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Serotonin-Induced Short- and Long-Term Sensitization in the Crab *Chasmagnathus*

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AGGIO, J., A. RAKITÍN AND H. MALDONADO. *Serotonin-induced short- and long-term sensitization in the crab* Chasmagnathus. PHARMACOL BIOCHEM BEHAV 53(2) 441-448, 1996.—On sudden presentation of a passing shadow (a visual danger stimulus), the crab *Chasmagnathus granulatus* responds with a running reaction that we term an escape response (ER). A single administration of 5-HT in a dose range between 0.005 and 0.1 μg/g enhances the ER at 30 min, and a dose of 10-15 μg/g has a similar effect for at least 24 h. The classical 5-HT antagonist cyproheptadine (CYP) given within a dose range 0.01-2.0 μg/g has no effect on ER at 30 min, but 0.5 μg/g blocks the 5-HT-induced short-term sensitization. An enhancing effect of CYP on the ER is shown at 24 h with 1.0-2.0 μg/g doses. An explanation of this results in terms of a model similar to that proposed for *Aplysia*, that is two sensitizing processes linked in parallel and mediated by two different types of 5-HT receptors is discussed. Results indicate that the sensitizing effect of 5-HT is confined to high responders, thus suggesting that crabs of a same population have different degrees of sensitivity to the drug according to their different degrees of reactivity to the visual danger stimulus.

Crab Serotonin Cyproheptadine Sensitization

THE CRAB, *Chasmagnathus granulatus*, reacts to a shadow passing overhead with an escape response that habituates quickly upon repetition of the shadow stimulus. This habituation lasts for several days (5,43). Ongoing research on this long-term habituation, aimed at analyzing mechanistic and theoretical aspects, has been under way for the last 10 years. Included in this analysis have been studies on its specificity (26,37,43), on its adaptive value (43), on its relation with age (41), and circadian cycle (35), on the role of opioids in acquisition [e.g., (15,38,42,44)], and on the effect of protein synthesis inhibitors on memory consolidation (33,34).

Besides habituation, short- and long-term sensitization processes in *Chasmagnathus* have been also reported. The iterated presentation of a screen similar to that used in this work, but with a different spatial relation with the animal (it approaches and then recedes from the crab), has both a short- and long-term enhancing effect on the escape response (1,37,39). Based on these results and parallel studies on the role of opiates in habituation of *Chasmagnathus*, a search for a possible biochemical first messenger involved in sensitization, is pertinent. Several reports suggest that serotonin (5-hydroxytryptamine, 5-HT) might be a good candidate. In vertebrates, 5-HT has been generally considered associated with anxiety and stress, because an increase in its activity appears to induce anxiety and mimic stress, while a decrease seems to have opposite effects (17,18). However, little is known of how 5-HT works in behaving vertebrates, in contrast with the progress made in invertebrates (24). Thus, it has been shown that 5-HT administration induces short- and long-term sensitization of both tail-siphon- and gill-withdrawal reflex in *Aplysia* (6); produces synaptic facilitation at type B cell connections in *Hermissenda* (40); causes lobsters to stand tall on the tip of their walking legs with their claws open in front of them and their abdomen loosely tucked downward, namely, the so-called serotonergic posture (similar to that displayed by several decapod crustaceans when engaged in agonistic encounters) (19,24,25,32); induces prolonged spontaneous swimming in the medicinal leech (47); produces increased ventilatory and heart rates (46), glycemia (27), and locomotor behavior to the detriment of their photonegative behavior during daylight hours (30) in the crab, *Carcinus maenas*; enhances the optokinetic response in the crab, *Leptograpsus variegatus* (11); and augments the eye withdrawal reflex in another crab, *Cancer magister* (2).

Notwithstanding, all these results suggesting that 5-HT may be involved in sensitization or in the excited state of

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invertebrates (45), not all evidence supports this view. Opposite results have been reported in the cockroach (16), crayfish (13,14), and bees (10).

The purpose of the present article is to test the hypothesis that 5-HT induces short- and long-term sensitization of the escape response of *Chasmagnathus* to a passing screen.

Throughout this work we term sensitization the enhancement of the escape response due to drug administration. Short-term sensitization is that disclosed 30 min after injection and long-term sensitization that shown 24 h after injection.

