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The Behavioral Response of Spiny Lobsters to ATP: Evidence for Mediation by P_2-like Chemosensory Receptors

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Abstract. The results of both behavioral and electrophysiological studies with the California spiny lobster, *Panulirus interruptus,* support the hypothesis that a locomotory response evoked by ATP in seawater may be mediated by chemoreceptors akin to P_2-type purinoceptors. Behavioral results consistent with this hypothesis are: (1) an activity sequence of ATP > ADP > AMP or adenosine; (2) the behavior is also evoked by ATP analogs with modifications in both the adenine and ribose moieties; and (3) the slowly degradable analogs, β,γ-methylene ATP and β,γ-imido ATP (AMPPNP) are active. Extracellular recordings from single chemosensory cells show that ATP-sensitive cells are present in the antennule of *P. interruptus* and exhibit marked similarities to the P_2-like chemoreceptors identified earlier in *P. argus.* Although the ranked order of behavioral activity to ATP and eight analogs parallels that measured physiologically, important differences include: (1) AMPPNP is relatively more active physiologically; and (2) the behavioral sensitivity to ATP, ADP, and AMP is greater than that measured physiologically. Since degradation of ATP proceeds rapidly in animal flesh and in seawater, it is proposed that ATP may represent a particularly appropriate signal molecule for foraging by the lobster as it is indicative of recently injured or freshly killed organisms.

Introduction

Receptors for purine nucleotides, referred to as purinergic receptors or purinoceptors, occur in various internal tissues of mammals (see Burnstock, 1978). One type of purinergic receptor, the P_2-type, is most sensitive to the nucleotide adenosine triphosphate (ATP) (Burnstock, 1978). Interestingly, chemoreceptors stimulated by ATP, which exhibit properties similar to P_2-type receptors, have been demonstrated electrophysiologically in the olfactory organ of the Florida spiny lobster, *Panulirus argus* (Carr *et al.,* 1986). However, their role in the chemically mediated behavior of *P. argus* has not been explored.

Recently, in the California spiny lobster, *P. interruptus,* ATP was shown to be a potent chemoattractant, evoking a locomotory response associated with the recognition and finding of food (Zimmer-Faust, 1987; Zimmer-Faust and Dyson-Hudson, in prep.). When the effectiveness of adenosine and adenine nucleotides was compared, the potency sequence for the locomotory response was: ATP > ADP > AMP or adenosine. This sequence was identical to that found by Carr *et al.* (1986) in the physiological studies with *P. argus.*

Collectively, the above findings provided the impetus for initiating an integrated behavioral and physiological study to ascertain if the behavioral response of *P. interruptus* to ATP might be mediated by P_2-like chemoreceptors. In this study, we have extended the behavioral data for *P. interruptus* using a series of ATP analogs to permit comparison with the physiological results from *P. argus.* We also demonstrate physiologically that *P. interruptus* has ATP-sensitive chemoreceptors which exhibit major similarities to those characterized in *P. argus.* Together, our findings reveal that *P. interruptus* does have P_2-like chemoreceptors which may mediate its behavioral response to ATP.

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Materials and Methods

Collection and maintenance of animals

Specimens of *P. interruptus* were captured by hand on reefs near Santa Barbara, California. Before use in behavioral studies, lobsters were held for 7 to 14 days in large circular tanks. A continuous flow of seawater maintained the temperature at 15 to 17°C, and a 12:12 D:L cycle was imposed. Only hard-shelled animals (n = 108) of 60 to 72 mm carapace length were used; each was tattoo marked on the ventral sternites for individual recognition (Kuris, 1971). Lobsters were fed live mussels (*Mytilus californianus* and *M. edulis*) and sea urchins (*Strongylocentrotus purpuratus*) *ad libitum;* all food was removed 24 h before tests.

Specimens of *P. interruptus* used for electrophysiological studies were shipped by overnight courier from Santa Barbara to the Whitney Laboratory (St. Augustine, Florida) where they were held together in a tank with a flow-through seawater supply maintained at 14 to 16°C. Specimens of *P. argus* were collected in the Florida Keys and held at ambient seawater temperature in flow-through tanks at the Whitney Laboratory. All animals were fed a diet of fish, squid, and shrimp.

