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Specificity of Odorant-evoked Inhibition in Lobster Olfactory Receptor Neurons

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Abstract

Lobster olfactory receptor neurons, like those of many animals, use two modes of olfactory signaling, excitation and inhibition to code olfactory information. Inhibition appears to act through two distinct ionic mechanisms. Here we show that neither ionic mechanism is odor-specific, providing further support for the emerging understanding that there are no inhibitory odorants *per se*, but rather that the action of a particular odorant is inherent in the olfactory receptor cell on which an odorant acts.

Key words: olfaction, lobster, sensory transduction, ORNs

Introduction

Two modes of olfactory signaling, excitation and inhibition, are used by olfactory receptor neurons (ORNs) in diverse animal species to code olfactory information, including lobster (McClintock and Ache, 1989), *Drosophila* (Dubin and Harris, 1997; deBruyne *et al.*, 2001) and other insects (Akers, 1992; Shields and Hildebrand, 2001), squid (Lucero *et al.*, 1992), mudpuppy (Dionne, 1992) and other amphibians (Vogler and Schild, 1999; Sanhueza *et al.*, 2000), catfish (Kang and Caprio, 1995) and rat (Duchamp-Viret *et al.*, 1999). If, as it appears, such dual signaling is a ubiquitous feature of olfactory organization, the question arises as to the functional significance of having opponent input in olfaction.

Important to knowing the functional significance of having inputs of opposite polarity in olfaction is to know whether there are inhibitory odorants *per se* or whether the action of a particular odorant is inherent in the receptor cell on which the odorant acts. The emerging understanding is that there are not inhibitory odorants *per se*, since one odorant can have opposite physiological effects on different ORNs. Examples of this include arginine, proline and cysteine, which can have opposite electrophysiological effects on different lobster ORNs (McClintock and Ache, 1989), and methionine, arginine, alanine, glutamic acid and ATP, which primarily suppress spontaneous firing in catfish ORNs but also excite other cells (Kang and Caprio, 1995). An especially elegant recent demonstration furthered this understanding by showing that in *Drosophila* it was possible to confer the ability of an odorant to inhibit an ORN by exchanging the native receptor for one normally expressed in a cell that was inhibited by that odorant, indicating that it is the receptor itself that confers the direction of the output of the cell (Hallem *et al.*, 2004).

In some animals inhibition appears to work through two distinct ionic mechanisms, raising the possibility of there being mechanism-dependent odorant specificity to inhibitory signaling. One such instance is lobster ORNs, in which odorants can inhibit the cells by activating a K^+ conductance or suppressing a resting Cl^- conductance (Doolin *et al.*, 2001). By pharmacologically blocking one or the other outputs, it is possible to determine whether odorants are specific to a particular inhibitory conductance. Here, we confirm previous evidence that single odorants can have opposite electrophysiological effects on different lobster ORNs, and extend our previous understanding by showing that none of the odorants tested were specific for either of the two ionic conductances. These findings provide further support for the idea that the action of a particular odorant is determined by the receptor cell and not by the odorant.

Materials and methods

Animals and preparation

Adult specimens of the Caribbean spiny lobster, *Panulirus argus*, were collected in the Florida Keys. They were maintained

in the laboratory in running seawater on a mixed diet of frozen fish, squid and shrimp until used. ORNs were recorded *in situ*, as detailed elsewhere (Doolin and Ache, 2002). Briefly, the olfactory organ (the lateral filament of the antennule) bearing the olfactory (aesthetasc) sensilla was cut into sections of four annuli. Each section was split longitudinally in a dorsal ventral plain and an individual hemisection containing aesthetascs was pinned to silicone elastomer in the bottom of a recording chamber so as to allow simultaneous access to both the outer dendrites and the somata for drug/odorant delivery and electrophysiological recording, respectively. The recording chamber was filled with *Panulirus* saline (PS) after treating the cells sequentially with L-cysteine-activated papain (Sigma Type IV, 0.17 mg/ml in PS) for 1 min and trypsin (1 mg/ml in PS) for 1 min to digest away the sheath surrounding the somata of the ORNs. The recording chamber was mounted on the stage of an upright microscope (Axioskop, Carl Zeiss, Inc.) and somata were viewed with conventional bright-field optics using a ×40 long working-distance water-immersion objective (Zeiss 440091).

