DEFENSE MECHANISMS IN NOTASPID SNAILS: ACID HUMOR AND EVASIVENESS

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Summary

Notaspid snails are known for their defensive skin secretion of sulfuric acid (pH 1–2) in response to noxious stimuli. We observed acid secretion and behavior in five notaspid species, and studied them in detail in Pleurobranchae californica. All species secreted acid in response to skin abrasion or compression. Moreover, all species showed stereotypic avoidance behavior to acidified sea water less acidic (pH 2–3) than their own secretions. In Pleurobranchae, secretion could also be stimulated by dilute solutions of taurine, $10^{-5}$–$10^{-2}$ mol L$^{-1}$. Secretion began at the stimulated region and spread slowly for about a minute following stimulation. Local contraction and transient edema of the skin were associated with acid secretion. In de-ganglionated preparations secretion could be caused by ortho-dromic stimulation of body wall nerves, by mechanical stimulation or by taurine.

These data suggest that acid secretion is a positive feedback process modulated by inhibitory paths and coordinated by both central and peripheral nervous systems. A picture emerges of a defensive secretory response that provides an additional noxious stimulus initiating or potentiating avoidance behavior. The data also suggest a potential role for taurine release from injured tissue and the existence of specific nociceptive neural pathways regulating complex behavior. In addition to deterring extraspecific predation, acid secretion could regulate interactions between animals of the same species.

Introduction

With the evolutionary dwindling and loss of their protective shells and burrowing habits, the opisthobranch gastropods have elaborated a variety of chemical defenses against predation (reviewed by Harris, 1973; Thompson, 1988). One notable defense utilized by some opisthobranchs is the secretion of sulfuric acid from special skin cells in response to mechanical stimuli. Such acid secretion is a particular characteristic of the notaspid opisthobranchs, in which mechanical stimulation induces secretion of mucus as acidic as pH 1–2. Fish, crabs and other generalized carnivores do not accept the skin of such animals, although they readily take the internal organs. The bulk of the work in this area has been

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Although the physiology and ethological context of acid secretion have received appreciable attention, aspects remain to be explored. In the course of examining feeding stimulants in different species of notaspideans on the Isle of Man, a mis-made solution was found to stimulate stereotypic avoidance behavior. The cause was found to be mild acidity, weaker than that of the animal’s own mucus secretion. Exploring the contexts in which acid secretion occurs, and the role of acid-sensory feedback in the regulation of the animal’s behavior, we made further observations that emphasize the importance of acid secretion in notaspid behavior. In the present report we present an account of the roles of tactile and chemical factors in stimulating acid secretion, the coupling of acid secretion to aversive behavior, regulation of secretion by central and peripheral mechanisms, and the probable neural organization of avoidance behavior. The observations suggest that acid secretion may play a large part in the animals’ neuroethology and behavioral ecology.

Materials and methods

Two specimens of Pleurobranchus membranaceus (Montagu) were captured by diving and trawling at depths of 15–60 m in waters local to the University of Liverpool Marine Laboratory at Port Erin, Isle of Man. Twelve specimens of Berthella plumula (Montagu) were collected from tide pools near Port Erin. Eight specimens of Berthellina citrina (Ruppell and Leuckert) were collected by Dr R. Fay (Pacific BioMarine, Inc.) diving in waters near Los Angeles. Two specimens of Pleurobranchus strongi (MacFarland) were also taken by Dr Fay trawling at depths of 100–200 m. Thirty-four Pleurobranchaea californica (MacFarland) were supplied by Pacific BioMarine and by Michael Morris of SeaLife Supply, Inc. Animals were kept in running sea water (Isle of Man) or in Instant Ocean (Illinois).

Behavioral observations were made at 13–15°C either in the tanks that housed the animals or in large glass dishes. Behavioral responses to solutions were recorded after control applications of sea water and subsequent tests of experimental solutions. Solutions of taurine at concentrations of $10^{-6}$–$10^{-2}$ mol l$^{-1}$ were made with Instant Ocean buffered to pH 7.5 with 10 μmol l$^{-1}$ Hepes. Acidified sea water was made by adjusting small volumes of Instant Ocean to pH 2–3 with HCl. No difference in response was seen when H$_2$SO$_4$-acidified sea water was used in pilot experiments, and HCl was used in the bulk of the experiments. Controls for mechanical effects of application of acid and taurine solutions were carried out by applying 1–1.5 ml of sea water over 3–5 s with a Pasteur pipet. This had no effect, so acid or taurine solutions were tested in the same way. Mechanical stimulation was delivered either by scratching of the skin with the tip of a Pasteur pipet or Dumont no. 5 forceps, deeply enough to indent.
the skin by 0.5–1 mm, or by transient compression of the skin with forceps lasting about 1–2 s.