Behavioral assays

Individual lobsters were assayed for locomotory responses to chemical solutions in rectangular aquaria, 30 × 30 × 13 cm, a size shown previously to permit both careful control of stimulus flow and rapid testing, without inhibiting behavior (Zimmer-Faust and Case, 1983). Earlier studies revealed excellent agreement between the locomotory responses evoked by chemostimulants in natural habitats and in the small aquaria used for assays in the current study (*e.g.,* Zimmer-Faust and Case, 1982; Zimmer-Faust *et al.,* 1984). Seawater entered each aquarium at a flow rate of 2 ml/s from a head-tank maintained under constant hydrostatic pressure. A three-way valve was used to introduce a 25-ml volume of each test solution into the seawater flow. Dilution associated with stimulus delivery was determined in 18 trials by introducing a fluorescent dye (sodium fluorescein) and continuously monitoring fluorescence using optical fiber probes attached to the antennules of unrestrained animals (see Zimmer-Faust and Stanfill, 1986; Zimmer-Faust *et al.,* in press). Maximum concentrations contacting the antennules were determined to be 7.57×10^{-3} ($\pm 3.62 \times 10^{-3}$ S.D.) times the injected concentration, with dye peaks attained 29.8 s (±5.8 s S.D.) after initial dye input. Concentrations presented herein are corrected for this dilution.

A locomotory response by *P. interruptus* is defined as forward ambulatory movement to a distance greater than one carapace length. Previously, ATP was found to stimulate other behaviors associated with appetitive feeding (Zimmer-Faust, 1987); however, these other behaviors were more variable than forward ambulatory motion. Individual lobsters were tested only once every 72 to 96 h for a maximum of 5 tests during a 20-day period. Animals were put into experimental aquaria 45 to 60 min prior to testing and usually settled within 30 to 40 min. Observations were initiated 1 min before introduction of a chemical solution and continued for 4 min afterwards; lobsters were tested only if they were inactive during the first minute of observation. Each assay consisted of a randomized presentation of a test or control stimulus with the exception that identical solutions were never repetitively presented to the same animal. All trials were conducted according to a double-blind protocol. At least 20 different lobsters were assayed with each test solution; seawater alone was the control.

Electrophysiological recordings

Extracellular recordings were made from ATP-sensitive sensory cells in the olfactory organ (lateral filament of the antennule) of both *P. interruptus* and *P. argus*. The preparation was similar to that used by Carr *et al.* (1986) and has been described in detail (*e.g.,* Gleeson and Ache, 1985). The essential features of the preparation were as follows. An excised, lateral filament was placed in an olfactometer and maintained via arterial perfusion with *Panulirus* saline. Selected volumes of chemical stimuli were injected into a carrier stream of artificial seawater (ASW) which continuously flowed through the tuft of olfactory sensilla on the filament at a rate of 3 ml/min. Suction electrodes were used to obtain action potential recordings from the axons of individual sensory cells innervating the sensilla. These recordings were made from the antennular nerve which was exposed at the proximal end of the filament and separated into small bundles within a bath of *Panulirus* saline.

ATP-sensitive cells were identified by their initiation of action potentials (impulses) following the introduction of a search stimulus of ATP (10 μM, *ca.* 20 μl) into the carrier flow of ASW; ASW alone was presented in an identical manner as the control. The response of a cell to defined chemicals was determined by injecting a 190 μl volume of each test substance into the carrier flow. Conductivity measurements showed that this volume generated a stimulus profile in the olfactometer that reached the injected concentration within one second and remained constant for approximately two seconds before beginning to wash out. [Note: due to an error in conductivity measurements, the stimulus profile reported previously (Carr *et al.,* 1986) overestimated the time a stimulus remains at the injected concentration.] Intervals of

4 min were maintained between stimulus presentations, during which time ASW continuously flowed over the filament. In each trial, stimuli were presented in a random sequence except in dose-response determinations where an ascending concentration series was used. Single cell responses were recorded using an amplitude/time window discriminator, the output of which was monitored with a microprocessor to measure and store the time intervals between impulses for on-line or subsequent display and analysis of the response. In this report, cell responses are expressed as the total number of evoked impulses.