Recording and data analysis

Current-clamp recordings were made from the soma of cells using conventional whole-cell patch-clamp recording. Patch pipettes were fabricated from borosilicate filament glass (1.50 mm o.d., 0.86 mm i.d.; Sutter Instrument Co.), and fire-polished to a tip diameter of 1 μm. Pipettes with resistances of 5–9 MΩ when filled with patch pipette solution (see Solutions) formed seals with resistances of 4–10 GΩ. Signals were recorded with a commercial amplifier (Dagan 3900), low pass filtered at 1 kHz (−3 dB; four-pole Bessel filter), directly digitized at 2–5 kHz, and analyzed using pClamp 8 software (Axon Instruments, Inc.). A reference electrode was connected to the bath solution through a 3 M KCl/agar bridge. All potentials were corrected for junction potentials between the pipette tip and the indifferent electrode. During the course of the experiments the series resistance was <10 MΩ. After obtaining a whole-cell patch, the resting membrane potential of most cells was at or near threshold for the generation of action potentials (−40 mV), causing those cells to discharge high frequency action potentials. When necessary to eliminate action potentials in the voltage traces, current was injected (56 ± 5.3 pA, n = 28) to adjust the membrane potential to −80 mV. Individual ORNs were exposed to one or more odorants and the resulting receptor potential, if any, was measured for peak amplitude and polarity. Odorant delivery was achieved using a 'spritzer' type olfactometer (Doolin and Ache, 2002). Experiments were carried out at room temperature (20–22°C). Data were reported as the mean ± SEM.

Solutions

PS consisted of (mM) 457.7 NaCl, 13.4 KCl, 13.6 $CaCl_2$, 9.8 $MgCl_2$, 14 Na_2SO_4, 3 HEPES, 1.9 glucose, pH 7.4. Intracellular solution consisted of (mM) 180 KCl, 30 NaCl, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 696 glucose, pH 7.2. Anthracene-9-carboxylic acid (9-AC) was prepared as a stock solution (50 mM in DMSO), diluted as needed in PS and the pH was adjusted to pH 7.4. 4-Aminopyridine (4-AP, 10 mM) was dissolved in PS and pH adjusted to pH 7.4. Odorants were L-proline, L-arginine and L-cysteine, prepared as 2 mM solutions in PS, with the pH adjusted to 7.4 as necessary. These compounds were selected to increase the probability of evoking hyperpolarizing receptor potentials since they were previously shown do so in lobster ORNs (Michel *et al*., 1991). The stated concentration of the solutions delivered by the olfactometer reflects a 50% dilution imposed by the delivery system (Doolin and Ache, 2002). Odorants therefore were effectively delivered at 1 mM. While this concentration is at the upper end of the concentration range likely to be experienced by the cells in real life, it was selected to assure we would not miss small magnitude responses that otherwise would be hidden in the noise. All inorganic salts were purchased from Fisher Scientific, Inc. All organic chemicals were obtained from Sigma Chemical Co. except for HEPES, which was obtained from Research Organics, Inc.

