Stimulus-Response Relations and Stability of Mechanoreceptor and Motor Neurons Mediating Defensive Gill-Withdrawal Reflex in Aplysia

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SUMMARY AND CONCLUSIONS

1. A weak or moderate-intensity tactile stimulus delivered to the siphon skin of Aplysia californica elicits a defensive reflex withdrawal of the gill and siphon into the mantle cavity. The reflex undergoes both short- and long-term habituation and sensitization and has, therefore, been used as a model system to examine various forms of learning. In this paper we describe studies of the response properties of the sensory and motor neurons of the reflex during repeated stimulation at rates that produce habituation.

2. The sensory neurons are slowly adapting mechanoreceptor cells whose frequency of discharge is a monotonic function of controlled-force punctate stimuli delivered to the skin. The majority of the stimulus-response relations could best be described by exponential functions.

3. We examined the stability of the sensory neuron responses in two ways: with punctate stimuli of varying intensity and with water jets of varying intensity.

4. With repeated punctate stimulation at rates which produce habituation in the intact animal the mechanoreceptor discharge showed no decrement. This stability was observed over a 10-fold range of intensities.

5. Weak or moderate intensity water-jet stimuli to the skin also gave stable responses but stronger stimuli caused the mechanoreceptor response to fatigue.

6. We examined the stability of the motor responses by using intracellular depolarizing current pulses to produce repetitive bursts of action potentials in gill motor neurons while monitoring the gill contractions with a strain gauge, photocell, or videotape recorder. The photocell and strain gauge were alternatively used in the same experiment. Gill contractions monitored with the photocell were stable, whereas those monitored by the strain gauge showed decrement. An independent measure of gill contraction, videotape recording, confirmed the results obtained with the photocell and showed that the gill contractions following repeated intracellular depolarization of the motor neurons were stable.

INTRODUCTION

A weak or moderate-intensity tactile stimulus applied to the siphon skin or the mantle shelf of Aplysia californica elicits a defensive withdrawal reflex of the gill and siphon into the mantle cavity (18, 24). This reflex undergoes both short- and long-term habituation and sensitization (8, 23, 24) and has, therefore, been used as a model system for studying these simple nonassociative forms of learning (5, 10, 11). In this and the companion paper we examine further the cellular mechanisms underlying these behavioral modifications. In this paper we describe studies of the response properties and stability of the sensory and motor neurons of the reflex during repeated stimulation at rates that produce habituation. In the second paper (5) we evaluate the contribution of individual sensory neurons to the reflex response produced by weak stimuli. In a further study (unpublished data) we will consider the synaptic changes in the connections between the sensory neurons, interneurons, and motor cells that accompany habituation and sensitization.

The sensory component of the reflex is mediated by two symmetric clusters of sensory neurons located in the abdominal ganglion. One cluster (the RE cells) consists of approximately 20 neurons which innervate the skin of the mantle.
The other cluster (LE cells) consists of approximately 24 neurons which innervate the siphon skin (4). The two clusters of sensory neurons play a critical role in transmitting information about peripheral events to the central nervous system. We have here focused primarily on the LE cluster. They provide most of the tactile input from the siphon skin (5) and make direct excitatory connections with interneurons and gill and siphon motor neurons in the abdominal ganglion (5, 10). In a previous study we described the receptive field and some of the response properties of these mechanoreceptor sensory neurons (4). In the present paper we have extended the analysis by examining the stimulus response characteristics of these cells and their stability to repeated stimulation. By making intracellular recordings from the cell bodies of individual mechanoreceptor neurons, we have found that the firing rate of the sensory neurons is a monotonically increasing function of the intensity of the tactile stimulus over a range of intensities that elicit the behavior and that the sensory discharge remains stable at repetition rates that produce habituation in the intact animal.

The motor component of the reflex which is centrally mediated consists of 13 central motor neurons, 6 for gill, 6 for siphon, and 1 for gill and siphon (6, 20). Using a quantitative videotape analysis we have focused on the motor neurons of the gill component of the reflex and have examined the stability of the gill contractions produced by direct intracellular stimulation of identified motor neurons. Stimulation of the gill

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**FIG. 1.** Experimental preparation used to examine mechanoreceptors. The siphon skin along with the abdominal ganglion with the intact siphon nerve are removed from the animal and pinned out on the flat surface of an experimental chamber with a Sylgard base. Punctate controlled-force stimuli are delivered to the skin with a feedback-controlled electromechanical stimulator (1) while intracellular recordings are made from the cell bodies of individual primary sensory mechanoreceptor neurons in the abdominal ganglion.
motor cells at rates that produce marked habituation in the intact animal produced stable motor responses.

These findings extend earlier studies on this reflex (4, 9, 19) and indicate that habituation does not involve to any significant degree either receptor or motor fatigue. A preliminary account of portions of this work has previously been presented (2).

METHOIDS

Preparations

The preparation we used to examine the mechanoreceptor cells (Fig. 1) was similar to that previously described (4). In most cases the dissected siphon skin and abdominal ganglion with its intact siphon nerve were transiently bathed in a solution of high magnesium (elevated 4 times normal to 220 mM) and low calcium (reduced by 10% to 1 mM). This procedure reduced reflex contractions during pinning and facilitated the pinning of the skin on the flat surface of a 50-ml experimental chamber. The solution was added several minutes before the skin was to be pinned and washed out immediately after the pinning was completed. The preparation was then rested for approximately 1 h before beginning the experiment. In most cases the chamber was continuously perfused with seawater at 15°C supplemented with dextrose, amino acids, and vitamins (4). Preparations were rejected if the siphon skin was distorted or damaged.

