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PROLINE INHIBITION OF A SEA ANEMONE ALARM PHEROMONE RESPONSE

BY NATHAN R. HOWE

Hopkins Marine Station of Stanford University, Pacific Grove, California, U.S.A.

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SUMMARY

1. L-proline, by itself or in animal tissue extracts, inhibits the response of the sea anemone *Anthopleura elegantissima* to the alarm pheromone, anthopleurine.

2. The effect of proline is mediated by a receptor that is specific for the structure and configuration of the part of the L-proline molecule containing the carboxyl and imino groups.

3. Proline inhibition is competitive, in the sense that the effects of a given proline concentration can be overridden by an increase in anthopleurine concentration.

4. The magnitude of proline inhibition increases with proline concentration and decreases as the duration of exposure to proline increases.

5. Neither the final conducting system mediating the alarm response nor the responding muscles are inhibited by proline. Inhibition presumably occurs at or soon after the level of anthopleurine receptors.

6. Proline inhibition may resolve the potential conflict between *Anthopleura*'s mutually exclusive feeding and alarm pheromone responses.

INTRODUCTION

The simultaneous stimulation of potentially conflicting reflexes is undoubtedly a frequent occurrence for animals in their natural environments. In animals with well-developed central nervous systems such conflicts are almost always resolved so that one reflex is entirely suppressed and the other expressed. Increasing the stimulus strength for the suppressed reflex either has no effect or exchanges the suppressed and expressed reflexes. This either-or property has been considered one of the most important evolutionary developments of the central nervous system (Bullock & Horridge, 1965). Most evidence for mutual exclusion between incompatible reflexes is from vertebrates, but recent studies show that the same capacity is well developed in 'higher' invertebrates (Davis, Mpitsos & Pinneo, 1974).

How animals that lack central nervous systems respond to conflicting stimuli has not been extensively investigated. Rushforth (1965) in studies with *Hydra* found that the feeding activator, glutathione, inhibited contractions usually evoked by light and mechanical stimuli. Ross & Sutton (1964) showed that the sea anemone *Stomphia* became relatively insensitive to food while swimming and, conversely, that prior

exposure to food extracts inhibited the swimming response. In neither of these two systems has a further analysis of the mechanisms of inhibition been undertaken.

The response to anthopleurine (Howe & Sheikh, 1975; Howe, 1976), an alarm pheromone from the sea anemone *Anthopleura elegantissima*, provides a relatively simple system for investigating the effects of conflicting stimuli on cnidarian behaviour. When anthopleurine combines with receptors on the tentacles of *A. elegantissima*, a characteristic alarm response is evoked. Tentacles bend sharply away from the mouth, mesenteric retractor muscles contract to shorten the column, and the marginal sphincter smoothly closes up the top of the column (Howe, 1976). When *Anthopleura* feeds, L-asparagine from the prey causes tentacles in contact with the prey to bend toward the mouth (Lindstedt, 1971). Not only is a fully alarmed animal incapable of feeding, but the contractions of tentacle muscles evoked by feeding activators and anthopleurine are antagonistic. The present study was undertaken to see how *A. elegantissima* resolves the potential conflict between the feeding and alarm responses when stimuli for both are present simultaneously.

MATERIALS AND METHODS

Anemones from the same asexually produced clone (Francis, 1973) were maintained in glass bowls in the laboratory as previously described (Howe & Sheikh, 1975). Animals were starved for at least 1 week but no more than 4 weeks before any experiment. To measure responses to anthopleurine the sea water supply line to an animal bowl was removed, and 12 min later 0·5 ml of a solution of anthopleurine in distilled water was mixed into the bowl. Animals were observed for 28 s following the administration of pheromone; animals showing any degree of rapid downward bending of the tentacles were considered to have responded positively. To measure the effect on the alarm response of a substance other than anthopleurine, 0·5 ml of a distilled water solution of that substance was mixed into an anemone bowl 10 min before the administration of anthopleurine. Tests with anthopleurine were repeated no more frequently than once every 2 h, a period of time adequate for full recovery of the alarm response (Howe & Sheikh, 1975). Median effective concentrations (EC_{50}) were determined graphically by the method of Litchfield & Wilcoxon (1949).