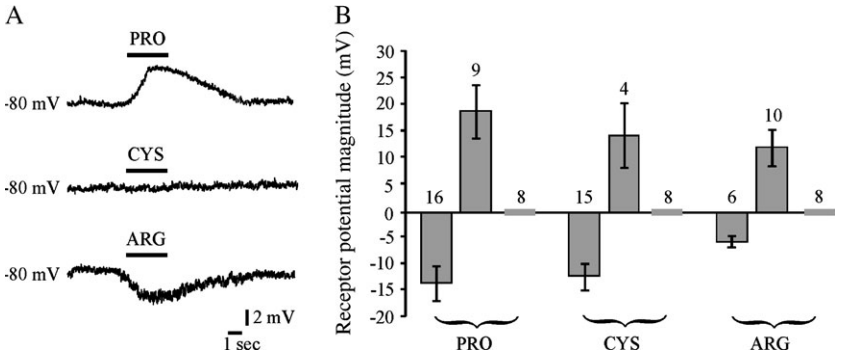

Figure 1 All three odorants (1 mM concentrations of proline, cysteine and arginine) had the potential to evoke either depolarizing or hyperpolarizing receptor potentials in different cells. **(A)** Three traces from three ORNs exposed to three different odorants, illustrating variation observed with the evoked responses (bars represent 3 s odorant exposure). E_m = −80 mV. **(B)** Bar graph of average magnitude of each type of response seen with the three odorants.

Results

As expected, different odorants elicited hyperpolarizing and depolarizing receptor potentials in the same cell from resting membrane potentials of −80 mV (Figure 1A). Any one odorant, in turn, elicited hyperpolarizing and depolarizing receptor potentials in different cells (Figure 1B). Proline (1 mM) hyperpolarized 16 of 33 cells by −13.5 ± 3.3 mV from a membrane potential of −80 mV, depolarized 9 of the 33 cells by 18.1 ± 6.0 mV and had no measurable effect on the remaining eight cells (Figure 1B). Cysteine (1 mM) hyperpolarized 15 of 27 other cells by −12.2 ± 2.5 mV from a membrane potential of −80 mV, depolarized four cells by 13.9 ± 6.2 mV, and had no measurable effect on the remaining 8 cells (Figure 1B). In a different subset of 24 ORNs, arginine (1 mM) hyperpolarized six cells by −5.7 ± 1.1 mV from a membrane potential of −80 mV, depolarized 10 cells by 11.6 ± 4.1 mV and had no detectable effect on the remaining eight cells (Figure 1B).

In a population of 23 ORNs tested with all three odorants, no clear structure–activity relationship was associated with receptor potentials of either polarity (Figure 2). Of 18 ORNs that were hyperpolarized by at least one odorant, proline evoked the strongest (greatest peak magnitude) response in 44% of the cells (n = 8), cysteine in 44% of the cells (n = 8), and arginine in 11% of the cells (n = 2). The same also held true for depolarizing responses. Of 13 ORNs that were depolarized by at least one odorant, proline evoked the strongest response in 45% of the cells (n = 5), cysteine in 27% of the cells (n = 3) and arginine in 36% of the cells (n = 4). One of the 13 cells responded equally to cysteine and arginine. Overall, however, the incidence of hyperpolarization appeared to be greater than the incidence of depolarization in the sample population.

Given evidence that two different, pharmacologically selective ionic mechanisms underlie hyperpolarizing receptor potentials in lobster ORNs (Doolin *et al*., 2001), we looked for evidence of mechanism-dependent odorant specificity. 9-AC was used to block Cl^- dependent inhibition (hyperpolarizing receptor potentials), while 4-AP was used to block K^+ dependent inhibition, both at concentrations shown to be effective on the respective conductance (Doolin *et al*., 2001). Due to limitation such as cell longevity, the effect of the blockers on each odorant was necessarily assessed on different groups of ORNs. Arginine hyperpolarized 13 of 30 cells by −6.1 ± 1.7 mV from a membrane potential of −80 mV (Figure 3). Pretreatment (1 min) with 9-AC (500 μM) reduced the peak amplitude of the hyperpolarization in 6 of the 13 cells to 15.8 ± 14.1% of its initial magnitude. Pretreatment (1 min) with 5 mM 4-AP reduced the peak amplitude of the hyperpolarization in 5 of the 13 cells to 27.7 ± 11.1% of its initial magnitude. Both drugs were effective on the remaining two cells. Pretreatment with either 9-AC (500 μM) or 4-AP (5 mM) reduced the peak magnitude of the hyperpolarization to 36.6 ± 17.9 and 51.5 ± 19.2% of its initial magnitude, respectively.

