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SENSORY AND MOTOR NEURONES RESPONSIBLE FOR THE LOCAL BENDING RESPONSE IN LEECHES

BY WILLIAM B. KRISTAN, JR.

Department of Biology, University of California, San Diego, La Jolla, California 92093

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SUMMARY

1. Intracellular recordings were made from identified mechanosensory neurones (T and P cells) and longitudinal muscle motor neurones of leeches *Hirudo medicinalis* and *Macrobdella decora* while the skin was electrically stimulated to produce local bending responses.

2. The stimulus intensity required to produce local bending was found to activate the mechanosensory neurones at physiological firing rates. For a given stimulation frequency, intracellular activation of the mechanosensory neurones produced the same local bending response as did skin stimulation. Hyperpolarization sufficient to block the propagation of the afferent impulses into the central nervous system eliminated the local bending response to skin stimulation.

3. Stimulating identified longitudinal muscle motor neurones at frequencies observed during the local bending response produced body wall movements similar to those seen in local bending. Hyperpolarization of the motor neurones to block impulse initiation abolished local bending.

4. Mechanosensory neurone to longitudinal muscle motor neurone connexions were demonstrated to be effective and reliable, but polysynaptic for all but the previously documented monosynaptic connexions from mechanosensory neurones onto the L motor neurone (Nicholls & Purves, 1970).

5. It is concluded that the previously identified mechanosensory and motor neurones are exclusively responsible for the local bending response.

INTRODUCTION

The way in which a leech responds to tactile stimulation depends upon the intensity and location of the stimulus (Gee, 1913; Kristan, McGirr & Simpson, 1982). At threshold, mechanical or electrical stimulation produces a local bending response, during which there is longitudinal shortening on the side of the stimulus and relaxation on the side away from the stimulus, a movement which pulls the stimulated skin away from the site of stimulation. At higher intensities, mechanical stimulation elicits shortening, curling, whole-body bending, or swimming, depending upon the site of stimulation along the length of the body. At very strong stimulus intensities, mechanical stimulation produces a violent writhing response at all stimulus sites.

As a first step in the characterization of these responses, the neuronal basis of local

bending has been investigated. In the preceding paper (Kristan *et al.* 1982), the identified mechanosensory cells, T (touch) and P (pressure) (Nicholls & Baylor, 1968), were implicated as the sensory cells for these responses because the threshold for electrical activation of these neurones in dissected preparations was similar to the electrical threshold for behavioural responses in intact animals. Since the longitudinal muscles produce shortening of the animal, their motor neurones presumably provide the motor output for the behaviour. A total of nine pairs of excitatory and four pairs of inhibitory motor neurones are known to cause contraction and relaxation of the longitudinal muscles in each segment (Stuart, 1970; Ort, Kristan & Stent, 1974; Sawada *et al.* 1976). All motor neurones innervate only portions of the longitudinal musculature. For instance, cell 3 excites only a narrow band of longitudinal muscle fibres near the dorsal midline (Ort *et al.* 1974) whereas the L cell excites muscle fibres in the entire lateral half of each segment (Stuart, 1970).

This report characterizes the responses of sensory and motor neurones during the local bending reflex. Data is presented in support of three major conclusions. First, the previously identified sensory and motor neurones account for the entire local bending reflex. Secondly, the initial part of the response is caused by monosynaptic connexions of T and P mechanosensory cells on to L cells (Nicholls & Purves, 1970). Thirdly, the prolonged contraction on the side of stimulation as well as the relaxation on the side opposite the stimulus result from the activation of unidentified interneurones.