For measurements of the electrical stimulus threshold of the through-conducting system in *Anthopleura*, anemones were forced to settle on plastic discs from which insulated platinum electrodes 8 mm apart projected 0·4 mm. Electrodes were connected to a Grass Model 5B Stimulator, adjusted to deliver shocks of 10 ms duration 1 s apart. At the beginning of each 15 s period a series of five shocks was administered, with voltage increased by 0·2 V for each series of shocks. Animals were observed for the first rapid symmetrical contraction, indicating activation of the through-conducting system (Pantin, 1935), and threshold voltage was recorded for each animal.

Anthopleurine, isolated and crystallized as previously described (Howe & Sheikh, 1975), was stored in 10^{-4} M aqueous solution at −4 °C. Crude animal tissue extracts were made by homogenizing fresh, blotted tissue in 39 volumes distilled water, centrifuging the homogenate (12000 *g*, 15 min, 0 °C), then diluting the supernatant

liquid as necessary with distilled water. Protein concentrations of tissue extracts were measured by the method of Lowry *et al.* (1951). For quantitative proline assays, fresh whole *A. elegantissima* and squid mantle tissue were lyophilized, ground, and 100 mg of each powder extracted in 10 ml of water (1 h at 20 °C) and then centrifuged (500 *g*, 20 min). The supernatant liquids were adjusted to 50% (v/v) ethanol, cooled (2 h, −4 °C), then filtered and analysed for L-proline without prior hydrolysis on an automatic amino acid analyser. L-proline (Calbiochem, A grade) was recrystallized twice from hot ethanol before use. All other chemicals were obtained commercially and used without additional purification. One-dimensional chromatography was performed on Whatman No. 1 paper in *n*-butanol–acetic acid–water (4:1:5). For two-dimensional chromatography, Eastman cellulose thin-layer sheets were developed first in the above solvent system, dried overnight, then developed in water-saturated phenol (NH_3 atmosphere) in the second dimension.

RESULTS

Crude extracts of squid mantle tissue at concentrations equal to or greater than 5×10^{-4} g wet tissue per litre of anemone bowl water evoked a brief (1–5 s) period of tentacle writhing, followed by a slight lateral expansion of the oral disc that persisted for the duration of the test. Those concentrations of squid extract also abolished the alarm response to 8×10^{-10} M anthopleurine (for H_0 = no effect: $\chi^2 > 10$, $P < 0{\cdot}005$). Neither mouthward bending of tentacles nor opening of the mouth was observed at any extract concentration used, since activation of the feeding responses requires simultaneous chemical and mechanical stimuli (Lindstedt, 1971). To see if the inhibitory effect of squid extract was unique to squid, extracts of bivalve mantle, gastropod foot, crab leg muscle and sea urchin gonad were tested. All extracts abolished the alarm response except that from urchin gonad, which had no effect at any concentration. Expressed as a function of extract protein concentration, the effects of the inhibitory tissue extracts did not differ significantly. To test the possibility that one of *Anthopleura*'s chemical feeding activators was responsible for inhibition of the alarm response, solutions of L-asparagine and reduced glutathione were administered at concentrations equal to and ten times greater than those reported to be maximally effective in eliciting the feeding response (Lindstedt, 1971). Neither compound had an inhibitory effect.

Extracts from ground, lyophilized squid mantle were then treated to determine some of the properties of the inhibiting substance. The inhibitory substance had a molecular weight less than 3500 (dialysis with Spectrapore 3 membrane) and was stable to heat 100 °C, 1 h) and acid hydrolysis (1 N-HCl, 100 °C, 1 h). One-dimensional paper chromatography revealed that inhibition corresponded with a single spot with R_F and staining properties (ninhydrin) characteristic of the α-imino acid proline. L-proline was tested for inhibitory activity, and the results are plotted in Fig. 1. L-proline is a powerful inhibitor of the alarm response to 8×10^{-10} M anthopleurine, with an EC_{50} of approximately 5×10^{-9} M and a significant inhibitory effect at $1{\cdot}25 \times 10^{-9}$ M ($t = 2{\cdot}45$, $P < 0{\cdot}05$, one-tailed test). Inhibitory concentrations of L-proline caused lateral expansion of the oral disc and appeared to reduce the frequency of spontaneous contractions of both tentacle longitudinal muscles and oral disc radial muscles.