GENERAL METHOD

Animals

Animals were adult male *Chasmagnathus granulatus* 2.1–3.4 cm across the carapace, weighing 17 ± 0.2 g (n = 60), collected regularly every 15 days from water less than 1 m deep in the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina, and transported to the laboratory, where they were lodged 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 14 L : 10 D illumination cycle (lights on 0700–2100 h). Experiments were carried out during the first week after the animal's arrival, between 1000 and 1700 h, from November to June (i.e., late spring, summer, and fall). Each crab was used in one experiment.

Apparatus

The experimental device is described in detail elsewhere (38). Briefly, the experimental unit was the actometer: 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, which allowed it to cover the entire opening in 2.3 s. The shadow produced by the screen movement provoked the crabs' 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. Such signals were amplified, integrated during the recording time of 9 s (i.e., the duration of a trial) and translated into numerical units ranging from zero to 3060, before being processed by a computer. Thus, the scores were proportional to the velocity and number of the oscillations recorded during the 9 sec.

The escape response to a shadow began with the crab running in an attempt to move away from the passing screen. 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. As a result, 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 vehicle, i.e., distilled water containing ascorbic acid 10^{-4} M, or the drug solution prepared within 30 min of use. Ascorbic acid was used to prevent spontaneous oxidation of the amine (16). We currently use 50 μl of distilled water as vehicle because such a volume of dw proved not to affect crab's responsiveness or drug action (15). Crabs were injected through the right side of the cephalothoracic-abdominal membrane by means of a syringe and needle fitted with a sleeve to limit the 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 serotonin (5-HT) and cyproheptadine (CYP) both purchased from Sigma Chemicals, St. Louis, MO.

In short-term experiments (i.e., those in which the effect of the drug was evaluated 30 min after administration), crabs were placed in the actometers immediately after injection and a two-trial testing session with 180-s intertrial interval was performed after a 30-min adaptation time. A trial lasted 9 s and consisted of passing the screen four times over the actometer, while recording the crab's activity during the entire trial time, a procedure rutinely used in our laboratory (5,15,26). In long-term experiments (i.e., those in which the effect of the drug was evaluated 24 h after administration), animals were individually housed in plastic containers immediately after injection and remained there for 24 h, after which they received the two-trial testing session.

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. Rationale for this selection is that crabs with a slow righting reaction show a low responsiveness to a large diversity of stimuli. For example, they respond very little or don't respond at all either to an habituating stimulus as the passing screen or to sensitizing stimuli as 6 V electrical shock or a screen approaching and then receding from them. Besides, at a later time, they usually exhibit unhealthy symptoms. No more than 10% of tested crabs were eliminated on this basis.

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

To determine the effect of 5-HT or CYP on crabs' reactivity to the passing screen, testing performances of drug- and vehicle-injected groups (EXP and CT, respectively) were compared. This method of evaluating a drug effect is now currently used in our laboratory (38). However, it differs from that previously adopted (28). In previous work, 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 base-

line, two trials were required, an amount of stimulation that sufficed to induce a change in reactivity possibly masking the effect of the drug under study. Unless otherwise noted, throughout this article a group (EXP or CT) includes 40 crabs.

Data Analysis and Statistics

The reactivity of each animal was assessed by adding the scores recorded during the two testing trials. A wide range of reactivity was found within a same group, with some crabs obtaining a minimum score of 50 and others obtaining maximal scores of 3000.

Despite this variability, each individual crab kept roughly the same response level in a reactivity rank after diverse treatments, as has been observed during habituation training and water or drug injections (15,33,34). In other terms, the relative level of response of a crab within its own group seems to represent an individual behavioral feature. On this account and considering that at least for some drugs the effect seems to depend on the crab's level of reactivity (15), the statistical analysis was performed comparing the scores of entire groups or scores corresponding only to the 20 higher ranked values (high responders) or the 20 lower ones (low responders).

Because ranking of response was used, a nonparametrical statistical test was preferred: the Mann-Whitney (alpha = 0.05, one tailed).

RESULTS

Short-Term Sensitization

Experiments in this section were designed to assess the effect of several doses of 5-HT on the escape response evaluated 30 min after drug administration.

A first series of four experiments was performed, each one testing a different dose (0.005, 0.01, 0.1, or 1.0 μg/g). Each experimental group (EXP) and its respective control group (CT) were simultaneously run using crabs belonging to the same capture effort.