The preparation used to examine the motor cells has been previously described (9). The gill and siphon, left attached to the abdominal ganglion via the branchial, genital and siphon nerves, were pinned with the gill ventral side up to the Sylgard (Dow Corning) floor of an experimental chamber. This chamber was constantly perfused with seawater at room temperature (20–22°C). To examine habituation of reflexly evoked gill contractions, intact animals were restrained in a small (2 liter) aquarium also at room temperature (20-22°C). The solution was added several minutes before the skin was to be pinned and washed out immediately after the pinning was completed. The preparation was then rested for approximately 1 h before beginning the experiment. In most cases the preparation was then rinsed once with seawater at 15°C supplemented with dextrose, amino acids, and vitamins (4). Preparations were rejected if the siphon skin was distorted or damaged.

Recording techniques

BEHAVIORAL TECHNIQUES. Gill contractions were measured using three different techniques: 1) Photocell. A photocell was imbedded in the recording chamber floor and a weak-intensity light was shone on the entire gill surface but not on the photocell directly, so that movements of the gill allowed light to directly strike the photocell and uncover an area which was proportional to the amplitude of gill contraction (for details see ref 24). 2) Strain gauge. Gill contractions were also measured by the method of Peretz et al. (22) using a strain gauge (Grass FT103) attached with 005 suture thread to the tip of a gill pinnule or, in some cases, to the gill efferent vein by means of a loop of suture thread. 3) Videotape analysis. Video recordings of gill contractions were obtained by directing a video camera (Panasonic WV-342) at a mirror placed above the dorsal view of the gill. A continuous timing signal from a video timer (GYRR type G77) was simultaneously recorded. Recordings were displayed on an 8-inch screen of a video monitor (Panasonic type WV-930) driven by a Sanyo recorder which permitted both slow-motion (one-seventh real time) and single, stop-frame analysis of gill contractions. Gill contractions were quantified in the following manner. The planar area of the gill, as viewed from the monitor screen, was outlined on a clear transparency, first with the gill at rest (1 s prior to stimulation) and at peak contraction. The transparencies were traced on onion skin paper, cut out, and weighed. The amplitude of a contraction was quantified as the percent reduction in weight of the tracing of the contracted gill compared to the relaxed gill. This method provides a quantifiable estimate of the reduction of gill area during contraction.

ELECTROPHYSIOLOGICAL TECHNIQUES. Single-barreled microelectrodes were used to make intracellular recordings from the cell bodies of the mechanoreceptor neurons using conventional electrophysiological techniques. Double-barreled microelectrodes were used in studying the motor cells, one barrel for recording membrane potential and the other for passing intracellular current. Data were simultaneously recorded on an FM tape recorder (Hewlett Packard 3960) at 15/16 inches/s and a Brush 440 pen recorder.

Mechanical stimulation

Two types of mechanical stimuli were utilized. 1) Water-jet stimuli of 800 ms were delivered to the skin with a commercially available Water Pik (Teledyne). In one series of experiments with the pinned-out skin, the receptive field of the mechanoreceptor was initially tested. The water jet was then directed toward the site of maximum sensitivity and positioned between 5 and 20 mm from the skin, usually perpendicular but in some cases at more oblique angles. At these distances and with the intensity settings used the effective pressure at the skin ranged between 70 and 600 g/cm² as determined by directing the water jet at a calibrated miniature pressure transducer (Pitran PTM2, Stow Laboratories). In another series of experiments with the unpinned skin the water jet was directed toward the general region of a sensory cell's receptive field, previously determined by probing with a moderate-intensity von Frey hair. The water jet was positioned 15
mm from the tip of the skin and adjusted to deliver a pressure 254 g/cm². In both series of experiments the preparation was then rested for 15 min after the initial probing before a new stimulus series was initiated. 2) Punctate stimuli were delivered with a feedback-controlled electromechanical stimulator which could deliver controlled force stimuli to the skin (1). This stimulator has a force transducer in series with the probe tip. As a result it provides a continuous monitor of the actual force which is delivered to the skin. In all cases 800-ms-step force stimuli were utilized which were of identical duration as the water-jet stimuli used in previous behavioral studies (8, 23, 24). The intensity of the stimulus varied between 0.24 and 4.2 g (the maximum output force of the stimulator). For convenience we have expressed the force measurements in terms of grams (gram-force). To convert these values to pressure in grams per square centimeter, a more appropriate measure, one can simply divide by the probe area. Thus, for a probe diameter of 0.56 mm and, therefore, a probe area of $7.5 \times 10^{-3}$ cm², a force of 1.0 g produces a pressure of 400 g/cm². The transducer was calibrated by lowering the probe onto a piece of Sylgard placed on the loading pan of an electronic balance (Mettler PN 1210). In the experimental situation the probe of the stimulator was positioned perpendicular to the skin and lowered to a site which responded well to von Frey hair stimulation. The stimulation site was not, however, necessarily the site of highest sensitivity. The threshold was determined and the cell was rested for 15 min before a training session was begun. Each testing period was usually initiated with a weak, barely threshold stimulus which was repeated once every 30 s for 10 trials. After a 15-min rest, a higher intensity series was delivered. After each 15 min rest, the intensity was increased at approximately equal steps until the mechanoreceptor response appeared to plateau or the limit of stimulator was reached. The entire procedure was then repeated, but with decreasing-intensity steps.

