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Pulsed Magnetic Field Induced ''Analgesia'' in the Land Snail, *Cepaea nemoralis*, and the Effects of μ, δ, and κ Opioid Receptor Agonists/Antagonists

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THOMAS, A. W., M. KAVALIERS, F. S. PRATO AND K.-P. OSSENKOPP. *Pulsed magnetic field induced ''analgesia'' in the land snail,* Cepaea nemoralis*, and the effects of μ, δ, and κ opioid receptor agonists/antagonists*. PEPTIDES **18**(5) 703–709, 1997.—A brief exposure to a pulsed magnetic field (Cnp: patent pending) had significant antinociceptive or ''analgesic'' effects in the land snail, *Cepaea nemoralis*, as evidenced by an increase in the latency of response to a warmed (40°C) surface. This analgesia was in part opioid mediated being significantly reduced, but not eliminated: by the prototypic opiate antagonist, naloxone; the μ (mu) opioid receptor directed antagonists, naloxazine or β-funaltrexamine, and the δ (delta) opioid receptor directed antagonists, naltrindole-5′-isothiocyanate or ICI 174,864. However the Cnp induced analgesia was unaffected by the κ (kappa) opioid receptor directed antagonist, nor-binaltorphimine. The δ_1 and δ_2 opioid receptor directed agonists, (DPDPE, [D-Pen^2,D-Pen^5]enkephalin), (deltorphin, [D-Ala^2,Glu^4]), respectively, also had significant differential analgesic effects, supporting a functional δ opioid receptor mediated enkephalinergic mechanism in *Cepaea*. These results suggest that this specific pulsed magnetic field (Cnp) elicits significant analgesic effects through mechanisms that, in part, involve δ and, to a lesser extent, μ opioid receptors. © 1997 Elsevier Science Inc.

Pulsed magnetic field Analgesia Snail Opioid Deltorphin DPDPE Naloxone Naloxazine 5′-NTII Nor-binaltorphimine β-Funaltrexamine ICI-174,864

THERE is growing evidence that weak magnetic fields can have a variety of behavioral effects in animals. Relatively weak time-varying magnetic fields, especially those in the extremely low frequency (ELF) range (<300 Hz), have been shown to affect behavioral responses, such as; reduce exogenous opiate and endogenous opioid peptide mediated analgesia in various species, including that of humans (4,14,15,22).

Although initial attention was primarily focused on vertebrates, there is considerable evidence that magnetic fields also affect opioid mediated responses in invertebrates (14). Opioid peptides are similarly involved in the modulation of various functions, including that of thermal escape behaviors (nociceptive response), in vertebrates and invertebrates. The prototypic opiate agonist morphine, as well as endogenous opioid peptides, can enhance the latency of response of terrestrial molluscs, such as the snail *Cepaea nemoralis*, to a thermal stimulus in a manner analogous to the anti-nociceptive or analgesic responses reported in mammals (3,13,29). Also, the prototypic opiate antagonist, naloxone (32), as well as more specific antagonists can reduce these opioid induced responses.

These opioid mediated antinociceptive responses of *Cepaea* have provided a valuable model with which to investigate the effects of magnetic field exposure. Results of studies with *Cepaea* have shown that sinusoidal ELF magnetic fields can reduce morphine, enkephalinase induced and enkephalin mediated analgesia. In addition, results of more recent investigations have also indicated that certain other types of magnetic fields may either increase or actually induce analgesia (16,34).

Time-varying repeated-pattern magnetic fields, or pulsed magnetic fields (complex neuroelectromagnetic pulse (Cnp),

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patent pending), also have been shown to exert a number of biological effects, again, possibly through alterations of opioid activity (8,23,35). Results of preliminary investigations revealed that a brief (15 min) exposure of *Cepaea* to the Cnp induced an antinociceptive response that was partially sensitive to the prototypic opiate antagonist, naloxone (34). This suggested that the analgesic effect of this specific pulsed magnetic field may, at least partially, involve opioid systems.

