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Opiate Effect on the Threat Display in the Crab *Carcinus mediterraneus*

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BERGAMO, P., H. MALDONADO AND A. MIRALTO. *Opiate effect on the threat display in the crab* Carcinus mediterraneus. PHARMACOL BIOCHEM BEHAV **42**(2) 323–326, 1992. – The well-known defense response of a crab (laterus merus display, LMD) was easily evoked in *Carcinus mediterraneus* by striking the cephalothoraxic protogastric region between the eyestalks. Following a program aimed at investigating the regulatory action of diverse neuromodulators on the LMD of this crab, a study on the role of opioids was started by testing the effect of morphine administration. Injection of morphine HCl (MP) (40, 50, 60, 70, or 100 μg/g) produced a dose-dependent reduction of the LMD so elicited that dissipated with the postinjection time. Only MP doses higher than 50 μg/g were effective 30 min after drug administration. The MP-induced inhibition of LMD was blocked by a 4.8-μg naloxone HCl/g dose injected 10 min before MP. These results and those previously obtained as the action of GABA on the LMD of this crab are discussed in connection with results reporting a similar effect of these drugs on another agonistic item of behavior in the crab *Chasmagnatus granulatus*. The possibility of demonstrating habituation of the LMD to an iterated stimulation in *C. mediterraneus* and of using such a process to elucidate the acting paths of the drugs is discussed.

Crustacea Opioids Morphine Naloxone Defense response

TWENTY-FOUR items of agonistic behavior have been identified during intraspecific encounters of the male crab *Carcinus mediterraneus* (1,2). Among them, the most frequent and conspicuous item proved a ritualized posture in which both chelae are spread out literally and the carapace is raised on the flexed walking legs. Such a reaction was first described in *Carcinus maenas* by Bethe (3) and called *lateral merus display* (LMD) by Wright (23).

The LMD may easily be elicited in *C. mediterraneus* by gently prodding with a plastic stick between the eyestalk (protogastric region) (5), whereas in *Chasmagnatus granulatus*, a crab used also in studies on agonistic behavior (10,12,20,21), only a strong electric shock, delivered through electrodes chronically impaled in the carapace, can consistently evoke a LMD in laboratory conditions (10). By contrast, another well-defined agonistic item, to so-called escape response (12,16,17), is far easier to evoke in the latter crab than in the former one.

Accordingly, a study on the mechanisms involved in the regulation of the escape response has been conducted with *C. granulatus* (12,20,21), results showing an inhibitory effect of both GABA and morphine but acting along wholly different paths: GABA on the efferent limb and morphine on perception. On the other hand, a program aimed at studying the action of diverse neuromodulators on the LMD of *C. mediterraneus* is in course. The role of the GABA chloride ion channel component was analyzed, results suggesting that GABAergic sites other than peripheral ones are involved in the regulation of this agonistic posture (5). The purpose of the present article, in line with such a program, is to investigate the role of opioids on the LMD of this crab, starting by testing the effect of morphine administration.

It is worthwhile to stress that the inhibitory effect of morphine, naloxone reversible, on the invertebrate behavior was reported the first time in 1982 (9,11). Later studies confirmed this effect in different arthropods (10,14,22,24) and radioimmunoassay evidence indicated the existence of enkephalin analogs (6,8,18,19,22) and native peptides with opioid-like activity (13,15).

GENERAL METHOD

Animals

Adult, male *C. mediterraneus* crabs, 3.1–3.5 cm body length, 4.6 g/cm mean weight quotient, were used in this study. Upon receipt from the supplier (Fishing Service of the Stazione Zoologica "Anton Dohrn," Naples, Italy), crabs were kept in running seawater tanks (28 × 28 × 10 cm), 20 animals to each tank. The light-dark cycle in the stabularium corresponded to those of the season. Animals were fed on the day of capture with pieces of anchovy (*Engraulis encrasicholus*) and used in only one experiment in the following 2 days.

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C. mediterraneus lives on the sandy bottom of harbors and estuarine environments. It is taxonomically affined to *C. maenas* (Atlantic species) (7), which does not live in the Mediterranean Sea. It is easy to get year-round and maintain in stabularium. Experiments of the present study were performed between January and April (seawater temperature: 14–15°C; pH: 7.4–7.5).

