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Modulation of the Escape Response by [D-Ala^2]Met-Enkephalin in the Crab *Chasmagnathus*

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GODOY, A. M. AND H. MALDONADO. *Modulation of the escape response by [D-Ala^2]Met-enkephalin in the crab* Chasmagnathus. PHARMACOL BIOCHEM BEHAV **50**(3) 445–451, 1995.—The synthetic opioid analog [D-Ala^2]Met-enkephalin (DAME) significantly reduces the escape response to a danger stimulus in the crab *Chasmagnathus granulatus* when administered within the dose range 0.01–1.0 μg/g. There is no reduction with lower or higher doses, thus suggesting a U-shaped dose-response curve. A 0.1-μg/g dose of naloxone has no effect per se on the response, but when it is administered together with DAME, it completely blocks the decremental effect of this drug and an escape response generally higher than that of control is observed. An explanation for these results in terms of a possible dual action of DAME is offered. In addition, we present evidence for different degrees of opiate sensitivity among crabs of the same population in relation to their different degrees of reactivity to the visual danger stimulus.

Crab Met-enkephalin Habituation Opioid

ON SUDDEN presentation of a passing shadow, the crab *Chasmagnathus granulatus* responds with a running reaction in an attempt to escape, which quickly decreases over trials. The result is a fast habituation that lasts for at least 5 days (3,24). An increasing number of studies have been performed in our laboratory focused on the parametric conditions of this habituation process, as well as on the probable role of opioids in the acquisition and/or retention of the habituated response (28,37,43,44). Thus, it has been shown that morphine administration produces dose-dependent, naloxone-reversible reduction in the level of the escape response to a danger visual stimulus (28) and that this effect may be accounted for by a central drug action (42,43). In addition to these results, two lines of evidence support the view that habituation of *Chasmagnathus* to such a stimulus involves the modulatory action of an endogenous opioid mechanism. First, after naloxone pretreatment an enhancement of the escape response emerged over trials, slowing down the short-term habituation (37), and second, repeated exposure to a given danger stimulus produced a transient, naloxone-reversible, inhibitory effect on the subsequent escape response to a different stimulus type (38,44).

However, the conclusion that habituation in *Chasmagnathus* might be modulated by an endogenous opioid mechanism is open to criticism because: 1) the drug used in these studies was morphine, for which the presence in crustaceans has not been reported, and 2) exceedingly large doses, namely non-physiological doses (14), were required to inhibit the studied response, a finding also observed with other arthropods (27,34,47).

Since 1981 several studies have suggested the presence of enkephalins in crustacea. Enkephalin-like immunoreactivity was determined in photoreceptors, axons, and perikarya of the spiny lobster *Panulirus interruptus* (29), in the sinus gland of the shore crab *Carcinus maenas* (8,17), in the eyestalk of the crab *Uca pugilator* (9), and in the brain and eyestalk of the crab *Geocarcinus lateralis* (22). Additionally, the involvement of Met-enkephalin in pigment regulation of *Uca pugilator* (21) and of Leu-enkephalin in blood glucose regulation of *Carcinus maenas* (39) has been reported. Recently, isolation and sequence analysis of Leu- and Met-enkephalin from thoracic ganglia of *Carcinus maenas* has been performed, demonstrating that they are structurally identical to the compounds found in vertebrates (25).

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On the basis of these data, we hypothesized that the endogenous agent of the proposed opioid mechanism of *Chasmagnathus* would be one or both enkephalins, for which action has been mimicked in previous experiments by the exogenous administration of morphine. The purpose of the present article is to test whether Met-enkephalin exerts an inhibitory effect on the escape response to a visual danger stimulus in this crab.

Given the extremely short life of enkephalins in arthropod hemolymph (5), we tested [D-Ala^2]Met-enkephalin (DAME), a synthetic analog that has proven to be a potent agonist in vertebrates, binding to opiate receptors and inducing opiate-like effects in either in vitro (6) or in vivo assays, the latter including behavioral responses (46).

