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Effect of Morphine and Naloxone on a Defensive Response of the Crab *Chasmagnathus granulatus*

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LOZADA, M., A. ROMANO AND H. MALDONADO. *Effect of morphine and naloxone on a defensive response of the crab* Chasmagnathus granulatus. PHARMACOL BIOCHEM BEHAV 30(3) 635–640, 1988.—Male crabs (*Chasmagnathus granulatus*) exhibited a defensive response (DR) to an electric shock (8 V, 50 Hz, 1 sec). The DR so elicited was used as a model for studying the antinociceptive effect of morphine. Injections of morphine-HCl (MP) (25, 50, 100 and 150 μg/g) were administered and the DR was examined at 2, 7.5, 15, 30, 45 and 75 min post-injection. (a) MP produced a dose-dependent reduction of the crab's sensitivity to the nociceptive stimulus. (b) A 100 μg/g dose of MP caused a 50% response inhibition with an injection-shock interval of 30 minutes, but no inhibition occurred when the same dose was administered with 1.6 μg/g of naloxone-HCl, suggesting that MP acts through an opiate receptor. (c) The ED_{50} at 2 min post-injection was roughly 33 μg/g and the threshold dose was estimated to be 6.8 μg/g. These doses are lower than ED_{50} values reported for other arthropods (90 to 930 μg/g) and approach those of vertebrates. (d) The peak MP effect was reached quickly, within 2 min post-injection. The duration of the MP effect was calculated to be 45.0–75.0 min depending on the dose, and an indirect estimate of half-life elimination was 15.7 min. These values are remarkably lower than those reported for vertebrates. The shorter duration of the MP peak effect is attributable to a greater permeability of the arthropod blood-brain barrier as compared to that of verebrates.

Arthropods Antinociceptive effect Defensive response Morphine pharmacokinetics

ANTINOCICEPTIVE effects of opiates in arthropods were first described in 1982 [8,11] and confirmed in later reports [12,24], all employing a defensive behavior as end point. In turn, the existence of opiate receptors in animals of this phyllum was suggested by several radioimmunoassay studies [3, 6, 7, 13, 15, 19, 22].

Most behavioral studies concluded that morphine potency in arthropods is much less than that of vertebrates, ED_{50} values ranging from 90 to 930 μg/g in the former vs. 0.3 to 10.0 μg/g in the latter [14, 17, 23]. High ED_{50} levels in arthropods have been attributed to slow drug diffusion because of their open cardiovascular system, to the possibility of faster drug degradation by hemolymph, to a blood-ganglion barrier that prevents easy morphine entry, and/or to the peculiar kinetics of arthropod opiate receptors. However, all these assumptions can only be correctly tested by analyses of the time-dose-response for morphine action. Reported ED_{50} values have most likely been overestimated, since it cannot be taken for granted that they were assessed at peak drug action levels.

The purpose of the present work was to pursue a systematic study of the antinociceptive effect of opiates on arthropods, with particular emphasis on the morphine-induced response inhibition as a function of dose and time.

METHOD

Animals

The animals were adult male crabs (*Chasmagnathus granulatus*), 2.8–3.0 cm across the carapace, collected from water less than 1 m deep in the *rias* of San Clemente del Tuyu, Argentina, and transported to the laboratory where groups no greater than 20 animals each were lodged in glass tanks (35×48×27 cm). Each tank had a box with aerated water and a small container for food (pellets for rabbits, NUTRIMENTS SA). The tanks were kept in an isolated room (the house-room) at constant temperature (23–25°C). Crabs remained in the tank for 1–5 days and were used only in one experiment. The average wet weight was initially determined as 17.3 g (SE: 0.2; n=60) and the dose (μg/g) of the drug to be injected calculated according to this mean.

Animals were collected on 18 different days of the year, from April to December 1986. Crabs coming from the same capture are said to belong to the same *population*.

