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Anti-nociceptive activity of nitric oxide synthase inhibitors in the mouse: dissociation between the effect of L-NAME and L-NMMA

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Abstract—The anti-nociceptive effect of selective inhibitors of nitric oxide synthase has been assessed in a formalin-induced paw-licking model in mice. L-N^G-Nitro arginine methyl ester (L-NAME) but not L-N^G-monomethyl arginine (L-NMMA) exhibited anti-nociceptive activity in both the early and late phases of paw licking following intraperitoneal administration. The effect on the late phase response was more pronounced. L-NAME (0·1–100 μg) and L-N^G-nitro arginine base (L-NOARG; 10 μg) but not D-NAME (10 μg) were also anti-nociceptive following intracerebroventricular administration. L-NAME (10 μg) administered by this route did not influence locomotor activity. L-NMMA was inactive at doses up to 40 μg by this route. At higher doses (75–200 μg) L-NMMA produced a similar and non-dose related reduction in early/late phase paw-licking time. D-NMMA (100 μg) was inactive. The greater anti-nociceptive effect of L-NAME in this model accords with recently published biochemical data indicating that L-NAME is several orders of magnitude more potent than L-NMMA as an inhibitor of brain nitric oxide synthase. These data support the use of L-NAME as a selective tool to investigate the central pharmacological effects of nitric oxide.

Nitric oxide (NO) biosynthesis occurs in a number of non-neuronal, mammalian tissues including vascular endothelial cells, platelets, leucocytes, fibroblasts, Kuppfer cells and as yet unidentified cell types within the adrenal cortex and lung (Moncada et al 1989). Additionally, NO may serve as a neurotransmitter both in the peripheral (non-adrenergic, non-cholinergic, NANC) (Bult et al 1990; Gibson et al 1990; Tucker et al 1990) and central (Garthwaite et al 1988; Garthwaite 1991) nervous systems. Within the brain the NO synthase enzyme involved has been purified (Knowles et al 1989) and its regional distribution within different parts of the rat brain determined (Bredt et al 1990; Forstermann et al 1990).

Despite these not inconsiderable advances in our understanding of the biochemistry of the brain NO system, the functional significance of NO in the central nervous system remains to be established. For example, Garthwaite and colleagues have suggested that NO produced within cerebellar neurones under the influence of *N*-methyl-D-aspartate (NMDA) type glutamate receptors may play a part in the development of synaptic plasticity in this part of the immature rat brain (see Garthwaite 1991; Southam et al 1991). In addition, we have demonstrated that L-N^G-nitro arginine methyl ester (L-NAME), a selective inhibitor of NO synthase, exhibits a potent and long-lasting anti-nociceptive action in the mouse by what is apparently a naloxone-independent central mechanism (Moore et al 1991).

Recent evidence has suggested that cerebellar NO synthase appears to be exquisitely sensitive to inhibition by L-NAME when compared with other inhibitors of this enzyme such as L-N^G-monomethyl arginine (L-NMMA) (East & Garthwaite 1990; Lambert et al 1991). For this reason we considered it worthwhile to compare the anti-nociceptive effect of L-NAME (and its base L-N^G-nitro arginine, L-NOARG) with that of L-NMMA in an attempt to determine whether the reported disparity in biochemical properties of the two inhibitors was correlated with differences in their biological activity in the brain. Some of these results have been communicated in preliminary form to the British Pharmacological Society (Babbedge et al 1991).

Materials and methods

The methods employed in this study have been fully discussed elsewhere (Moore et al 1991) and will thus be outlined only briefly. Male LACA mice (28–35 g) were used for all experiments. Animals were allowed free access to food and water until transported to the laboratory at least 1 h before the experiment. All experiments were conducted in the period between 1300 and 1700 h in normal room light and temperature (22 ± 2°C).

The anti-nociceptive effect of L-NAME, D-NAME, L-NOARG and L-NMMA was assessed using the formalin-induced paw licking procedure essentially as described by Hunskaar & Hole (1987). Animals were injected sub-plantar in one hindpaw with formalin (5%, 10 μL). The duration of paw-licking (an index of nociception) was measured 0–5 min and 15–30 min after formalin administration. L-NAME or L-NMMA (50 mg kg^{-1}) were administered intraperitoneally (i.p.) 15 min before formalin injection. In separate experiments, L-NAME (0·1–10 μg), D-NAME (10 μg), L-NOARG (10 μg), L-NMMA (10–200 μg) or D-NMMA (100 μg) was administered intracerebroventricularly (i.c.v.) as described by Oluyomi (1989) again 15 min before formalin injection. Control animals received an appropriate volume of 0·9% w/v NaCl (saline, 0·1 mL/10 g, i.p.; 5 μL, i.c.v.).

Locomotor activity was determined after i.c.v. administration of L-NAME (10 μg). Animals were injected with L-NAME or an

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appropriate volume of saline (5 μL) and placed individually in automated activity cages (Ugo, Basile, Italy). Results are expressed as counts obtained over a 30 min period from 15–45 min following drug administration.