METHOD

Animals

Animals were adult male *Chasmagnathus granulatus* 2.1–3.4 cm across the carapace, collected from water less than 1m deep in the rias (narrow coastal inlets) of San Clemente del Tuyu, Argentina, and transported to the laboratory, where they were housed in plastic tanks (35 × 48 × 27 cm) filled to 2 cm depth with water, at a density of 35 crabs per tank. Water used in tanks and other containers during the experiments was prepared with hw-Marinex (Winex-Germany), salinity 1.0–1.4%, pH 7.4–7.6. Crabs were fed rabbit pellets (Nutrientes SA) every 3 days, and after feeding the water was changed. The holding and experimental rooms were maintained within a temperature range of 19–24°C and a 14L:10D illumination cycle (light on 0700–2100 h). Experiments were carried out during the first week after the animal's arrival, between 1000 and 1700 h, from December to June (i.e., summer and fall). Each crab was used in one experiment.

Apparatus

The experimental device is described in detail elsewhere (37). Briefly, the experimental unit was the actometer, which is a bowl-shaped plastic container with a steep concave wall and a circular central flat floor 10 cm in diameter, covered to a depth of 0.5 cm with water. The crab was placed in the container that was suspended by three strings from an upper wooden framework (23 × 23 × 30 cm) and illuminated by a 10-W lamp placed 30 cm above the animal. An opaque rectangle screen (25 × 7.5 cm) could be moved horizontally across the upper border of the framework by a motor at an angular speed that allowed it to cover the entire opening in 2.3 s. The shadow produced by the screen movement provoked a crab's running response and, consequently, container oscillations. A stylus was centrally cemented to the outside bottom of the container and connected to a piezoelectric transducer. Container oscillations induced electrical signals through the transducer, which were proportional to the velocity of the oscillations (4). Such signals were amplified, integrated during the recording time (9 s), and translated into numerical units ranging from zero to 1530, before being processed by a computer. Thus, the scores were correlated proportionally to the velocity and number of the oscillations recorded during the 9 s.

The escape response to a shadow in the actometer consisted in the crab starting to run in an attempt to move away from the passing screen, a reaction that we term escape response. However, because the steep concavity of the circular wall prevented the animal from climbing up, each running effort was confined to the flat center of the container in such a way that the escape response during a single trial looked like a series of flights from the center toward the base of the wall.

The experimental room had 40 actometers, isolated from each other by partitions. To avoid unobserved malfunctioning, the actometers were periodically calibrated against one another by throwing small lead balls from the upper border of the framework to the center of the container and recording the score for 9 s. A noticeable uniformity of scores was obtained.

A computer was employed to program trial sequences, trial duration, and intertrial intervals, as well as to record escape scores during experimental events.

Experimental Procedure

Each crab was administered 50 μl of distilled water or drug solution. Because such a volume of distilled water does not affect the animal's responsiveness (23), we currently use it as vehicle. Crabs were injected through the right side of the cephalothoracic-abdominal membrane, by means of a syringe and needle fitted with a sleeve to control depth of penetration to 4 mm, thus ensuring that the injected solution was released roughly at the center of the pericardial sac. Drugs used were [D-Ala^2]methionine-enkephalin (DAME) and naloxone-HCl (Nx), both purchased from Sigma Chemicals.

Immediately after injection, the animal was placed in the actometer and, after a 30-min adaptation period, a two-trial testing session was performed with 180-s intertrial interval. A trial lasted 9 s and consisted of passing the screen four times over the actometer, recording the crab's activity during the entire trial time.

Before animals were used in an experiment, they underwent a selection test: each crab was turned on its back and only animals that immediately returned to their normal position were used. The reason for this selection is that crabs with a slow righting reaction show a low responsiveness to a large diversity of stimuli (e.g., they respond very little or don't respond at all either to the habituating horizontally passing screen or to the sensitizing 6-V electrical shock or vertically approaching shadow). Besides, at a later time, they usually exhibited unhealthy symptoms. No more than 10% of tested crabs were eliminated.

The crab's baseline responsiveness to the passing screen proved remarkably consistent up to 10 days after arrival, but on occasion an animal coming from different capture efforts presented differences in response level. Therefore, only crabs belonging to the same capture were used in each experiment.

To determine the effect of DAME on crabs' reactivity to the passing shadow, the performances during the two-trial testing of drug- and distilled water-injected groups (n = 40) were compared. This method of evaluating a drug effect is now currently used in our laboratory (37). However, it differs from that previously adopted (28). In previous works, the difference between pre- and postinjection reactivities in the experimental group was compared with that of the control group. This early method proved inconvenient when tested in pilot experiments for this study. In fact, to obtain a reliable preinjection baseline, two trials were required, an amount of stimulation that in the present animal populations was sufficient to induce such a large fall in reactivity that the potential inhibitory effect of this drug became masked.