Experimental Device

Two pins of stainless steel (E-E', Fig. 2) were implanted through and A and A' in the carapace, at a depth no

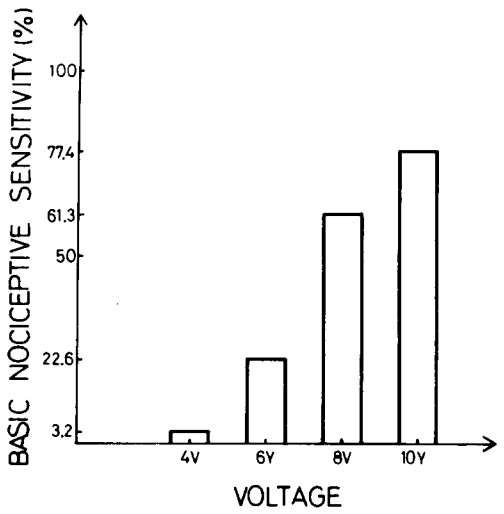

FIG. 1. Basic nociceptive sensitivity vs. voltage.

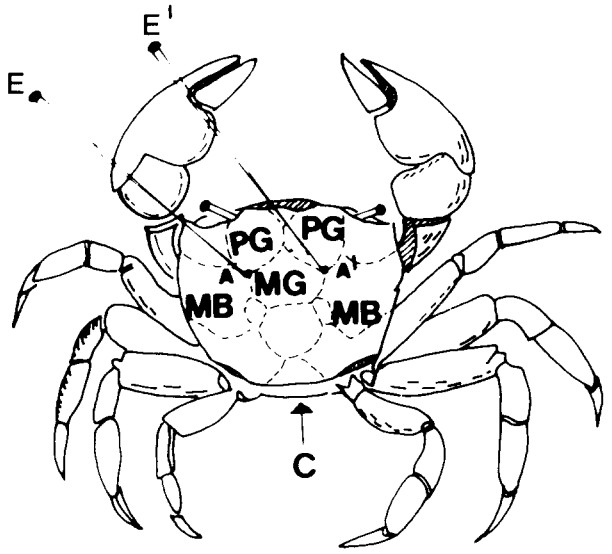

FIG. 2. Diagram of a male crab *Chasmagnathus granulatus*. A, A': points where the electrodes E-E' are implanted. Three regions on both sides of the carapace converging in A-A' are indicated: the protogastric region (PG), the mesogastric region (MG) and the mesobranchial region (MB). C: cephalothorax-abdomen membrane point where the injection is given.

greater than 3 mm. A and A' are two small depressions in the confluence of three regions of the carapace, i.e., the protogastric (PB), the mesogastric (MG) and the mesobranchial (MB) regions [1]. The pins were fixed in the cuticle and connected to the output of an electric stimulator. Implanted crabs were individually placed into a plastic container with a 1 cm layer of water, illuminated by a 10 W lamp. There were 40 containers in the *experimental room* at a constant temperature (23–25°C).

Injection

After 30 min of adaptation in the experimental room, each crab was quickly injected (1–2 seconds) by means of a Hamilton syringe through the cephalothoracic-abdominal membrane (Fig. 2, C). Injections consisted of 100 μl of the vehicle, i.e., distilled water (d.w.), or a solution of the drug to be tested. Pilot experiments in which d.w. was replaced by 2% w/v NaCl as vehicle failed to produce any alteration in the results, a finding in agreement with those reported for other arthropods [11, 12, 24].

Trial and Defensive Response

Each trial consisted of a single 1 sec, 50 Hz, 4, 6, 8 or 10 V shock, given at 2, 7.5, 15, 30, 45, or 75 min post-injection, and was administered once per animal.

The end point adopted here was an all-or-nothing display in which both chelae were spread behind the ocular peduncles, held in an extended position, and the carapace elevated on the flexed walking legs, hereinafter called the *defensive response* (DR). This response is a well known reaction of a crab in nature when facing an immediate danger or receiving a strong aversive stimulus. The presence or absence of the defensive response elicited by a shock in the laboratory was defined by simple observation. Previous checks have indicated higher interobserver reliability (90–99%).

An animal that displayed a defensive response following shock is called a *responding animal.*

Basic Nociceptive Sensitivity and Defensive Response Inhibition

The percentage of responding animals injected with d.w. is called the *basic nociceptive sensitivity* (BNS). This baseline sensitivity is defined as CR/N $\times$ 100, where CR (control responders) is the number of responding animals and N the total number of tested crabs.