Data analysis

In the vast majority of cases, the sensory neuron discharges only persisted for the duration of the stimulus (see RESULTS). In order to account for those few cells which continued to discharge after the stimulus was terminated, stimulus-response plots were constructed by defining the response as the total number of spikes elicited between the time the mechanical stimulus was applied and up to 5 s after the stimulus was withdrawn. Data were used for stimulus-response plots if at least four series of stimuli of 10 trials each at different force intensities were available.

The stimulus-response plots were fitted with each of the three mathematical functions:

$$\text{linear } R = a(S - T) \quad (1)$$

$$\text{power } R = a(S - T)^b \quad (2)$$

$$\text{exponential } R = a(1 - e^{-b(S - T)}) \quad (3)$$

where $R$ represents the response (see above), $S$ the stimulus strength in grams, $a$ and $b$ parameters of the various equation, and $T$ the threshold parameter.

A nonlinear least-squares curve-fitting routine was applied (13) to the stimulus-response data in order to estimate the unknown parameters in each of the three equations. The equation which yielded the lowest residual error was selected to best describe the data. The threshold parameter was constrained to be positive and in the case of equations 2 and 3, to be less than the lowest force value applied to the skin in that stimulus-response series. Statistical analyses and linear regression (Fig. 3) for the pooled stimulus response data were performed with programs written in BASIC on a PDP11 computer (Digital Equipment Corp.).

RESULTS

Response of mechanoreceptor neurons to mechanical punctate stimuli

The primary receptive field of the defensive gill-withdrawal reflex, the siphon, mantle shelf, and purple gland skin is innervated by two symmetric clusters of primary sensory neurons whose cell bodies are located within the abdominal ganglion. One cluster of neurons, the RE cluster, innervates the anterior mantle shelf and purple gland, while the other cluster, the LE cluster, innervates the posterior mantle shelf and siphon skin (4).

In the following experiments we have focused on the I.F cluster neurons and describe their response to punctate stimuli. While water jet stimuli are generally used in behavioral studies on the intact animal, they are not sufficiently precise for a quantitative analysis of the mechanoreceptor response properties. The intensity of the water-jet stimuli striking the skin is difficult to quantify since the effective force is a function of the orientation and distance of the water-jet nozzle from the skin. The unusual geometry of the siphon skin and the limited working distance of the experimental chamber prevent these parameters from being precisely controlled from cell to cell and preparation to preparation. In addition, the force delivered to the skin is not uniform across the field of stimulation, but varies as a function of the distance from a given site on the skin to the center of the water-jet stream.
FIG. 2. Typical responses of one mechanoreceptor neuron to 800-ms-step forces delivered to the skin. The upper traces are intracellular recordings from the cell body of a mechanoreceptor neuron, while the lower traces are the waveforms of the actual force stimuli delivered to the skin using the electromechanical stimulator illustrated in Fig. 1. Data are samples from the experiment of Fig. 4F. The sensory discharge is a monotonically increasing function of the stimulus intensity.

The punctate stimulator (see Methods) is a feedback-controlled instrument which is capable of delivering precisely controlled force stimuli of predetermined amplitudes and durations to a large extent independent of skin thickness and geometry (Fig. 1). The 0.5-mm probe permits small restricted regions of the skin to be investigated.

STIMULUS-RESPONSE RELATIONS. As a first step in understanding the details of the processing of a tactile stimulus which initiates the gill-withdrawal reflex, we examined the mechanoreceptor stimulus-response relationships in an attempt to determine a quantitative fit between the sensory discharge and the intensity of a tactile stimulus. With this information in hand we could then compare the types of transformations taking place at the primary sensory level with those taking place at the central integrative and effector organs of the reflex (see below and ref 5).

A common feature of slowly adapting mechanoreceptor neurons is that their discharge frequency is proportional to the intensity of a given tactile stimulus (12). We investigated the response of the LE mechanoreceptor neurons to 800-ms controlled-force step stimuli delivered to the skin at various intensities in both ascending- and descending-intensity series. Figure 2 illustrates typical responses of the intracellular discharges recorded from one mechanoreceptor neuron, while various intensities of controlled force stimuli, each an 800-ms step, were delivered to the skin. The sensory discharge is a monotonically increasing function of the intensity of the tactile stimuli and, as previously noted, the discharge exhibits a characteristic irregular interspike-interval variability (4).

In some instances the mechanoreceptor neurons gave prolonged several second discharges to the 800-ms-step stimulus. These situations were quite rare; the discharge typically persisted only throughout the duration of the stimulus and shows a brief off-response (Fig. 2 and ref 3).

The data of Fig. 2 indicate that the sensory discharge is a graded (approximately linear) function of the intensity of the tactile stimuli. We next pooled the stimulus-response data to examine the generality of these observations and to determine what mathematical function might best describe the data. Figure 3 illustrates the pooled stimulus-response data from 11 cells each in different preparations. Each point represents the average of between 10 and 40 responses at a given intensity depending on the number of ascending- and descending-intensity series presented (see Methods). There is considerable variability in the data but the entire curve was well described by an exponential function (solid curve). The slope of the curve indicated that it might also be described by a second-order piecewise linear function. We therefore divided the data into a number of different regions and found that when the force was less than or equal to 2.3 g, the linear function with the highest correlation coefficient was obtained (R = 2.7(S - 0.21); r = 0.86). The remaining data points were fit with a second linear function (R = 1.14(S + 2.02); r = 0.26).
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FIG. 4 Stimulus-response relations: 13 stimulus-response relations from 11 different mechanoreceptor neurons; 10 were obtained with testing period interstimulus intervals (ISI) of 30 s. One cell in addition to being tested at an ISI of 30 s (L), was also tested at ISI of 10 (K) and 60 (M) s. Once cell (J) was tested at 10 s ISI. Circles on graphs represent data points, while the smooth curve is the predicted curve generated by the best-fitting mathematical function. N represents the number of samples averaged to obtain each data point. The majority of stimulus-response relations were best fit by exponential functions.