MATERIALS AND METHODS

All data presented are from experiments performed on *Hirudo medicinalis*. (Similar unpublished results were obtained using *Macrobdella decora*.) For ease of dissection and experimental manipulation, segments from the middle of the animal (10–14) were used routinely, although anterior and posterior segments gave similar results. Recording and stimulating techniques were as described in the previous paper (Kristan *et al.* 1982). Large-bore suction electrodes (I.D. = 0·5 mm) were used exclusively for electrical stimulation of the skin because the localization of these electrodes could be better judged and more accurately maintained than could the localization of the wire electrodes used in behavioural experiments (Kristan *et al.* 1982). A standard train of stimulus pulses of 2–12 V, consisting of ten 1 ms biphasic pulses, was used to elicit reflex bending. That the dorsal contraction was not due to direct stimulation of the body-wall musculature was always tested at the end of each experiment by applying these same voltages after all nerves to the body wall were cut; below 20 V, no direct activation of body wall musculature was ever observed. Longitudinal muscle tension was measured with Biocom 1030 tension transducers attached by hooks and thread to the body wall. Responses of the transducers were linear over 0–6 g, the range of tensions generated by leech muscles in this study. Movements of the body wall were always observed visually, to be sure that longitudinal muscles were the only muscles contributing to the recorded tension changes. When the contractions of dorsal and ventral longitudinal muscles were monitored in the same body-wall preparation (e.g. Fig. 1), it was often necessary to make a longitudinal cut between the dorsal and ventral body-wall regions, sparing the peripheral nerves, to separate the

dorsal body-wall movements from ventral movements. In comparing contraction amplitudes under two or more conditions, as in Figs. 2–5, 7, 8 and 10, the effects of variability were diminished by repeating each condition several times and comparing the mean amplitudes. To minimize the effects of progressive changes, contractions were elicited no more frequently than once every 3 min and the conditions to be compared were presented in different orders each time they were repeated.

Identification of extracellularly recorded impulses, measurements of impulse frequency and determination of maximum amplitude and rate of rise of tension were performed on taped recordings played back at one-quarter the recording speed onto a Brush 260 chart-recorder. Maximal rate of rise was determined by drawing a straight line tangent to the initial rise of the tension recording and measuring the slope of the line in milligrammes per second.

RESULTS

(1) *The local bending response in a ganglion/body-wall preparation*

To confirm that the local bending reflex could be elicited in a single ganglion, all nerves and connectives to a ganglion were cut except the nerves to the body wall on one side (Fig. 1A). The standard train of 10 electrical pulses at 10 Hz was delivered through a suction electrode attached to the dorsal skin and the tension generated by both the dorsal and ventral longitudinal muscles was measured. At low voltages, no responses were recorded. As the stimulus intensity was increased to 5 or 6 V, the dorsal longitudinal muscles contracted and the ventral longitudinal muscles initially contracted, then relaxed (Fig. 1B). This dorsal contraction and ultimate ventral relaxation appeared to be the same response as the local bend reflex seen in the whole animal at low stimulus intensities (Fig. 1A in Kristan *et al.* 1982).

To quantitate the response at various stimulus intensities, the preparation was further simplified by cutting all nerves to the body wall except the dorsal branch of the posterior nerve, designated DP (Ort *et al.* 1974), and the tension was monitored in the dorsal longitudinal muscle (Fig. 2A). As graphed in Fig. 2B, below some stimulus intensity (in this case, 5 V), the dorsal longitudinal muscles produced no tension. As the stimulus voltage was increased above the response threshold, the tension increased until, above some voltage (in this case, 7 V), there was no further increase in tension over the range of voltages used.

(2) *Mechanosensory cell contributions to the local bending response*

In the preparation shown in Fig. 1A, the only identified tactile sensory neurones that retain their connexions to their receptive fields are one T cell, one P cell and one N cell, which send their processes through the DP nerve to innervate the dorsal skin (Nicholls & Baylor, 1968). This preparation was used to test whether these sensory neurones constitute the *entire* sensory input for this reflex.

(A) *Activation of identified mechanoreceptors by electrical stimulation of the skin.* Comparison of Figs 2(B) and 4(D) in the previous paper (Kristan *et al.* 1982) shows that the shape of the curve relating behaviour to stimulus strength is very similar to the curves relating T and P cell activation to stimulus strength, except for the shift to higher stimulus strengths required in the saline used for the ganglion/body-wall