Relevant physiological results obtained for *P. argus* (Carr *et al.*, 1986) are included in some figures and summarized in the text to facilitate their comparison with the results obtained with *P. interruptus*.

Chemical solutions

All chemicals were from Sigma Chemical Company. Structural formulae of ATP and the analogs included in the study are shown in Figure 1. For behavioral assays, solutions were prepared immediately before tests in membrane-filtered (0.45 μm) seawater and adjusted to pH 7.8. Aliquots were stored on dry ice and warmed to ambient seawater temperature just prior to use. Solutions for physiological studies were prepared as stocks in ASW, adjusted to pH 7.8, stored at −70°C, and aliquots were brought to room temperature just prior to use.

Results

Behavior

Figure 2 shows the results of the behavioral assays comparing the stimulatory activity of ATP and nine structurally related substances. Although ATP was the most stimulatory substance, all of the analogs, except for 8-bromo-ATP and adenosine, were significantly more effective than seawater alone (G-test for Independence with Williams' correction: $G \geq 7.60$, d.f. = 1, $P < 0.01$, all comparisons). We observed no responses to seawater in 66 trials. The rank order of behavioral activity for these analogs parallels that previously measured physiologically in ATP-sensitive cells of *P. argus* (Fig. 2). A Kendall's Tau analysis of these ranks revealed a highly significant association ($\tau = 0.721$, n = 10, $P < 0.008$). The most notable exception to this ranking concerns the stable analog, β,γ-imido ATP (AMPPNP), which in the physiological tests proved to be more stimulatory than ATP itself. Behaviorally, the relative activity of ADP and AMP was greater than that measured physiologically; however, the behavioral activity induced by these analogs was significantly less than that for ATP (G-Test with Williams' correction: $G \geq 4.31$, d.f. = 1, $P < 0.05$, both comparisons).

To characterize the dose-response (D-R) relationships of selected analogs, behavioral assays were conducted over a range of concentrations with ATP and with three analogs having structural modifications in either the nitrogenous base, the ribose, or the triphosphate moiety. The results revealed that the slopes of the D-R curves for ATP and the analogs were not significantly different (Fig. 3). This finding is consistent with the notion that the actions of these substances are mediated by a common population of receptors. The rank order of potencies found in this analysis was ATP > dATP > CTP ≅ AMPPNP. This ranking confirmed the order of activities observed earlier with the single-dose determinations (see Fig. 2).

Physiology

Physiological studies revealed that *P. interruptus*, like *P. argus*, has a distinct population of olfactory cells that are selectively activated by ATP. In both species, the response of these cells is characterized by a short burst of impulses, the duration of which is generally only a few hundred milliseconds in spite of the fact that the stimulus is present for several seconds (Fig. 4A). A comparison of the D-R relationships for these cells reveals similar sensitivities in the two species. In *P. interruptus*, however, the D-R function exhibits a significantly steeper slope [Test for Parallelism (Tallarida and Murray, 1981): $P < 0.05$], with a response maximum of approximately 17 impulses (Fig. 4B); the cells in *P. argus* show a maximum response of about 11 impulses.

Comparisons of the stimulatory capacities of the adenine nucleotides and adenosine on the ATP-best cells of both species show marked similarities. In both species the activity sequence is ATP ≫ ADP > AMP and adenosine (Fig. 5A). In both species ADP is a very poor stimulant and, like AMP and adenosine, is virtually inactive. Moreover, the ATP-best cells in both species show greater responses to the slowly degradable analog, AMPPNP, than to ATP itself (Fig. 5B). A similar specificity for ATP in the cells of both species was also indicated in trials in which 10 μM glutamate, taurine, betaine, and glycine were each individually tested. None of these substances elicited responses from any of the nine cells examined in *P. interruptus*, and only glycine evoked a response (a single impulse) in one of the seven cells tested in *P. argus*.