The extrapolated threshold for both the linear and exponential curves were nearly identical to the threshold of 0.25 g obtained directly in a previous study (4).

We next examined the stimulus-response relations of individual neurons to test the generality of the pooled stimulus-response data. Figure 4 illustrates 13 stimulus-response plots from 11 mechanoreceptor neurons in 11 different experiments. Cells A-J and L were tested at 30-s intervals. The stimulus-response plots of Fig. 4K, L, M represent the same cell but were obtained using different rates of stimuli; 4K was obtained with an interstimulus interval of 10 s, 4L, 30 s, and 4M with an interval of 60 s. Data points on Fig. 4J were obtained with an interstimulus interval of 10 s. Of the stimulus-response plots eight could be best fit by exponential functions, three by power functions, and two by linear functions. The stimulus-response plot of one additional cell was quite unusual and was not analyzed since it probably represented a rapidly deteriorating preparation. This cell gave very weak responses to all stimulus intensities, the higher intensity stimuli actually giving weaker responses than the lower ones.

STABILITY OF MECHANORECEPTOR DISCHARGES. The gill-withdrawal reflex undergoes profound habituation with repeated elicitation (24). It was, therefore, of interest to examine the stability of the response of the mechanoreceptor neurons to determine to what degree changes in their responsiveness contribute to habituation. Figure 5A illustrates the response of one cell to 800-ms-step force stimuli delivered to the skin once every 30 s. Figure 5B illustrates the pooled data from 124 testing periods on 21 cells in 17 experiments, where 10 consecutive stimuli were presented once per 30 s. Each cell contributed an average of about six testing periods and the stimulus intensity varied between 0.24 and 4.2 g, with a mean of 1.9 g. The 30-s interstimulus interval was chosen since stimuli delivered at these rates and longer produce habituation in the intact and isolated preparation (5, 24). Each cell was rested at least 15 min before a training session was initiated. The total number of spikes elicited from the first stimulus in each
A typical intracellularly recorded discharges from one mechanoreceptor neuron to 800-ms punctate stimuli delivered to skin at rates of once per 30 s. Shown are the response to the 1st, 5th, and 10th stimuli. B: plot of mechanoreceptor stability; 100% corresponds to a mean discharge of 3.8 impulses (see text for further details). N = number of sessions. The mechanoreceptor neurons show no decrement to repeated punctate stimulation.

series was scored as 100%. The summary graph indicates that the mechanoreceptor neurons do not fatigue at these rates of elicitation. Therefore, fatigue of the response of these mechanoreceptor neurons cannot account for the behavioral decrement of the reflex which, after 10 trials, is usually reduced to about 25% of control (see Fig. 16). In fact, the sensory discharge appears to show a slight increment in responsiveness, that is, the third and subsequent stimuli produce a slightly higher discharge in the sensory neurons than that produced by the first stimulus. But this effect is small and the increased response beyond the first stimulus is not significant at the 0.05 level (two-tailed t test for nonindependent groups).

STABILITY AS A FUNCTION OF INTENSITY. When data were pooled over a wide range of stimulus intensities the mechanoreceptor responses appear to respond in a stable manner and perhaps increment slightly to repetitive stimuli (Fig. 5). We next examined if some decrement or facilitation might be detected as a function of stimulus intensity. Figure 6 illustrates data where the various stimulus intensities have been divided into nine different force categories over a 10-fold range from 0.5 to 4.5 g. Averaged-response curves for stimuli within each range are given. For eight of the nine categories, there was no statistically significant difference (at the 0.05 level; two-tailed t test for nonindependent groups) between the first response and last response of each series. In one case (1.0 ≤ F < 1.5 g) the last response was statistically greater (P < 0.05) than the initial response.

LONG-TERM STABILITY. We next examined if the discharges from one stimulus training session of 10 stimuli could affect the mechanoreceptor discharges when 10 identical stimuli were presented to the skin while recording from the same cell 15 min later. We showed above that the mechanoreceptors cannot account for the short-term habituation obtained in a single training session. This test examines the sensory neuron stability in repeated training sessions. The responses of the mechanoreceptors to the first stimulus of the second series were sometimes higher than the responses of the mechanoreceptors to the first stimulus of the initial training session. This carry-over was more likely to occur at weaker intensities (Fig. 7) or if the responses
in first stimulus series were themselves increasing, but it did not occur in all cases and, in general, the effects were always small and not statistically significant at the 0.05 level (t test for nonindependent groups). There was also no difference in the total number of impulses elicited during the first series and the second series 15 min later. For example, the mean of the total number of impulses elicited in each of the first series was 47.9 ± 14.6 (mean ± SD; N = 17 different cells), whereas the mean for the second series was 47.8 ± 32.1. The intensity of the stimuli used in these 34 series varied between 0.57 and 3.55 g.