Opioid peptides are considered to exert their effects through three main types (and subtypes) of receptors, delta ($\delta_{1,2}$), mu ($\mu_{1,2}$) and kappa (κ_{1-3}). The delta opioid receptor directed peptide, methionine enkephalin, has been immunocytochemically localized in various ganglia in *Cepaea* (30) as well as other species of snails (7). In addition the specific enkephalinase inhibitor, SCH 34826, has significant antinociceptive effects in *Cepaea* (31), likely involving augmented enkephalin activity at delta opioid receptors. Similarly, evidence from rodents supports the involvement of δ_1 and δ_2 opioid receptors in the mediation of enkephalin sensitive analgesia (11,19). This suggests the existence of functional enkephalinergic systems in *Cepaea* that are associated with the mediation of antinociception.

In the present study, we considered the possible opioid receptor(s) associated with the ''analgesic'' effects of a brief (15 min) exposure of *Cepaea* to a Cnp magnetic field. We examined the effects of: the prototypic opiate antagonist, naloxone (32), the selective δ antagonist ICI 174,864 (1), the specific δ_2 antagonist naltrindole-5′-isothiocynate (5′-NTII) (26), the μ opioid receptor antagonists, naloxazine (9) and β-funaltrexamine, and a κ opioid receptor antagonist, nor-binaltorphimine (25) on the Cnp-induced analgesic responses of *Cepaea*. In addition, in view of the evidence for functional enkephalinergic systems in *Cepaea*, we considered in more detail the delta opioid receptor mediated antinociceptive responses. We examined the effects of the specific δ_1 and δ_2 opioid receptor agonists, [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (21) and [D-Ala^2,Glu^4] (deltorphin) (6,17), respectively, and various antagonists on the nociceptive responses of *Cepaea*.

METHOD

Animals

Snails were collected from old field sites in London, Ontario which did not have overhead or underground electric transmission lines (<0.01 μT ambient magnetic fluctuation). The animals were held in a terrarium (ambient fluctuating magnetic fields <0.4 μT) under indirect natural, and fluorescent, lighting at an approximate 12 h L:12 h D cycle, at 20 ± 2°C, with lettuce available ad lib.

Assessment of Nociception

As the activity of gastropods is affected by their state of hydration (33), all snails were allowed to fully hydrate under a saturated atmosphere at 20 ± 2°C before being tested.

Individual fully-hydrated snails were placed on a warmed surface (''hotplate''; 40 ± 0.2°C) and the latency of their ''escape from'' or ''avoidance of'' the thermal stimulus, or more appropriately ''withdrawal reflex'', was determined. The avoidance behavior was a characteristic elevation of the anterior portion of the fully extended foot, the behavioral endpoint being the time the foot reached maximum elevation (13). After displaying this aversive, or more appropriately, ''nociceptive'' response (5,12), individual snails were quickly removed from the thermal surface. The hotplate, which does not produce any magnetic fields, consisted of an aluminum water jacket with a stainless steel top (33 × 33 cm) with water pumped through it from a circulating water bath.

Experiment 1: Experimental Procedures

At midphotophase separate groups (n = 10–12 per group) of hydrated snails were injected with either the δ_1 selective agonist, DPDPE (0.001, 0.01, 0.05 or 0.10 μg/1.0 μl saline; Research Biochemicals, Natick, MA), the δ_2 agonist, deltorphin (0.001, 0.01, 0.05 or 0.10 μg/1.0 μl saline; Research Biochemicals, Natick, MA) or 0.9‰ saline vehicle (1.0 μl). Response latencies of the snails were determined prior to, and 15, 30, 45 and 60 min after injection.

Other groups of hydrated snails (n = 10–12 per group) were injected with either the prototypic opiate antagonist, naloxone (1.0 μg/1.0 μl saline; Sigma, St. Louis, MO), the δ antagonists, ICI 174,864 (1.0 μg/1.0 μl saline; Research Biochemicals, Natick, MA), the δ_2 antagonist 5′-NTII (1.0 μg/1.0 μl saline; Research Biochemicals, Natick, MA) or saline vehicle (1.0 μl) 15 min prior to receiving either DPDPE (0.10 μg/1.0 μl saline), deltorphin (0.10 μg/1.0 μl saline) or saline vehicle (1.0 μl). Response latencies of the snails were determined prior to the peptide or saline injections, as well as 15, 30, 45 and 60 min after the treatment. The doses and time courses of the antagonists, which had no evident effects on basal response latencies, were established in prior (13,14,31) and pilot studies.