In many cases skin pH was estimated with colored indicator paper (pHydrion) to within 1 pH unit. More precise measurements were made with a round-tipped pH electrode. The exposed hemispherical surface was 4 mm in diameter and could be held by hand entirely in contact with the skin. The electrode was connected to a Corning model 125 pH meter, whose output was fed to a Gould 220 chart recorder.

To compare the roles of central and peripheral nervous systems in mediating acid secretion, the circuemesophageal nerve ring, consisting of the cerebropleural and pedal ganglia, was removed together with the buccal and visceral ganglia. The buccal mass and digestive and reproductive systems with their local ganglia were also removed to leave the muscular foot, mantle, tail and head region with their associated peripheral ganglia and nerve plexuses. Such preparations were lightly pinned down in sea water in cooled, waterjacketed dishes for nerve stimulation and observations of behavior and skin pH. Cut distal ends of body wall nerves were stimulated with suction electrodes. Stimulation was performed at about 20% above the voltage thresholds (0.2–0.4 V) causing skin contraction at shock durations of 2 ms; test stimuli were delivered at 10 s⁻¹ for 1–3 s.

Results

Acid stimulation and aversive behavior

All the notaspid species showed an actively aversive, substratum locomotory response to stimulation with acid sea water. In its general features the response was very similar across species. Animals responded to acid stimulation of the oral veil by withdrawal of the oral veil head region, frequently followed by an aversive turn and rapid locomotion. A typical response is shown for \textit{P. californica} in the photographic sequence of Fig. 1. For a unilaterally applied stimulus, the subsequent turn was to the opposite side, mediated by shortening of the contralateral side of the body and foot. Typically, preceding and during the aversive turn the anterior margin of the foot was slightly raised from the substratum to show the ventral surface. This characteristic appeared to arise from strong, bilateral local withdrawal mechanisms; it occurred in such close correlation that we consider it practically diagnostic of active aversive behavior. Locomotion lasted for 3–10 min.

All parts of the body of the animals were sensitive to acid stimulation. Thus, stimulation of the foot or mantle caused both local contracture and curling of a broader expanse surrounding the stimulated area. Acid application to the gill and rhinophores caused rapid contracture of those structures. Application to the tail induced rapid tail shortening and forward locomotion.

An alternative acid-induced aversive behavior, the escape swim, was also shown by \textit{P. californica}. The escape swim is a fixed action pattern consisting of a sequence of dorsal and ventral flexions of the body that lifts the animal off the substratum in a clumsy, rocking swim (Davis and Mpitsos, 1971). In many respects this is similar to the escape swim of the nudibranch \textit{Tritonia} (Willows and Hoyle, 1969; Getting
In P. californica, the swim typically begins with a vigorous dorsal flexion with the foot leaving the substratum in a wave traveling antero-posteriorly; this action tends to catapult the animal vertically off the substratum.

Fig. 1. Behavioral response to stimulation with acid sea water. A ventral view of an animal attached to the side of the aquarium affords a view of the foot during avoidance. Stimulation of the right oral veil region (arrow) by 1 ml of acidified sea water (pH 2.25) in frame B causes local withdrawal and a contralateral turn in frames C–F. The shortening of the contralateral side of the animal's foot is clearly apparent. Frames were taken at 5–10s intervals.
The vault shown in Fig. 2 brought this 10 cm animal straight up by about one body length off the substratum. Escape swims were elicited by acid stimulation in trials on 14 out of 34 animals. While swims could be elicited by stimulation of oral veil and tail, the most effective site appeared to be the dorsal mantle. Oral veil withdrawal clearly preceded the swim when the latency from stimulus to swim was greater than a second or two; however, withdrawal appeared to be suppressed.

Fig. 2. A 10 cm specimen of Pleurobranchaea californica responds to a puff of acidified sea water (pH 1.95) by initiating a swim. B was taken 1–2 s after application of the stimulus from the pipet, which is visible. It shows the initiation of the first dorsal flexion of the swim while the animal still shows a unilateral local withdrawal, and has partly turned away from the stimulus as the swim begins.
during the swim. A ventral flexion completed the cycle. A swim episode often consisted of only a single cycle, but could consist of five or more cycles.