STIMULUS-RESPONSE VARIABILITY. The stimulus-response plots of Fig. 4 were obtained by pooling the data from both ascending- and descending-intensity series. When the ascending and descending series are plotted separately, we usually observed large, often significant differences. However, there was not any consistent pattern to this hysteresis. In some cases the descending-series responses were elevated from the ascending, in other cases the reverse was true, and in others the two curves crossed. These results are not surprising in light of the wide variability of the mechanoreceptor neurons to particular stimulus intensities (see Fig. 6). Figure 8 illustrates the variability in the stimulus-response relations for one mechanoreceptor neuron where two ascending and one descending series were obtained (numbers on plot represent order of stimulus presentation). Three of the four points of the first ascending series were above the first descending series but in the second ascending series, only two points were above the descending series, and three of four points were below the first ascending series. To test whether the general shape of our stimulus-response plots or the response variability was due to some artifact cause by the temporal pattern of the stimuli, we examined one cell’s stimulus-response properties with two different methods. The first technique was the one already described (see METHODS). In the second method, stimuli were presented every 30 s and the intensity incremented after each individual stimulus instead of the end of a testing period of 10 stimuli. Complete ascending and descending series were repeated 10 times to give the same number of total stimulus presentations with each method. Figure 9 illustrates the results obtained using the two methods on a single cell. The two plots are remarkably similar. It seems unlikely that such similarity would exist if the temporal pattern was the underlying cause of the hystereses. This experiment also tests if the long-term stability of the mechanoreceptors is affected by the temporal pattern of stimuli. A simple test was to count the total number of spikes for each ascending and descending series obtained by the second method. If temporal patterns were critical a change in the total number of spikes might be expected with each new series. No such effect was observed, which supports the notion that the stimulus-response characteristics and temporal stability of the mechanoreceptor are, at least within a limited range, independent of the temporal pattern of stimulus presentation.
Response of LE mechanoreceptor neurons to jets of seawater

Most previous behavioral studies of reflexly mediated gill contractions used repeated presentation of seawater jets to the siphon skin. In earlier work Kupfermann et al. (19) found no systematic decrease in the extracellular afferent volley recorded from the siphon nerve when the skin was stimulated with 800 ms duration jets of seawater at rates of 1 per minute. We have reexamined this question using intracellular recordings from individual mechanoreceptor neurons in the ganglion while similar water-jet stimuli were presented to the skin. We used two different preparations. In one the skin was unpinned to closely parallel the natural situation, while in the other the skin was pinned out (Fig. 1) to ensure that the stimuli were being delivered to the same region of the skin each time. The results of both studies essentially parallel the stability observed with punctate stimuli. In the experiments where the skin was unpinned the water-jet stimulator was positioned 15 mm from the tip of the siphon skin and directed toward the general region of a mechanoreceptor receptive field which had previously been determined by probing with a moderate-intensity von Frey hair. The water-jet stimulator was set to deliver a pressure of 254 g/cm² which is a moderate-intensity stimulus used in the intact preparations (7). In 22 cells from five experiments we found that the sensory neurons responded on the average with 2.1 ± 2.65 (mean ± SD) impulses per response. The range varied between 0 and 8 impulses, with a median of 1. In five cells from five different preparations we tested the response to repetitive stimuli delivered once every 30 s. While there was considerable response variability with each cell, there was on the average no decrement.

To more accurately characterize the response of the mechanoreceptor neurons to repetitive water-jet stimuli, we performed further experiments with the skin pinned out. With this procedure the same area of the skin could be repetitively stimulated and the distance from the water-jet nozzle to the skin could be held fixed for any given training session. In these experiments 63 training sessions on 25 cells in 21 different preparations were performed. On the average each cell was tested about 3 times. In 36 of the training sessions, stimuli were delivered once every 30 s and in 27, once every 60 s. By directing the water-jet at a pressure transducer (see Methods) we calculated that the effective water pressure at the skin probably varied between 70 and 600 g/cm² but because the intensity of the water-jet stimulus contacting the skin cannot be calibrated precisely (see above), we chose to examine the data with respect to the number of spikes elicited. This approach seemed justifiable since we showed earlier that the mechanoreceptor discharges are monotonic function of the intensity of a punctate stimulus (Figs. 2, 3, 4). Thus, we used a neuron’s discharge rate and the stimulus-response relationship determined from probe stimuli as an index of the intensity of the tactile stimulus. Using this method of calibration we found that at low discharge rates and presumably low intensities, the mechanoreceptors give stable responses to repetitive stimuli, but at higher initial discharge and presumably higher intensities, the mechanoreceptors show a slight decrement (Fig. 10A, B).

Figure 10 illustrates these findings for interstimulus intervals of 30 and 60 s. In each case the cells were classified according to the initial response in a given testing period. Units with similar initial responses were then averaged over each of the 10 responses and plotted as shown. For low initial discharge rates (less than 4 spikes) there was no statistically significant difference (0.05 level) between the first and last responses of the training period for either the 30- or 60-s interstimulus intervals (two tailed t test for non-independent groups). With higher initial discharges reflecting higher stimulus intensities, there was decrement. For example, when the initial discharge rates were between 4 and 6 spikes there was significant decrease of about 20–30% (P < 0.02).

The stability observed at weak discharge rates produced by the water jet on the pinned skin is consistent with data from the unpinned siphon
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Skin and with the earlier results from the punctate stimulator. However, decrement at higher discharge rates was not observed with the punctate stimulator (Fig. 6). Some of the discrepancy may be due to interactions which are established when multiple sites of a mechanoreceptor's field are simultaneously stimulated by the diffuse water jet. While we cannot account for the differences in the probe and water-jet data, what is clear is that experiments designed to examine central contributions to habituation should be performed with weak or moderate-intensity stimuli (water jet < 254 g/cm²; probe < 4.5 g).

