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European Journal of Pharmacology 482 (2003) 133–137
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Systemic galnon, a low-molecular weight galanin receptor agonist, reduces heat hyperalgesia in rats with nerve injury

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Received 7 July 2003

Abstract

We have examined the effect of systemically administered galnon, a novel low-molecular weight agonist of galanin receptors, on neuropathic pain-like behaviors in rats after photochemically induced partial nerve injury. Galnon is a galanin receptor ligand with moderate affinity to spinal cord membranes (K_D of 6 ± 0.6 μM). While intraperitoneally applied galnon produced no significant effect on mechanical or cold hypersensitivity, it dose-dependently prolonged heat withdrawal latency in nerve-injured rats. The effect of galnon was more potent on the injured side which has significantly shorter latency than the contralateral side. The anti-hyperalgesic effect of galanon was prevented by intrathecal M35, a galanin receptor antagonist. No side effects, such as sedation or motor impairment, were seen following systemic galnon treatment at the doses used. It is concluded that systemic galnon alleviated heat-hyperalgesic response in rats with partial sciatic nerve injury. This effect was likely to be mediated by activation of spinal galanin receptors.

Keywords: Agonist; Antinociception; Galanin receptor; Hyperalgesia; Neuropathy; Pain; Spinal cord

1. Introduction

The neuropeptide galanin may have a role in nociception at spinal level (see Xu et al., 2000 for review). Galanin-like immunoreactivity (LI) (Ch'ng et al., 1985; Skofitsch and Jacobowitz, 1985) and galanin receptor (ga11, ga12 and ga13) mRNAs have been found in dorsal root ganglion (DRG) neurons (Xu et al., 1996; Shi et al., 1997) and in dorsal horn interneurons (Parker et al., 1995; Gustafsson et al., 1996). Intrathecal (i.t.) administered galanin produces complex effects on spinal excitability with excitation at low doses and inhibition at high doses (Wiesenfeld-Hallin et al., 1988, 1989). Although the majority of studies using both electrophysiological and behavioral methods have shown an antinociceptive effect of galanin (Yanagisawa et al., 1986; Post et al., 1988; Nussbaumer et al., 1989; Wiesenfeld-Hallin et al., 1989, 1993), a hyperalgesic effect of this peptide has also been reported (Kuraishi et al., 1991; see Xu et al., 2000 for review).

Peripheral nerve injury produces increased expression of galanin in DRG neurons (Hökfelt et al., 1987) and we and others have shown that the inhibitory effect of exogenous and endogenous galanin appears to be increased after nerve injury (Wiesenfeld-Hallin et al., 1989, 1992; Verge et al., 1993; Ji et al., 1994; Shi et al., 1999; Flatters et al., 2002). In several models of neuropathic pain, spinally administered galanin has been shown to alleviate pain-like behaviors in rodents (Dray, 1999; Eaton et al., 1999; Hao et al., 1999; Yu et al., 1999; Liu et al., 2001). This has lead to the suggestion that agonists of galanin receptors may be novel analgesics.

Most galanin receptor ligands available today are peptides, vulnerable to enzymatic degradation and unable to penetrate the blood–brain barrier. Galnon (Fmoc-cycleohexylalanine-Lys-amidomethylcoumarin) is a low-molecular weight ligand of the galanin receptor that has been recently identified by application of a combinatorial library approach to the galanin pharmacophores (Saar et al., 2002). It has been shown that galnon penetrates the

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doi:10.1016/j.ejphar.2003.09.018

blood–brain barrier, displaces galanin from its binding sites in the spinal cord and exhibits anticonvulsant activity following systemic administration (Saar et al., 2002). The present study was conducted to examine the effect of systemic galnon on neuropathic pain-like behaviors in rats after photochemically induced partial sciatic nerve injury. In order to verify whether the observed effect of galnon was mediated by spinal galanin receptors, we have also tested the effect of intrathecal (i.t.) M35, galanin-(1–15)-Pro-bradykinin-(2–9), a high-affinity galanin receptor antagonist (Wiesenfeld-Hallin et al., 1992).