Figure 1 presents the performance of each EXP-CT pair. When data corresponding to all the animals of EXP were compared with those of CT (Fig. 1A), a significant incremental effect of 5-HT was found for 0.1 μg/g ($z = +1.83$, $p < 0.05$) but not for higher or lower doses, thus suggesting a U-shaped dose-response curve. When the highest and the lowest portions of the response rankings were considered separately, a significant effect was found on high responders with an effective dose range between 0.005 and 0.1 μg/g ($z = +2.33$, $p < 0.01$ for 0.005 μg/g; $z = +2.30$, $p < 0.01$ for 0.01 μg/g, and $z = +2.66$, $p < 0.01$ for 0.1 μg/g; Fig. 1b). No significant effect on low responders was found except for 0.1 μg 5-HT/g dose ($z = +1.72$, $p < 0.05$; Fig. 1c).

The foregoing results for high responders were confirmed during three replications. No instance of an enhancing effect on low responders was shown in any other case, and significant EXP-CT differences for whole group scores were found only with 0.01 μg/g.

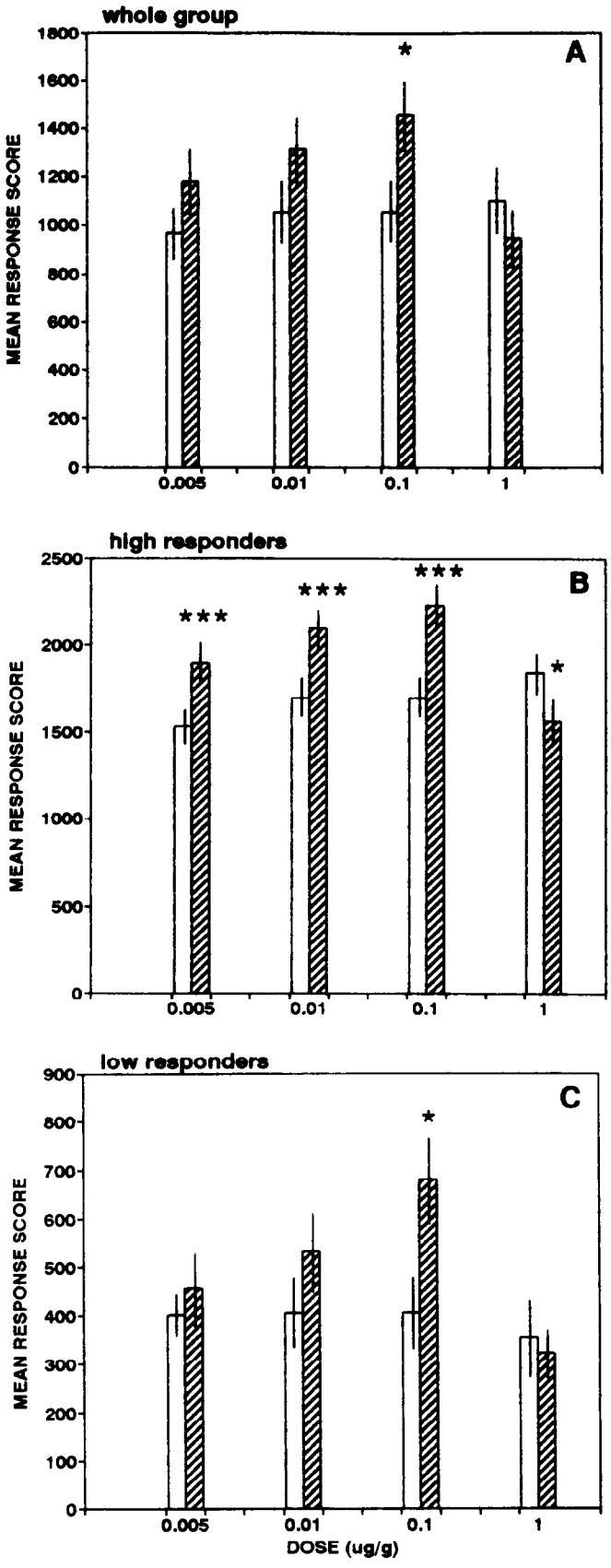

FIG. 1. 5-HT-induced short-term sensitization (30 min after injection). Ordinates: means ± SEM of two-trial testing blocks. Hatched bars: 0.005, 0.01, 0.1, and 1 μg 5-HT/g. White bars: vehicle-injected controls. (A) Whole group: all 40 animals. (B) High responders: the 20 higher ranked crabs in each group. (C) Low responders: the 20 lower ranked crabs in each group. $*p < 0.05$, $**p < 0.025$; $***p < 0.01$, Mann-Whitney *U*-test, comparisons between 5-HT- and vehicle-injected groups.