In experiments aimed at determining the antinociceptive effect of a drug, the term *group* stands for the number (N) of crabs that received the same treatment, and *set* for the number of groups belonging to the same population, each having the same total N and tested simultaneously in the experimental room. Each set had one d.w.-injected group (control group). The total number of crabs for a set was not determined beforehand, but was fixed on reaching 20–25 responding crabs in the control group. *Defensive response inhibition* due to drug action was defined as (1-ER/CR) $\times$100, where CR (control responders) is the number of responding animals of each set's control group, and ER (experimental responders) the number of responding animals of any treated group belonging to the given set.

Experimental design

Pertinent details are given when presenting results.

RESULTS

Effect of Stimulus Intensity on the BNS

One hundred and twenty-four crabs injected with d.w. were randomly distributed in four groups of 31 each. One group was given a 4 V shock; a second group, 6 V; a third group, 8 V; and a fourth group, 10 V. All shocks were administered 30 min after injection.

As shown in Fig. 1, reactivity to the shock increased with voltage.

Effect of Capture Season and Injection-Shock Interval on the BNS

Since there were 18 different populations spread over three seasons, the relation between *capture season* and basic

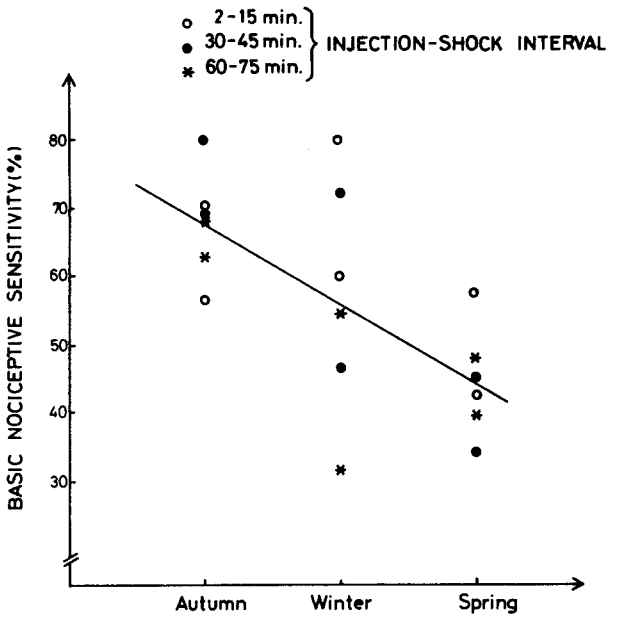

FIG. 3. Basic nociceptive sensitivity vs. capture season. Linear regression: $r^2=.41$, $t=3.25$, $p<0.005$.

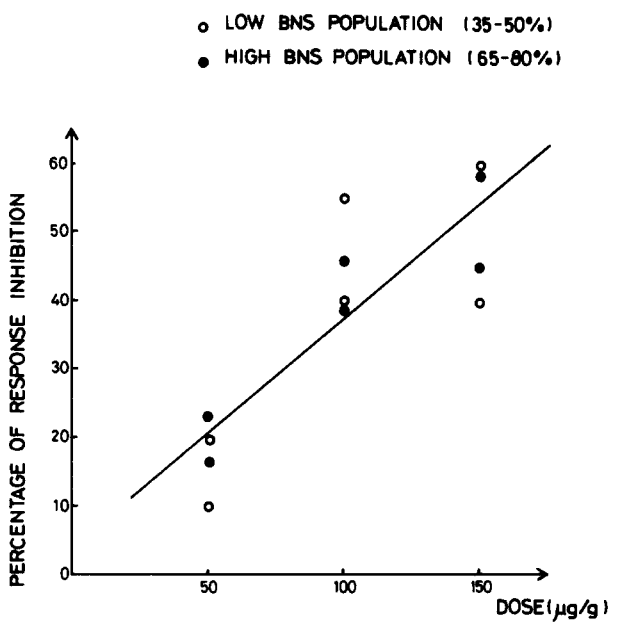

FIG. 4. Percentage of response inhibition vs. morphine dose. Linear regression: $r^2=.72$, $t=5.07$, $p<0.001$.