Stability of motor response produced by direct stimulation of gill motor neurons

We have previously reported that direct intracellular stimulation of the major gill motor neurons L7 and LDG, at rates that produce habituation in the intact animal (10- to 30-s interstimulus intervals) produced no decrement of gill contraction (9). Recently Jacklet and Rine (14, 15) reported that direct stimulation of the gill motor neurons at comparable rates (30-s interstimulus interval) produced decrement of gill contractions. The major difference between the two types of experiments was the method for measuring gill contractions. Jacklet and Rine used a strain gauge to measure gill contractions, whereas Carew et al. (9) used a photocell. To examine whether these differences in recording techniques might account for the differences in the results, we carried out a series of five experiments in which we recorded gill contractions using both techniques alternately in the same experiment. Each experiment consisted of three blocks of 10 stimuli each; each block was separated by 5 min. Depolarizing pulses were injected into the gill motor cell L7 (2 s duration) at 30-s intervals, causing the cell to fire at an average frequency of about 13 spikes per second. In the first block of 10 stimuli, gill contractions were measured with a photocell; in the second block, contractions were measured with a strain gauge; and in the third block, contractions were again measured with a photocell. The results are shown in Fig. 11. As we had previously observed (9) gill contractions as measured with a photocell were variable (notice the large standard error of the mean for each response) but showed no net tendency to decrease at these slow rates of stimulation. However, in the same preparation, when gill contractions were measured with a strain gauge, comparable stimulation of cell L7 produced decrement in the amplitude of the gill contractions. When we returned to the photocell we again observed no decrement.

For statistical analysis the data were normalized: for the series of five experiments each response was expressed as a percentage of the initial response in the block of 10 stimuli. The mean response amplitude for all 10 stimuli was then computed and constituted a single score for each block. A Friedman two-way analysis of variance revealed a significant difference \( P < 0.0085 \) in the mean response amplitude across the three blocks of stimuli. Subsequent sign tests showed that the gill contractions measured with a strain gauge (block two) were significantly less \( P < 0.031 \) than those measured with a photocell (blocks one and three). The response amplitudes for blocks one and three in which the

![FIG. 10. Response to water-jet stimuli. Discharges have been separated into categories according to the number of impulses (R) in the initial response. Responses for each stimulus number in each category were then averaged. Data points are means ± SE; N = number of responses in each category. A: ISI = 30 s. B: ISI = 60 s.](image-url)
FIG. 11. Stability of gill contractions: two methods of measurement. Gill contractions (open circles) produced by direct intracellular stimulation of gill motor cell L7 (number of spikes in solid circles) were recorded alternately in the same experiment by means of A, a photocell; B, a strain gauge; and C, again with a photocell. Response amplitudes are normalized by expressing each response in a run as a percentage of the initial response amplitude of that run. Data from five experiments are expressed in means ± SE. Responses measured with the strain gauge show decrement.

The pooled data from all five experiments are

- photocell was used were not significantly different from each other.

These data show that the decrease in response observed with the strain gauge was not due to deterioration of the preparation since the contractions subsequently measured with a photocell (in block three) were stable. Another interpretation of these results is that the recording of gill contractions with a strain gauge partially restrains the gill (or at least a gill pinnule) and forces it to work against an unphysiological load. The gill pinnule is a delicate organ that has very little mass (see 9) and normally never has to work against such a load. Therefore, the depression observed with the strain gauge, rather than reflecting true habituation, may actually reflect either peripheral fatigue or alteration of the normal contractile properties of the gill pinnule muscle.

Another possible interpretation of the data shown in Fig. 11 is that the strain gauge is a more sensitive measuring device than the photocell. Thus, changes in response amplitude undetected by the photocell, might still be detected by the strain gauge. This alternative is unlikely. In experiments in which gill contractions were simultaneously measured with both strain gauge and photocell, contractions are always more readily detected by the photocell than the strain gauge, at the gain used for experiments described above. Small contractions are often seen on the photocell while the strain gauge recorded no movement. In addition, the time course of recovery from a full gill contraction is always much faster when measured with a strain gauge than with the photocell, presumably because the strain gauge exerts some pull on the gill when it is fully contracted.

However, to insure that photocell was accurately measuring the actual response amplitude of gill contractions, we carried out another series of five experiments while quantitatively measuring gill contractions with a video tape recorder. Gill contractions were produced as before by direct stimulation of cell L7. Spikes were recorded on an FM tape recorder and contractions were simultaneously recorded on the videotape recorder. The contractions were then measured by single stop-frame analysis for quantification (see METHODS).

There were two runs in each of five experiments. Each run consisted of 10 depolarizing pulses applied to L7 at an average frequency of 19 spikes per second for approximately 2 s, with an interstimulus interval of 30 s. Runs were only accepted for analysis if the difference in spike number between the 1st and 10th stimuli was three spikes or less. All runs were separated by at least 30 min. An example of our results is shown in Fig. 12. Although there is some variability from one stimulus to another in the amplitude of the gill contraction, there is no net tendency of the response to decrease with repeated stimulation. The percent reduction of gill area for the 1st, 5th, and 10th contraction in the example shown in Fig. 12 is 20.1, 17.0, and 19.3%, respectively.

The pooled data from all five experiments are
shown in Fig. 13. In each experiment two runs were pooled by computing the mean contraction exhibited during each of the 10 stimuli. Thus the results of each experiment consisted of the mean of the two runs in that experiment. The mean responses for all five experiments (for each of the 10 stimuli) were then computed (Fig. 13). A t test of correlated means comparing the mean amplitude of the 1st and 10th contractions revealed no significant difference between them ($t = 0.168$). Although the data are variable, as indicated by the relatively large standard errors of the mean, they show that there is no net tendency of the gill contractions to decrease.