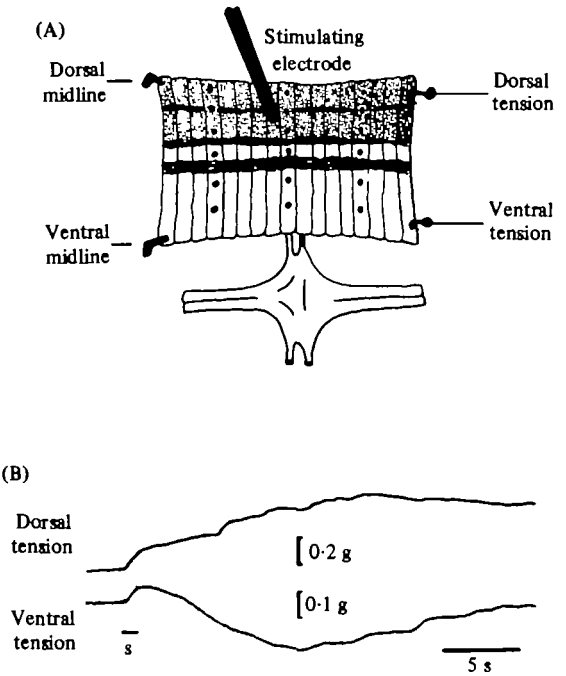

Fig. 1. Measurement of the local bending response in a ganglion/body-wall preparation. (A) Drawing of the isolated body wall and ganglion preparation. The body markings shown are those of *Hirudo*. The six black dots along three of the annuli indicate the locations of the sensilla. The ganglion was attached to the body wall by all nerves on one side. Electrical stimuli were delivered by a large bore suction electrode (about 0·5 mm diameter) into which a bleb of skin was aspirated. The ganglion and skin are drawn to different scale to allow showing details of both on the same drawing. Ganglia were about 0·5 mm across and the annuli (the area between two adjacent vertical lines) were about 1 mm wide in the animals used. (B) Dorsal and ventral muscle tension recordings. The time at which the skin was stimulated (1 ms pulses at 10 Hz for 1 s) is indicated by the bar with the 's' beneath it.

preparation. To test directly the relation between sensory cell activation and behavioural response strength, intracellular recordings were obtained from the T, P and N cells with dorsal receptive fields while stimulating the dorsal skin and recording dorsal longitudinal muscle tension. As previously documented (Kristan *et al.* 1982), T and P cells had very similar electrical thresholds, although the T cell would typically follow each stimulus in the train at a lower voltage than did the P cell. For instance, as shown in Fig. 2(C), the T cell responded to every electrical pulse in the standard train at all voltages above 5 V, whereas the P cell required an intensity of at least 7 V to respond to all pulses. Neither cell produced multiple impulses at intensities up to 12 V, the maximum intensity used in these experiments.

That the T and P cells provide the entire sensory input for the local bending response is suggested by the strong correspondence between the production of impulses in these neurones and the amount of tension generated in the dorsal longitudinal muscle. As shown in Fig. 2(B), at stimulus intensities less than 5 V, neither sensory cell was activated and, correspondingly, no change in tension was recorded. From 5·0 to 5·2 V, only the T cell was stimulated and little tension was generated.