Procedure

Animals were moved from the stabularium to the laboratory, where they underwent a selection test: Each crab was turned on its back and only animals that immediately returned to their normal position entered the experiment. The rationale behind this selection is that crabs with a slow righting reaction show a low reactivity to diverse stimuli (Maldonado, personal communication). Once a crab was selected, it was placed in a glass cylinder (14 cm in diameter, 7 cm high) containing 230 ml seawater and illuminated from one side by a lamp focused on the cylinder. After a 10-min adaptation time in the glass cylinder, each crab was submitted to a pretesting trial, which consisted of striking the cephalothorax protogastric region between the eyestalks. The described procedure was previously used (5). A plastic tube (25 cm length, 7 mm internal diameter) containing a solid bar of the same length (6 mm diameter, 10 g weight) that was held in place by a pin at the distal end was neared gently to the glass cylinder. When the proximal end of the tube was near the top of the glass cylinder and over a point roughly midway between the eyestalks, the pin was extracted so that the bar fell striking the carapace. The animal could not detect the approach of the tube because of the strong lateral light focused on the cylinder. A response to the strike was considered an LMD only when the chelae spread behind the ocular peduncles, that is, the so-called full LMD according to Wright's classification (23). Pilot experiments showed that with a full LMD when submitted to a pretesting trial such a trial could be successfully repeated 8–10 times over 30 min.

Animals that failed to produce the full LMD were discarded and those that reacted were immediately injected with either 100 μl as already reported (5,20,21) of vehicle, that is, distilled water (DW), or a solution of the drug to be tested, namely, morphine HCl (Carlo Erba) or naloxone HCl (Endo Laboratories Inc.), colored with neutral red to ensure the correct injection inflow (5,12). The injection was given by means of a Hamilton syringe through the membrane between the cheliped and the first walking leg coxa as already described (5).

EXPERIMENT 1

The purpose of this experiment was to study the relationship between the LMD and diverse doses of morphine at various intervals after injection.

METHOD

One-hundred and twenty crabs were randomly distributed in 6 groups of 20 each and injected with DW or 40, 50, 60, 70, or 100 μg morphine/g, respectively. The effect of each dose of morphine at 10, 20, and 30 min after injection was calculated as follows: LMD inhibition % = (1-*NR*/20) × 100, where *NR* stands for the number of crabs showing LMD.

RESULTS AND DISCUSSION

As shown in Fig. 1, morphine administration produced a dose-dependent inhibition of the LMD to the mechanical stimulus. Moreover, the effect of morphine declined with time. However, whereas the inhibitory effect of morphine at a dose of 40 μg/g disappeared completely after 20 min, with 100, 70, or 60 μg/g there was a significant effect even at 30-min postinjection. Statistical analysis confirmed these findings. The correlation between dose and inhibition level, expressed by the contingence coefficient *C*, was significant ($p < 0.001$) at the three injection-trial intervals (at 10 min,