2. Materials and methods

2.1. Binding studies

Two adult male rats (Sprague–Dawley, 150–200 g, B&K Universal, Sollentuna, Sweden) were decapitated and the lumbar spinal cord was rapidly dissected and divided into dorsal and ventral parts. The tissue was homogenized on ice with a teflon-glass homogenizer (10 strokes at 695 rpm) in 0.32 M sucrose buffered with 5 mM Hepes (pH 7.4). The homogenate was diluted 10 times in sucrose buffer and centrifuged at $1000 \times g$ at 4 °C for 10 min. The obtained supernatant was further centrifuged at $10\,000 \times g$ at 4 °C for 45 min and the pellet was resuspended in Hepes buffered Krebs–Ringer solution (HKR) (5 mM Hepes, 137 mM NaCl, 2.68 mM KCl, 2.05 mM $MgCl_2$, 1.8 mM $CaCl_2$; 1 g/l glucose), pH 7.4, supplemented with 0.05% (w/v) bovine serum albumin (BSA) and protease inhibitor cocktail (1:300 dilution) (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined according to Lowry method modified by Peterson (1977). Displacement experiments were performed in a final volume of 400 μl 5 mM HKR buffer, supplemented with 0.05% BSA containing 50–100 pM porcine [^{125}I]galanin (Amersham Biosciences, UK), 70–100 μg lumbar dorsal spinal cord membrane preparation and varying concentrations of galnon (10^{-11}–10^{-4} M). Galnon was dissolved in HKR buffer containing 10% DMSO. Samples were incubated for 30 min at 37 °C in a shaking water bath. Incubation was terminated by the addition of 2×10 ml ice-cold HKR buffer, supplemented with 0.05% (w/v) BSA, followed by rapid filtration over presoaked (0.3% (w/v) polyethylenimine solution) Whatman GF/C glass fiber filters (Whatman International, Mainstone, UK). Radioactivity retained on the filters was determined in a gamma counter (Packard Instrument, Meriden, CT). All tubes and pipette tips used for galnon solutions were silanized before the experiments (dichlorodimethylsilane). Nonspecific binding was determined as the part of total binding of [^{125}I]galanin that could be displaced with 1 μM galanin. IC_{50} values for rat galanin and galnon were calculated using program Prism (GraphPad Software, San Diego, CA) and converted into K_D values using the equation of Cheng–Prusoff.

2.2. Photochemically induced sciatic nerve ischemia

For behavioral studies, adult male Sprague–Dawley rats (Mollegård, Denmark) weighing 250–300 g at the time of surgery were used. The experiments were carried out according to the Ethical Guidelines of the International Association for the Study of Pain and were approved by the local research ethics committee.

The technique for producing sciatic nerve ischemic injury has been described in detail (Kupers et al., 1998). The rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and the common sciatic nerve was exposed at mid-thigh level. The part of the sciatic nerve just proximal to trifurcation was irradiated with a laser beam for 2 min with a tunable argon laser (Innova model 70, Coherent Laser Products Division, Palo Alto, CA, USA) operating at 514 nM. The light was focused into a 0.3-mm beam via a light optics system (Melles Griot, USA). The rat was positioned so that the laser beam was perpendicular and transversal to the exposed nerve. Immediately before the irradiation, erythrosin B (Aldrich, 32.5 mg/kg dissolved in 0.9% saline) was injected i.v. via a catheter inserted into a jugular vein. After irradiation, the wound was closed in layers. The nerve was exposed on the contralateral side, but not irradiated.

2.3. Implantation of i.t. catheters

After anesthesia with methohexital (Brietal, Lilly), a catheter (PE 10, o.d. 0.61 mm) was inserted into the subarachnoid space through a guide cannula connected to a 20-gauge needle which punctured the dura at the level of the cauda equina. The catheter was then carefully implanted rostrally, aiming its tip at the lumbar enlargement. The location of the catheter was tested 24 h before the pharmacological experiments by assessing sensory and motor blockade after i.t. injection of 7 μl lidocaine (50 mg/ml, Astra).

2.4. Behavioral tests

A set of calibrated von Frey hairs was used to assess mechanical allodynia. The rats were put in chambers with metal mesh floors. von Frey hairs were applied in ascending order on the plantar surface of the hindpaw at a frequency of 1/s. The lowest force at which the animal withdrew the paw or vocalized in at least two out of three trials was taken as mechanical response threshold. The highest intensity of stimulation used was 73 g as stronger stimuli lifted the paw.

