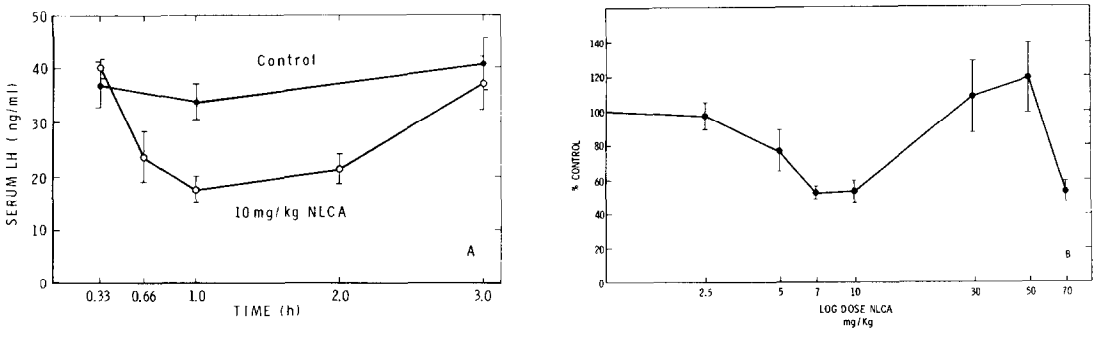

Fig. 3. Effect of NLCA on serum LH levels of male rats. Panel A: time course. Panel B: dose response. Values shown are the means ± S.E.M.; N = 6.

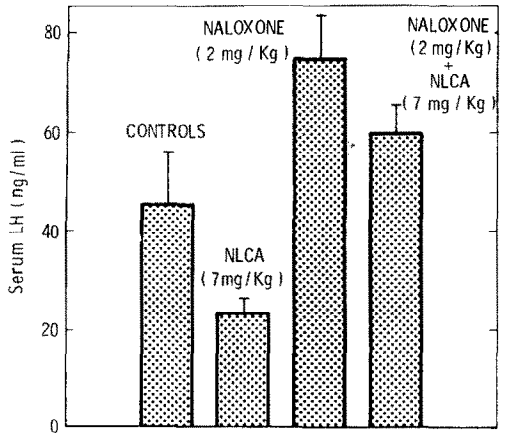

Fig. 5. Naloxone reversal of the effect of NLCA on serum LH levels in male rats. Values shown are the means ± S.E.M.; N = 10.

dose tested. The larger dose required to produce a comparable IC_{50} in the castrates, as opposed to the normal male rat, is in agreement with the results obtained with morphine (T. J. Cicero, unpublished observations).

DISCUSSION

The data presented in this paper indicate that NLCAs behave like opiate agonists both *in vivo* and *in vitro*. In our *in vitro* studies, we found that NLCA inhibited naloxone binding by 50 per cent (IC_{50}) at a concentration of approximately 7.8×10^{-7} M, in the absence of NaCl. Comparing this with the IC_{50} for other opiate agonists, it appears that NLCA is about as potent as codeine and propoxyphene, but is two orders of magnitude less effective than morphine [20, 22]. These data suggest that NLCA is a relatively modest, but nevertheless effective, opiate agonist under these *in vitro* conditions. DNLCA and MNLCA also appear to be opiate agonists, but are less effective than NLCA. The conclusion that

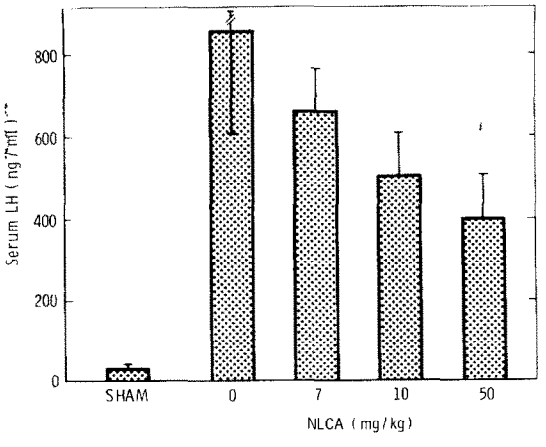

Fig. 6. Effect of NLCA on serum LH levels of castrated male rats. Values shown represent the means ± S.E.M.; N = 6.

NLCA and its structurally related analogues are opiate agonists is supported by the observation that there was a substantial sodium shift in the IC_{50} values which is characteristic of all opiate agonists. Indeed, the magnitude of the sodium shift observed with the NLCAs is comparable to that observed with other opiate agonists [20].

