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Evaluation of Hypothermia-Induced Analgesia and Influence of Opioid Antagonists in Leopard Frogs (*Rana pipiens*)

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SUCKOW, M. A., L. A. TERRIL, C. F. GRIGDESBY AND P. A. MARCH. *Evaluation of hypothermia-induced analgesia and influence of opioid antagonists in Leopard frogs.* PHARMACOL BIOCHEM BEHAV **63**(1) 39–43, 1999.—Hypothermia results in diminished voluntary muscle activity, and is frequently used as a means of providing deep anesthesia to ectotherms and some mammals. In ectotherms, however, it is unclear if hypothermia produces true pain insensation. A needle-probe thermometer was used to demonstrate in frogs (*Rana pipiens*) that local hypothermia (9°C) could be induced by placement of a tourniqueted leg into ice water (6°C) for 10 min in contrast to the contralateral nontourniqueted leg (21.8°C) kept out of ice water. Analgesia was tested by placement of dilutions of acetic acid on the rear leg. Further tests using groups of 10 frogs demonstrated that frogs with local hypothermia tolerated greater concentrations of acetic acid (mean acetic acid test score = 11) than morphine (10 mg/kg)-treated (9.6) or nontreated (5.8) frogs. Additional studies showed that morphine analgesia was blocked with naloxone doses as low as 0.01 mg/kg and hypothermia-induced analgesia at 10 mg/kg. Naltrexone blocked morphine analgesia at dosages as low as 0.01 mg/kg and hypothermia-induced analgesia at 0.10 mg/kg. In summary, this study demonstrates that hypothermia induces significant analgesia in an amphibian, and that this analgesia is partially blocked by naloxone and naltrexone, suggesting that the effect is mediated at least partially by opioid receptors. © 1999 Elsevier Science Inc.

Analgesia Amphibian Frog Hypothermia Opioid

TEMPERATURE is an important environmental component in body functions of most ectotherms. Amphibians can undergo and tolerate body temperature changes of 30°C on a daily basis, and up to 35°C on a seasonal basis (3). The ability of amphibians to tolerate greatly lowered body temperatures and the subsequent reduced metabolic and motor activity is a characteristic which is often exploited when using amphibians in studies involving surgical manipulation. Although anesthesia for such procedures can be easily induced by administration of chemical agents such as tricaine methanesulfonate (MS-222), it is common practice to immobilize amphibians by means of hypothermia (4,9). Similarly, hypothermia has been used for anesthesia of neonatal rodents undergoing experimental surgical procedures (16).

Although commonly used, the ability of hypothermia to induce true pain insensation versus paralytic immobilization in ectotherms, such as amphibians, is unclear. In addition, the mechanism(s) underlying hypothermia-induced analgesia are undefined (11).

The purpose of the present study was to evaluate the ability of hypothermia to induce analgesia in a candidate amphibian species, the leopard frog (*Rana pipiens*). In addition, the involvement of opioid substances in hypothermia-associated analgesia was examined.

METHOD

Animals

Leopard frogs, 47.1 ± 8 g, were obtained from Connecticut Valley Biological Supply (Northhampton, MA) and housed in a flow-through tank that provided access to a water-filled

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polycarbonate shoebox cage and to nonaqueous sites, such as rocks located on the floor of the tank. The source of the water in which the frogs were maintained was known to be free of contaminants. Illumination was maintained on a 12-h light:dark cycle, while the average room temperature was 19.7 ± 0.4°C. Frogs were provided live crickets (Fluker's Cricket Farm, Baton Rouge, LA) twice weekly. A 2-week acclimation period was observed before frogs were placed on study. Use of the frogs was approved by the Institutional Animal Care and Use Committee.

Test Compounds

Morphine sulfate (Elkins-Sinn, Cherry Hill, NJ) was used at 10 mg/kg diluted in sterile normal saline. Naloxone hydrochloride (Astra Pharmaceuticals, Westborough, MA) was used at 0.01 and 10.0 mg/kg diluted in sterile normal saline. Naltrexone hydrochloride (Sigma Chemical Co., St. Louis, MO) was used at 0.01 and 0.10 mg/kg diluted in sterile normal saline.

Induction of Local Hypothermia

Groups of five frogs each had the left leg tourniqueted with a tightly wound rubber band held in place by a weighted alligator clip (Fig. 1). The tourniquet was placed to minimize the flow of cooled blood from that leg and thereby maintain local hypothermia. An empty steel can with an opening cut in the plastic lid was filled with ice water (6°C), and the tourniqueted left leg passed through the opening in the lid and into the water for either 2 min, 5 min, 10 min, or 15 min (Fig. 2) while the frog was covered with damp paper towels and hand held in place. The internal temperatures of both the tourniqueted and nontourniqueted legs immediately after removal from the ice water was measured using a needle-probe thermometer (Yellow Springs Instrument Co., Yellow Springs, OH).