The response to cold was tested with ethyl chloride (Medikema, Perstorp, Sweden), which was briefly (< 1 s) sprayed on the plantar surface of the hind paw. The responses were scored as the following: 0 = no response, 1 = startle-like response, no hindpaw withdrawal, 2 = brief withdrawal of the stimulated hindpaw, 3 = sustained or repeated withdrawal of the stimulated hindpaw, brief licking or shaking and 4 = prolonged withdrawal, shaking and

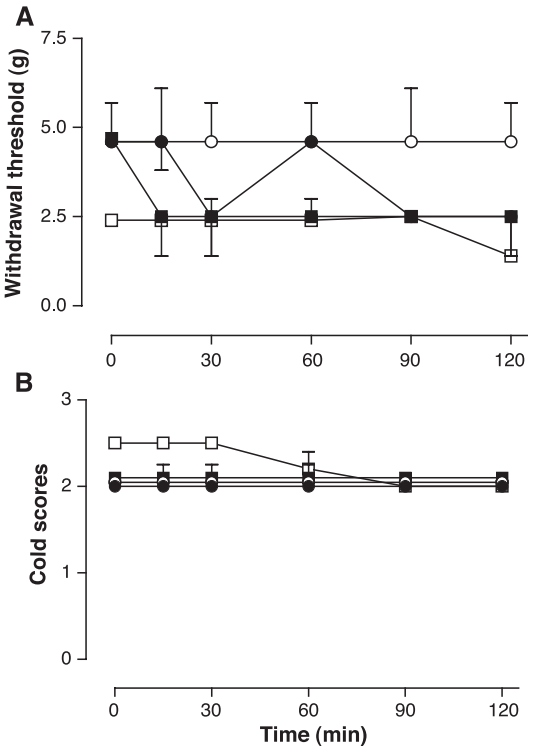

Fig. 1. Effect of vehicle (open circles, $n=6$), 1 (open squares, $n=7$), 2 (filled circles, $n=7$) or 5 mg/kg (filled squares, $n=7$) i.p. galnon on paw withdrawal threshold to mechanical stimuli (A) and cold response scores (B) in nerve injured rats for the paw ipsilateral to the injury. The data are expressed as median ± M.A.D. The Wilcoxon signed ranks test did not show any significant differences at any time point among the groups.

licking of the hindpaws, vocalization and generalized aversive reactions.

The response to heat was tested with a modification of the Hargreaves method (Hargreaves et al., 1988, Ugo Basile, Italy). The rats were lightly held and the plantar surface of the hind paw was stimulated with a radiant heat source through a transparent plastic floor and the latency to withdrawal of the stimulated paw was measured.

2.5. Drugs

Galnon was synthesized according to the method described previously (Saar et al., 2002) and dissolved in 20% DMSO in Cremopher EL (Sigma). Galnon at various concentrations was injected i.p. in a volume of 1 ml/kg. M35 was synthesized on a model 431A peptide synthesizer (Perkin-Elmer/Applied Biosystems) as previously described (Langel et al., 1992) and dissolved in saline. M35 was injected i.t. in a volume of 10 μl followed by 15 μl saline to flush the catheter.

2.6. Statistics

The results from von Frey hair and cold tests were presented as median ± median-derived absolute deviation (MAD) and were analysed with the Wilcoxon signed ranks test. The results from the heat test were presented as mean ± S.E.M. and analysed with ANOVA with repeated measures followed by Dunnett test or unpaired *t*-test.

3. Results

Galnon displaces [^{125}I]galanin from the spinal cord galanin receptors with moderate affinity, K_D of 6i ± 0.6 μM (data not shown). This is well in accordance with the previously characterized affinity of galnon in rat hippocampus (Saar et al., 2002).