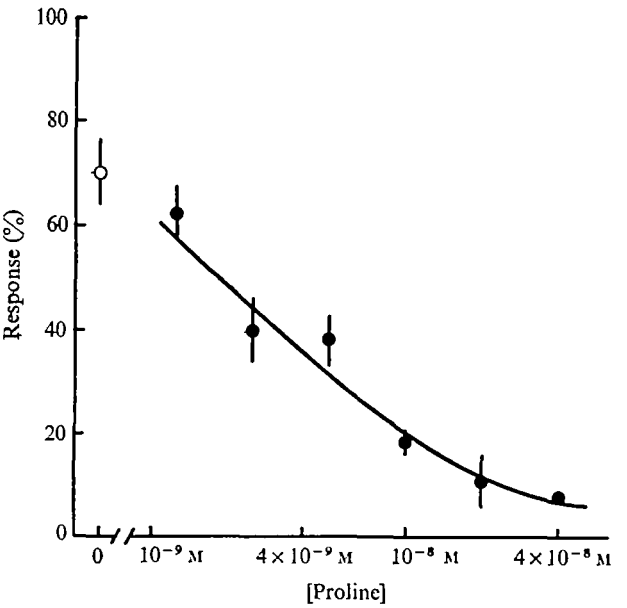

Fig. 1. Alarm response inhibition by L-proline. L-proline was administered 10 min before 8×10^{-10} M anthopleurine. Vertical bars indicate standard errors of the means. The curve was fitted graphically on probability paper. The point furthest to the left (open circle) shows the percent responding in the absence of proline.

To discover whether L-proline was the only inhibitory substance in squid tissue, a methanol extract of lyophilized squid mantle was chromatographed in two dimensions. Duplicate chromatograms were prepared with test spots of pure L-proline for each dimension on one chromatogram. After final development and drying, the chromatogram with test spots was stained with ninhydrin. Proline from the squid sample migrated as a single spot, well separated from other stained spots. The spot corresponding with proline was scraped from the unstained chromatogram and eluted in 7 ml of water. Except for a 1 cm border around the proline spot, the entire remainder of the chromatogram was then removed and eluted in 7 ml of water. When both eluates were compared with a distilled water control for alarm inhibition, only the proline spot was effective ($\chi^2 = 5{\cdot}83$, $P < 0{\cdot}025$), a result indicating that proline is the only substance in tissue extracts that inhibits the alarm response.

Among the possible models for the inhibitory action of proline the most likely were: (i) that proline combined directly with the anthopleurine receptor (either at the anthopleurine site itself or at some other site) and thereby prevented the binding of the pheromone or (ii) that proline combined with its own receptor to activate a conducting system that inhibited the transmission of alarm response impulses. If proline acts to block the binding of anthopleurine to its receptor, the magnitude of inhibition should depend on the proline concentration but not upon the duration of exposure to proline. If, on the other hand, proline exerts its effect via a separate receptor and conducting system, one might expect proline inhibition to habituate. To test for habituation, aqueous proline was introduced at a constant rate into the sea water supply system for 24 bowls of anemones, so that a proline concentration of approximately 8×10^{-8} M was maintained. At 5 min intervals after first exposure to