Evaluation of Analgesia

Groups of eight frogs were assigned to cohorts for evaluation of hypothermia-induced analgesia. Test groups included immersion of the tourniqueted left leg (TLL) in room temperature water for 10 min, immersion of the TLL in ice water for 10 min, morphine (10 mg/kg) administered intraperitoneally (IP), immersion of the nontourniqueted left leg in room temperature water for 10 min, and treatment with saline (pH 7.4) IP. Morphine and saline were administered 10 min prior to acetic acid testing.

Analgesic, nociceptive effects of treatments were measured using the acetic acid test procedure developed by Pezalla (14). Acetic acid, serially diluted to 10 strengths equally spaced on a logarithmic scale from 0.26 to 15.0 M, was numbered from 1 to 11 with increasing concentration. Glacial acetic acid was assigned the number 11. The NT, or nociceptive threshold, was defined as the number of the lowest concentration of acetic acid, delivered in dropwise (30 μl) fashion to the left rear leg of the frog, that induced a wiping response by either the acetic acid-treated or untreated contralateral leg. To evaluate the ability of the right leg to respond to nociceptive stimuli when the TLL was exposed to ice water, groups were tested by applying acetic acid to the right rear leg after the TLL had been exposed to ice water. In addition, the ability of the right leg to respond to nociceptive stimuli in the immobilized TLL was evaluated by applying the acetic acid to the left leg with the TLL physically restrained with ends of the tourniquet fastened to a balsa wood board.

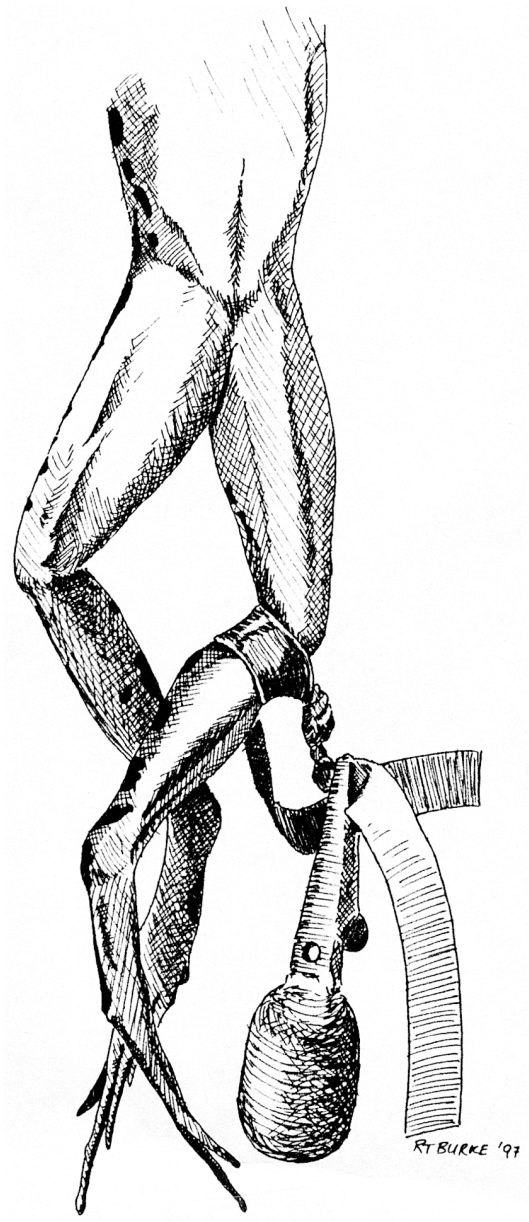

FIG. 1. Frog with a tourniquet held in place with a weighted alligator clip on left leg. The tourniquet was placed to minimize the flow of cooled blood from the leg and thereby maintain local hypothermia. The weighted clip was placed to keep the leg from being pulled from the ice water.

The acetic acid test was performed immediately following removal of the TLL from the ice water bath. In frogs treated IP with either morphine or saline, the acetic acid test was performed 10 min after administration of the test substance.

All frogs were observed for 15 min after acetic acid testing and returned to the room temperature water tank for any behavior suggestive of response to the nociceptive stimulus of acetic acid treatment.