In the two specimens of *Pleurobranchus membranaceus* observed, 5 and 7 cm in length, acid stimulation induced the swimming behavior characteristic of this animal (Thompson and Slinn, 1959). The swim is an alternating contracture of the lateral halves of the broad foot; this propels the animal, ventral surface up, in a rocking motion. It differs markedly from the swim of *P. californica* in its motor pattern and in its long (many minutes to hours) duration. An adequate stimulus for initiating swimming in the two animals studied was simply removing the foot from the substratum; swimming ceased when the animal was gently pressed to attach its foot to the substratum. Thus, acid stimulation may have caused swimming, in part or in full, by causing body contraction and a consequent loss of contact between foot and substratum. The other species studied showed only the substratum locomotor response and did not swim.

**Acid secretion caused by mechanical and chemical stimuli**

The stimulation of acid secretion by mechanical stimuli was confirmed for all species by application of pH indicator paper to the mantle skin following prodding with a finger or Pasteur pipet. In each case the color change of the indicator paper reflected an acidification to around pH 1–2.

Skin acidification was localized to a spreading area around the stimulation site. The occurrence and time course of acid secretion were documented for *P. californica* with a pH electrode held next to the skin surface. In the unmolested animal, either quiescent or actively locomoting in sea water of pH 7.4–8.3, the skin surface pH was slightly more acid than the milieu, in the range 7.10–8.2 (N=4). Scratching the dorsal mantle with a pipet tip or forceps, or transient compression with a pair of forceps, caused acidification recorded with latencies of 2–5 s, with acidification rapidly peaking in the pH range 1.55–1.80 (Fig. 3A). All stimuli that induced acid secretion caused local and spreading contracture around the stimulated area. In larger animals (>15 cm in length) a transient blistering of the skin was evident at the stimulated area and the blister grew in diameter up to several centimeters over some tens of seconds. The blister was evident within 5 s of stimulation as a raised edematous area slightly lighter in color than the surrounding skin. In two animals, moving the pH electrode between blister and surrounding skin showed that the peak pH response was localized on the blister area, falling off sharply at its periphery. Blistering subsided over 5 min. Blistering coincided approximately with the local contracture of the skin. For one response in a blistered area, the electrode read a maximum acid response of pH 1.55; 1 cm outside the blister periphery the reading was pH 4.45, and 6 cm away the reading was 7.52, close to the pH of the ambient sea water.

Taurine, a feeding stimulant for many marine carnivores, has an opposite effect in *P. californica* and acts as a feeding deterrent and noxious stimulus. Taurine concentrations as low as $10^{-5}$ molL$^{-1}$ can cause aversive behavior (Huang and Gillette, 1985). We tested taurine in the present study and found that it also causes
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Fig. 3. Skin acidification following mechanical and taurine stimulation. (A) Following a scratch to the mantle (arrow) with the tips of a pair of forceps, the skin under the electrode acidified by over 6 pH units. The scratch occurred about 1 cm away from the electrode. The long latency until the fastest acidification reflects the slow propagation of the response from the stimulus site. (B) After placement of the pH electrode on the skin (left-hand arrow), approximately 1 ml of a solution of $10^{-2}$ mol\(\text{l}^{-1}\) taurine in sea water was pipetted at the site over about 3 s (right-hand arrow). The acidification response was rapid and attained a recorded amplitude of nearly 6 pH units.

Acid secretion (Fig. 3B). The action of taurine occurred at relatively low concentrations; skin acidification occurred at concentrations as low as $10^{-6}$ mol\(\text{l}^{-1}\). The effect of taurine on acid secretion paralleled its effects in inducing aversive behavior (local withdrawal and aversive locomotion), and in being effective when applied anywhere on the body surface.

Taurine caused transient skin blistering with a shorter latency than mechanical stimulation; in one animal application of $10^{-2}$ mol\(\text{l}^{-1}\) taurine to the back caused evidence of a blister with a latency of less than 1 s.

Acidic sea water that caused aversive motor responses also induced an acid secretion. For the experimental record shown in Fig. 4, pH2.1 sea water was

Fig. 4. Stimulation of skin acidification by acid stimulation. Several milliliters of acid sea water (pH2.1) slowly directed at the left tentacle/oral veil region of a quiescent animal (first arrow) evoked at first little skin acidification and little behavioral response. However, after about 40 s a rapid skin response occurred, accompanied by a vigorous contralateral turn and initiation of locomotion (beginning at the second arrow). The initial small acidification is probably due to the acid stimulus.
Fig. 5. Skin mucus retains acidity. After recording an acidification response on the mantle of *Pleurobranchaea californica*, the electrode was drawn away from the animal into the ambient sea water (beginning of record). There, the pH recorded by the electrode alkalized only very slowly. When the electrode was quickly withdrawn from the water, wiped with a tissue and returned to the sea water (arrow), its response was markedly swifter.

pipetted near the pH electrode held on the left dorsal surface of the oral veil. With nearly 50 s latency, an acid secretory response began which distinguished itself from the stimulus in attaining a greater acidity: pH 1.80. The acid secretory response was accompanied by a strong aversive turn and rapid locomotion.