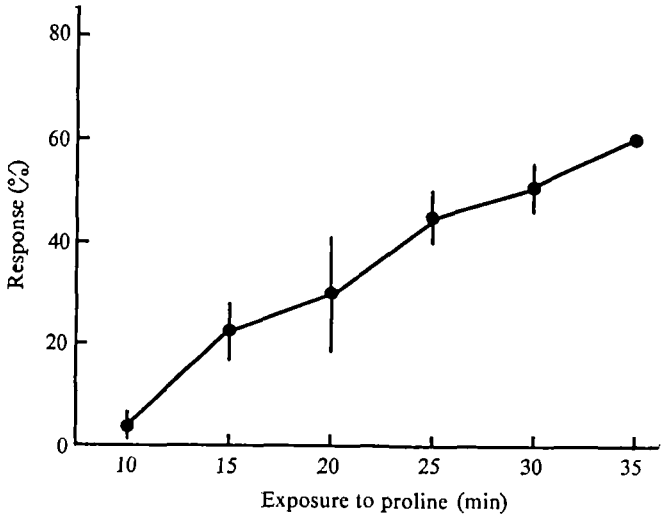

Fig. 2. Habituation to proline inhibition. Animals were tested for responses to 8×10^{-10} M anthopleurine after the indicated durations of exposure to 8×10^{-10} M L-proline. Means of three or four determinations are plotted with vertical lines to indicate standard errors.

proline, four bowls (with a total of 21–23 animals) were disconnected from the proline/sea water supply, allowed to stand for 5 min, then tested for the alarm response to 8×10^{-10} M anthopleurine. The experiment was repeated four times. The results, plotted in Fig. 2, show that as the duration of exposure to proline increases, the ability to respond to anthopleurine is steadily regained. After 35 min of exposure to proline, the proportion of responding animals differs only slightly from that observed in the absence of proline (approximately 70%). This result indicates that proline inhibition habituates and is probably mediated by a specific receptor and conducting system.

There are several possible stages in the mediation of the alarm response at which the inhibitory input of the proline system could act: at the anthopleurine receptor, at the motor neurone/effector level, or at some intermediate stage. Since the alarm response is mediated by an identified through-conducting system (Howe, 1976), it was possible to test the second of these three alternative hypotheses by testing for a proline-induced increase in the electrical threshold of the through-conducting system. Two anemones from the same clone were prepared for electrical stimulation. In each of six threshold determinations one animal served as a sea water control and the other was exposed to a proline concentration of 2×10^{-8} M or 2×10^{-7} M, concentrations that strongly inhibit the alarm response (Fig. 1). Control through-conducting system threshold voltage was low and stable for both animals ($4{\cdot}1 \pm 0{\cdot}1$ V (S.D.) and $2{\cdot}9 \pm 0{\cdot}3$). Threshold voltage was not altered by exposure to either proline concentration. This result suggests that proline inhibits the alarm response prior to activation by anthopleurine of the through-conducting system.

To determine the specificity of the proline receptor, chemical analogues of L-proline were tested for inhibition of the alarm response to 8×10^{-10} M anthopleurine. Fig. 3 shows a structural diagram of L-proline surrounded by those of the analogues tested. Each analogue differs from proline by a single addition, deletion, substitution, or change in configuration. The EC_{50} was determined for each compound that had an inhibitory