It is noticeable that 1.0 μg 5-HT/g dose showed a depressant effect on the response of the high responders ($z = -1.76$, $p < 0.05$). Such a tendency was even more manifest when higher doses were employed (5 or 10 μg/g) because after the amine injection most of the crabs displayed the serotonergic posture that prevents the animals from running. As with lobsters, the crabs' claws were opened and held forward; walking legs were kept directly underneath the body, with the distal joints slightly flexed so that animals stood high off the substrate (25). These effects appeared one to several minutes after injection and were dose dependent. With 5 or 10 μg/g of 5-HT, the immobilization was complete up to an hour, so animals were not exposed to the testing session. A dose of 1 μg/g had a much milder effect on posture and mobility, the animals being able to move even immediately after receiving the amine, so that they could be tested 30 min after the injection.

Long-Term Sensitization

In the following experiments the effect of 5-HT on the escape response was tested 24 h after injection using an experimental design as above.

Preliminary experiments showed no effect at 24 h of doses that proved to induce enhancement of the escape response at 30 min (0.01 and 0.1 μg/g). Therefore, higher doses were tested to induce long-term sensitization.

Figure 2 shows results corresponding to the two-trial testing block of experiments in which EXPs were injected with 1, 5, 10, 15, 20, 30, or 40 μg 5-HT/g compared with that of the respective CTs. An enhancing effect on the response level of high responders was apparent for EXPs given 10 and 15 μg/g ($z = +2.14$, $p < 0.025$ and $z = +1.89$, $p < 0.05$, respectively, Fig. 2B). One of the doses (10 μg/g) also had an effect on the whole group ($z = +1.79$, $p < 0.05$, Fig. 2A) and the low responders ($z = +1.93$, $p < 0.05$, Fig. 2C). Three replications with the same series of doses confirmed these results concerning high responders, but no instance of an enhancing effect on the whole group or on low responders was observed. This result agrees with the preceding conclusion that low responders are less susceptible to the modulatory action of 5-HT on the escape response, though in the present case both high and low responders displayed the serotonergic posture with all the doses higher than 1.0 μg/g.

A cursory inspection of Fig. 2 suggests a U-shaped dose-response curve because the highest doses did not show the stimulating effect observed with lower doses. The EXP group injected with 40 μg/g showed a reactivity significantly lower than that of CT group in all three analyses ($p < 0.001$, $z = -3.13$, -3.88, and -3.34 for whole group and high and low responders, respectively, Figs. 2A, B, and C).

Maintenance of the Response Ranking

The above analyses showed that 5-HT has a mild effect or no effect at all on hyporeactive crabs, suggesting that low responders may be generally unsusceptible to the drug action. This result is precisely opposite to that obtained with Met-enkephalin (15). This conclussion assumes that the condition of being hyporeactive preceded the drug treatment and was not its outcome. Therefore, it is necessary to evaluate the possibility that 5-HT produces so gross a change in the response level that, for instance, previous high responders become low responders or vice versa.

As explained in general methods, a pretesting session could have a generalized depressant effect on the crabs in the testing