All solutions were injected with a 2.0 μl microsyringe (No. 75, Hamilton, NV) in either the vicinity of, or directly in, the mantle cavity into the haemocoel. Injections were made on the basis of 1.0 g body mass. The body mass of snails, without shells, range from 0.7 to 1.3 g.

Experiment 2: Experimental Apparatus

In the magnetic field and sham exposures, groups of 10–15 snails were placed in a polypropylene container (12 cm square, 5 cm high) in the center of three mutually orthogonal Helmholtz coils (1.2 m for the coil that generated a vertical field and 1.1 m and 1.0 m for the coils that generated horizontal fields. Details of the coils and amplifiers are provided in Prato et al.(28)). A computer driven 8-bit resolution digital to analog converter (S. Koren, Neuroscience Research Group, Laurentian University, Sudbury, Ontario) was used to produce the pulsed waveforms. Magnetic fields were measured with a fluxgate magnetometer (model FGM-3D1) and a field monitor (model ELF-66D) both Walker Scientific, Worcester, MA.

Magnetic Field Exposure Conditions

The 15 min magnetic field exposures consisted of a specific low frequency pulsed magnetic field (Complex neuroelectromagnetic pulse (Cnp, patent pending) (Fig. 1)) set to 100 μT peak amplitude in the vertical direction.

Sham exposures consisted of a three-dimensionally (3-D) zeroed earth field (Helmholtz coils tuned to oppose the Earth's magnetic field to within ±0.1 μT, horizontal component = 14.7 μT, vertical component = 43.3 μT.)

Experimental Procedures

At midphotophase separate groups (n = 15 per group, with several replicate groups per treatment) of hydrated snails were exposed to either a specific pulsed magnetic field (Cnp) or sham magnetic field for 15 min. Response latencies of the snails were determined prior to (Pre), immediately after (0) and 15, 30 and