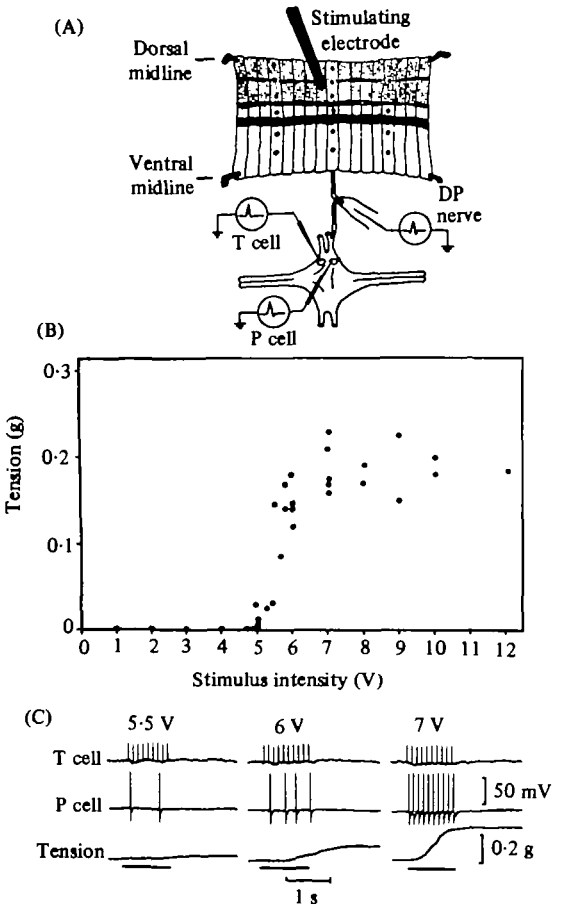

Fig. 2. Strength of the dorsal contraction part of local bending as a function of electrical stimulus intensity delivered to the skin. (A) Preparation used, showing two intracellular electrodes and one *en passant* suction electrode in place on the dorsal branch of the posterior root, i.e. on the DP nerve. (B) Maximal tension generated at stimulus intensities of 1–12 V in a single preparation. (C) Intracellular recordings from the dorsal T and P cells as well as tension recordings in response to skin stimulation of 5·5, 6 and 7 V. In all cases, ten pulses were delivered to the skin at 10 Hz during the times indicated by bars beneath the tension recording.

Between 5·2 and 7·0 V there is progressive increase in the number of P-cell impulses produced (as seen in Fig. 2C), and a corresponding increase in muscle tension was recorded. From 7 to 12 V there was no further increase either in the number of mechanosensory cell impulses nor in the muscle tension generated. The voltage threshold for activation of the N cell with a dorsal field was 30 V in this preparation. In fact, since in no case was the N cell threshold less than 12 V in any preparation, it never contributed to the responses elicited in this study.

This experiment was repeated in four preparations. Although the voltage threshold varied from 4 to 6 V in different preparations, in all cases there was a similar correspondence between the degree of T and P cell activation and longitudinal tension generation.

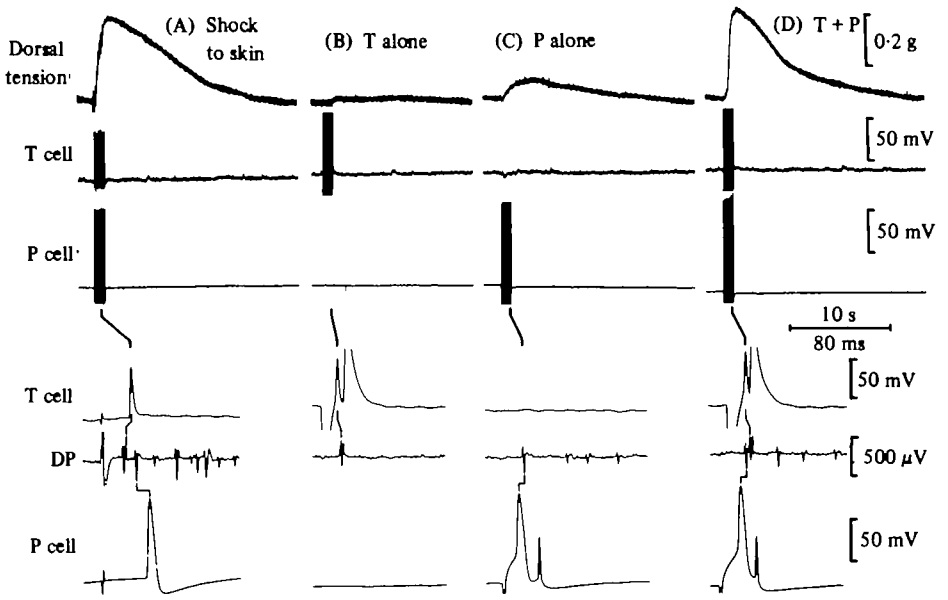

Fig. 3. Comparison of tension generated in response to T and P cell stimulation with tension generated in response to skin stimulation. The preparation used was that shown in Fig. 2(A). In all cases, the intracellular activity of the dorsal T and P cells were recorded, along with the extracellular activity in the DP nerve and the tension generated in the dorsal longitudinal muscle, in response to a train of 10 stimuli at 10 Hz. (The second upward deflection in the T cell recordings in B and D are very large stimulus artifacts.) The top three traces are at slow speed, to show the entire contraction. The bottom three traces show the response of the T and P cells to one of the ten stimuli applied in the four stimulus trains shown. The extracellular impulses in the DP nerve that correspond to the intracellular impulses in T and P are indicated. In (A) the stimulus train was applied to the dorsal skin via a suction electrode. In (B–D) identical stimulus trains were elicited via intracellular electrodes in the T cell (B), the P cell (C), or both the T and P cells (D).