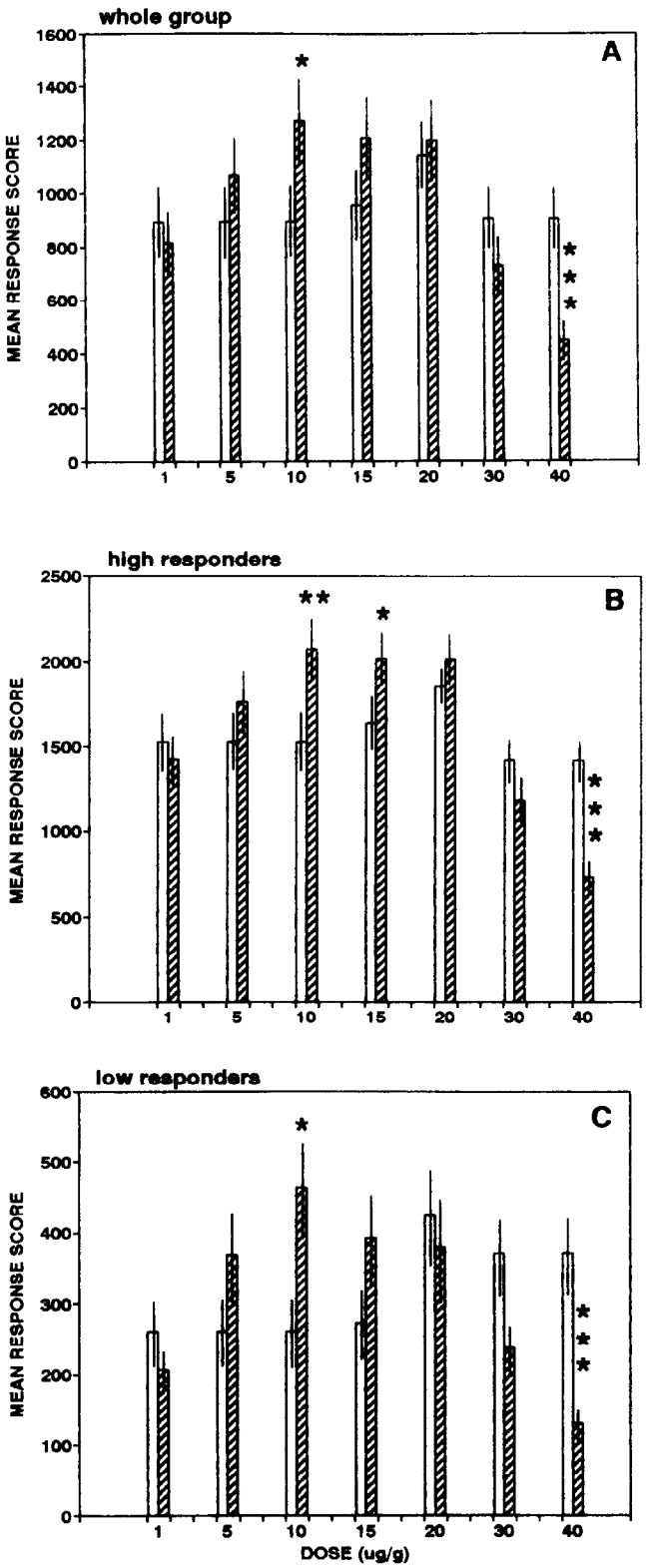

FIG. 2. 5-HT-induced long-term sensitization (24 h after injection). Hatched bars: 1, 5, 10, 15, 20, 30, and 40 μg 5-HT/g. Statistics and symbols as in Fig. 1.

response, thus masking EXP-CT differences. However, pretesting is required to determine whether the response ranking is or not maintained after drug administration.

To address this issue, the following two experiments were performed.

In the first experiment, two groups of crabs (n = 60 per group) were given two pretesting trials separated by 180 s and stored in individual containers for 24 h. Then, they were injected with vehicle or 0.01 μg 5-HT/g and given 30 min after injection of the usual testing session of two trials. To evaluate if the response ranking is maintained, the response rankings of the two-trial block of pretesting with that of testing were compared for each group using a Spearman rank order test. The correlation coefficient was significant for both groups ($p < 0.001$), $rs = 0.77$, $t = 10.44$, for the control, and $rs = 0.59$, $t = 6.37$ for 0.01 μg/g of 5-HT. Therefore, neither vehicle nor 5-HT injected 30 min before testing seem to drastically alter the response ranking.

A second experiment was carried out to test whether the ranking is maintained 24 h after drug administration. Two groups of animals (n = 80 per group) received a pretesting session as above, were injected immediately afterwards with vehicle or 10 μg 5-HT/g and then placed in the individual containers for 24 h. After such time they were given the usual testing session. A Spearman test showed that animals maintained their rank (vehicle: $rs = 0.61$, $t = 4.69$, $p < 0.0005$; 5-HT: $rs = 0.43$, $t = 2.92$, $p < 0.005$).