To directly compare the stability of gill contractions produced by direct motor cell activation with the habituation of the gill contractions produced by repeated reflex activation, we carried out a final series of experiments. Five animals were restrained, one at a time, in a small aquarium filled with aerated artificial seawater (for details of methods, see ref 6 and 24). The parapodia and mantle were retracted and a small platform was placed under the gill. After a rest of at least 1 h the gill-withdrawal reflex was elicited by 10 repeated moderate-intensity jets of seawater to the siphon at an estimated intensity of 254 g/cm² (for calibration procedures and details, see METHODS and ref 6 and 21) using an interstimulus interval of 30 s. As described previously, a videotape recording of gill contractions was made. An example of the results is shown in Fig. 14. The first stimulus produced a large contraction (a 59.6% reduction of gill area). With repeated stimuli the response habituated: the 5th and 10th stimuli produced a response of 25.6 and 6.9% reduction of gill area, respectively. The data from all five experiments are shown in Fig. 15. The mean initial response was 68.9% of gill area—a near maximum contraction. By the 10th stimulus, the response decremented to a mean value of 15.7% of gill area. A sign test comparing the last response to the first showed that there was significant decrement ($P < 0.031$) of gill withdrawal.

In order to permit a direct comparison of the

![Fig. 12](image-url)  
**Fig. 12.** Example of stability of gill contractions as measured with videotape recorder. Direct stimulation of L7 (1.8-s pulse, approximately 17 spikes per second) produces a rather small gill contraction (STIM 1) which constitutes a 20.1% reduction of gill area (see METHODS). Dashed lines indicated the relaxed gill 1 s prior to stimulation; solid lines indicate peak of gill contraction. With repeated stimulation of L7 (10 stimuli; ISI = 30 s) there is no significant tendency of gill contractions to decrement: the 5th and 10th responses, which are illustrated, constitute a 17.0 and 19.3% reduction of gill area, respectively.

![Fig. 13](image-url)  
**Fig. 13.** Stability of gill contractions: summary of videotape recorder experiments. Summary of five experiments in which gill contractions produced by direct intracellular stimulation of L7 were quantified by means of a videotape recorder analysis. Data are expressed in means ± SE. There are no significant differences among the responses across all 10 trials.
FIG. 14. Example of response decrement produced by repeated reflex activation of gill contractions. The gill-withdrawal reflex was elicited by jets of seawater to the siphon (see METHODS). Stimuli were delivered at the same intervals as direct activation of cell L7 in Figs. 11-13 (10 stimuli; ISI = 30 s). Responses were measured with a videotape recorder. The 1st, 5th, and 10th responses from a single experiment are illustrated. Dashed lines indicate relaxed gill; solid lines are the gill at peak of contraction. The first stimulus elicits a near-maximal reflex response: 59.6% reduction of gill area. With repeated stimulation the reflex exhibits pronounced habituation: the 5th and 10th stimuli constitute a 25.6 and 6.9% reduction, respectively. The gill in this illustration appears slightly different than that shown in Fig. 12 because this is a view of the dorsal surface of the gill in the intact animal; the irregular border on the left is the retracted mantle shelf. Figure 12 shows the ventral surface of an excised gill and mantle preparation (see METHODS for details of the two preparations).

actual amplitudes of contraction produced both by direct motor cell activation and by reflex activation, we have used the same scale for Figs. 13 and 15. The contractions produced by stimulation of L7 at an average frequency of about 19 spikes per second are relatively small (about 18% reduction of gill area) compared to those produced by reflex activation in which the initial response is about 69% reduction of gill area. In order to further compare the responses produced by direct motor cell activation to the responses produced by reflex activation, the data from Figs. 13 and 15 have been normalized by expressing each response as a percentage of the mean initial response. Those data are shown in Fig. 16. As this summary figure illustrates repeated motor cell responses, although variable, are relatively stable, whereas the repeatedly elicited reflex responses habituate to about 25% of their initial value.

DISCUSSION

Stability of mechanoreceptor neurons

Kupfermann et al. (19) have previously found in the semi-intact preparation that the extracellular recorded afferent discharge from the siphon nerve remained constant when the siphon skin was repeatedly stimulated with water-jet stimulation. The present results confirm this finding utilizing intracellular recordings from the cell bodies of the LE mechanoreceptor neurons. These neurons provide most if not all of the tactile input from the siphon skin to the abdominal ganglion (5). In addition, in an isolated preparation the effects of punctate mechanical stimulation were also explored. Parallel studies using an isolated preparation with only the gill attached revealed that the reflex-mediated gill contractions and the amplitude of complex PSP in the motor neurons show marked amplitude decrease with identical stimuli that produce stable responses in the sensory neurons (5). Since these mechanoreceptor sensory neurons make significant contributions to the complex PSP in the motor neurons and the resultant gill contractions, it is clear that sensory fatigue makes little or no contribution to the habituation of the reflex.

While the mechanoreceptor discharges are on the average stable with repeated stimulation, there is much variability in the responses of in-
Fig. 16. Comparison of stability of gill contractions produced by direct motor cell activation and by reflex activation. Data from Figs. 13 and 15 have been normalized for purposes of comparison by expressing each response as a percentage of the initial response. Data are expressed as means ± SE. Although gill contractions produced by direct motor cell activation (open circles) are variable, there is no significant tendency to show decrement with repeated stimuli. This contrasts sharply with gill contractions produced by reflex activation (solid circles), which show significant decrement ($P < 0.031$) with repeated stimuli.