(B) *Intracellular stimulation of identified mechanoreceptors.* The dorsal shortening caused by dorsal skin stimulation was compared to the shortening caused by identical impulse bursts brought about by intracellular stimulation of the mechanoreceptors (Fig. 3). The relative contributions of T and P cells was tested by stimulating T and P cells individually. In all cases, the response to T cell stimulation (Fig. 3B) was less than the response to P cell stimulation (Fig. 3C). Often, the T cell produced no reflex response at all. However, the response to stimulation of both cells was always greater than the linear sum of the responses to the individual neurones (Fig. 3D). Therefore, even though it exerted little or no reflex effect on its own, T cell activity significantly increased the strength of the contractions produced by the P cell. Usually (6 of 10 cases tested), as can be seen by comparing Fig. 3(A) and (D), the maximal contraction resulting from stimulation of these two sensory neurones was equal to that produced by skin stimulation. (The slower relaxation rate in Fig. 3(A) as compared to Fig. 3(D) was insignificant; there was great variability in this feature of contractions.) In some cases, however, the tension generated in response to intracellular stimulation was significantly less than the response to skin stimulation. This opened the possibility that there might be other neurones, in addition to the dorsal T

and P cells, that were activated by electrical stimulation of the skin and thereby contributed to the reflex contraction. Alternatively, there could have been some difference between the effects of impulses entering the ganglion via the afferent processes as compared to those generated by passing current into the somata. Two possible differences were tested in further experiments.

One difference between peripherally and centrally initiated impulses might be their relative timing onto some common postsynaptic neurone. It is possible, for instance, that centrally initiated impulses would arrive relatively synchronously whereas peripherally initiated impulses would arrive asynchronously. This possibility was tested by varying the time between centrally initiated T and P impulses from 0 to 90 ms in 10 ms steps. There were some differences in response amplitude at different impulse intervals, but the differences were small and inconsistent from preparation to preparation. These experiments show that up to 50 ms differences in the relative timing of T and P cell impulses produced comparable excitation of the dorsal longitudinal motor neurones.

A second difference between peripherally and centrally initiated impulses might be the accessibility of the impulses to the sensory cell presynaptic terminals. For instance, impulses entering the ganglion over the axon might reach all presynaptic terminals whereas the intracellularly initiated impulses might fail to pass all the neuritic branch points and reach only some of the terminals (Yau, 1976). If this were true, subthreshold depolarization of the mechanosensory cell somata might facilitate conduction through the branch points and produce a full-sized local bending response. Fig. 4 shows that this manipulation did indeed increase the effectiveness of the sensory neurone impulse train and made intracellularly initiated impulse trains as effective as the peripherally initiated trains. In this case, the T cell was depolarized slightly during the time that both it and the P cell were fired (Fig. 4A) in the same pattern as during skin stimulation (Fig. 4C). When superimposed on the sustained subthreshold depolarization of the T cell, intracellular stimulation of T and P cells elicited a dorsal shortening response comparable to that elicited by skin stimulation, whereas intracellularly stimulated firing without the depolarization of the T cell produced a significantly smaller contraction (Fig. 4B). By such sustained depolarization of the T cell, all ten preparations tested produced dorsal longitudinal muscle contractions in response to T plus P cell stimulation that were as strong as the contractions in response to skin stimulation. That this presynaptic depolarization may be a normal concomitant of sensory cell stimulation is suggested by the depolarization of the T cell seen during the peripherally evoked impulse train (Fig. 4C).

(C) *Hyperpolarization of identified mechanoreceptors during local bending.* Another test of the contribution of the identified mechanoreceptors to local bending was to measure the amount of local bending produced by skin stimulation, then repeat the same skin stimulus while blocking incoming impulses in one or both mechanoreceptors. This blockage was accomplished by passing large (5–10 A) hyperpolarizing currents into mechanoreceptor somata throughout the period of dorsal skin stimulation. Fig. 5 shows the results of hyperpolarizing a dorsal P cell. During small hyperpolarizations (Fig. 5B), the action potentials increased in size, presumably because the membrane potential was further from their peak ionic equilibrium potential, but the reflex contraction was undiminished. During larger hyperpolarizations, the action potentials