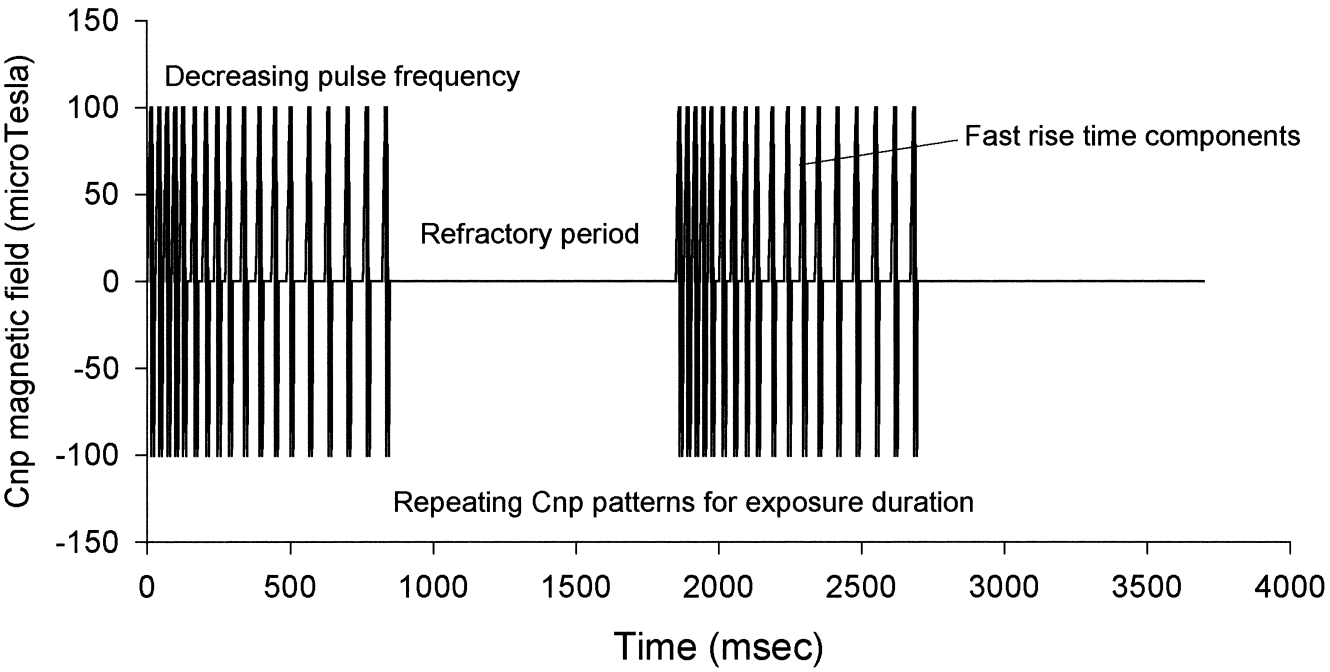

FIG. 1. The complex neuroelectromagnetic pulse (Cnp) incorporates three basic components: (i) the pulse sequence starts at a specific set of frequencies; (ii) the frequency of the pulse is then either decreased, or increased, to alter the entrained endogenous system in a preferred direction; (iii) a refractory period allows for a partial stabilization of endogenous frequencies. The Cnp sequence is continually repeated during the exposure period.

60 min after exposure. One individual carried out the exposure conditions, while a second experimenter in a separate room determined the response latencies.

Other groups ($n = 10-15$ per group, with several replicate groups per treatment) of hydrated snails were injected with either the prototypic opiate antagonist, naloxone (1.0 μg/1.0 μl saline), the general δ_1 and δ_2 opiate antagonist, ICI 174,864 (1.0 μg/1.0 μl saline), the specific δ_2 antagonist, 5′-NTII (0.1 μg/1.0 μ saline), the μ opiate antagonist, β-funaltrexamine (1.0 μg/1.0 μl saline; Research Biochemicals, Natick, MA), the μ_1 opiate antagonist, naloxazine (1.0 μg/1.0 μl ethanol vehicle), the κ opiate antagonist, nor-binaltorphimine (1.0 μg/1.0 μl saline; Research Biochemicals, Natick, MA), saline (1.0 μl) or ethanol vehicle (1.0 μl) prior to being exposed to either the Cnp or sham condition for 15 min. Additional groups of snails received no injections. Naloxone, ICI 174,864 and 5′-NTII were injected 15 min prior to exposure, naloxazine and β-funaltrexamine 24 h before exposure, and nor-binaltorphimine 2 h before exposure. Response latencies of the snails were determined prior to the injections, prior to the Cnp or sham exposure (for the 2 and 24 h prior injections) and immediately, 15, 30 and 60 min after exposure.

Data were analyzed with one and two-way repeated measures analyses of variance (ANOVA) using The Statistical Package for Social Sciences (SPSS 7.0). Post-hoc analyses were done with Tukey's HSD test. All hypotheses tests used $\alpha = 0.05$ as the criterion for significance.

RESULTS

Experiment 1

The delta-opiate receptor agonists, DPDPE (δ_1) ($F(1,75) = 87.51$, $p < 0.001$, $\text{Eta}^2 = 0.54$) and deltorphin (δ_2) ($F(1,74) = 34.35$, $p < 0.001$, $\text{Eta}^2 = 0.32$) elicited significant increases in response latency indicative of the induction of analgesia (Fig. 2A–B). The effects of both agonists were linearly dose dependent with 0.1 μg having a significantly greater effect. As well, maximum effects of both agonists were evident at 15–30 min post-injection with a decline toward basal levels by 60 min post-injection (DPDPE, $F(3,75) = 273.35$, $p < 0.001$, $\text{Eta}^2 = 0.92$, Fig. 2A) (deltorphin, $F(3,74) = 216.89$, $p < 0.001$, $\text{Eta}^2 = 0.80$, Fig. 2B). There were no significant differences in pre-injection latencies. Saline vehicle injection had no significant effect on response latency.

Analysis of variance on the response latency revealed a significant overall interaction of the agonist/antagonist combinations ($F(2,58) = 110.27$, $p < 0.001$, $\text{Eta}^2 = 0.79$) (Fig. 3A–B). Both DPDPE ($F(1,72) = 697.52$, $p < 0.001$, $\text{Eta}^2 = 0.91$) and deltorphin ($F(1,72) = 310.70$, $p < 0.001$, $\text{Eta}^2 = 0.81$) induced significant analgesia as compared to injection of saline vehicle. Pre-treatment with naloxone significantly ($p < 0.05$) reduced, but did not block, the antinociceptive effects of DPDPE or deltorphin. ICI 174,864 also reduced ($p < 0.05$), but did not block, the antinociceptive effects of DPDPE or deltorphin. Pre-treatment with 5′-NTII (δ_2 antagonist) did not significantly affect the DPDPE (δ_1 agonist) induced antinociception (Fig. 3A). However, 5′-NTII (δ_2 antagonist) completely blocked the deltorphin (δ_2 agonist) induced antinociception (Fig. 3B).