TABLE 1

EFFECT OF NALOXONE ON NOCICEPTIVE SENSITIVITY AND MORPHINE-RESPONSE INHIBITION

Set	Groups	Number of Responding Crabs	Percentage of Response Inhibition
A	a. CONTROL	30	—
	b. 18 NX	29	3.4%
B	a. CONTROL	30	—
	b. 1.6 NX	30	0.0%
C	a. CONTROL	30	—
	b. 100 MF	17	43.4%
	c. 100 MF+1.6 NX	29	3.4%

nociceptive sensitivity (BNS) was studied. Batches of 30 crabs selected from each population received an 8 V shock at different intervals after d.w. injection.

Figure 3 illustrates the correlation between BNS and the three capture seasons of the year. Different symbols stand for three different injection-shock intervals (2–15 min, 30–45 min and 60–75 min). A 3×3 ANOVA performed on the BNS values showed that *capture season* proved to be a source of variation significantly greater than individual differences, $F(2,9)=5.26$, $p<0.025$. On the other hand, there was no significant difference in levels due to the *injection-shock interval* or any significant interaction between factors.

Thus, crabs collected at different seasons had different baseline sensitivity, decreasing linearly from autumn to spring.

Effect of Morphine and BNS

The experimental design included 16 groups of crabs distributed in 4 sets of 4 groups each: a *control group*, a *50 MP group*, injected with morphine-HCl 50 μg/g (SAPORITI, Argentina), a *100 MP group* with 100 μg/g and a *150 MP group* with 150 μg/g. Each set came from a different population. Two sets belonged to populations showing high BNS values (65–85%), and two sets to populations with low values (35–50%). An 8 V shock was given at 30 min post-injection.

Figure 4 shows the group performance. A 2×3 ANOVA on response inhibition percentages showed that *morphine-HCl dose* proved to be a significantly greater source of variation than individual differences, $F(2,6)=15.23$, $p<0.01$, but there was no significant difference in levels due to BNS or interaction between factors. Linear regression performed on these values (Fig. 4) illustrates the morphine dose-dependent reduction of crabs reactivity.

Effect of Naloxone on Nociceptive Sensitivity and on Morphine-Induced Response Inhibition

The experimental design included three sets. Set A comprised one *control group* and one *18 NX group* injected with 18 μg/g solution of naloxone-HCl (Sigma, St. Louis, MO); set B, one *control group* and one *1.6 NX group* with 1.6 μg/g of naloxone-HCl; set C, one *control group*, one *100 MP group* with 100 μg/g of morphine-HCl, and one *100 MP+1.6 NX group* with 100 μg/g of morphine-HCl plus 1.6 μg/g of naloxone-HCl. Throughout, an 8 V shock was given at 30 min post-injection. Thirty control responders (CR) were used in this experiment for each set.

Table 1 shows the results. Despite a high 18 μg/g naloxone dose, there was no effect on the level of nociceptive sensitivity (set A). Injection of morphine-HCl 100 μg/g (group C,b) produced a drop in the sensitivity statistically similar to 50% (normal curve test for proportions). A 1.6 μg/g dose of naloxone-HCl antagonized morphine-HCl 100 μg/g (group C,c). The difference between inhibition in group C,b and C,c is highly significant (standard normal variable $z=3.66$; $p<0.001$).