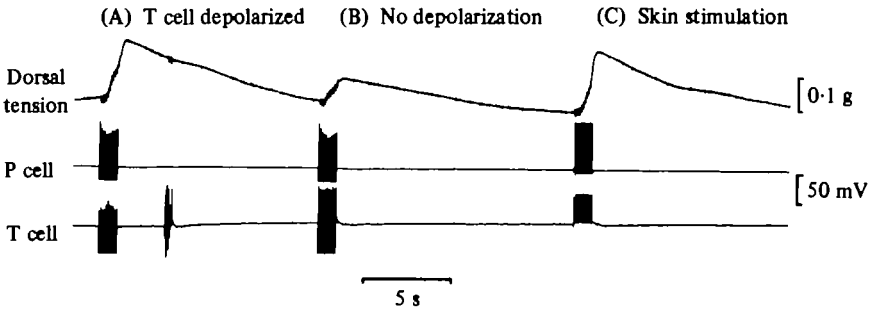

Fig. 4. Comparison of the contractions caused by T and P cell stimulation to those caused by stimulation of the skin, with and without subthreshold depolarization of the T cell. The preparation used was that shown in Fig. 2A. (A) The T cell was depolarized to just below impulse threshold with 1·8 nA of injected current. Both the T and P cells were then caused to fire a train of ten impulses by passing short pulses of depolarizing currents intracellularly. (The vertical lines starting at about 5 s after the start of the T cell recording, with no corresponding vertical lines in the P-cell recording, are switching artifacts and are not impulses in the T cell.) (B) Dorsal tension generated in response to intracellularly evoked trains of 11 T and P cell impulses without depolarization of the T cell. (C) Tension generated by stimulation of the dorsal skin at 7 V, which caused 11 impulses in both the T and P cells.

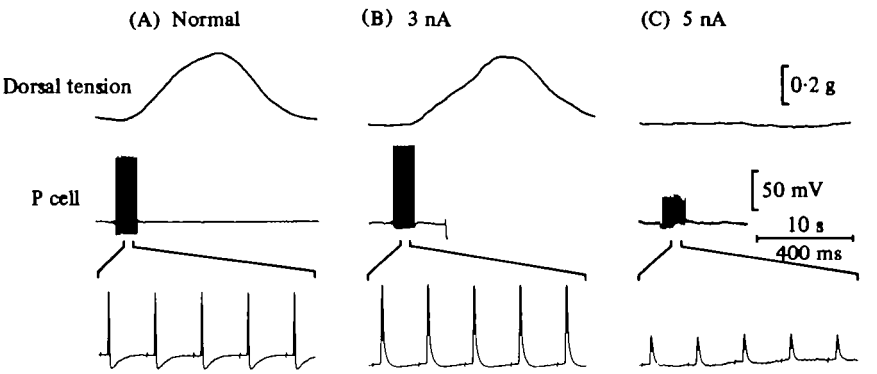

Fig. 5. Tension generated in dorsal longitudinal muscles in response to electrical stimulation of the dorsal skin with the dorsal P cell hyperpolarized by various amounts. The preparation was that shown in Fig. 2A. In all cases, stimuli were 6 V pulses delivered to the same spot on the skin. In (A) no current was passed into the P cell; in (B) 3 nA of hyperpolarizing current was passed; in (C) 5 nA of hyperpolarizing current was passed. In all cases, the amplitude calibration in the expanded traces are the same as shown for the slower traces. The P cell recordings in (B) and (C) are cut short because the current injection level was changed and the bridge became unbalanced; in neither case, however, did the P cell produce impulses during this period of current adjustment, as judged by DP nerve recordings. The short vertical lines before each impulse in the expanded traces are stimulus artifacts caused by the electrical pulses delivered to the skin.

in the P cell became smaller in a single step and the reflex contraction disappeared (Fig. 5C). The quantal decrease in action potential amplitude indicated that the impulses were blocked at some point distant from the soma and that this blockage eliminated local bending. Similar hyperpolarizations in the T cell produced much smaller, although quantal, decreases in the amplitude of local bending.

(D) *No effect on local bending of other sensory neurones.* Other impulses were seen occasionally that were time-locked to each stimulus pulse, but only when the stimulating electrode was directly on a sensilla (Kristan *et al.* 1982). For the most part, this complication was avoided by applying the stimulating electrodes to annuli in which no sensilla are located. However, even when the electrodes were placed on sensilla, the bending reflex caused by skin stimulation could still be matched by intracellular stimulation of the T and P cells. Hence, the other axons, presumably some type of sensory afferents from the sensilla (Kretz, Stent & Kristan, 1976; Freisen, 1981), do not contribute to the bending reflex.