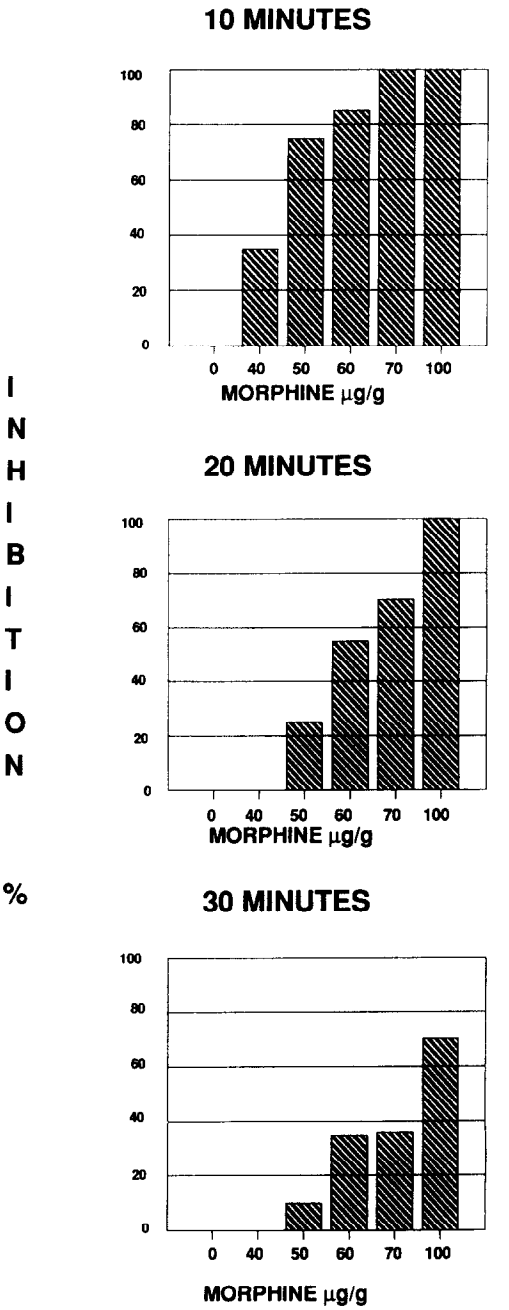

FIG. 1. Dose-dependent morphine-induced inhibition of LMD. Effect of each morphine dose at 10, 20, and 30 min after injection. LMD inhibition % = (1-*NR*/20) × 100, where *NR* stands for the number of crabs showing LMD. Ordinates: mean LMD inhibitory % values.

$C = 0.5$, $X = 33.6$; at 20 min, $C = 0.64$, $X = 68.5$; at 30 min, $C = 0.47$, $X = 28.3$). In addition, when all the data of the same interval were pooled there was a high correlation between injection-trial interval and inhibition ($C = 0.38$, $X = 51.0$, $p < 0.001$).

Thus, morphine injections produced a dose-dependent reduction in the crab's LMD to mechanical stimulation. It is noteworthy that at 30-min postinjection only doses higher than 50 μg/g continued to exert an inhibitory effect. This result is in keeping with two conclusions drawn in an earlier study on the effect of morphine on arthropods (2,10): The dose required to induce a significant effect on responding to aversive stimuli is higher in arthropods than in vertebrates (1) and morphine clearance seems to be faster in the former than in the latter.

EXPERIMENT 2

The aim of this experiment was to test the effect of naloxone on the morphine-induced inhibition of the LMD.

METHOD

In this case, the procedure consisted of administering two 50-μl injections of morphine with a 10-min between-injection interval.

The rationale for a double injection was to have the opportunity to inject two drugs separately in time. In addition, this testing session consisted of eight instead of three trials. The LMD inhibition was calculated as follows: recovery time = $FT \times 10$ min, where FT stands for the trial number at which the crab showed LMD for the first time during the testing session.

Pilot experiments revealed no significant difference in recovery time between a group ($n = 20$) given a single injection (100 μl of 100 μg morphine/g) and another administered a double injection (2 × 50 μl of 50 μg morphine/g). Animals that were double injected with DW presented LMD in all eight trials. Two series of experiments were run separately: Experiment 2a and Experiment 2b. In Experiment 2a, 60 crabs were randomly assigned to 3 groups of 20 each: DW + DW (two DW injections); DW + 50MP (a DW injection followed by 50 μg morphine/g); and NX + 50MP (4.8 μg naloxone/g followed by 50 μg morphine/g). The 4.8-μg naloxone/g dose was chosen because preliminary experiments showed it to be the maximum dose without agonist effects. In Experiment 2b, 3 groups of 20 crabs each were formed (DW + DW; DW + 100MP; NX + 100MP).

RESULTS AND DISCUSSION

Figure 2 shows the mean recovery times obtained in each group of Experiment 2a (upper panel) and Experiment 2b (lower panel). LMD inhibition, that is, a recovery time longer than that of a DW group, occurred with both 50- (and 100-μg morphine/g doses. Indeed, with the Mann-Whitney test significant differences were observed between both DW + 50MP vs. DW + 100MP vs. DW + DW ($U = 30$ and 0, respectively; $p < 0.002$, two tailed). No inhibition was observed when crabs were administered naloxone 10 min before a 50-μg morphine/g injection (NX + 50MP vs. DW + 50MP, $U = 60$, $p < 0.002$; NX + 50MP vs. DW + DW, $u = 0.140$, $p > 1.0$) In addition, the naloxone preinjection significantly reduced the LMD inhibition induced by 100 μg morphine/g (NX + 100MP vs. DW + 100MP, $U = 72.5$, $p < 0.002$), although the effect of morphine was not abolished entirely