The acidity of the skin caused by mechanical and chemical stimulation persisted for over 5 min, but could be rapidly returned to near the pH of the ambient sea water by a gently directed flow of fresh sea water. Thus, secretion of acid was a transient event, and the persistence of skin acidification was probably a function of the animal's mucus acting as a diffusion barrier, and possibly as a buffer. This interpretation is supported by observations that mucus stuck to the pH electrode retained acidity for a prolonged period (Fig. 5). This, in turn, suggests that the transfer of acidified mucus to a potential predator would leave an after-image of the defensive response that would linger for some time following an encounter, perhaps reinforcing avoidance learning by the predator.

*Central nervous control of acid secretion*

The potential role of the central nervous system in mediating acid secretion was investigated by observing the effect of nerve stimulation on skin acidity. Observations were made in three preparations in which the brain, buccal, pedal and visceral ganglia were dissected out. The distal end of a cut body wall nerve, which, when intact, originated from the pleural lobe of the cerebropleural ganglion, was drawn into a suction electrode for stimulation while skin pH was recorded. The body wall nerves innervate a large area of the animal's dorsal mantle. The skin of the denervated region was more acid than that of the intact preparation by more than 1 pH unit and remained so for over an hour. The pH did not recover when the skin was rinsed, indicating that cutting the nerve caused a long-lasting, tonic release of acid.
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5.14
pH

Stimulation

6.67

Fig. 6. Acid secretion in response to nerve stimulation in a preparation from which the central nervous system had been removed. The body wall nerve innervating the dorsal mantle was stimulated for 3 s at 10 Hz (bar), which caused rapid acidification at a short latency. The skin pH continued to decrease more slowly over the next 20 s until it reached a plateau.

Stimulation of the body wall nerve caused mantle contraction with a latency of a fraction of a second, estimated visually, followed by rapid skin acidification (Fig. 6). The effects of nerve stimulation are consistent with a direct contribution of central efferent effectors to acid secretion. However, these results could also be obtained indirectly, if the body wall contraction caused by nerve stimulation were to excite peripheral mechanoreceptive paths which, in turn, were to stimulate acid secretion. In either case, the results suggest central nervous control of acid secretion.

Peripheral nervous control of acid secretion

The peripheral nervous system of molluscs is capable of mediating certain simple behavioral reflexes autonomously. The deganglionated *P. californica* preparation was found to respond to both mechanical and chemosensory stimulation with acid secretion. This was shown in four separate experiments where the skin pH response was recorded following stimulation (Fig. 7). These results indicate that the peripheral nervous system may make a substantial contribution to acid secretion, and that sensory–motor paths may exist at the periphery to couple mechanoreception and chemoreception to acid secretory cells. In summary, the data are consistent with the neural organization of acid secretion by interaction of the peripheral neural plexus and the central nervous system.

Discussion

The experiments and observations reported here are simple; however, they relate to an appreciable body of previous observations on notaspid physiology and behavior. The results have shown that acid secretion in notaspideans is a more complex action than was expected from what seemed at first to be a simple, reflexive defense response. An interpretation has emerged of a context of neural pathways within which acid secretion is stimulated and causes feedback into the animal’s behavioral repertory of defensive withdrawal and locomotion.
Fig. 7. Sensitivity of the acid response to taurine and mechanical stimulation is retained following removal of the central nervous system. (A) After a stable pH record had been obtained from the dorsal mantle, the pH electrode was transiently lifted to enable a rapid compression of the underlying skin with forceps (arrow). (B) Application of 10^{-2} \text{mol} \text{l}^{-1} \text{taurine} (arrow). The records are from different preparations.

Acidic sea water applied anywhere on the animal’s body provoked local contracture of the skin. Applied to the tail or oral veil, acid stimuli typically evoked defensive withdrawal of those regions and, in quiescent animals, initiated ciliary locomotion (substratum locomotion in these species is by the unidirectional sweep of myriad cilia on the foot; Jones, 1975) or escape swimming. Applied to the oral veil, acidic sea water induced aversive turning away from the direction of the stimulus. The overall sensitivity of the body to acidic stimuli contrasts with the chemosensory sensitivity to food stimuli shown by *P. californica*, which is restricted to the oral veil, rhinophores and mouth area (Davis and Mpitsos, 1971; Lee and Liegeois, 1974).