Discussion

This study shows that the behavioral response to ATP exhibited by the California spiny lobster, *P. interruptus*, may be mediated by chemoreceptors related to the P_2-

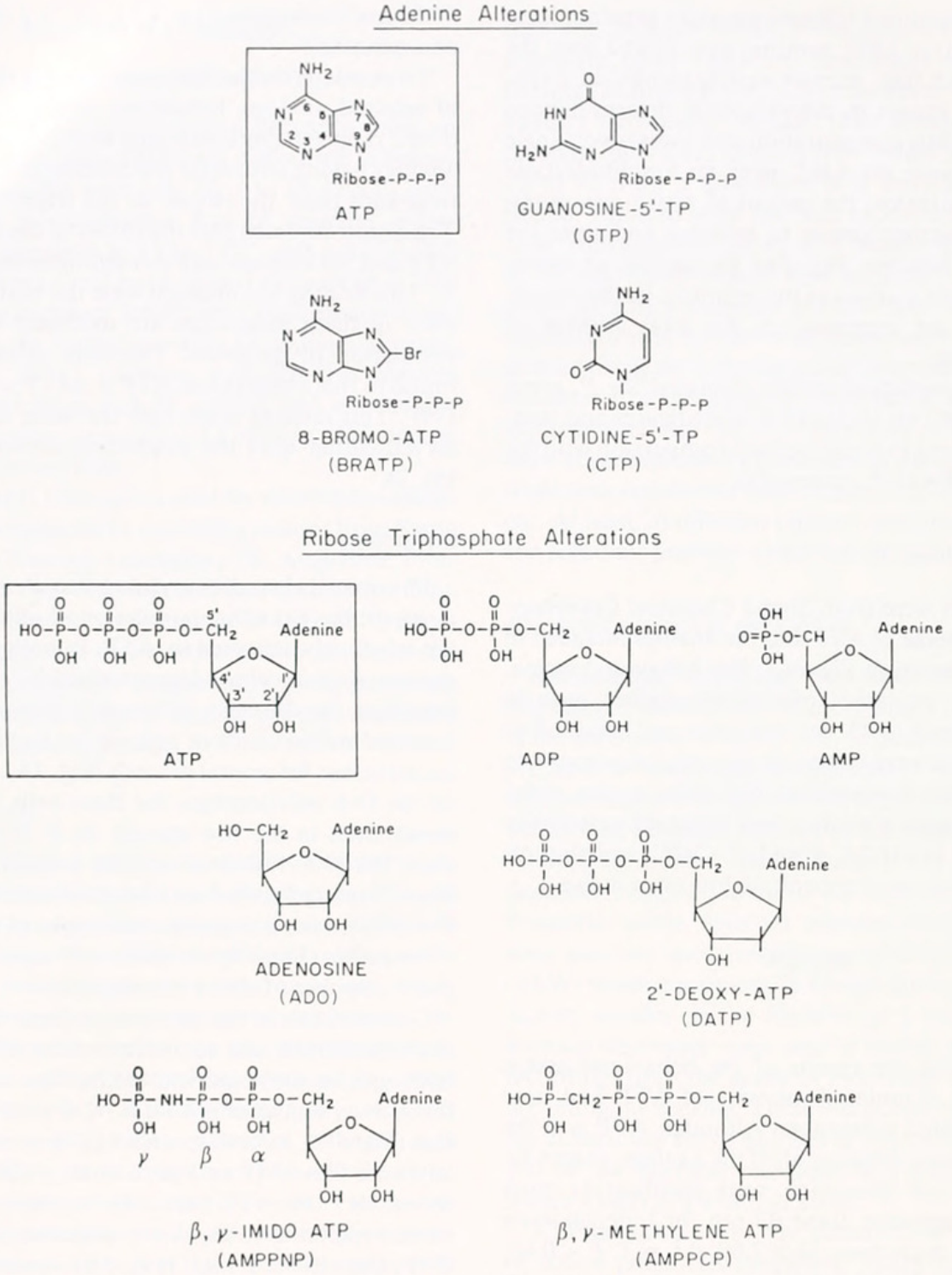

Figure 1. Structural formulae of ATP and analogs tested physiologically and/or behaviorally in *Panulirus interruptus.*

type purinoceptors described by Burnstock (1978). Data supporting this hypothesis include: (1) structure activity relationships (SAR) for the behavior which show congruence with the SAR for P_2-like chemoreceptors previously described in *P. argus* (Carr *et al.*, 1986); and (2) the electrophysiological identification of ATP-sensitive chemoreceptors in *P. interruptus* that are virtually identical in their sensitivity, specificity, and temporal response characteristics to those described in *P. argus.*