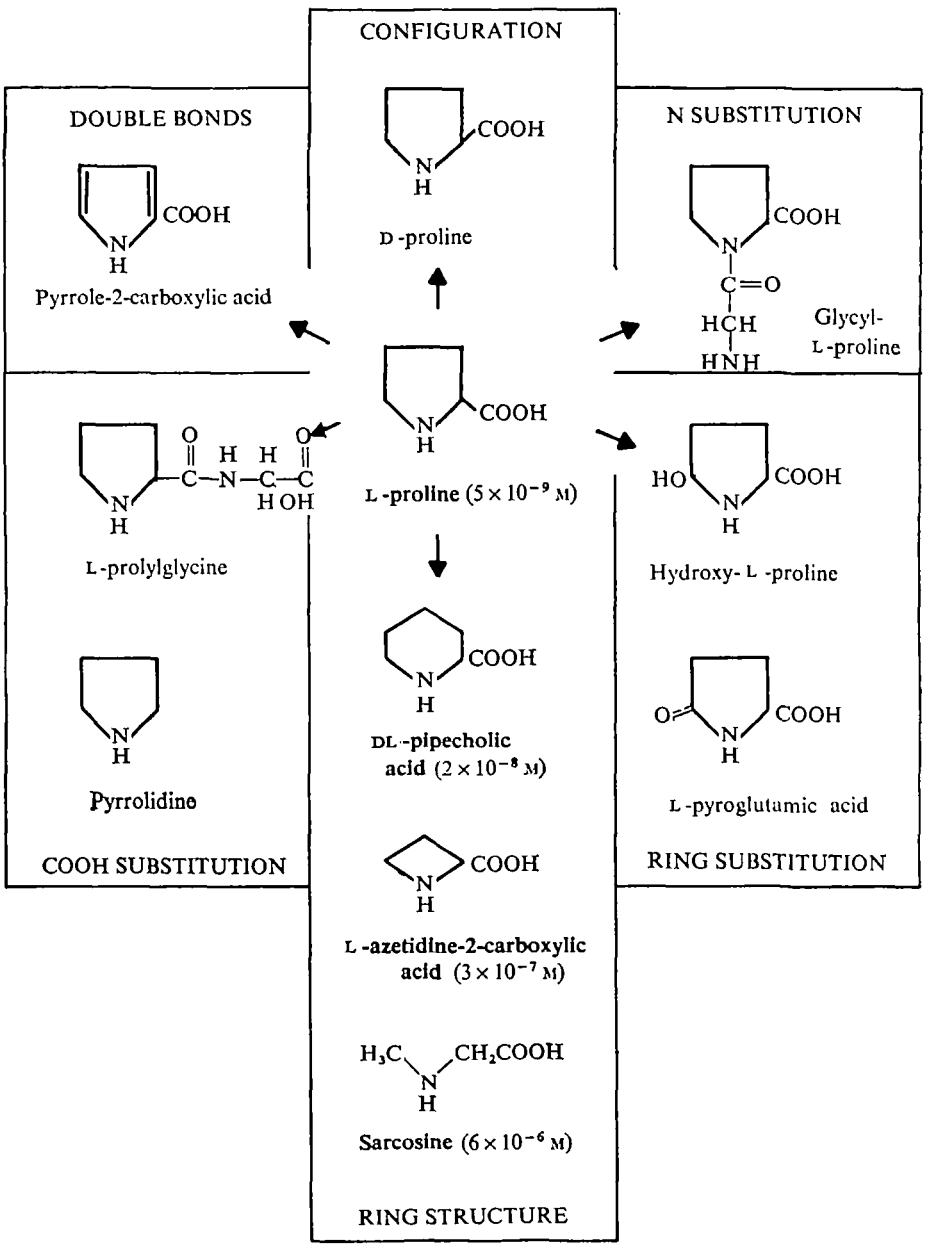

Fig. 3. Specificity of the proline receptor. L-proline analogues were tested for the ability to inhibit the response to 8×10^{-10} M anthopleurine. Compounds that were inhibitory at or below 2×10^{-5} M are labelled in bold type with EC_{50} indicated parenthetically.

effect at or below 2×10^{-5} M, and those compounds are labelled in bold type. Substitutions for hydrogen at the 3-carbon (hydroxy-L-proline, L-pyroglutamic acid) or imino (glycyl-L-proline) positions abolished inhibition, as did the addition of ring double bonds (pyrrole-2-carboxylic acid) or a change in the configuration of (D-proline), the addition to (L-prolylglycine), or the deletion of (pyrrolidine) the carboxyl