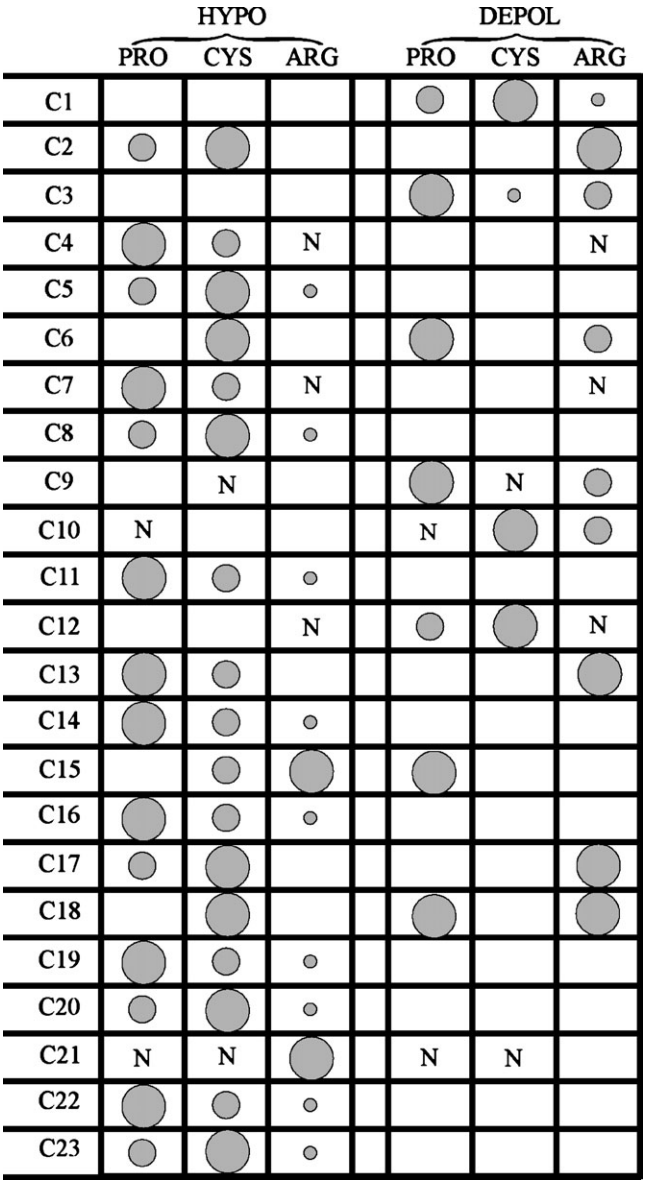

Figure 2 Chart of 23 cells showing the magnitude and polarity of responses evoked by three odorants (1 mM concentrations of proline, cysteine and arginine). The filled circles of three different sizes relate to relative magnitude of the odorant-evoked responses and 'N' represents no response to an odorant. The largest circles indicate the largest evoked responses of a certain polarity for a cell. Mid-sized circles represent evoked responses that were between 94% and 50% of the largest magnitude while the smallest circles represent responses in individual cells that were <50% of the largest receptor potential of the same polarity. Equally sized circles represent responses evoked with an equal magnitude (within 5% of each other) in the same cell. The absence of a circle in a box indicates the odorant was not tested.