Data Analysis and Statistics

The reactivity of each animal was assessed by adding the scores recorded during the two testing trials. A 180-s intertrial interval ensures that the response level of the second trial is not largely depressed by the influence of the first one, as found for very short intervals (38). A wide range of reactivity was found within the same group, oscillating, roughly, between a minimum score of 200 and a maximum of 3000.

As is shown in the Result section, it was relevant to take into account the response value ranking so, unlike previous studies in our laboratory, a nonparametric statistical test was used to asses the experimental and control group performances (Mann-Whitney test, alpha = 0.05, one-tailed).

RESULTS

A pilot experiment included two groups of *Chasmagnathus* (n = 40): a distilled water-injected group (the control group, CT) and a group administered 1.0 μg DAME/g (the experimental group, DAME). When considering the entire groups, DAME caused a decrement in the experimental group response, although this difference was statistically nonsignificant (Fig. 1A). When the animals in each group were ranked according to their response score, a highly significant response impairment was observed for the 20 lowest ranked scores of DAME compared to those of CT ($z = -2.68$, $p < 0.005$), whereas no difference was observed between the 20 highest scores of each group (Fig. 1B). These portions of groups are termed low responders and high responders, respectively, throughout this work. A screening of comparisons of successive samples of 20 ranked scores from lowest to highest values (Fig. 1C) showed that the DAME-induced response reduction decreased as more animals with increasing reactivity were included in each step. The between-group difference was no longer significant for steps 13–32 upward.

In two following experiments, a series of DAME doses was tested. Crabs were administered 0.001, 0.01, 0.1, or 1.0 μg DAME/g in one experiment and 5.0 and 10.0 μg DAME/g in the other. Each experiment had its own control group.

Figure 2A and B presents the performance of each DAME group and their respective control group. When data corresponding to all the animals in DAME groups were compared with those of their respective control (Fig. 2A), a significant decremental effect of DAME was found for 0.1 μg/g ($z = -2.8$, $p < 0.005$) but not for higher or lower doses, thus suggesting a U-shaped dose-response curve. When the analysis considered separately the lowest and the highest portions of the response rankings (Fig. 2b), a significant effect was found on low responders, with an effective dose range between 0.01 and 1.0 μg/g and a maximum drug action around 0.1 μg/g ($z = -3.37$, $p < 0.001$ for 0.01 μg/g; $z = -4.25$, $p < 0.001$ for 0.1 μg/g, and $z = -3.52$, $p < 0.001$ for 1.0 μg/g), although no significant effect was found for high responders. A screening of comparisons by successive steps of 20 ranked scores from lowest to highest values (Fig. 2C) showed that for doses 0.01 and 1.0 μg/g the effect of DAME decreased with steps until it was no longer significant, and for 0.1 μg/g the effect was always significant although significance decreased with steps. In contrast, no significant effect in any step of the screening was found with doses other than those in the effective range (i.e., 0.001, 5.0, and 10.0 μg/g).

The foregoing results were confirmed by three replications; in one instance the dose 1.0 μg DAME/g showed the maximum inhibitory effect. Although experiments were conducted through different months, no seasonal influence on results was observed.