The opiate-like properties of NLCA inferred from these *in vitro* studies were confirmed in an *in vivo* assay as well. Cicero *et al.* [14, 15] have described an *in vivo* assay, based upon the testosterone and LH-depleting properties of the narcotics, which can be used effectively to assess structure–activity relationships and other receptor-mediated actions of the narcotics. Moreover, it has also been shown recently that, whereas opiate agonists depress serum LH and testosterone, opiate antagonists increase the serum levels of both hormones [21, 23]. Hence, this technique permits an assessment of structure–activity relationships and a differentiation of opiate agonists and antagonists. Our results with this technique demonstrate that NLCA is, under *in vivo* as well as *in vitro* conditions, an opiate agonist, since LH and testosterone levels were substantially reduced subsequent to its administration.

Several questions might be raised concerning the dose–response curve for NLCA with respect to depressions in serum LH levels. First, an inspection of the data presented in this paper (Fig. 3B) indicates that no dose of NLCA in the normal male animal suppressed serum LH levels by more than 50 per cent. This is somewhat perplexing, since other opiate agonists are fully able to depress serum LH levels below this level. While the reasons for this are not entirely clear, one explanation seems plausible. In the normal male rat, the hypothalamic-pituitary-gonadal axis is massively suppressed by the negative feedback control of testosterone. For example, if this negative feedback control is removed by castration, serum LH levels rise more than fifteen to twenty times, indicating that basal levels of serum LH are under very rigid control by testosterone (Fig. 6; Ref. [15]). The induction of any further statistically significant decreases in serum LH might be extremely difficult to achieve under these conditions. This conclusion appears to be valid in the case of the potent opiate agonist, morphine, and its structurally related analogues, and would certainly seem to be equally valid for the modest opiate agonist NLCA. It may be that had we been able to inject higher doses of NLCA (greater than 70 mg/kg), we might have been able to reduce serum LH levels somewhat further, but this was not possible because of the limited solubility of the compound. A further difficulty with the dose–response curve shown in Fig. 3B is that LH levels were depressed by doses of NLCA up to 30 mg/kg, but inexplicably they rose to control levels between 30 mg/kg and 50 mg/kg. We have no simple explanation for this paradox, but it may be that the NLCAs exert opiate-like effects at low doses because of their relatively high affinity (as compared to other receptors) for opiate receptors, whereas at some higher doses they may bind to receptors for which they have less affinity but nevertheless exert some action. In this connection, the possibility that these substances have weak catecholamine-like properties,

and an affinity for catecholamine-uptake mechanisms, has been demonstrated [24]. It seems plausible that this weak catecholamine-like activity may predominate at higher NLCA concentrations, and thus override the opiate-like effects of the drug. This conclusion is supported by the fact that, at least in some studies, dopamine has been found to lead to increases in luteinizing hormone releasing hormone (LHRH) [25]. These observations, however, are entirely speculative at this point and more definitive studies are required.

On the basis of the results presented in this paper, it seems reasonable to conclude that NCLAs exert opiate-like activities both *in vivo* and *in vitro*. The principal question raised by these observations is whether NLCAs play a normal role in the function of the hypothalamic-pituitary-gonadal axis in the male rat or human. Although the substances have been found to occur in the normal rat and human brain [1, 2], it is not clear whether they occur in sufficient abundance to have a meaningful impact upon the neuroendocrine control of this axis. On the basis of the doses required to inhibit both naloxone binding to rat brain membranes or serum LH levels, it might be predicted from these studies that under normal conditions these substances would be relatively ineffective. However, it should be recognized that NLCA was administered systemically, and, hence, significant metabolism more than likely occurred. In addition, we have not ascertained how much NLCA actually penetrated the blood brain barrier and reached target regions in brain. It is difficult by simply measuring the tissue content of the NLCAs to determine its concentration at critical sites within the brain and within the neurons involved. However, even if the normal levels of these substances are insufficient to modulate activity in the hypothalamic-pituitary-gonadal axis, it might be speculated that the higher levels of these compounds found under phenylketonuric-like conditions or in Parkinsonian patients on L-dopa chemotherapy might, in fact, influence neuroendocrine systems. There is some evidence, in fact, which indicates that L-dopa chemotherapy alters serum levels of a variety of hormones [26–28], but there has been an absence of good systematic studies. Clearly, future research should be directed at examining the neuroendocrine status of PKU patients and those Parkinsonian patients maintained on L-dopa chemotherapy. Furthermore, the formation of NLCAs in areas of dopaminergic innervation (e.g. the striatum) might have an influence on dopamine synthesis and release, has been suggested for other opiates [29–31].

In conclusion, we have demonstrated that NLCA and, to a lesser extent, MNLCA and DNLCA are opiate-like compounds. Studies are currently under way to characterize further their opiate-like properties.

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