Cysteine hyperpolarized 13 of 24 ORNs by −11.8 ± 1.5 mV from a membrane potential of −80 mV (Figure 4). Pretreatment (1 min) with 9-AC (500 μM) reduced the peak amplitude of the hyperpolarization in 6 of the 13 cells to 28.8 ± 17.7% of its initial magnitude. Pretreatment (1 min) with 4-AP (5 mM) reduced the peak amplitude of the hyperpolarization in 4 of the 13 cells to 25.9 ± 10.0% of its initial

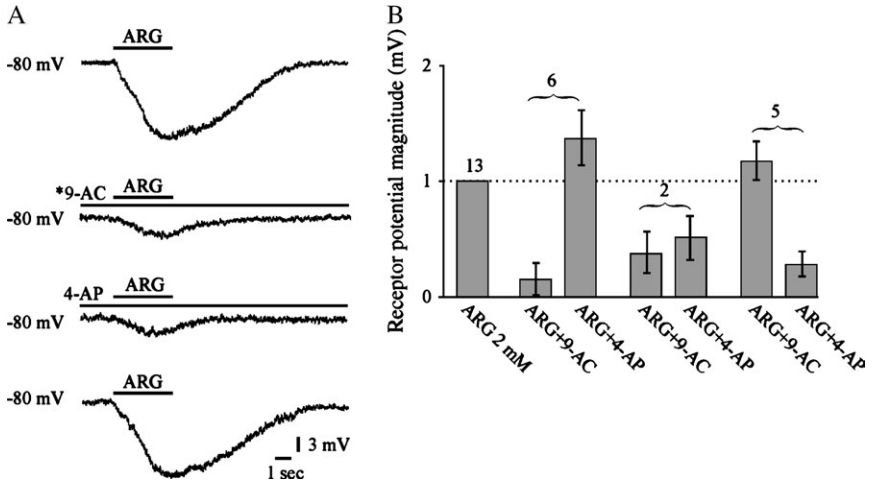

Figure 3 Arginine-evoked hyperpolarization exhibits 9-AC and 4-AP sensitivity in some cells. **(A)** Four traces from the same ORN showing that both Cl^- and K^+ channel blockers (500 μM 9-AC and 5 mM 4-AP, respectively) can block an evoked hyperpolarization in the same cell. E_m = −80 mV. Shorter bar: 3 s exposure. Long bar: exposure to drug indicated. *In this and all subsequent figures with 9-AC pretreatments, current was injected to adjust pre-odorant E_m to −80 mV. **(B)** Bar graph showing the proportion of odorant-evoked hyperpolarization blocked by 9-AC (500 μM), 4-AP (5 mM) or both blockers (n = 13).

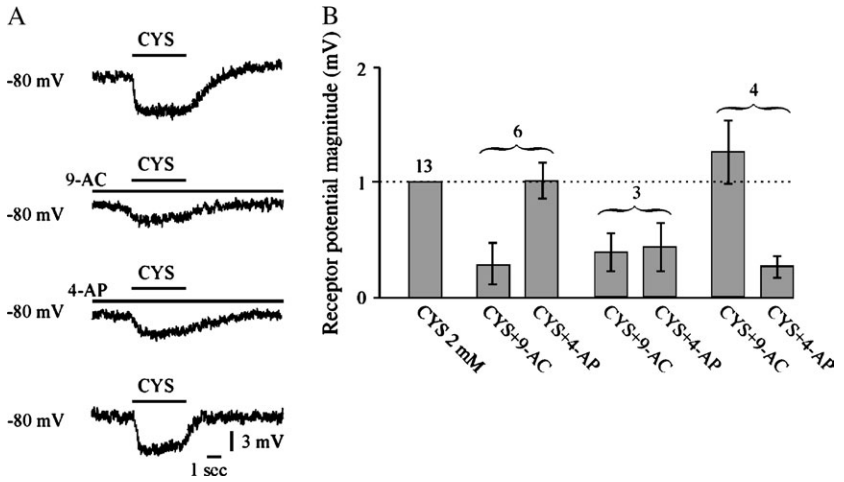

Figure 4 Cysteine-evoked hyperpolarization exhibits a 9-AC sensitivity, which may co-exist with a 4-AP sensitivity in some ORNs. **(A)** Four traces from the same ORN showing that both Cl^- and K^+ channel blockers (500 μM 9-AC and 5 mM 4-AP, respectively) can block an evoked hyperpolarization in the same cell. E_m = −80 mV. Small bar: 4 s exposure. Long bar: exposure to drug indicated. **(B)** Bar graph showing the proportions of odorant-evoked hyperpolarization blocked by 9-AC (500 μM), 4-AP (5 mM) or both blockers (n = 13).