All drugs were purchased from Sigma and dissolved in saline on the day of use. Results indicate mean ± s.e.m. Statistical significance of differences between groups was determined by an unpaired Student's *t*-test. A probability (*P*) value of 0·05 or less was taken to indicate statistical significance.

Results

L-NAME (50 mg kg^{-1}) administered intraperitoneally produced significant anti-nociception in both the early (63·0 ± 6·8 s, compared with 102·0 ± 8·0 s in control animals, n = 11, $P < 0·05$) and late (93·8 ± 21·1 s, compared with 171·0 ± 21·2 s in control animals, n = 14, $P < 0·05$) phases of the formalin-induced paw-licking response. In contrast, L-NMMA did not exhibit anti-nociceptive activity (early phase: 100·5 ± 11·0 s, compared with 98·7 ± 8·9 s, n = 8, $P > 0·05$; late phase: 184·5 ± 26·5 s, compared with 183·6 ± 36·5 s, n = 8, $P > 0·05$).

L-NAME was also anti-nociceptive following i.c.v. administration (Fig. 1). For comparison, similar anti-nociception was achieved following administration of 1 μg L-NAME i.c.v. and 50 mg kg^{-1} i.p. Interestingly, L-NOARG (10 μg) also reduced early and late phase paw-licking time with a potency similar to that of its methyl ester salt whilst D-NAME (10 μg) was devoid of activity (Fig. 1). In contrast, L-NMMA failed to influence the late phase paw-licking at doses up to 40 μg administered i.c.v. At higher doses, L-NMMA (75–200 μg) caused a significant but not dose-related anti-nociceptive effect in both the early and late phases of the paw-licking response (Fig. 2). D-NMMA (100 μg) was inactive (Fig. 2).

L-NAME (10 μg) administered i.c.v. did not influence mouse locomotor activity over the period (15–45 min post-injection) representing the duration of the hindpaw-licking time in formalin-treated animals (854 ± 120 counts, compared with 1020 ± 182 counts, saline-treated controls, n = 6–8, $P > 0·05$).

Discussion

These experiments confirm and extend the results of a previous study in that L-NAME produces a potent and stereoselective anti-nociceptive effect in a formalin-induced paw-licking assay in the mouse (Moore et al 1991). Although not utilized in the present study we also reported a similar anti-nociceptive effect of i.p. administered L-NAME in two other models of nociception in this species, namely acetic acid-induced abdominal constriction and foot withdrawal on a hot plate (Moore et al 1991).

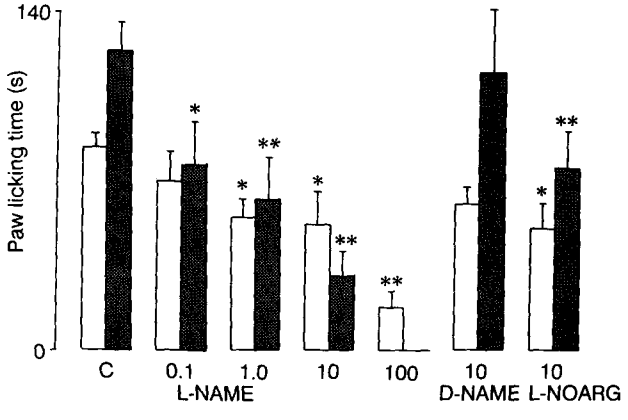

FIG. 1. Paw-licking time in mice pretreated i.c.v. with L-NAME, D-NAME or L-NOARG (μg), 15 min before intraplantar formalin injection. Control animals (C) were pretreated with saline (5 μL). Open columns indicate paw-licking time in the early phase whilst stippled columns indicate paw-licking time in the late phase. n = 5–11, $*P < 0·05$, $**P < 0·01$.

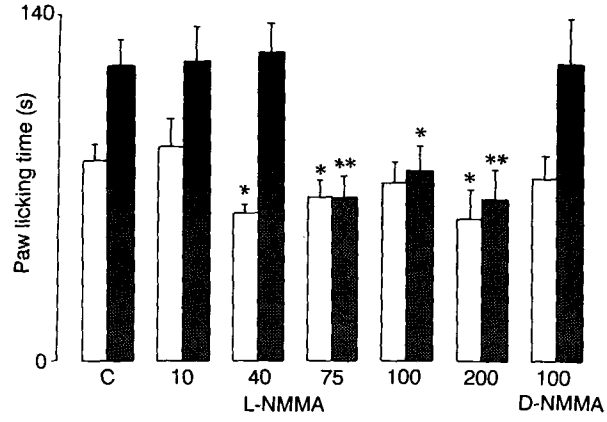

FIG. 2. Paw-licking time in mice pretreated i.c.v. with L- or D-NMMA (μg), 15 min before intraplantar formalin injection. Control animals (C) were pretreated with saline (5 μL). Open columns indicate paw-licking time in the early phase whilst stippled columns indicate paw-licking time in the late phase. n = 5–11, $*P < 0·05$, $**P < 0·01$.