Experiment 2

Exposure to the Cnp produced a significant increase ($F(1,116) = 1205.41$, $p < 0.001$, $\text{Eta}^2 = 0.91$) in the latency of the foot-lifting response, indicative of the induction of analgesia (Fig. 4). Post-hoc analyses revealed that significant analgesia was induced from 15–75 min post Cnp exposure ($p < 0.05$),

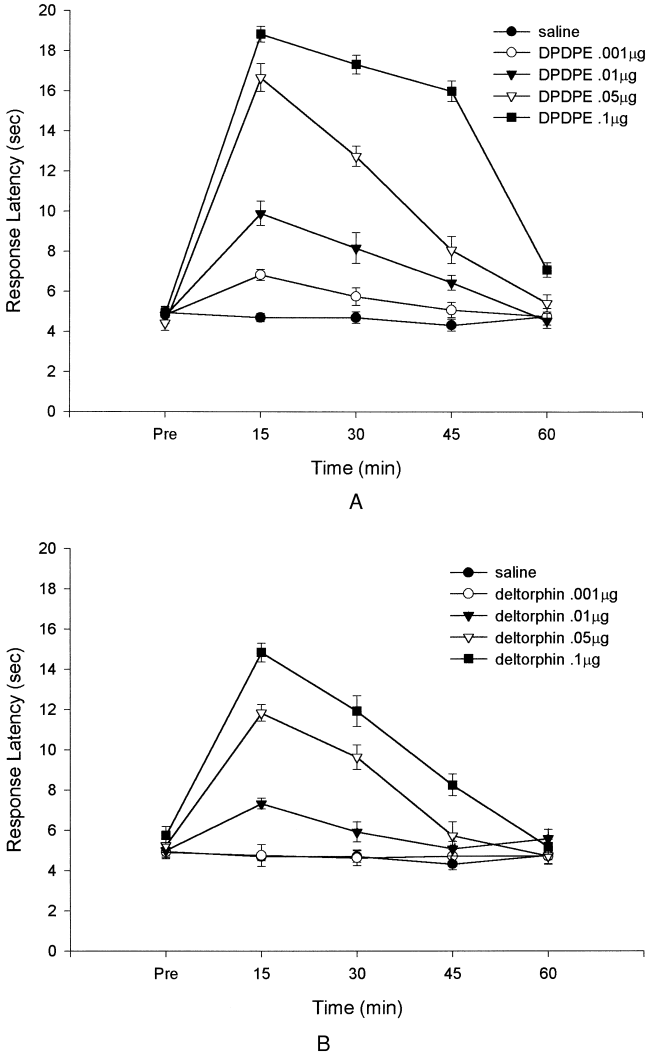

FIG. 2. Effects of (A) [D-Pen^2,D-Pen^5]enkephalin (δ_1 agonist) (DPDPE; 0.001, 0.01, 0.05 or 0.10 μg) and (B) [D-Ala^2,Glu^4]deltorphin (δ_2 agonist) (deltorphin; 0.001, 0.01, 0.05 or 0.10 μg) or saline vehicle (1.0 μl) on the thermal (40°C) response latencies of individual hydrated snails. Response latencies were determined prior to (Pre) and after injection. $n = 10-12$ snails per group. Error bars represent the standard error of the mean (SEM).

with maximal analgesia evident at 15 to 45 min. Sham exposure conditions had no significant affect on response latencies.

There were no significant differences found within the pre-exposure latencies, or the sham exposed latencies ($n = 423$ and $n = 211$, respectively) (Fig. 5).