Hence, the categories of low and high responders used during the above experiments, either in those of short- or long-term sensitization, would actually correspond to the behavioral category to which an animal belongs, regardless of the treatment. These results are in keeping with those previously reported for crabs injected with Met-enkephalin (15) or with protein synthesis inhibitors (33, 34).

Modulatory Action of the Antagonist CYP

To investigate the receptors that mediate the modulatory action of 5-HT on the escape response, the classical antagonist CYP was used (4,36).

The effect of CYP on reactivity was evaluated through two series of experiments designed as above. In the first series, EXPs were injected with 0.001, 0.01, 0.1, 1.0, 2.0, or 3.0 μg CYP/g and tested at 30 min; in the second series, animals were administered 0.5, 1.0, 2.0, and 3.0 μg/g and tested 24 h after injection.

Groups injected with 3.0 μg/g showed a deep lowering of response at 30 min, and 24 h after injection these crabs exhibited appendage losses, a phenomenon termed autotomy (12), and several deaths were found.

Figure 3 (left side) exhibits results for groups tested at 30 min. No significant differences between EXP and CT groups were disclosed for any dose. When the analysis considered separately scores of high and low responders, results similar to those found for the entire group were obtained (data not shown). Thus, the antagonist CYP has no effect per se on responding to the danger stimulus when tested 30 min after injection.

Crabs injected with 2.0 μg CYP/g and tested 24 h after injection (Fig. 3, right side) showed scores statistically higher than that of CT ($z = +2.25$, $p < 0.025$), but no significant difference for EXP vs. CT was disclosed with the other two doses. When the analysis considered high and low responders separately, similar results were found (data not shown).

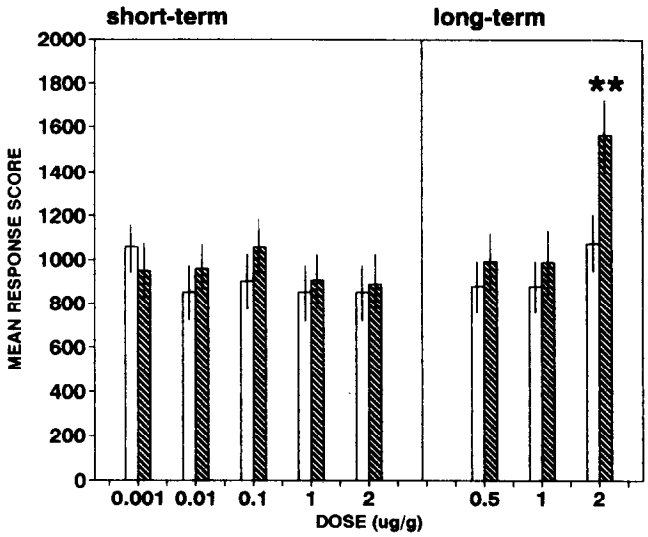

FIG. 3. Effect of CYP on the escape response. Hatched bars: CYP, white bars: vehicle. Left side: short-term evaluation (30 min); 0.001, 0.01, 1, and 2 μg CYP/g. Right side: long-term evaluation 24 h); 0.5, 1, and 2 μg CYP/g. Only whole groups are shown. Statistics and symbols as in Fig. 1.

No serotonergic posture was exhibited with any CYP dose.

Replications of these experiments confirmed the long-term enhancing effect of 2.0 μg CYP/g. In two instances, 1.0 μg/g induced a significant increase of the response when scores of high responders were compared.

Thus, CYP shows no effect on the escape response when tested at 30 min but exhibits a positive modulatory action when crabs are tested 24 h after injection of a dose equal to or higher than 1.0 μg/g.

Effect of CYP on 5-HT–Induced Sensitization

To test if the 5-HT–induced short-term sensitization is mediated by a CYP-sensitive receptor, the following experiment was performed.

One hundred and sixty crabs were randomly assigned to four groups of 40 animals each: CT (control), CYP alone (0.5 μg/g), 5-HT alone (0.01 μg/g), and CYP + 5-HT (0.5 μg CYP/g plus 0.01 μg 5-HT/g) and tested for short-term sensitization.

Figure 4 depicts results of this experiment. A significant difference between CT and 5-HT alone was disclosed when scores of either the whole group or high responders were compared ($z = +1.96$, $p < 0.05$; $z = +3.04$, $p < 0.025$; Figs. 4A and B, respectively) but this difference disappeared when 5-HT and CYP were injected together (CT vs. CYP + 5-HT). Thus, the increasing effect of 5-HT on responding at 30 min appears to be mediated by CYP-sensitive receptors.