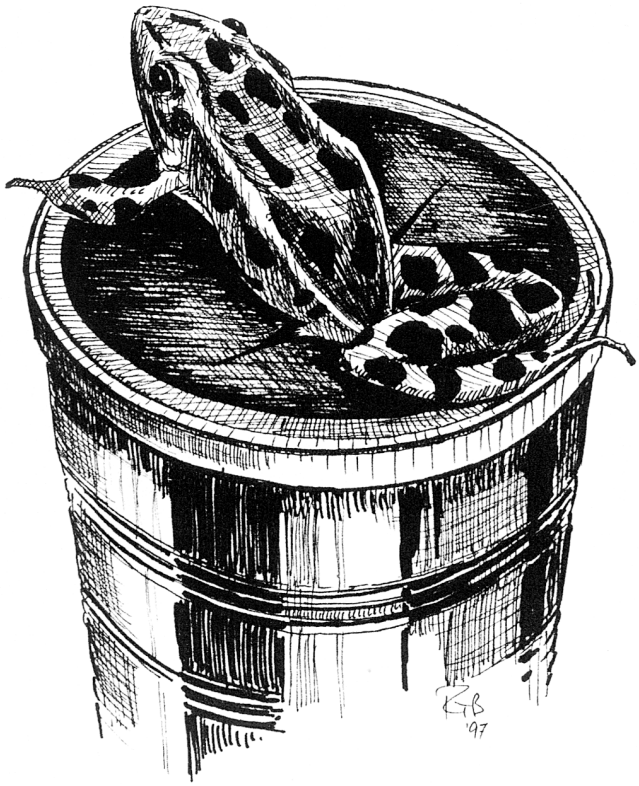

FIG. 2. Frog with left leg submersed in container filled with ice water. Local hypothermia is consistently induced after 10 min of ice water exposure.

Evaluation of Opioid Influence on Hypothermia-Induced Analgesia

Opioid influence on hypothermia-induced analgesia was evaluated by IP administration of naloxone or naltrexone to frogs prior to acetic acid testing. In frogs treated with IP morphine, opioid antagonists were administered 10 min prior to morphine. Similarly, in ice water-exposed frogs, antagonists were administered 10 min prior to exposure of the TLL to ice water. In this way, all frogs underwent acetic acid testing 20 min after treatments were initiated.

Statistical Evaluation

Data were analyzed by one-way analysis of variance, followed by the Multiple *t*-test for comparisons. Significance was set at $p \leqslant 0.05$. Data are presented as mean (±SEM) acetic acid test scores.

RESULTS

Induction of Analgesia by Hypothermia

Pilot studies done to determine the optimal ice-water exposure time showed that local hypothermia (internal temperature of 9.0 ± 0.2°C for the tourniqueted leg and 21.8 ± 0.3°C for the nontourniqueted leg) could be consistently induced in the tourniqueted leg by submersion of this leg in ice water for 10 min. Internal temperatures of the leg ranged from 15.0 to 18.3°C for legs exposed to ice water for 2 or 5 min. Measurements taken 5, 10, and 15 min after removal from the ice water and return to a tank containing room temperature water determined that the internal temperature of both legs equilibrated to room temperature water (19.7 ± 0.4°C) within 15 min in all frogs tested.

Mean acetic acid test scores for the various treatment groups are summarized in Fig. 3. As expected, frogs treated with morphine tolerated significantly greater ($p \leqslant 0.01$) acetic acid concentrations than untreated frogs. No significant difference in mean acetic acid test scores was found between groups in which the TLL was immersed in room temperature water versus physical restraint of the TLL, demonstrating the ability of the contralateral (right) leg to respond to nociceptive stimulation of the left leg. In addition, these scores were not significantly different from frogs that had acetic acid applied to the right rear leg following submersion of the TLL in ice water, demonstrating the ability of the right leg to respond to nociceptive stimuli. In contrast, frogs that underwent immersion of the TLL in ice water had significantly greater ($p \leqslant 0.01$) mean acetic acid test scores (average of 11) than all other groups.

Frogs that underwent unilateral immersion of the TLL in ice water responded to acetic acid only with the right leg, and the left leg appeared functionally immobile during acetic acid testing. Frogs that did not undergo unilateral hypothermia of the TLL responded to acetic acid treatment by both ipsilateral and contralateral wiping of the acetic acid-treated leg. Frogs that had been treated with either dosage of naloxone or 0.10 mg/kg of naltrexone prior to induction of hypothermia responded to acetic acid treatment by wiping of the TLL with the contralateral leg.

No abnormal behaviors nor behaviors suggestive of nociceptive perception were recorded in any frogs during observation for 15 min following acetic acid testing and return to a tank of fresh water.