The stimulation of the oriented locomotory response in *P. interruptus* by adenine nucleotides and adenosine shows a potency sequence of ATP > ADP > AMP or adenosine. This behavior is also evoked by ATP analogs with modifications in the adenine moiety (*e.g.*, GTP, CTP), the ribose moiety (2'-deoxy ATP), and the triphosphate moiety (AMPPNP, AMPPCP) (see Fig. 2). However the analog 8-Bromo-ATP is only a weak behavioral stimulant. These SAR are consistent with the hypothesis

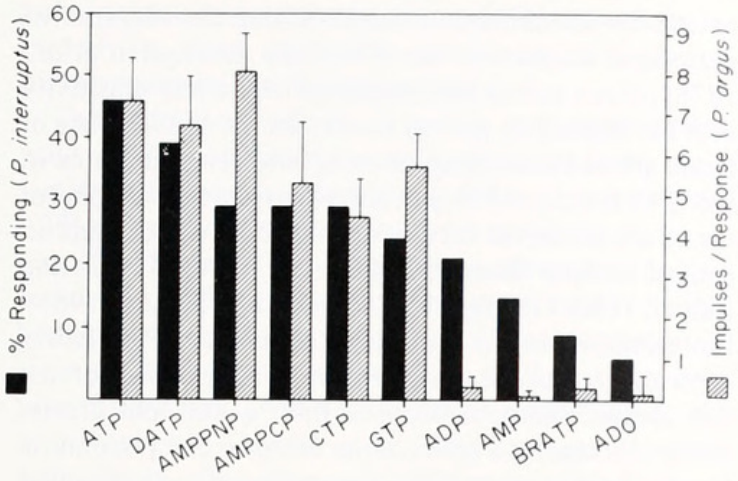

Figure 2. Relative activities of ATP and analogs as determined by behavioral assays in *Panulirus interruptus* (solid bars) and by physiological recordings from ATP-best cells in *P. argus* (hatched bars). In behavioral experiments each compound was tested on at least 20 lobsters at a concentration of 2.3 μM. In physiological studies each compound was tested at 100 μM on at least six cells; bars represent mean responses ± SEM. The physiological data are derived in part from Carr *et al.*, (1986). Abbreviations as in Figure 1.