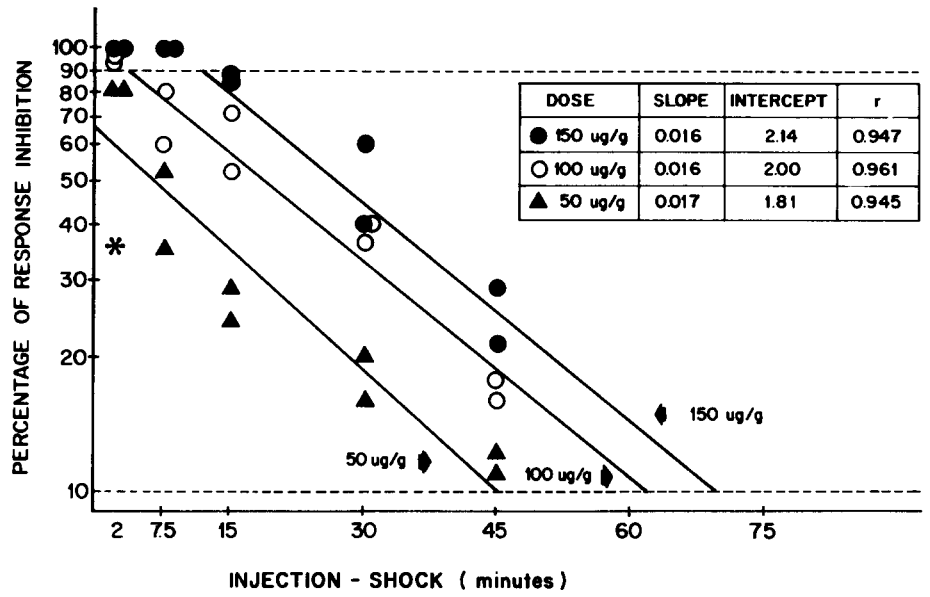

FIG. 5. Morphine-induced response inhibition vs. time. Linear regressions (log of response inhibition % vs. injection-shock interval) were performed on response inhibition percentages between 90 and 10. *Stands for a group that received 25 μg/g of morphine-HCl in a set with 2 minutes of injection-shock interval.

Duration of Morphine-Induced Inhibition

Experiments in this section were carried out to study morphine-induced response inhibition as a function of injection-shock interval.

There were 12 sets of 4 groups each: one *control group*, one *50 MP group* (morphine-HCl 50 μg/g), one *100 MP group* (100 μg/g) and one *150 MP group* (150 μg/g). Groups of the same set had the same injection-shock interval. There were 6 different intervals (2, 7.5, 15, 30, 45 or 75 min), so that two sets of groups corresponded to each interval. An 8 V shock was applied.

Figure 5 depicts a linear regression for each dose (log of response inhibition percentage vs. injection-shock interval) performed on values of response inhibition between 90 and 10%, i.e., excluding percentages statistically similar to 100 and to zero (normal curve test for proportions, two tails), respectively. The goodness of fit was high for all three regressions (0.82–0.92), suggesting a mono-exponential decrease of the response inhibition over time. A test of homegeneity for the regression coefficients [5] rendered a common slope for the 3 curves ($F=0.18$), indicating an additive dose effect when asymptotic values of response inhibition were disregarded.

According to the respective equations of Fig. 5, 50% inhibition was achieved 6 min after a 50 μg/g dose, at 18.8 min after 100 μg/g and at 27 min after 150 μg/g. The ED_{50} at 2 min was roughly 33 μg/g, an estimate stemming from results obtained with the two 2-min groups of 50 μg/g and a third 2-min group of 25 μg/g (* in Fig. 5). Duration of the morphine effect (TD), i.e., the x-intercept of the lines, was calculated to be 45.0 min for the 50 μg/g dose, 61.4 min for 100 μg/g, and 69.8 min for 150 μg/g.

Figure 6 shows that TD. vs. log(dose) is represented by a straight line whose equation is TD=52.3 log(dose) − 43.7. In a mono-compartmental model, dose doubling is known to cause a lengthening of TD equal to the half-life [21], so that an indirect estimate of this value could be calculated from the regression equation of Fig. 6: $t^1/_2$ = TD (100 μg/g) − TD (50 μg/g) = 15.7 minutes. Furthermore, this equation enables us to estimate the threshold dose (Qmin), i.e., the value of the abscissa at the curve intersection: Qmin = antilog 43.7/52.3 = 6.8 μg/g.

DISCUSSION

Defensive Response as a Model to Measure Opiate Action

The defensive response of *Chasmagnathus granulatus* elicited by an electric shock correlates with the stimulus strength (Fig. 1). This finding suggests that the measure of reactivity to this stimulus should also reflect variations in nociception, enabling the response to be used as an assay model for opiate agonists and antagonists.

Seasonal Variability and Baseline Sensitivity

Seasonal variability of basic nociceptive sensitivity (BNS) is clearly illustrated in Fig. 3. However, results presented in Fig. 4 demonstrate that percentages of morphine-induced response inhibition are independent of BNS values. Therefore, animals coming from different captures can be used, provided that control BNS values are carefully evaluated for each population.