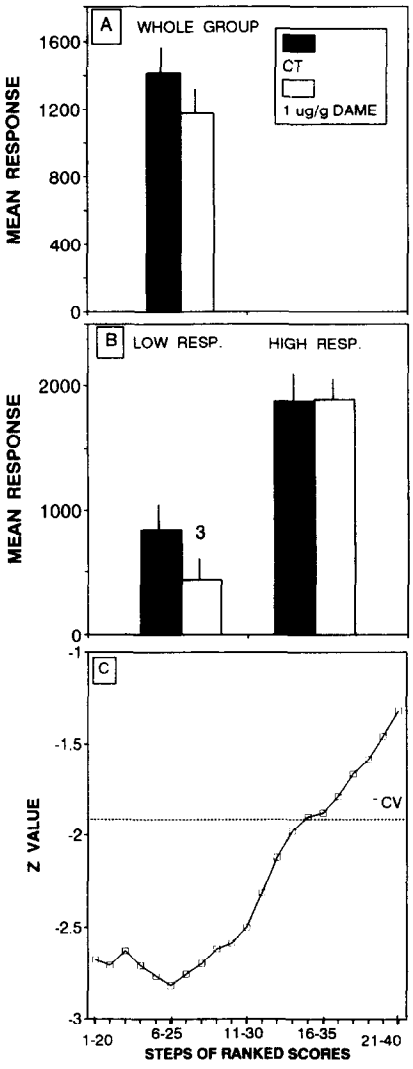

FIG. 1. Mean response score of the two-trial block of CT group (administered distilled water) vs. DAME group (1.0 μgDAME/g). (A) Whole group, n = 40. (B) Low and high responders, n = 20 each ($^3p < 0.005$, Mann-Whitney test). (C) Screening of CT-DAME comparisons. Abscissae: successive samples of 20 ranked scores of each group, from lower to higher response values. Ordinates: z value (Mann-Whitney test statistics) from each comparison. $-z$ for CT > DAME; $^-$CV, tabulated critical value below which DAME response is significantly lower than CT response. The height of each bar is the mean ± SEM.

The above screening of comparisons showed that DAME has a mild effect or no effect at all on hyperreactive crabs, suggesting that extremely high responders may be generally unsusceptible to the drug action. However, such an interpretation assumes that the condition of being hyperreactive preceded the drug treatment and was not its outcome. Therefore, it is necessary to dismiss the possibility that distilled water or DAME produce such a gross change on the response level that, for instance, previous low responders become high responders or vice versa.

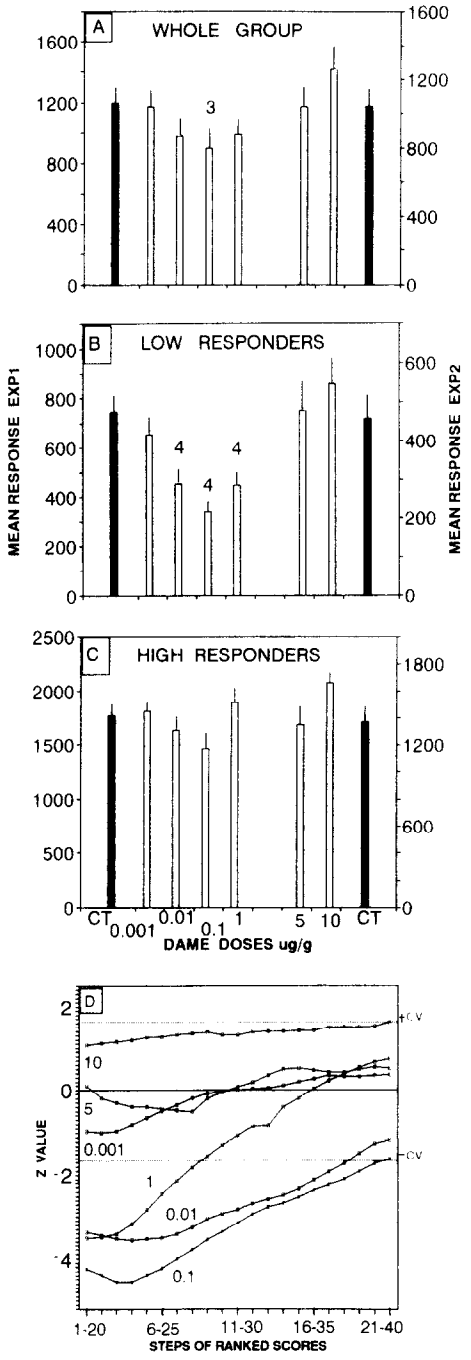

FIG. 2. Mean response scores of the two-trial block of EXP groups injected 0.001, 0.01, 0.1, and 1.0 μg DAME/g in one experiment (ordinates on the left) and 5.0 and 10.0 μg DAME/g in another experiment (ordinates on the right). Black bars stand for CT groups; white bars stand for EXP groups. The height of each bar is the mean $\pm$ SEM. CT bars were adjusted to the same height to better observe the dose-response relationship. Numbers express the significance of comparisons between each DAME group and its respective control by the Mann-Whitney test ($^3p < 0.005$; $^4p < 0.001$). (A) Whole groups, $n = 40$. (B) Low responders, $n = 20$. (C) High responders, $n = 20$. (D) Screening of CT-DAME comparisons of 20 ranked scores; $-z$ for CT > DAME, $-$CV as in Fig. 1C; $+z$ for DAME > CT, $+$CV, tabulated critical value over which DAME response is significantly higher than CT response.