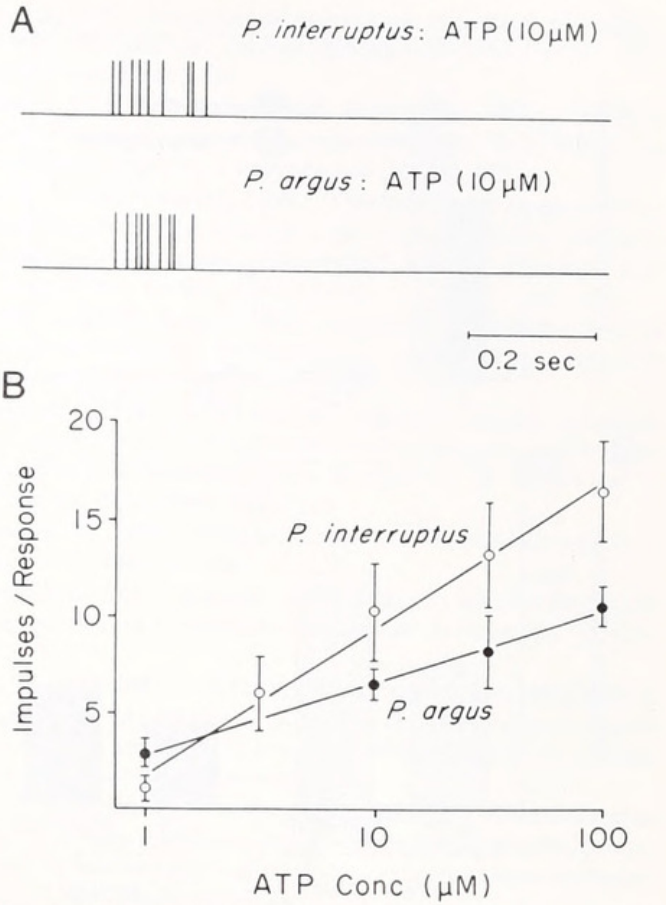

Figure 4. Response properties of ATP-sensitive cells in two lobster species. A. Computer-generated impulse trains depicting typical response profiles following stimulation with ATP. B. Dose-response functions for stimulation with 1 to 100 μM ATP. For each species, the points represent mean values ± SEM for 11 ATP-sensitive cells. Data for *Panulirus argus* are from Carr *et al.* (1986).

that the locomotory behavior is mediated by chemoreceptors akin to the P_2-type purinoceptors that have been found by various workers to exhibit the following SAR: (1) potency sequence of ATP > ADP > AMP or adenosine (Burnstock, 1978); (2) broad sensitivity to nucleotide triphosphates including those with modifications in both the adenine and the ribose moieties (Maguire and Satchell, 1981; Phillis and Wu, 1981; Lukacsko and Krell, 1982); (3) tolerates modifications in the triphosphate moiety such as those represented by the slowly degradable analogs, AMPPNP and AMPPCP (Burnstock and Kennedy, 1985); (4) does not tolerate the deletion of a phosphate group (*e.g.*, as in ADP) (Lukacsko and Krell, 1982); and (5) often not strongly stimulated by the analog, 8-Bromo-ATP (see Maguire and Satchell, 1979).

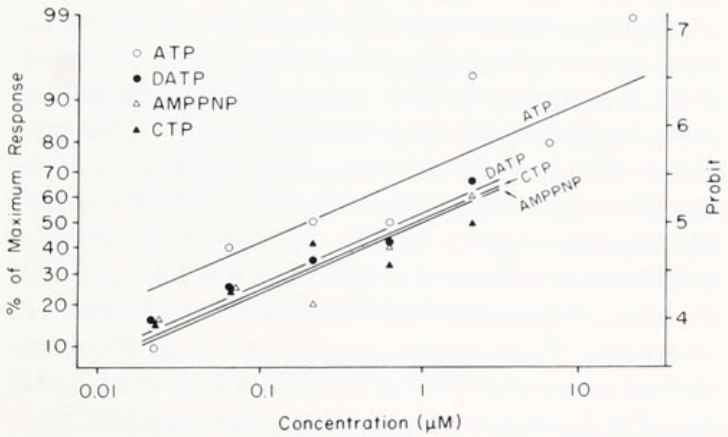

Figure 3. Dose-response relationships for ATP and analogs tested behaviorally in *Panulirus interruptus*. Each point represents data from at least 20 lobsters. The procedure of Potency Probit Analysis (PPA) (Daum and Givens, 1963) showed that the slopes of the individual regression lines are not significantly different, and can be depicted as the parallel lines shown above. Relative potencies obtained by PPA were: ATP = 1.0; 2'deoxyATP (DATP) = 0.257; CTP = 0.188*; β,γ-imidoATP (AMPPNP) = 0.177*. Asterisks indicate potencies significantly less than ATP ($P < 0.05$).

The physiological recordings from the antennules of *P. interruptus* clearly showed that receptor cells selectively sensitive to ATP and related analogs do exist. Furthermore, these cells exhibit marked similarities to the ATP-sensitive cells described earlier in *P. argus* (Carr *et al.*, 1986). A unique property of these cells in both species is that the response is characterized by a brief burst of impulses. Each response terminates abruptly after only a few hundred milliseconds even when the chemical stimulus is continuously introduced over a period of several seconds (see Fig. 4). These "phasic" responses indicate that the cells are rapidly adapted, or desensitized, by stimulatory molecules. This rapid desensitization contrasts markedly with the longer, more "tonic," responses exhibited by other types of antennular chemoreceptor