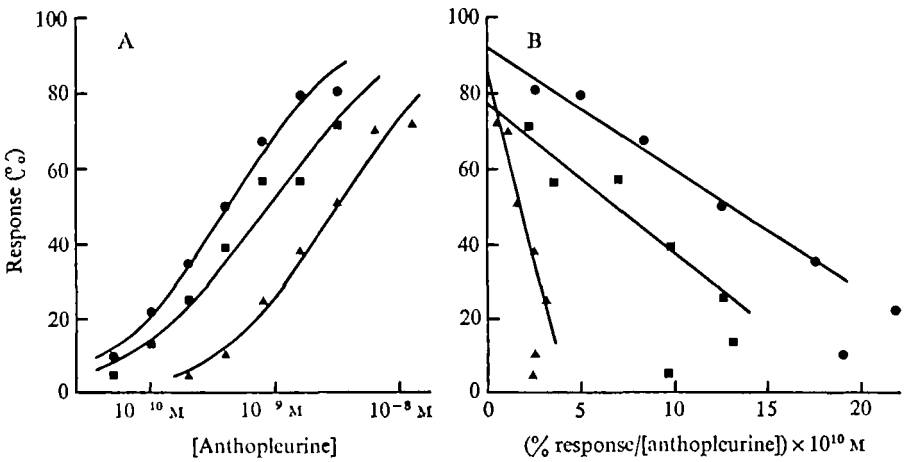

Fig. 4. Effects of two proline concentrations on the anthopleurine dose-effect relationship. (A) Comparison of log concentration–response curves for anthopleurine alone (circles) and anthopleurine in the presence of 4×10^{-8} M (squares) or $1{\cdot}6 \times 10^{-8}$ M (triangles) L-proline. (B) Data from Fig. 4A replotted by the Eadie method with the same symbol conventions. Lines were fitted by least squares to the five strongest responses for each treatment. Slopes of the lines were used to make two independent estimates of an inhibitor rate constant for L-proline (see text).

group. Compounds that differed from L-proline only in the size of the ring were inhibitory and can be located in Fig. 3 in a column directly beneath L-proline. DL-pipecholic acid with a six-member ring was the most active L-proline analogue ($EC_{50} = 2 \times 10^{-8}$ M), followed by L-azetidine-2-carboxylic acid with a four-member ring ($EC_{50} = 3 \times 10^{-7}$ M). Even sarcosine, an acyclic α-imino acid, essentially lacking the 3- and 4-position carbons of proline, had some inhibitory activity, though its EC_{50} (6×10^{-6} M) was more than 1000-fold greater than that for L-proline. It appears, therefore, that the receptor mediating alarm response inhibition is quite specific for L-proline and that the specificity resides in the part of the proline molecule corresponding with the structure of sarcosine.

To see whether proline operates by blocking the alarm response or by competitively raising the anthopleurine threshold, the dose-effect curve for anthopleurine alone was compared with similar curves from the same animals exposed to low and high proline concentrations. The results are presented in Fig. 4A. Neither the maximum proportion of animals showing the alarm response nor the slope functions for the three curves differ significantly. Instead, it appears that proline raises the anthopleurine threshold in much the same way that a competitive inhibitor acts upon an enzyme reaction. If one explores this analogy further, however (as Lenhoff (1965) has done with glutathione-induced feeding with *Hydra*), it begins to break down. In Fig. 4B the data from Fig. 4A have been replotted by the Eadie method for enzyme-substrate reactions. This plot (V_s vs $V_s/[S]$) is equivalent to Lineweaver–Burk plots and was chosen to permit the use of the data from Fig. 4A. Lines were fitted by least squares to the points representing the five strongest responses for each treatment, since points at the low end of the response scale are likely to have been affected by a threshold phenomenon (Lenhoff, 1965). The slope of the control line, $-3{\cdot}26 \times 10^{-10}$ M, represents the negative rate constant for the association of anthopleurine and its

receptor. Though the data still appear to support the competitive inhibition model (note the similar y-intercepts but different slopes for the three lines in Fig. 4B), the inhibition rate constants for proline, calculated by the method of Hofstee (1955) from the slopes for low and high proline concentrations, differ widely ($1{\cdot}8 \times 10^{-8}$ M and $3{\cdot}2 \times 10^{-9}$ M, respectively). It appears, then, that proline competitively raises the anthopleurine threshold, that the magnitude of proline's effect depends in a relatively complex way upon its concentration, and that single enzyme kinetics do not furnish an appropriate model for the interaction of proline and anthopleurine.