magnitude. In the remaining three cells, pretreatment with either 9-AC (500 μM) or 4-AP (5 mM) reduced the peak magnitude of the hyperpolarization to 38.6 ± 16.9% and 43.4 ± 21.2% of its initial magnitude, respectively.

Proline hyperpolarized 9 of 20 cells by −12.7 ± 2.7 mV from a membrane potential of −80 mV (Figure 5). The amplitude of the response in 4 of the 9 cells was reduced to 22.6 ± 14.7% of its initial magnitude by a pretreatment (1 min) with 9-AC (500 μM). In 2 of the 9 cells, the amplitude of the response was reduced to 37.0+11.5% of its initial magnitude by a pretreatment (1 min) with 4-AP (5 mM). Finally, the amplitude of the response in the three remaining cells was reduced by pretreatment with either 9-AC (500 μM) or 4-AP (5 mM) to 41.8 ± 12.6 and 54.7 ± 17.1% of its initial magnitude, respectively.

Pooling results from the 35 odorant-evoked hyperpolarizations of the previous three experiments (Figures 3–5) revealed that 46% (n = 16) were reduced by pretreatment with 9-AC to 22.7 ± 15.7% of their initial magnitude, 23% (n = 8) were reduced by a pretreatment (1 min) with 4-AP (5 mM) to 30.3 ± 10.7% of their initial magnitude, and 31% (n = 11) were reduced by both pretreatments to 39.3 ± 16.2 and 49.7 ± 19.3% by 9-AC and 4-AP, respectively.

Discussion

Finding that all three odorants in the test panel hyperpolarized some ORNs and depolarized others from the same membrane potential agrees with results of earlier studies on this

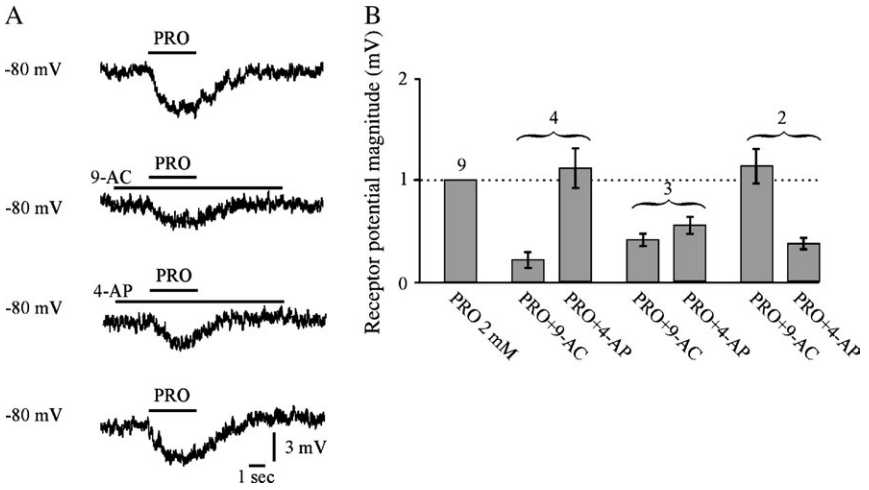

Figure 5 Proline-evoked hyperpolarization exhibits a 9-AC sensitivity, which may co-exist with a 4-AP sensitivity in some ORNs. **(A)** Four traces from the same ORN showing that both Cl^- and K^+ channel blockers (500 μM 9-AC and 5 mM 4-AP, respectively) can block an evoked hyperpolarization in the same cell. E_m = −80 mV. Small bar: 3 s exposure. Long bar: exposure to drug indicated. **(B)** Bar graph showing the proportions of odorant-evoked hyperpolarization blocked by 9-AC (500 μM), 4-AP (5 mM) or both blockers (n = 9).