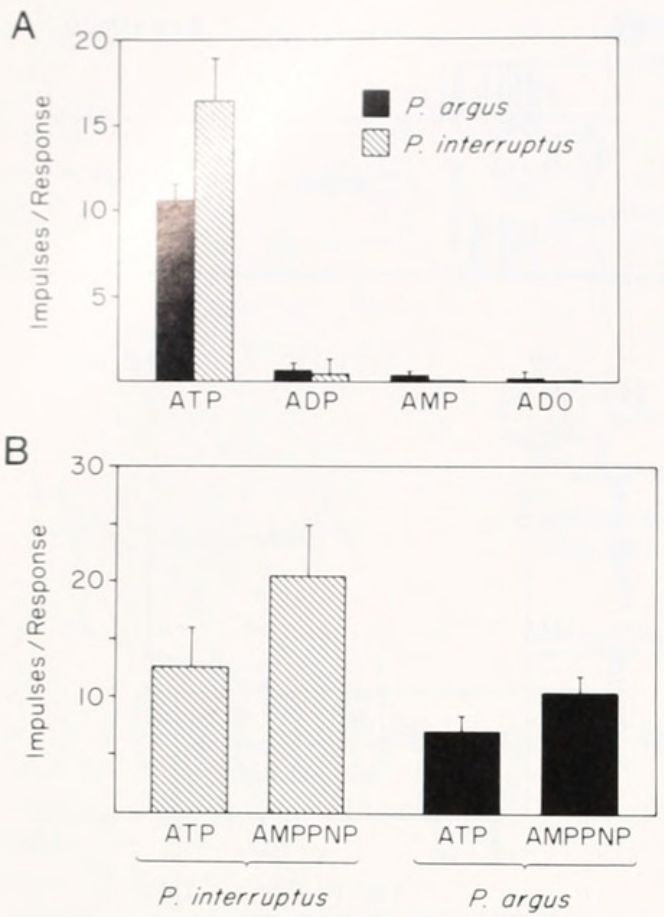

Figure 5. A. Relative activities of adenine nucleotides and adenosine on ATP-sensitive cells of two lobster species. Each compound was presented at a concentration of 100 μM. Bars represent mean responses ± SEM for 11 cells in *Panulirus interruptus* and at least 6 cells in *P. argus*. B. Relative activity of 10 μM ATP and 10 μM β,γ-imidoATP (AMPPNP) on ATP-sensitive cells. Bars are mean values ± SEM for four cells in *P. interruptus* and six cells in *P. argus*. Data for *P. argus* taken from Carr *et al.* (1986).

cells; *e.g.*, see responses of taurine-best cells (Fuzessery *et al.*, 1978) and AMP-best cells (Derby *et al.*, 1984). Since P_2-type purinoceptors are also rapidly desensitized by ATP and related analogs (Burnstock and Kennedy, 1985), it would appear that the ATP-sensitive chemoreceptors exhibit yet another property characteristic of P_2 receptors.

An important discrepancy in the behavioral and physiological data for *P. interruptus* is that the behavioral sensitivity to ATP is approximately 30-fold greater than that determined physiologically for the ATP-sensitive cells (compare D-R curves for ATP in Figs. 3 and 4B). If our physiological and behavioral sensitivity measurements are both accurate, this discrepancy would appear to be inconsistent with the hypothesis that these cells mediate the ATP-stimulated locomotory response; *i.e.*, suggesting that an as yet unidentified population of chemoreceptors with a lower threshold for ATP is responsible for inducing the behavior. However, another possible explanation for the difference could be that convergence of peripheral neurons in the CNS (van Drongelen *et al.*, 1978) plays a role in enhancing the behavioral sensitivity to ATP. Indeed, in several insect species, convergence of many pheromone receptor cells onto fewer neurons in the CNS is believed to give rise to extremely high pheromone sensitivity as measured both behaviorally and in central neurons (Boeckh and Boeckh, 1979; Mankin and Mayer, 1983; Olberg, 1983; Boeckh and Selsam, 1984). For example, in the American cockroach, *Periplaneta americana*, Boeckh and Selsam (1984) demonstrated that deutocerebral neurons exhibit a 100-fold greater sensitivity than the pheromone receptor cell population that projects to them. This was attributed to the fact that the central neurons receive inputs from many peripheral receptor cells and, consequently, can respond to concentrations that activate only a small fraction of the peripheral cells. Hence it is conceivable that a similar amplification occurs in the CNS of the lobster to produce behavioral thresholds for ATP that are lower than predicted from the observed physiological sensitivities of the receptor cells. Finally, because we do not have information on ATP-sensitive chemoreceptors associated with other appendages such as the walking legs (*e.g.*, Derby and Atema, 1982), it is quite possible that inputs from these appendages may contribute importantly to the behavioral response.