Individual neurons. In some cases a neuron would show a decrease in the elicited activity over one stimulus series, while the same cell in another stimulus series might show an increase in response. The fact that this variability of individual neurons is not reflected in the complex PSP in the motor neurons and the resultant gill contractions may be due to extensive convergence of the mechanoreceptor neurons onto their follower motor neurons (5, 10; unpublished observations). The integrative properties of the motor neuron postsynaptic membrane average the discharges from a number of different mechanoreceptor neurons, and thus reduce the effects of variability of individual mechanoreceptor discharges.

While the mechanoreceptor cells are stable at all ranges of punctate stimulation used, they do show fatigue at higher intensities when water-jet stimuli are used. Thus, in addition to the decrement of the central synaptic connections of these sensory neurons (5, 10, 11), fatigue of the mechanoreceptor neurons could account for some of the habituation of the reflex at stronger stimulus intensities.

These findings support the argument (6, 21) that the examination of the central contribution to habituation of this reflex requires using weak or moderate-intensity water-jet pressures (less than 250 g/cm²). A corollary to these findings is that at strong stimulus intensities, additional peripheral components may also contribute both to the behavior and to its depression with repeated stimulation (see also ref 20–22). However, the situation may be counterbalanced by the fact that this reflex also undergoes sensitization (7, 24). Thus, strong stimuli to the siphon will activate other pathways which facilitate the central synaptic connections (10, 11) and, therefore, may counteract the sensory decrement by enhancing central synaptic transmission. This feature may explain why strong stimuli habituate more slowly than weak stimuli despite the decrease in sensory neuron response.

Stimulus-response relations of mechanoreceptor neurons

The Aplysia LE mechanoreceptor neurons, like other invertebrate and vertebrate mechanoreceptor neurons, give slowly adapting discharges, the frequency of which is proportional to the intensity of the tactile stimulation. Previous studies on slowly adapting mechanoreceptors in a variety of preparations using controlled displacement stimuli have failed to find a single mathematical function which best fit the stimulus-response data from all units tested (16, 17, 25). In the present experiments we have used controlled-force stimuli in order to reduce the variability produced by differing mechanical properties of the skin and thus generate data that might be described with a single mathematical function. The stimulus-response plots of Fig. 4 appear to indicate that this was not the case. The curves which best fit the data over the entire range could be either a linear, exponential, or power function. But in many cases the differences between the best fits were small and given the inherent variability in the mechanoreceptor discharge properties, the similarity of the various curves is surprising.

While the entire range of the stimulus-response data is best fit by different functions for different cells and the pooled stimulus-response data is best fit by an exponential function, it appears that most cells could be well fit by linear functions in a more restricted but still behaviorally significant range (5) of stimulus intensities (see Fig. 4). Indeed the pooled data are well fit by two linear functions; one for forces less than 2.3 g (Fig. 3) and the other for forces greater than 2.3 g. Over this same intensity range increasing the intensity of the stimulus produces graded increase in the response to the mechanoreceptor neurons, in the size of the complex PSP amplitude and in the resultant gill contractions (5).
Stability of motor response

In their initial examination of the neuronal basis of habituation of the gill-withdrawal reflex, Kupfermann et al. (19) found that direct stimulation of the motor cell L7 produced comparable contractions both prior to and immediately following habituation of the reflex. These data indicated that habituation did not involve a decrease in efficacy of the motor cell since it was fully capable of producing a contraction even though the reflex was dramatically reduced. Subsequently Carew et al. (9) extended these observations, showing that gill contractions produced by repeated direct stimulation of either of the two major motor neurons L7 and LDG, at rates that produce habituation in the intact animal did not exhibit decrement. Both Kupfermann et al. (19) and Carew et al. (9) used a photocell to measure gill contractions.

Recently Jacklet and Rine (14, 15) have reported that repeated activation of the motor cell L7 produces gill contractions that decrease progressively. A major difference between their experiments and our earlier experiments is the way in which gill contractions were measured. Jacklet and Rine used a strain gauge and we used a photocell. To examine whether this difference in recording technique might account for the difference in our results, we carried out a series of experiments in which we used both a strain gauge and a photocell alternately in the same experiment. Identical repeated stimulation of cell L7 produced stable gill contractions when they were measured with a photocell, and decrementing contractions when a strain gauge was used. Thus it appears that the strain gauge itself might be interacting with the gill contraction, either making the gill contract against an unphysiological load or perhaps influencing the contractile properties of the pinnule muscle to which the strain gauge is attached.

To resolve the issue of motor stability clearly required a third, independent behavioral technique which permits a quantitative assessment of gill contraction. We therefore used the closed-circuit television analysis. The results confirmed our earlier observations with the photocell and indicate that the gill motor neurons produce stable contractions when repeatedly activated under the conditions of our experiments. Quite recently Jacklet and Rine (15) and Lukowiak (21a) have independently also observed stable gill contractions to repeated activation of gill motor cells.

Assessing motor neuron stability the way we have done here, using repeated direct stimulation, is a severe test of stability. The motor cells do not normally fire throughout the repeated trials of a habituation run. Typically the motor cells respond briskly only to the first two to four stimuli in a run. As the afferent input onto the motor cells decrements by the third or fourth stimulus, the motor cells’ response is usually reduced significantly. Thus, the motor cells never fire a constant number of spikes for 10 stimuli.

In summary, at rates of stimulation that produce habituation in the intact animal, there is a stable response of the mechanoreceptors to tactile stimuli and stable motor response from the motor neurons. These results support the idea that the critical site of plasticity underlying habituation is the set of synapses between the mechanoreceptor neurons and their follower cells (see ref 5).

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