A two-way analysis of variance, exposure condition (2) by type of injection (9), revealed that there was a significant increase in response latency, hence induction of analgesia, in the Cnp exposed group ($F(1,405) = 830.71$, $p < 0.001$, Eta^2 = 0.67). There was a significant main effect of the injection type (various opioid antagonists, vehicles and uninjected condition) ($F(8,405) = 11.75$, $p < 0.001$, Eta$^2 = 0.19$). However, the interaction of exposure condition and injection type was also significant ($F(8,405) = 12.04$, $p < 0.001$, Eta$^2 = 0.19$). This significant interaction was further examined with a one-way analysis of variance on the response latency of the Cnp exposed snails across the various injection types ($F(8,203) = 17.59$, $p < 0.001$). Post hoc analysis (Tukey's HSD) revealed that neither the injections of the vehicle solutions (saline, ethanol) nor the nor-binaltorphimine (κ antagonist) significantly decreased the Cnp induced analgesia. The μ and δ antagonists, however, produced a significant decrease in the Cnp induced analgesia. In all cases, there was still a significant Cnp induced analgesic response after treatment with μ or δ opioid antagonists ($p < 0.05$). There were no significant differences between the levels of Cnp induced analgesia evident after the μ and δ antagonist injections.

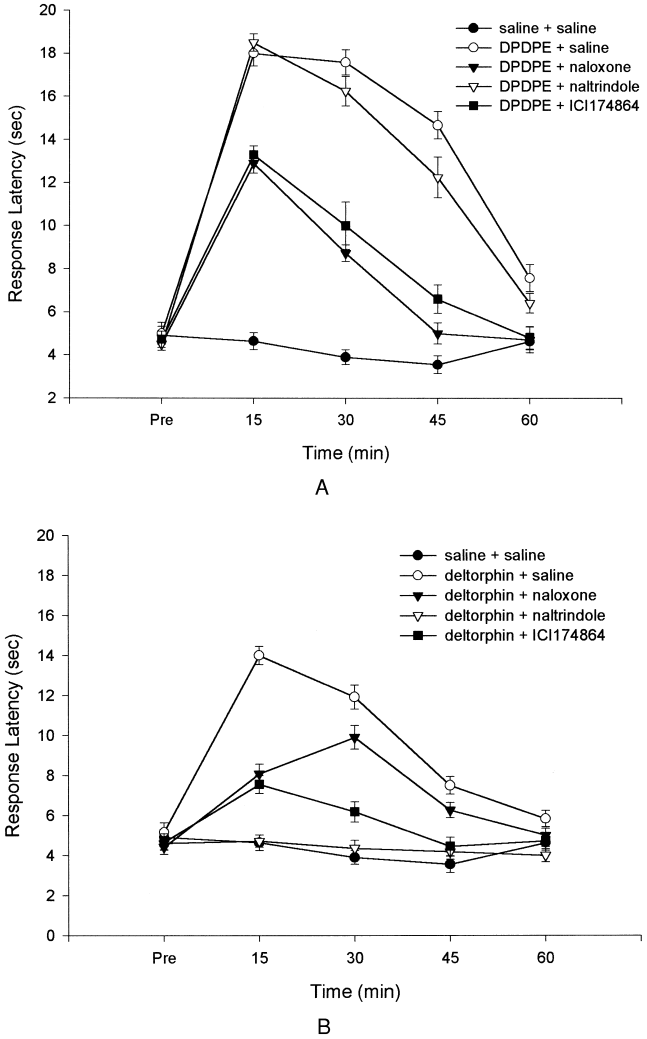

FIG. 3. Effects of pretreatment with either naloxone (1.0 μg), ICI 174,864 (1.0 μg), 5′-NTII (0.10 μg) or saline vehicle (1.0 μl) on the thermal (40°C) response latencies of individual hydrated snails injected with either (A) the δ_1 agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 0.10 μg) or (B) the δ_2 agonist [D-Ala^2,Glu^4]deltorphin (deltorphin, 0.10 μg). Response latencies of snails just receiving saline vehicle (2 injections of 1.0 μl each) are also shown. The antagonists were injected 15 min prior to the agonist treatment. Response latencies were determined prior to (Pre) and after the δ_1 and δ_2 agonist treatments. $n = 10-12$ snails per group. Error bars represent the SEM.