Reversal of Analgesia by Naloxone and Naltrexone

Results of opioid influence on hypothermia-induced analgesia are summarized in Fig. 4 (naloxone, 10.0 mg/kg) and 5

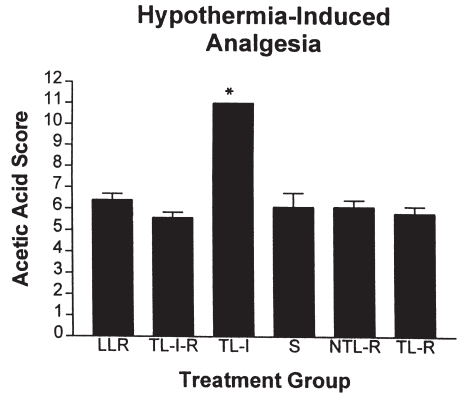

FIG. 3. Frogs exposed to ice water develop significant analgesia as measured by the acetic acid test. LLR = left leg manually restrained but not exposed to ice water; TL-I-R = left leg tourniqueted and exposed to ice water but right leg tested with acetic acid; TL-I = left leg tourniqueted and exposed to ice water; S = saline administered IP; NTL-R = left leg not tourniqueted and exposed to room temperature water; TL-R = left leg tourniqueted and exposed to room temperature water. Bars represent the mean acetic acid test score for the group with the standard error of the mean above each bar. Significance ($p \leqslant 0.05$) is indicated by the "*" character.

(naltrexone, 0.10 mg/kg). Mean acetic acid test scores of frogs treated with either dosage of naloxone (0.10 mg/kg or 10.0 mg/kg) or naltrexone (0.01 mg/kg or 0.10 mg/kg) followed by immersion of the leg in room temperature water were not significantly different from scores of frogs that did not receive naloxone or naltrexone prior to submersion of the TLL in room temperature water, indicating that neither drug produced analgesia at these dosages. Similarly, these scores were not significantly different from those of frogs receiving morphine and either dosage of naloxone or naltrexone, demonstrating complete blockage of morphine-induced analgesia. In contrast, frogs treated with both dosages of naloxone prior to immersion of the TLL in ice water demonstrated lower mean acetic acid test scores compared to ice water-exposed frogs not receiving naloxone. This difference was significant ($p \leq 0.05$) for frogs treated with 10.0 mg/kg, but not those treated with 0.10 mg/kg, of naloxone. In addition, these scores were significantly greater ($p \leq 0.05$) than frogs not exposed to ice water, indicating that blockage of hypothermia-induced analgesia by naloxone was not complete. Nociceptive responses in these frogs were characteristic, but subjectively less robust than positive nociceptive responses observed in other treatment groups.

Frogs treated with 0.10 mg/kg of naltrexone prior to immersion of the TLL in ice water had significantly lower ($p \leq 0.05$) mean acetic acid test scores than ice water-exposed frogs not treated with naltrexone (Fig. 5). Frogs treated with 0.01 mg/kg of naltrexone did not have significantly lower mean acetic acid test scores compared to frogs that only underwent immersion of the TLL. In addition, scores from frogs pretreated with 0.10 mg/kg of naltrexone were significantly greater ($p \leq 0.05$) than scores from frogs not exposed to ice water, demonstrating partial blockage of hypothermia-induced analgesia by naltrexone. Nociceptive responses in these frogs were subjectively less robust than positive nociceptive responses observed in other treatment groups, as for naloxone-treated frogs.

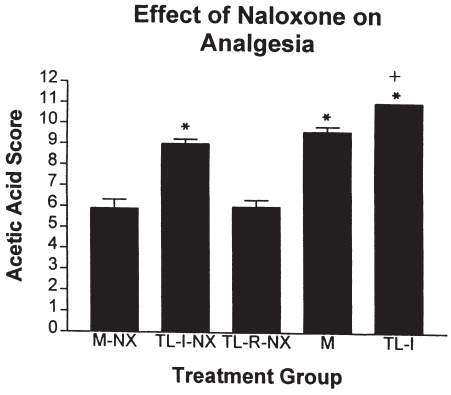

FIG. 4. Pretreatment with naloxone (10.0 mg/kg) partially reverses hypothermia-induced analgesia. M-NX = pretreatment with morphine (10 mg/kg) and naloxone; TL-I-NX = pretreatment with naloxone, left leg tourniqueted and exposed to ice water; TL-R-NX = pretreatment with naloxone, left leg tourniqueted and exposed to room temperature water; M = pretreatment with morphine; TL-I = left leg tourniqueted and exposed to ice water. Bars represent the mean acetic acid test score for the group with the standard error of the mean above each bar. Significance ($p \leq 0.05$) compared to the M-NX and TL-R-NX groups is indicated by the "*" character and by the "+" character when compared to all other groups.