Two significant discrepancies are evident when comparisons are made between the behavioral and physiological results obtained with the ATP analogs used in the current study. First, ADP and AMP are better stimulants of the behavioral response in *P. interruptus* than would be predicted from the physiological studies which revealed that both of these nucleotides are virtually nonstimulatory to the ATP-sensitive cells (see Fig. 2). Again, convergence, as discussed above, may result in the behavioral sensitivity to these analogs; also the integration of inputs from other receptor cells is likely since substances presented in the behavioral assays are not restricted to interacting exclusively with the ATP-sensitive cells described herein. Indeed, Spencer (1986) demonstrated strong responses to ADP and AMP in multiunit recordings from antennular chemosensory neurons in *P. interruptus*. Furthermore, sensory cell populations exhibiting selective sensitivity to AMP (Derby *et al.*, 1984) and ADP (Carr *et al.*, in press) have been described in *P. argus*. A second discrepancy between the behavioral and physiological results is that the slowly degradable analog, AMPPNP, is more effective than ATP when tested physiologically (see Fig. 5B), but less effective than ATP when tested behaviorally (see Figs. 2, 3). This suggests that a lobster is capable of discriminating ATP from AMPPNP via some response difference of the ATP-cell population and/or via inputs from other cells which exhibit a differ-

ential sensitivity to these compounds. The mechanisms underlying these intriguing behavioral/physiological differences remain to be determined.

The widespread effects of purine nucleotides, especially ATP, upon visceral muscles of several of the lower vertebrates prompted Burnstock (1975) to propose that ATP may represent one of the most primitive transmitters. If ATP was in fact a primitive transmitter, then receptors for ATP might also occur among many invertebrate organisms as well. Indeed, there is now substantial evidence that receptors activated by ATP or related adenine nucleotides, are frequently represented among the invertebrates (*e.g.*, Mato *et al.*, 1978; Barber *et al.*, 1982; Yatani *et al.*, 1982; Carr and Thompson, 1983; Derby *et al.*, 1984; Chase and Wells, 1986; Derby *et al.*, 1987; Hoyle and Greenberg, 1988). In addition to the P_2-like receptors present on the olfactory organ of the two species of spiny lobsters, ATP-sensitive chemoreceptors are known to occur in several species of insects including the tsetse fly (Mitchell, 1976), the assassin bug (Smith, 1979; Friend and Smith, 1982), the black fly (Sutcliffe and McIver, 1979), the mosquito (Galun *et al.*, 1984, 1985), and others (see review by Friend and Smith, 1977).

The capacity of ATP to serve as a behavioral stimulant of the spiny lobster is probably related to the fact that this nucleotide occurs in high concentrations in fresh tissues of prey organisms such as crustaceans and molluscs (*e.g.*, see Carr and Derby, 1986). Good signal molecules in the sea should be those for which a high "signal to noise" ratio is maintained (*e.g.*, see Fuzessery *et al.*, 1978; Atema, 1985; Carr, 1987). Because processes that maintain very low background concentrations ("noise") of ATP in seawater do exist, this molecule may be a particularly appropriate signal for recognizing recently killed or injured prey organisms. Processes minimizing noise levels of ATP in seawater include nucleotidases in tissues that rapidly dephosphorylate ATP after death (see Zimmer-Faust, 1987), plus dephosphorylating enzymes present on the outer surfaces of many planktonic organisms which quickly degrade nucleotides released into the sea (Ammerman and Azam, 1985). Hence the presence of ATP in seawater could provide a reliable indicator that an injured or freshly killed organism is nearby.

Acknowledgments

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