A dose ratio of 5-HT/CYP as above (0.01/0.5) could not be used to explore the antagonist effect of CYP on 5-HT–induced long-term sensitization, because the minimum effective dose of 5-HT was 10 μg/g and a dose of CYP higher than 3 μg/g proved to have lethal effects. Further, 1.0–2.0 μg/g of CYP showed an enhancing effect on the ER at 24 h.

In summary, the short-term but not the long-term sensitizing effect of 5-HT was demonstrated to be blocked by CYP.

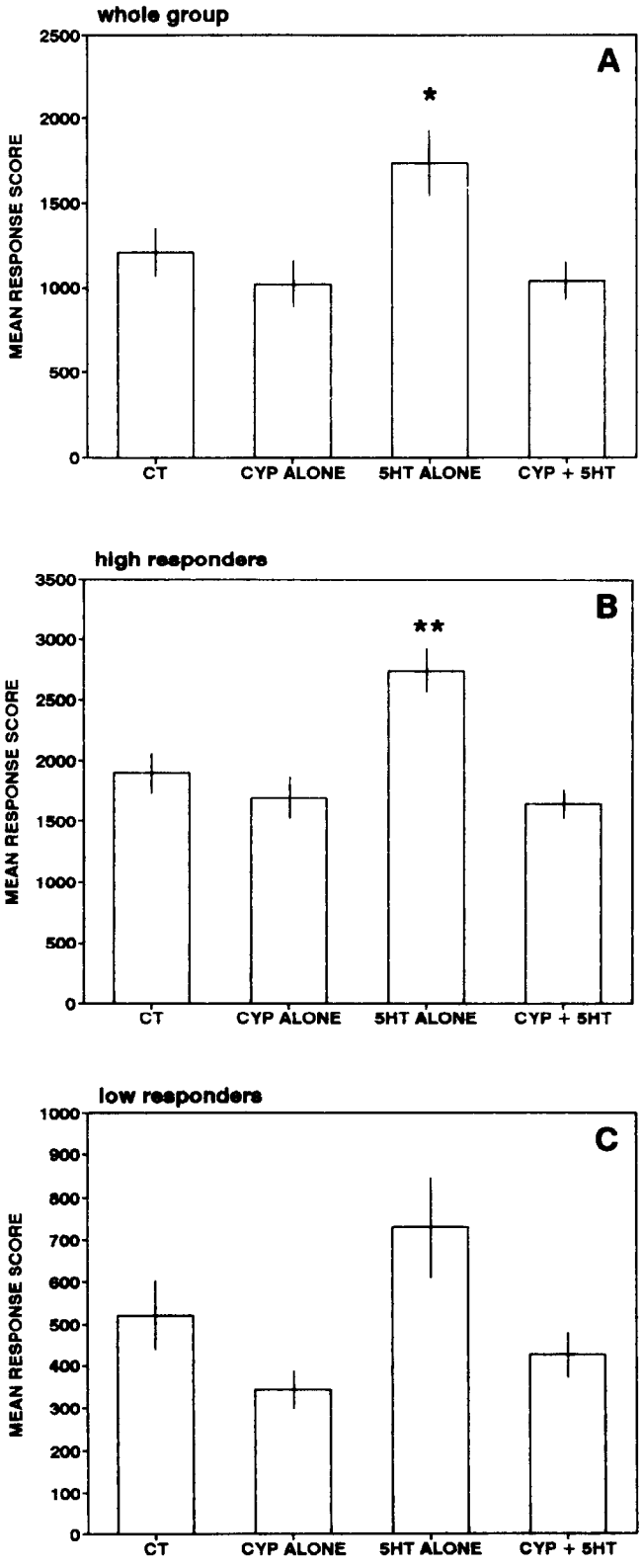

FIG. 4. Effect of CYP on 5-HT-induced short-term sensitization (30 min after injection). CT: vehicle, CYP alone: 0.5 μg CYP/g, 5-HT alone: 0.01 μg 5-HT/g, CYP + 5-HT: 0.5 μg CYP + 0.01 μg 5-HT/g. Statistics and symbols as in Fig. 1.