Morphine Seems to Act Through an Opiate Receptor

The data presented shows that morphine-HCl injections (25 to 150 μg/g) produced a dose-dependent reduction of crab sensitivity to the nociceptive stimulus (Figs. 4 and 5). A 100 μg/g injection caused a drop in BNS approaching 50%, but no inhibition occurred when the same dose was administered with 1.6 μg/g of naloxone-HCl (Table 1, C,c). On the other hand, naloxone showed no agonistic activity at either dose (Table 1, A,b and B,b). Thus, morphine seems to act through an opiate receptor.

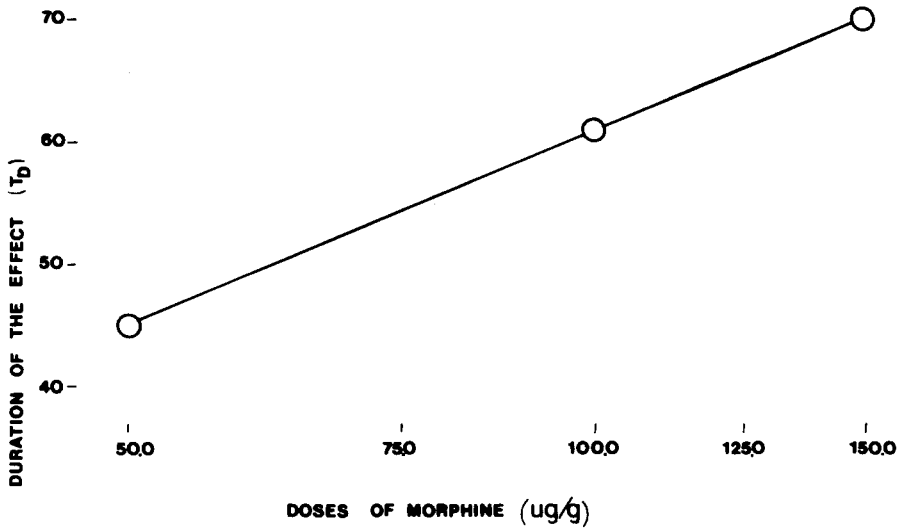

FIG. 6. Duration of the effect vs. morphine log (dose).

This set of findings is consistent with previous results reported for other arthropods, suggesting opiate systems may be widespead throughout the phyllum.

Analysis of Time-Dose-Response Curves. Comparison With Data Reported for Vertebrates

Peak morphine effect. Inspection of the fitted curves (Fig. 5) shows that the morphine effect peaked within 2 min post-injection. Comparable data from vertebrates indicate that peak effect occur at 20–30 min [17,20]. On the other hand, when lipid soluble opiates such as fentanyl were injected IV to vertebrates, the effect maximized within seconds after injection [9].

Thus, time values corresponding to the peak morphine effect for this arthropod are lower than those reported for vertebrates. However, values are similar when vertebrates received lipid-soluble opiates. This suggests that the arthropod blood-brain barrier is more permeable to morphine.

Duration of the effect and estimated half life. In agreement with calculations presented in the Duration of Morphine-Induced Inhibition section, duration of the morphine effect (TD) for the 50 μg/g dose was 45 min and the indirect estimate of half-life was 15.7 min.

When vertebrates are injected with morphine doses lower than 50 μg/g, the duration of the effect averages 4–5 hours [10, 16, 20]. Plasma concentration assessment indicates an average 3 hr half-life elimination for man following IV morphine [2,18] and roughly 2 hr after epidural administration [4].

This set of findings strongly suggests that arthropods have a much faster time course of elimination than vertebrates.

Drug potency. According to the respective equations of Figs. 5 and 6, the ED_{50} at 2 min was calculated to be 33 μg/g and the threshold dose (Qmin) 6.8 μg/g. Thus, the morphine potency estimated for arthropods resembles that reported for vertebrates, in disagreement with a conclusion put forward in previous papers [11, 12, 24].

To sum up, whereas morphine potency seems similar for these phylla, there is a noticeable difference in effect and elimination velocities.

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