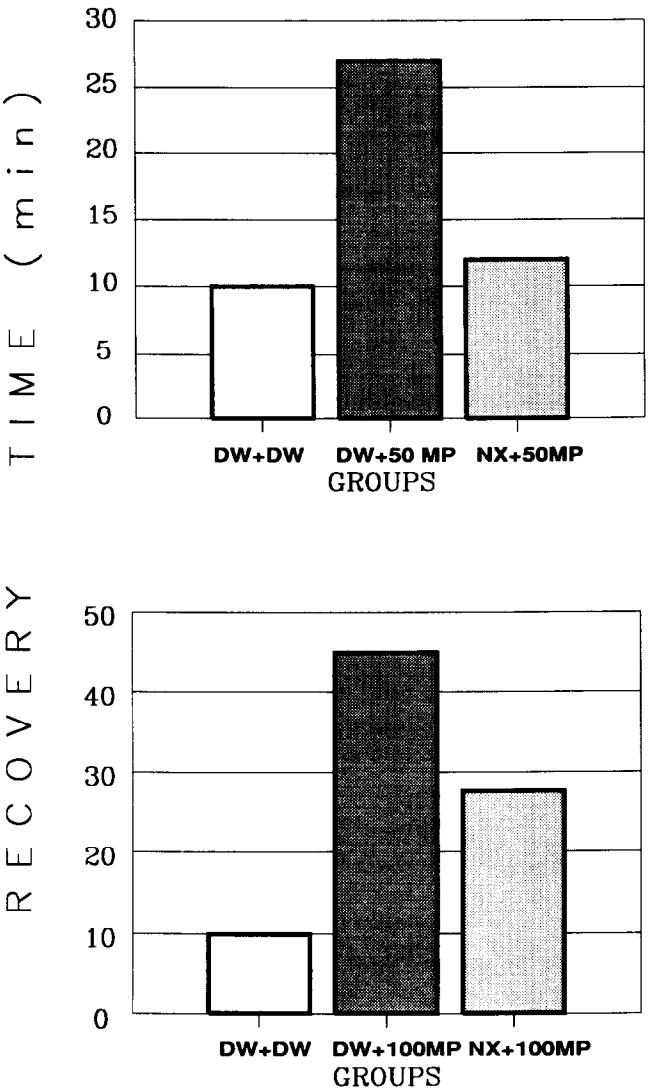

FIG. 2. Naloxone antagonizes the inhibitory effect of morphine. The LMD inhibition level was calculated by recovery time = $FT \times 10$ min, where FT stands for the trial number at which the crab shows LMD at the first time during an eight-trial testing session. Ordinates: mean recovery time values. Upper panel (Experiment 2a): DW + DW, two DW injections; DW + 50MP, DW followed by 50 μg morphine/g; NX + 50MP, 4.8 μg naloxone/g followed by 50 μg morphine/g. Lower panel (Experiment 2b): Groups as those of Experiment 2a except 100 μg morphine/g was used.

(NX + 100MP vs. DW + DW, $U = 30$, $p < 0.002$). Therefore, the morphine-induced inhibition of LMD was naloxone reversible.

It is worthwhile stressing that no morphine-injected crab showed LMD inhibition when tested at trial 8, that is, 80 min after injection.

GENERAL DISCUSSION

Results of the present study along with those of a previous one (5) lead us to conclude that both morphine and GABA abate the LMD elicited by mechanical disturbance in *C. mediterraneus*. Such a conclusion is in keeping with that stemmed

from studies in *C. granulatus* concerning the escape response to a danger stimulus (12,20,21) using similar doses and injection-test intervals. The morphine effect was also antagonized by naloxone except when this drug was injected at a shorter injection-test interval (10 min) (4). However, as regards the escape response a different path of action for both drugs was suggested, namely, that inhibitory effect of morphine, unlike that of GABA, could result from an interference with perception.

A study of the acting paths of both drugs on LMD would be extremely interesting because if results lead to a similar conclusion for both agonistic items this would suggest that morphine interferes with perception regardless of stimulus (visual or tactile stimulus) and of the type of response. Findings of the present and previous studies (1,2,5) indicate that *C mediterraneus* is a good candidate to address such a study. In fact, the induction of the LMD by mechanical disturbance, unlike the induction by a strong electric shock in *C. granulatus*, can be often repeated without showing harmful effects so that habituation of the LMD to an iterated stimulation might be built up in *C. mediterraneus*. Such a possibility proves especially interesting for the purpose of this research since habituation is a powerful tool to conclude, through behavioral analysis, if the inhibitory effect of a drug on a response is produced by interfering with perception or by acting on the efferent limb (20,21).

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