Rats exhibiting mechanical, cold and heat hypersensitivity 1 week after photochemically induced partial sciatic nerve injury were used in the study. The mechanical and cold hypersensitivity were present bilaterally, but were more profound on the ipsilateral than the contralateral side. The heat hypersensitivity was observed primarily in the ipsilateral side shown by significantly shortened paw withdrawal latency in comparison to the contralateral side. Galnon was injected i.p. at 1, 2 and 5 mg/kg and produced no observable

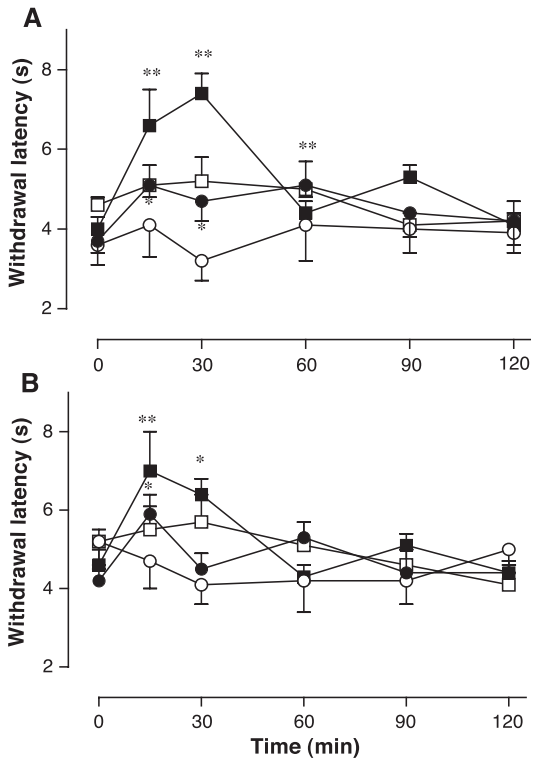

Fig. 2. Effect of vehicle (open circles, $n=6$), 1 (open squares, $n=7$), 2 (filled circles, $n=7$) or 5 mg/kg (filled squares, $n=8$) i.p. galnon on paw withdrawal latency to heat in the paw ipsilateral (A) or contralateral (B) to nerve injury. The data are expressed as mean ± S.E.M. ANOVA with repeated measures. A significant increase in response latency was observed following administration of 2 and 5 mg/kg galnon, but not 1 mg/kg galnon or vehicle, for both ipsilateral and contralateral paws. Individual comparisons were made with the Dunnett test, $^{*}p<0.05$. and $^{**}p<0.01$.

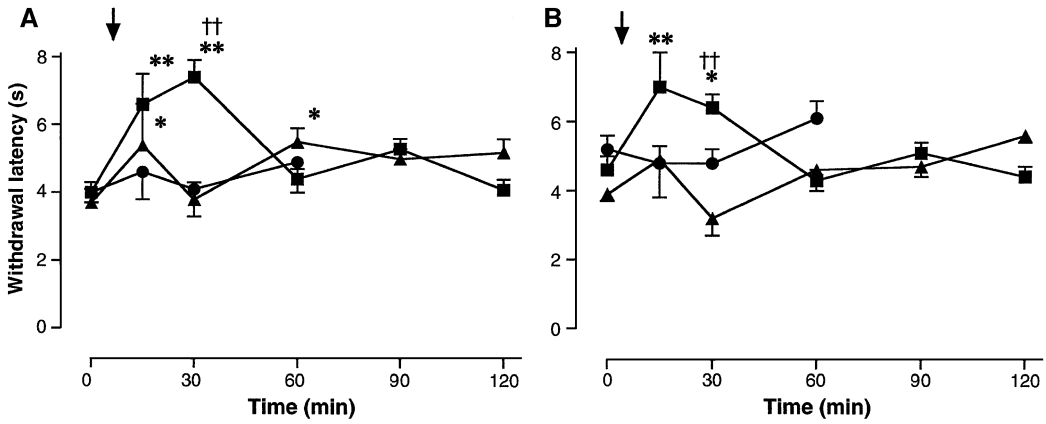

Fig. 3. Effect of 660 ng i.t. M35 on 5 mg/kg galnon's effect on heat paw withdrawal latency on the ipsilateral (A) and contralateral paws (B). M35 was injected 10 min (arrow) after i.p. galnon ($n=6$, triangles). Rats receiving only 5 mg/kg i.p. galnon ($n=8$, squares) or only 660 ng i.t. M35 ($n=5$, circles) were used as controls. The data are expressed as mean ± S.E.M. ANOVA with repeated measures indicating that there are significant interactions between groups and times on paws, indicating that M35 significantly reduced the effect of galnon. Individual comparisons were made with Dunnett test, $*p<0.05$ and $**p<0.01$ compared to control value and with unpaired t-test, $††p<0.01$ compared to the groups receiving M35 plus galnon. M35 alone did not significantly affected paw withdrawal latency up to 1 h after i.t. administration.