As explained in the Method section, a pretesting session could have untoward depressant effects on the crabs in the testing response. However, pretesting is required to determine whether the response ranking is or is not maintained after the drug administration. For such a purpose, the following experiment was carried out.

After a 30-min adaptation time in the actometers, crabs were given two pretesting trials separated by 180 s followed by an injection of distilled water or 1.0 μg DAME/g. Both groups were kept in the actometers for another 30 min and then underwent two testing trials with the same intertrial interval.

To compare the response ranking of the two-trial block of pretesting with that of testing, a Spearman rank order test was performed for each group. The correlation coefficient was significant for both groups ($p < 0.001$), $r = 0.65$, $t(38) = 5.2$ for the control, and $r = 0.63$, $t(38) = 4.9$ for 1.0 μg/g of DAME. Therefore, neither distilled water nor DAME seems to drastically alter the response ranking; hence, the categories of low and high responders used during the above experiments would actually correspond to the behavioral category to which an animal belongs regardless of the treatment.

To test whether DAME acts through opiate receptors to inhibit the crab's reactivity to a danger stimulus, the antagonist effect of naloxone (Nx) was tested.

The experimental design included eight groups. One group was administered 0.1 μg Nx/g; three groups received 0.1, 1.0, or 5.0 μg DAME/g; three groups were given the same DAME doses plus 0.1 μg Nx/g; and one group was injected with distilled water.

The analysis of the results, displayed in Fig. 3, showed that this dose of Nx has no effect per se on responding to the

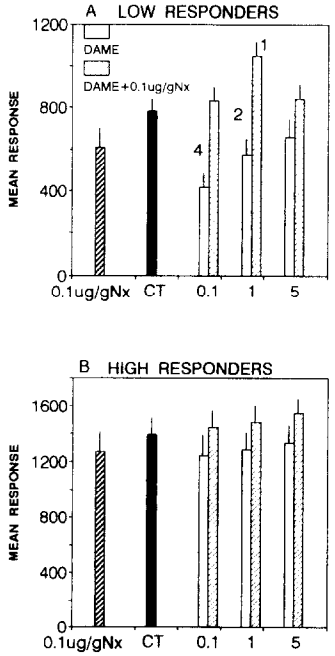

FIG. 3. Reversal of DAME effect by naloxone. Numbers express the significance of the comparisons between experimental and CT groups. Black bars stand for CT; white bars stand for DAME-injected groups; heavily stripped bars stand for Nx-injected groups; slightly stripped bars stand for DAME + Nx-injected groups. The height of each bar is the mean $\pm$ SEM. (Mann-Whitney test; $^1p < 0.05$; $^2p < 0.01$; $^4p < 0.001$). (A) Low responders, $n = 20$. (B) High responders, $n = 20$.

danger stimulus but, when administered together with DAME, antagonizes the decremental effect of DAME (0.1 or 1.0 μg/g) and results in a response generally higher than that of controls. Data from low responders gave $z = -3.41$, $p < 0.001$ for 0.1 and $z = -2.2$, $p < 0.005$ for 1.0 μg DAME/g, whereas differences were nonsignificant for 5.0 μg DAME/g, 0.1 μg Nx/g, 0.1 μg DAME/g plus 0.1 μg NX/g, or 5.0 μg DAME/g plus 0.1 μg Nx/g. The enhancing effect of Nx + DAME reached significance with 1.0 μg/g of DAME ($z = -1.82$, $p < 0.05$). For high responders no statistically significant difference was found, although the same tendencies as for low responders were observed.

DISCUSSION

The synthetic analog of Met-enkephalin, DAME, produces a dose-dependent and naloxone-reversible reduction, of the *Chasmagnathus* escape response to a shadow passing overhead. This effect parallels that obtained with morphine on the same behavioral response, thus supporting the hypothesis put forward in the Introduction, that Met-enkephalin may be an endogenous opioid for which the depressant action is mimicked by exogenously administered morphine. However, the effective DAME doses producing a reduction of the escape response (0.01, 0.1, and 1.0 μg/g) are noticeably smaller than the morphine doses found to exert the same effect (25, 75, and 100 μg/g) (28,42).