Although *Anthopleura*'s tissue fluids, like those of its prey, contain some proline, *Anthopleura* normally gives the alarm response to wounded or homogenized conspecifics. Two factors appear to be involved. First, *Anthopleura*'s proline content ($1{\cdot}2$ μM/g dry weight) is low, compared with its anthopleurine content (about 30 μM/g, recalculated from Howe & Sheikh, 1975) or with the proline content of its prey ($23{\cdot}5$ μM/g for squid mantle). Secondly, proline administered simultaneously with anthopleurine, as would occur if an anemone were wounded, has a considerably smaller inhibitory effect than if proline precedes anthopleurine. When 120 anemones were tested, $55 \pm 7\%$ (S.E.) gave an alarm response to 8×10^{-10} M anthopleurine administered simultaneously with squid extract. When the same squid extract preceded 8×10^{-10} M anthopleurine by 15 min, only $22 \pm 6\%$ (S.E.) responded, a significant reduction ($t = 5{\cdot}1$, $P < 0{\cdot}005$). Proline inhibition appears to have a slower time course than the response to anthopleurine.

DISCUSSION

Since *Anthopleura*, like all cnidarian polyps, is an essentially sessile carnivore, it is important that it be prepared to feed when the availability of prey is imminent. L-proline, by causing the lateral expansion of the oral disc and by reducing spontaneous contractions of the oral disc and tentacle muscles, may increase the likelihood of prey capture. A similar response to food extracts has been described for the sea anemone *Tealia felina* (McFarlane, 1970). L-proline is the first cnidarian preparatory feeding stimulus to be identified.

Since *Anthopleura*'s alarm response precludes feeding, it is of potential adaptive importance for the presence of food to reduce the likelihood of a response to anthopleurine. L-proline from the prey appears to perform this function. Rather than preventing the binding of anthopleurine, proline inhibits the alarm response by activating a specific proline receptor. Proline receptors have been reported to mediate the feeding responses of three species of cnidarians (cf. Lenhoff, 1974), and the specificity of the proline feeding receptor in the hydroid *Cordylophora* has been carefully examined (Fulton, 1963). The specificities of the proline receptors in *Cordylophora* and *Anthopleura* are nearly identical, a similarity that may indicate a common evolutionary origin.

The activation of *Anthopleura*'s proline receptor inhibits the alarm response at an early stage in the stimulus-response pathway, at any rate before activation of the through-conducting system. In this respect the proline inhibition system differs markedly from glutamic acid inhibition of circular muscle contraction in the sea anemone *Actinia* (Carlyle, 1974), where the inhibitory effect is exerted on or near the

muscle cells. It may be adaptively significant that proline, although reducing *Anthopleura*'s response to a distant threat by decreasing anthopleurine sensitivity, does not prevent protective responses to mechanical injury by inhibiting the through-conducting system and its associated musculature. It seems likely that the conducting system that mediates proline inhibition exerts its effect either upon anthopleurine receptors or upon interneurones that connect those receptors to the through-conducting system.

Recent electrophysiological studies on the excitation and control of prefeeding behaviour in *Tealia* have shown: (i) that food extracts activate chemoreceptors in the column ectoderm that generate impulses in an ectodermal slow conduction system (SS1) (Lawn, 1975), (ii) that activity in the SS1 inhibits spontaneous activity in and causes relaxation of the radial muscles of the oral disc (McFarlane, 1970; McFarlane & Lawn, 1972) and (iii) that prefeeding chemoreceptors show sensory adaptation after long periods of exposure to food extracts (McFarlane & Lawn, 1972; Lawn, 1975). Certain features of the effects of proline on *Anthopleura* are strikingly similar to the consequences of activation of the SS1 in *Tealia*. Proline causes oral disc expansion and inhibits spontaneous contractions of oral disc and tentacles, and the effect of proline on the alarm response diminishes with a time course similar to that of sensory adaptation in *Tealia*'s prefeeding response. On the basis of those similarities it seems reasonable to propose that the effects of proline on oral disc expansion and on the response to anthopleurine are mediated by the SS1.

Bullock & Horridge (1965) suggest that 'when our information is more complete it may be expected that lower and higher groups will differ markedly along a quantitative scale in the development of mutual exclusion, of concentration and scope of control of executive centers'. That prediction is borne out by this study. While it is clear that the proline system provides *Anthopleura* with a method for organizing two mutually exclusive behaviours in an adaptive way, it is equally clear that proline inhibition, since it is graded or competitive (Fig. 4), is not an example of the relatively sophisticated 'either-or' switch of higher animals.

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