side effect (irritation, sedation, motor abnormalities, etc.). In the paw ipsilateral to the injury, the response to mechanical or cold stimulation was not altered by galnon at any dose (Fig. 1), whereas paw withdrawal latency to heat stimulation for both paws was significantly and dose-dependently increased following galnon treatment (Fig. 2). The effect of galnon was stronger on the ipsilateral than the contralateral side (Fig. 2). Vehicle produced no effect.

In additional six rats, 660 ng M35 was administered i.t. 10 min after i.p. administration of 5 mg/kg galnon. This time frame was chosen as previously experience suggested that the effect of M35 after i.t. injection is quite brief. The antinociceptive effect of galnon was significantly reduced by M35 treatment at 30 min on both sides (Fig. 3). ANOVA with repeated measures also revealed an overall interaction between the two groups and time for both ipsilateral and contralateral paw (Fig. 3). Finally, i.t. administration of M35 at 660 ng alone produced no significant effect on paw withdrawal latency to heat stimulation (Fig. 3).

4. Discussion

The results indicate that systemically applied galnon produced an antihyperalgesic effect against heat stimulation in a rat model of neuropathic pain after partial nerve injury. The effect of galnon was reduced by i.t. administration of the galanin receptor antagonist M35. These results thus support the notion that galnon is a low-molecular weight galanin agonist with moderate affinity at galanin spinal cord receptors that is able to penetrate the blood–brain barrier and exert CNS effects (Saar et al., 2002). The results are also similar to previous finding showing that activation of galanin receptors in the spinal cord produces antinociception under normal conditions and after nerve injury (Post et al., 1988; Wiesenfeld-Hallin et al., 1993; Dray, 1999; Hao et al., 1999; Yu et al., 1999; Liu et al., 2001; Hua et al., 2002). The effect of galnon is stronger on the injured side than on the contralateral side. This may be due to an enhanced depressive effect of galanin in response to nerve injury (Wiesenfeld-Hallin et al., 1989; Flatters et al., 2002).

We have previously shown that i.t. galanin alleviated mechanical and cold allodynia after ischemic nerve injury (Hao et al., 1999). However, mechanical and cold allodynia-like behavior after nerve injury was not affected by i.p. galnon. This may be due to a low spinal concentration of galnon following systemic administration and/or by the moderate affinity of galnon to galanin receptors (Saar et al., 2002). The results further suggest heat hyperalgesia (mediated by C-fibers) is more sensitive to the effect of galanin receptor activation than mechanical allodynia (possibly mediated by A-β fibers). This supports previous results that in normal rats the inhibitory effect of galanin was preferential against C-fiber input (Wiesenfeld-Hallin et al., 1989; Xu et al., 2000). Furthermore, galanin over-expressing mice are hypoalgesic to thermal, but not mechanical, stimuli (Hygge Blakeman et al., 2001) whereas galanin receptor 1 knock-out mice exhibit hyperalgesia to heat, but not to mechanical, stimulation (Bleakman-Hygge et al., 2003). These recent results suggest that the galanin receptor 1 has an antinociceptive function.

Galnon which is the first systemically active galanin receptor agonist is acting as a receptor subtype nonselective ligand that are able to act at GALR1 and at other (GALR2 and GALR3) galanin receptors in the spinal cord and CNS. Similarly, M35 the chimeric peptidergic galanin receptor antagonist blocks all three galanin receptor subtypes and were able to antagonize the effect of galnon upon i.t. administration. The present data can thus partly be explained by galnon action at spinal galanin receptors, although contribution of brain galanin receptor to the effect cannot be excluded.

Acknowledgements

The present study is supported by Swedish Medical Research Council (07913 to ZWH and 12168 to XJX) and research funds of the Karolinska Institute.

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