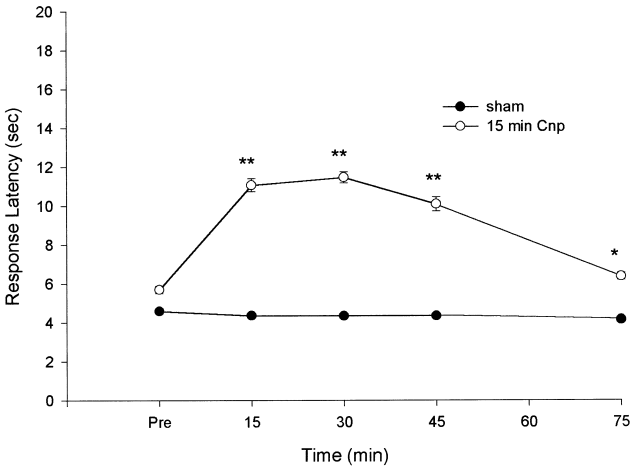

FIG. 4. Effects of a 15 min exposure to either a specific pulsed magnetic field (Cnp) or sham exposure condition on the thermal (40°C) response latencies of individual hydrated snails. Response latencies were recorded prior to (Pre) and after the 15 min Cnp and sham exposures. $n = 15$ snails per group, with several groups per treatment ($n = 120$). There were no significant differences between groups receiving the same treatment. Error bars represent the SEM. Where error lines are not visible they are embedded within the line symbol.

DISCUSSION

The results of the present study show that brief exposure to specific pulsed time-varying ELF magnetic fields (Cnps) have antinociceptive or ''analgesic'' effects in the snail, *Cepaea*. Exposure to a weak specific pulsed magnetic field resulted in a significant increase in the latency of the response of *Cepaea* to an aversive thermal surface, indicative of the induction of analgesia. This reinforces prior reports of specific pulsed magnetic fields having behavioral effects (8,23,35).

The effects of the Cnp were reduced, but not blocked, by the prototypic opiate antagonist, naloxone, the δ opioid receptor directed antagonists, ICI 174,84 or 5′-NTII, and to a lesser extent by the μ opioid receptor directed antagonists, naloxazine or β-funaltrexamine. However, the analgesic responses were unaffected by pretreatment with the κ opioid receptor antagonist, norbinaltorphimine. This indicates that the antinociceptive effects of the pulsed magnetic fields involve, at least partially, δ and μ opioid receptor directed mechanisms. The lack of a complete blockade of the antinociceptive response by the opioid antagonists also indicates that ''non-opioid'' as well as opioid mediated mechanisms are associated with the effects of the Cnp. Both opioid and non-opioid mediated analgesic responses are apparently phylogenetically conserved and expressed in both rodents and *Cepaea* (12).

In parallel, it was found that administration of either the δ_1 or δ_2 opioid receptor agonists, DPDPE and deltorphin, respectively, had significant dose-related antinociceptive effects in *Cepaea*. These decreases in nociceptive sensitivity are analogous to the analgesic effects of δ opioid ligands reported in laboratory rodents (11,19). The antinociceptive effects of the δ_1 agonist, DPDPE, were significantly reduced, but not completely blocked, by both the general opiate antagonist, naloxone, and the selective δ antagonist, ICI 174,864, with no significant effects of the specific δ_2 antagonist, 5′-NTII. The effects of the δ_2 agonist, deltorphin, were also significantly reduced by naloxone and ICI 174,864 and completely blocked by the specific δ_2 antagonist, 5′-NTII. This is consistent with the specificity of δ agonists and antagonists on nociception in rodents (11,19). This indicates that both δ_1 and δ_2 opioid receptors are present and likely involved in the mediation of antinociception in *Cepaea*. Previous studies with rodents, and to a lesser extent *Cepaea* and other molluscs, have established that highly specific μ and κ opioid antagonists have relatively little effect on δ opioid-induced antinociception (10,14). It should, however, be noted that there are suggestions of a possible functional association, or coupling, of μ and δ opioid receptors (24).