and other animals (see Introduction) showing that a given odorant can inhibit and excite different ORNs. Our finding increases the numbers of cells and odorants on which this conclusion can be based and provides further support to the idea that there are not inhibitory odorants per se in the lobster.

Finding that the number of odorants in the test panel that evoked hyperpolarizing receptor potentials in any one cell varied across cells, as with depolarizing receptor potentials, suggests that there is not a single type of receptor mediating hyperpolarizing receptor potentials in different cells. This idea is supported more formally by finding that the structure–activity relationship (the 'best' odorant for evoking hyperpolarizing receptor potentials) also was not consistent across cells. Variation in the magnitude of the hyperpolarizing receptor potentials was not due to summation of depolarizing and hyperpolarizing inputs since in no instance of single odorant stimulation did we observe a depolarizing receptor potential after blocking a hyperpolarizing one. We conclude, therefore, that at least within the resolution of a sample population, there is not a single type of receptor or limited subset of receptors that mediates hyperpolarizing receptor potentials. Given that odorant receptors are relatively specific for particular structural features of odorant molecules (Araneda *et al*., 2000), one might expect to see at least some hint of rank ordering of specificity in our sample if a restricted number of specific receptor types mediated inhibition. We cannot exclude, of course, that our sample size limited our ability to resolve less limited specificity in receptor types mediating the hyperpolarizing responses, specificity that would only emerge on comparison of a large number of cells.

Finding that none of the three odorants in the test panel appeared to evoke hyperpolarizing receptor potentials exclusively through a Cl^- or K^+ dependent mechanism is a novel contribution of our study. Even though odorants were not inhibitory (hyperpolarizing) *per se*, there could be mechanism-dependent specificity. Our results, however, suggest this is not the case, at least within the limits of resolution imposed by our sample size, and provide further support for the idea that there are not specific inhibitory odorants in the lobster. Our finding in the lobster contrasts with data from squid, the only other model investigated thus far in which more than one ionic mechanism appears to mediate odorant-evoked inhibition. In squid, two odorants have been identified that inhibit ORNs in an ionic-specific manner (Lucero *et al*., 1992; Danaceau and Lucero, 1998). In this instance, however, the odorants are behaviorally aversive and may have specialized signal function in the biology of the animal. We cannot exclude that odorants with specialized signal function could inhibit lobster ORNs in a mechanism-specific manner, but this idea does not appear to hold for more general odorants as the amino acids used in our test panel.

What, then, might be the functional significance of having dual inhibitory mechanisms that lack odorant specificity? Perhaps they are simply redundant, reflecting the importance of inhibition in olfactory coding, possibly serving as noise filters as they do in the brain (e.g. Fukai and Kanemura, 2001). Alternately, the two inhibitory inputs, while lacking odorant specificity, could be functionally distinct. For instance, each could have slightly different kinetic properties, one mediating 'fast' and the other 'slow' inhibition. Preliminary experiments to identify any consistent kinetic differences between Cl^- and K^+ mediated hyperpolarizing receptor potentials, however, gave negative results (unpublished data). Further work is clearly necessary to resolve this interesting question.

Collectively, our findings are consistent with the idea that there are not inhibitory odorants *per se* in the lobster, and that the response of a cell to a particular odorant is inherent

in the cell (including its receptor) and not the odorant. If so, as proposed earlier (Ache, 1994), inhibition may provide an additional degree of freedom to the combinatorial code on which odor recognition and discrimination is generally agreed to be based (e.g. Malnic *et al*., 1999).

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