DISCUSSION

A single administration of 5-HT in a dose range between 0.005 and 0.1 μg/g showed at 30 min an enhancing effect on the escape response that dissapeared after 24 h. On the other hand, no facilitatory effect was exhibited 30 min after injection of 10–15 μg 5-HT/g, because the rigid serotonergic posture impaired the response, but after 24 h an enhancing effect of such doses was manifest.

The sensitizing effect of 5-HT at both 30 min and 24 h as well as that of CYP at 24 h were confined mainly to high responders, that is, the effect proved to be mild or null on low responders. This rather intriguing fact should be commented in connection with a previous result (15), indicating that the depressant effect of Met-enkephalin on the escape response is stronger in low than in high responders. Thus, two subtypes of *Chasmagnathus* might tentatively be distinguished: on one hand, comparatively low responders, more sensitive to the depressant effect of enkephalins on the escape response and more prone to leave the running strategy to adopt other defensive reactions as immobilization or lateral merus display (48); on the other hand, comparatively high responders, more sensitive to the enhancing effect of 5-HT and more prone to show augmented escape response levels. Such distinction between low and high responders is a robust one, because individuals remain in the same category after diverse treatments.

The existence of behavioral subpopulations has been demonstrated in other animal models. In the crab, *Cancer magister*, the eye withdrawal reflex habituates during a session of 50 taps on the carapace, but in subsequent sessions animals become either habituated or sensitized (2). In an excitatory conditioning paradigm with *Drosophila melanogaster*, two subpopulations with diverse acquisition and extinction patterns were identified (21); further, it was possible to breed by divergent selection lines of flies that differ in this traits (32).

The long-lasting potentiation of the escape response can hardly be attributed to a residual effect of the drug because biogenic amines seem to be rapidly removed from hemolymph. The half-lives for catecholamines in *Eriocheir sinencis* are estimated to be between 10 and 40 min (20) and for 5-HT in *Carcinus maenas* to be 25 min (29). Neither can it be attributed to a secondary effect due to the serotonergic posture because this posture was seen in both high and low responders, whereas the latter did not show an enhanced response. Conversely, CYP given at a dose of 2 μg/g produced an augmentated response but no postural effects.

At first analysis, both the short- and long-term enhancing effect of 5-HT could be mediated by 5-HT receptors, though to induce long-term effect larger doses are needed in the same way as larger number of sensitizing trials or repeated 5-HT applications are required to pass from short- to long-term sensitization in *Aplysia* (3,22). However, the view that the two processes are linked in series through a single type of 5-HT receptor has been recently questioned as a result of experiments done on in vitro preparations of *Aplysia* (32), in which the effect of CYP on 5-HT-induced synaptic facilitation was studied. CYP proved to abolish the 5-HT-induced short-term but not the long-term facilitation.

Results presented in this article are not conclusive in this respect, mainly because of the impossibility to use the same 5-HT/CYP dose ratio in long- and short-term experiments. Nevertheless, it is worth noting that no dose of CYP was able to induce short-term sensitization, whereas 1.0–2.0 μg/g provoked an enhancement of the response much like 5-HT at 24 h. This result hints at the possibility that sensitization was

not mediated in *Chasmagnathus* by a single 5-HT receptor. Perhaps two 5-HT receptor subtypes are involved: one with which CYP interacts producing a minimal agonist activity and maximal inhibition of 5-HT-induced short-term facilitation; and another one, on which CYP displays agonist activity and no inhibition of 5-HT-induced long-term sensitization. An interpretative model with two 5-HT receptors was also put forward for sensitization in *Aplysia* (9), but differing as regards the long-term subtype because no long-term synaptic facilitation induced by CYP was shown in this mollusc. Furthermore, an alternative explanation in terms of the well-known antihistaminergic action of CYP [e.g., (23)] is possible. In effect, it has been showed that in crayfish, histamine mediates the presinaptic inhibition of the escape reaction (8).

Both short- and long-term sensitization of the escape response to a screen passing overhead can be induced either by 5-HT or by the iterated presentation of a screen that approaches and recedes from the crab, the so-called vertical stimulus (1,39). Further investigation is necessary to elucidate the relationship between these sensitizing procedures. CYP given before or after sensitization session with the vertical stimulus should be a useful tool of research to elucidate the mechanisms subserving sensitization.

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