The results obtained with the δ opioid agonists are consistent with the immunohistochemical localization of the endogenous δ opioid ligands leucine-, and in particular, methionine-enkephalin in *Cepaea* (5,30) and other molluscs (7). These results are also consistent with the electrophysiological and immunological studies supporting enkephalin mediated responses in snails and other molluscs (5,20). Present findings also agree with the demonstrations in *Cepaea* of significant antinociceptive effects of a specific enkephalinase inhibitor and methionine-enkephalin (31). Together, these findings support the presence of functional δ_1 and δ_2 receptor subtypes in *Cepaea* and further indicates the similarity of the expression and mediation of antinociceptive responses in rodents and *Cepaea*. These findings also suggest that Cnps may

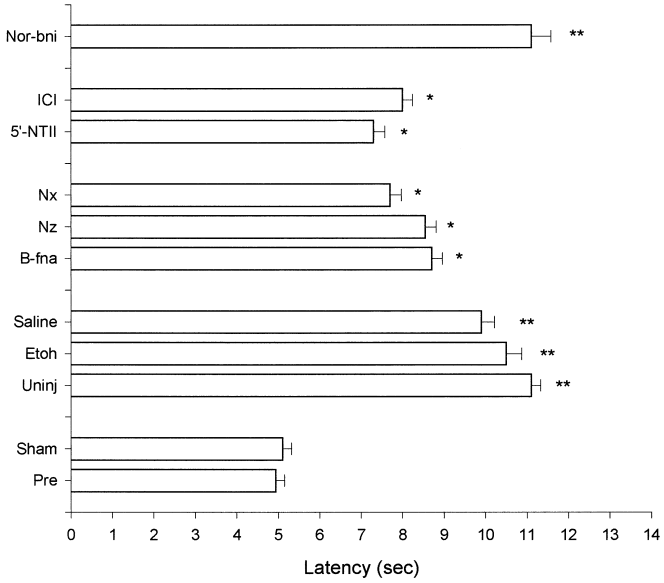

FIG. 5. Effects of pretreatment with either the general opiate antagonist naloxone [Nx] (1.0 μg, $n = 21$), the μ opiate antagonists naloxazine [Nz] (1.0 μg, $n = 22$), β-funaltrexamine [B-fna] (1.0 μg, $n = 22$), the δ opiate antagonists 5′-NTII (0.1 μg, $n = 22$), ICI 174,864 [ICI] (1.0 μg, $n = 12$), the κ opiate antagonist nor-binaltorphimine [Nor-bni] (1.0 μg, $n = 21$), saline vehicle [Saline] (1.0 μl, $n = 48$) or ethanol vehicle [Etoh] (1.0 μl, $n = 22$) on the thermal (40°C) response latencies if individual hydrated snails exposed for 15 min to either a Cnp or a sham exposure condition. Uninjected snails [Uninj] ($n = 22$) were also exposed to the 15 min Cnp or sham exposure condition. Naloxone, 5′-NTII and ICI 174,864 were injected 15 min prior to the Cnp or sham exposure. Naloxazine and β-funaltrexamine were injected 24 h, and nor-binaltorphimine was injected 2 h, prior to exposure. Response latencies were determined prior to (Pre- shown with all groups collapsed) and after the exposure. There were no significant differences between the sham groups ($n = 211$), hence all groups were collapsed for the figure, but not for analysis. $n = 10–12$ snails per group, with several groups per treatment. Error bars represent the SEM.

have similar actions in rodents involving, at least partially, δ opioid mechanisms. In this regard, weak 60 Hz magnetic fields, and the stronger magnetic fields associated with magnetic resonance imaging, have been shown to similarly attenuate exogenous opiate and endogenous opioid peptide mediated analgesia in *Cepaea* and rodents (14,27,28).

The specificity of the pulsed magnetic field used here was demonstrated in prior studies by the absence of any significant induction of analgesia by either a random frequency pulsed magnetic field or a pulsed magnetic field of a different design (34). These findings, combined with previous findings of significant magnetic field (60 Hz) attenuation of opioid analgesia (14,27,28), indicate that the Cnp effects do not arise from any non-specific magnetic field related ''stress'' response. Rather, they support a specific antinociceptive related effect of this particular pulsed magnetic field.

A number of possible mechanisms have been proposed for the biological effects of magnetic fields (27,28). Among these, resonance models have predicted both increases and decreases in opioid analgesia along with effects at specific frequencies (16). These actions have been suggested to include effects on metal ion binding proteins (e.g. Ca^{+2} and K^{+}) (18,27,28), both of which are associated with opioid actions (2).

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