Previous reports indicated that morphine acts centrally rather than on visual input and/or motor ability (42) and that this drug interferes with the decoding of the visual danger stimulus (43). No similar conclusion concerning DAME may be drawn from the present results, so further investigation is required to determine if the effects of both drugs are exerted through the similar mechanism. However, the likelihood that enkephalins are involved in the processing of visual information has been put forward for crustacea (25,40). Data showing immunoreactive enkephalins in ommatidia and diverse eyestalk ganglia (8,9,17,22,29), together with those describing the light-induced inhibition of neurons located in the X-organ-sinus gland system (11), hint at the possibility of an enkephalinergic control of circadian rhythmicity for some functions. It is assumed that enkephalins represent a link between light and the circadian rhythm in the release of the crustacean hyperglycemic hormone (CHH) (15,18,20) and the resulting daily fluctuations in blood glucose level, feeding, and locomotion (12). Consequently, it should be expected that there is a daily fluctuation in the escape response level of *Chasmagnathus* to a visual danger stimulus with a minimum of reactivity during the light phase. This prediction has recently been confirmed (36).

Serotonin has been reported to exert an action opposite to that of Met-enkephalin, namely an enhancing effect on the escape response (1). On the other hand, serotonin and Met-enkephalin have been considered good candidates as regulatory factors for the activity of the CHH-producing system (39,44), with the former facilitating the secretion of CHH (19) and the latter inhibiting the release of CHH from the sinus gland (16). Accordingly, it is tempting to speculate that the escape response to a visual danger stimulus could involve CHH. However, a recent result indicates that serotonin may increase glucose level independently of the eyestalk neurosecretory system (26).

Naloxone completely blocks the DAME-induced reduction in the response level. However, it is noticeable that the response of groups treated with DAME + naloxone is not only higher than that of DAME groups but also than that of control groups, a difference that reached significance in one case (Fig. 3A,B). A possible explanation for this result is as follows. DAME administration in the effective dose range would elicit the observed inhibitory process on the escape response but, in addition, an excitatory process by direct or indirect pathways, the former predominating over the latter, so the overall result is a diminished response. When DAME is administered together with naloxone, only the inhibitory process is blocked and an increased response is observed. According to our hypothesis on the existence of an opioid mechanism acting in habituation (37,38,44), an endogenous opioid substance would start being released from the first presentation of the habituating stimulus and its depressant effects would become more evident on later trials, inducing a response decrement. Thus, Nx treatment could exert no effect during the first few trials (as occurs in this study) but should provoke an arousal in the response when compared to control animals during later trials as habituation progresses. The latter has actually been proved in our laboratory (37). When DAME is injected, the internal state corresponding to the later trials is mimicked and effectively blocked by the opiate antagonist naloxone.

A dual effect of morphine has been reported for other species (31,33,41), the enhancing effect being explained by some authors as resulting from a direct action of opioids (2,31) whereas other authors attribute it to compensation for the depressant effect (13,35).

The dose-response relationship for DAME is represented by a U-shaped curve despite the fact that in classical pharmacology the ideal relationship between drug concentration and response magnitude is a hyperbolic function (10). However, the scientific literature shows many examples of a U-shaped type of curve, including studies on the effects of opioid agonists and antagonists on learning and behavioral responses of both crustacea and other species (30,37). To account for such a phenomenon, it has been suggested either that receptor fatigue occurs when doses greater than optimal are used (7) or that ascending and descending portions of the U-shaped curve represent opposing processes (32). In the present case, an explanation based on the proposed dual effect of DAME can be offered. Namely, it can be hypothesized that up to 1.0 μg DAME/g the depressant effect on the escape response masks the enhancing process, but that greater amounts of DAME (5.0–10.0 μg/g) induce an increase in the enhancing effect while the inhibitory process becomes masked. The ineffective dose, 0.001 μg DAME/g, may be too small to exert any effect on either of these processes.

An additional finding of this work is that the conspicuous inhibitory effect of DAME on the escape response of low responders proves to be mild or null on hyperreactive, high-responder crabs. In other words, there seems to be different degrees of opiate sensitivity among the crabs of the same population. This rather intriguing fact should be commented in connection with another result of our laboratory: the sensitizing effect of serotonin is stronger on high responders than on low responders (1). Therefore, two types of *Chasmagnathus* might tentatively be distinguished: on one hand, comparatively low responders, more sensitive to the depressant effect of enkephalins on the escape response to a visual danger stimulus and, on the other hand, comparatively high responders, more sensitive to the enhancing effect of serotonin. Of course, between these two extremes, there exists a gradient of sensitivity to each drug.

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