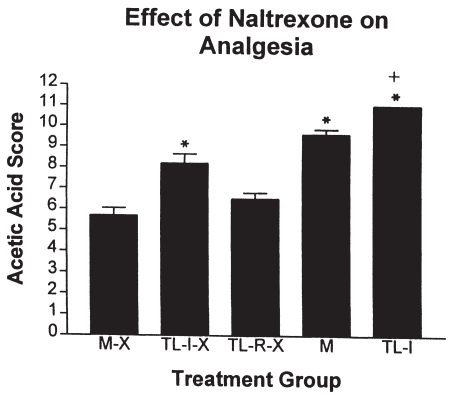

FIG. 5. Pretreatment with naltrexone (0.10 mg/kg) partially reverses hypothermia-induced analgesia. M-X = pretreatment with morphine (10.0 mg/kg) and naltrexone; TL-I-X = pretreatment with naltrexone, left leg tourniqueted and exposed to ice water; TL-R-X = pretreatment with naltrexone, left leg tourniqueted and exposed to room temperature water; M = pretreatment with morphine; LT-I = left leg tourniqueted and exposed to ice water. Bars represent the mean acetic acid test score for the group with the standard error of the mean above each bar. Significance ($p \leq 0.05$) compared to the M-X and TL-R-X groups is indicated by the "*" character and by the "+" character when compared to all other groups.

DISCUSSION

Induction of anesthesia by hypothermia is well described in mammals (1,5,17), and observations in humans support the notion that hypothermia can induce temporary analgesia (1,8). Depressed neuronal transmission returns with rewarming, suggesting that hypothermia would not supply analgesia to mammals upon rewarming.

Although hypothermia unquestionably induces immobilization of ectotherms, concomitant induction of analgesia has been presumed, but not proved. We have shown here that local analgesia can be induced by hypothermia in the leopard frog, a representative ectotherm. Insensation to topical acetic acid application was significantly greater in frogs made hypothermic in the tested leg than in all other groups of frogs, including those treated with morphine. The ability of the contralateral leg to respond to nociceptive stimuli in the hypothermic TLL suggests that this response is not due to a muted response related to paresis of the hypothermic TLL. This conclusion assumes that acetic acid penetrates the intact amphibian dermis to an equivalent extent in both cold and warm conditions.

The specific mechanism of hypothermia-induced analgesia is unclear. Studies in isolated rabbit sciatic nerves showed that there were no changes in morphology or functioning of nerves cryolesioned at −20°C (27). It therefore seems reasonable that the analgesia we observed in leopard frogs is not due to permanent hypothermic damage of the nerve.

Our observation that hypothermia-induced analgesia in leopard frogs is partially blocked by naloxone and naltrexone suggests that the analgesia may be mediated in part by opioid compounds. Amphibians appear to possess a well-developed endogenous opioid system (21). Endogenous opioids are present in the central nervous system in several amphibian species (6,10,19,20). Earlier articles reported that opioid binding sites in amphibians could be characterized as mu, delta, and kappa (7,13,18,19); however, more recent studies using selective opioid ligands show predominantly one binding site char-

acterized as kappa-like (2,12,25,26). Based on studies using type-selective opioid antagonists in leopard frogs, it has been suggested that mu, delta, and kappa opioids produce analgesia in amphibians at a single type of receptor, termed the "unireceptor" (23). The observations that hypothermia-induced analgesia is only partially blocked by opioid antagonists and that only the contralateral, but not the acetic acid-treated hypothermic leg, responded to the nociceptive stimulus in frogs pretreated with naloxone or naltrexone suggest that mechanisms other than the endogenous opioid system are also involved.

Exogenously administered opioids produce analgesia in leopard frogs (14,15,24). Morphine-associated analgesia is attenuated by the opioid antagonist naloxone, suggesting that the analgesic effect is mediated by opioid receptors (14). The potency of mu opioids is greater than delta opioids, which in turn, are more potent than kappa opioids following spinal administration to leopard frogs (22). The involvement of specific opioid receptors in hypothermia-induced analgesia has not been evaluated.

In summary, hypothermia induced by immersion in ice water produces analgesia in a representative amphibian. The resulting analgesia may be partially mediated by opioid substances. Further studies will be needed to more precisely define the mechansisms involved in this process.

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