Cutaneous acid secretion was evoked by the same mechanical and chemical stimuli (taurine and acid) that caused withdrawal contractions, aversive turns and locomotion. The acid response began at the stimulated site and spread over an adjacent area; blistering of the skin associated with acid secretion suggests that the secretion causes a local osmotic imbalance in the tissues involved. The acid secretory response of notaspideans to mechanical stimuli has been well characterized (Thompson and Slinn, 1959); the present observations both confirm previous ones and extend them to aversive chemical agents. Acid secretion appears to be a rather specific response to noxious stimuli.

The observations that acid secretion could be evoked in preparations without central ganglia by mechanical and chemical stimuli, and by orthodromic nerve stimulation, indicate that the control of acid secretion is exerted by both central and peripheral pathways. Either local sensory neurons could innervate acid secretory cells in the periphery, or the secretory cells themselves might be directly mechano- and chemoreceptive. The demonstration that central effector axons have access to the secretory cells implies that sensory inputs to the central ganglia exist to drive them. Thus, peripheral and central pathways resemble those
integrating a variety of sensory–effector actions in other molluscs (Rowell, 1963; Mpitsos and Lukowiak, 1986).

The data also imply that mechanisms exist to limit propagation of the acid response over the skin. Otherwise, a local response would be expected to spread over the entire animal through the positive feedback phenomenon of acid-induction of further acid secretion. The limiting mechanism could well be an inhibitory neural pathway.

**Acid secretion and aversive behavior**

Acid secretion is caused by the same stimuli that cause aversive behavior. At the same time, acidic sea water, more dilute than the acidic secretion from the animal, stimulates aversive behavior. Thus, acid secretion may be part of a positive feedback loop that contributes significantly to the regulation of behavior. Normally, noxious stimuli may act to cause acid secretion, which then stimulates or augments aversive behavior. Possibly, aversive behavior is entirely dependent upon acid secretion; however, it seems more likely that acid sensation acts in parallel with mechanosensory and other modalities to cause aversive movements.

It would appear that secreted acid acts as an excitant of a selectively nociceptive sensory pathway or of specially patterned sensory activity specifically inducing aversive behavior. The aversive turns caused by acid, taurine and mechanical stimulation are mediated by contracture of the body wall and foot on the side contralateral to stimulation. Since motor neurons causing turning and twisting movements in opisthobranchs are generally ipsilateral to the innervated muscle, this suggests that the putative nociceptive, sensory–motor paths within the central nervous system (CNS) decussate to stimulate contralateral motor neurons. This prediction provides a testable framework for the investigation of aversive behavior and its sensory regulation.

**The role of taurine**

The amino acid taurine is present in millimolar concentrations in marine invertebrate tissues, where it may act as an osmolyte. In the anaspid seaslug *Aplysia*, taurine perfused into pieces of body wall induces contractures like those caused by unidentified stressor substances released into the blood by mechanical and electrical stimulation (Cooper et al. 1989). To our knowledge, *P. californica* is the only animal responding to exogenous taurine with a strong aversive response; to many other marine predators taurine is a weak or neutral feeding stimulant (Grant and Mackie, 1974; Hara, 1982). Taurine and/or related substances are quite possibly natural mediators of acid secretion; released from skin cells by damage or depolarization (mechanisms of taurine release are reviewed by Huxtable, 1989), they could stimulate the acid secretory cells, either directly or through peripheral effects on neural paths. This hypothesis is testable by measuring taurine release.
Role of acid secretion in the chemical ecology of notaspids

Acid secretion is a highly effective defense against predation (Thompson and Slinn, 1959; Thompson, 1988), for which notaspids may well pay a high price in energy, and possibly also in stress to their own tissues. Potentially, acid secretion also has a role in maintaining individual dispersal in notaspids. This could be particularly important in *P. californica*, a notable cannibal. *P. californica* occasionally attack each other in the tanks in which they are maintained, and our informal observations are that a frequent response to interspecific contact is aversive behavior similar to that associated with acid secretion.

These results indicate a potentially pervasive and important role for acid secretion in the organization of the behavior of *P. californica*, and probably that of the other notaspids. In particular, the aversive behavior of *P. californica* elicited by taurine or acid stimulation is indistinguishable from that shown by animals trained in a food-avoidance associative conditioning paradigm (Mpitsos and Collins, 1975; London and Gillette, 1986) or exhibited after contact with attempted, noxious prey (Huang and Gillette, 1986). The present observations that aversive behavior may be stimulated by the animals' own acid secretion suggest that an important future task could be to examine the role of acid secretion in notaspid foraging behavior and conditioned food avoidance.

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References


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