That L-NAME clearly retains considerable anti-nociceptive effect following i.c.v. administration supports the hypothesis of a central mechanism of action and suggests a role for NO produced either in the brain or spinal cord in the mechanism of pain perception. L-NOARG was also anti-nociceptive following i.c.v. injection although lack of solubility in saline precluded the investigation of the anti-nociceptive activity of this inhibitor administered i.p. This result is perhaps not surprising in view of the similar potency of L-NAME and L-NOARG as inhibitors of vascular endothelial NO formation (Moore et al 1990) and NANC transmitter effects in the mouse anococcygeus preparation (Gibson et al 1990). Furthermore, L-NAME, at a dose which produced approximately 80% of the maximal anti-nociceptive effect, failed to affect mouse locomotor activity following i.c.v. administration. Similar results were obtained in a previous study in which L-NAME was administered i.p. (Moore et al 1991). For this reason we can conclude that the anti-nociceptive effect of L-NAME injected i.c.v. is not secondary to a reduction in gross locomotor activity.

A significant finding of the present study is the very weak anti-nociceptive effect of L-NMMA compared with L-NAME and L-NOARG. Indeed, L-NMMA administered i.p. failed to influence formalin-induced paw licking time. Following i.c.v. administration L-NMMA did cause a non-dose related anti-nociception but only at doses in excess of 40 μg. The lack of a clear dose-related response to L-NMMA makes it difficult to gauge accurately the relative anti-nociceptive effect of L-NAME and L-NMMA. However, by comparing single doses it would appear that 1 μg L-NAME is approximately equipotent with between 75 and 200 μg L-NMMA in the late-phase response. D-NMMA, which lacks the ability to inhibit the NO synthase enzyme (Moncada et al 1989), is devoid of activity in this model implying that the weak anti-nociceptive effect of L-NMMA may indeed be related to inhibition of central NO biosynthesis. However, we cannot exclude the possibility that the effect of L-NMMA may result from a stereoselective mechanism which is unrelated to inhibition of NO biosynthesis.

A considerable body of data is now available concerning the

relative potency of L-NAME and L-NMMA as inhibitors of NO synthase in mammalian cells. We have previously demonstrated that L-NAME is approximately 5 times more potent than L-NMMA in rabbit isolated aortic rings and in the rat perfused mesentery (Moore et al 1990). Furthermore, L-NAME is reportedly some 100 times more potent than L-NMMA as an inhibitor of NO synthase in cultured aortic endothelial cells (Gross et al 1990) and 30 times more potent in the adrenal cortex (Palacios et al 1989).

A similar or even greater discrepancy in potency has been reported for the two inhibitors in studies utilizing the neuronal NO synthase enzyme. For example, L-NOARG and L-NAME are each some 100 times more potent than L-NMMA as inhibitors of NANC relaxation in the mouse isolated anococcygeus preparation (Gibson et al 1990). Additionally, L-NAME is a potent inhibitor of NANC relaxation in the bovine retractor penis while L-NMMA is virtually without effect (Martin et al 1991). Furthermore, East & Garthwaite (1990) have suggested the existence of two NO synthase enzymes in the developing rat cerebellum which are inhibited by L-NAME with IC50 values of 6 and 600 nM compared with a single value of 2 μM for L-NMMA. Similarly, L-NAME was 200 times more potent than L-NMMA as an inhibitor of adult rat cerebellar NO synthase (Lambert et al 1991). In each of these latter studies NO biosynthesis was provoked in intact cerebellar slices by addition of NMDA. In contrast, Knowles et al (1989) observed that L-NAME and L-NMMA were approximately equal in potency using a partially purified, cytoplasmic preparation of rat cortex NO synthase. Thus, the difference in potency between the two inhibitors, which appears to be accentuated in brain tissue, may relate to a reduced ability of L-NMMA to cross biological, and in particular, neuronal membranes. Dwyer et al (1991) have recently reported that low doses of L-NAME produce an irreversible inhibition of brain NO synthase which persists for up to 5 days after injection. Such an effect provides an alternative explanation for the increased potency of L-NAME in brain tissue relative to L-NMMA and may also explain the remarkably prolonged (> 1 day) anti-nociceptive effect of L-NAME previously reported (Moore et al 1991).

The present data lend support to the numerous studies detailed above by demonstrating a functional as well as a biochemical disparity between L-NAME and L-NMMA in the central nervous system. Whether the two compounds differ in their ability to access the cytoplasmic enzyme in central neurons or whether the difference lies in the nature of the interaction with the enzyme (i.e. competitive or non-competitive) remains to be seen. These results support our previous proposal (Moore et al 1991) echoed by Dwyer et al (1991) that L-NAME will prove to be a useful tool with which to unravel the central biological effects of NO.

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