(3) *Motor neurone contributions to the shortening reflex*

Only two identified excitatory motor neurones, the L cell (Stuart, 1970) and cell 3 (Ort *et al.* 1974), reach the dorsal longitudinal muscles via the DP nerve. Hence, it is also possible to activate and hyperpolarize these motor neurones to test their contribution to the dorsal contraction in the bending reflex, using the preparation shown in Fig. 2(A). In Fig. 6 the dorsal skin was stimulated at an intensity just sufficient to activate the T and P cells, as monitored in the DP nerve recordings. This stimulation produced a depolarization in the two motor neurones, with a short burst of impulses in the L cell and a much longer impulse burst in cell 3 (Fig. 6A). The action potentials in the cell body were very small and difficult to distinguish from the barrage of excitatory synaptic potentials; however, their axonal impulses recorded in the contralateral DP nerve were large and distinguishable by matching them with positive deflexions in the intracellular record, as shown in the expanded recordings (Fig. 6B, C).

To evaluate the relative importance of the impulses in these two motor neurones, the tension generated in response to skin stimulation was measured, and then the stimulus was repeated while blocking impulses in each cell by injecting hyperpolarizing current (Fig. 7). These recordings indicate that the L cell contributed about two-thirds of the total response (Fig. 7B) and cell 3 contributed the remainder (Fig. 7C). When both cells were hyperpolarized (Fig. 7D), the response was completely eliminated. During these experiments, the DP nerve recording was monitored to be sure that impulses from other motor neurones did not increase their activity during the periods of stimulation. These results indicate that cell 3 and the L cell constitute the entire motor input to the dorsal longitudinal muscles via the DP nerve and that their effects sum linearly to produce the contraction.

The observation that the L cell produces fewer impulses than cell 3 during the reflex contraction but produces a stronger contraction implies that each L cell impulse must cause a stronger longitudinal muscle contraction than does each cell 3 impulse. This is borne out by the graphs shown in Fig. 8. To generate these graphs, the L cell and cell 3 were individually stimulated intracellularly with short depolarizing pulses at frequencies from 1 to 40 Hz until a maximal tetanic contraction was achieved.

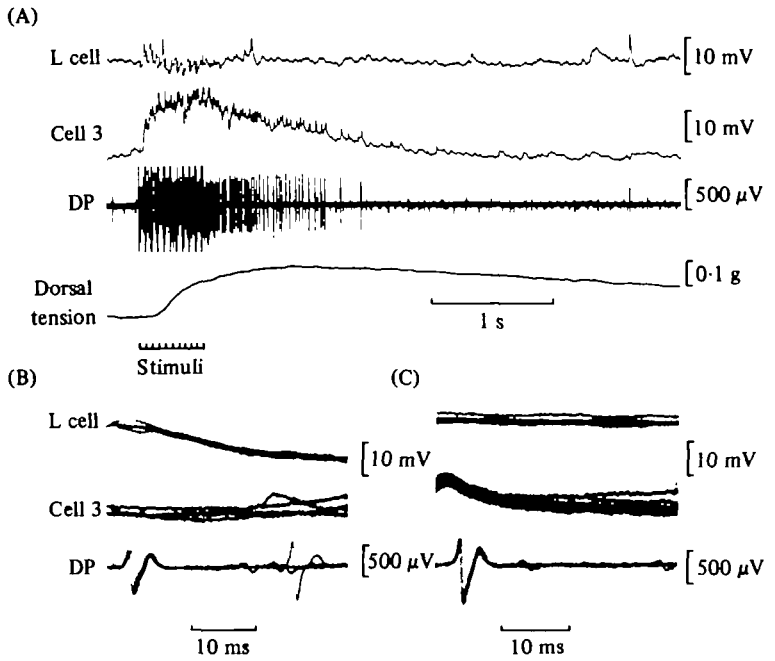

Fig. 6. Activity in dorsal longitudinal muscle excitatory motor neurones during the local bending reflex. The preparation is the same as that shown in Fig. 2 A. (A) Intracellular activity in the L cell and cell 3 during 10 Hz stimulation of the dorsal skin at 7 V for 1 s, as indicated by the markers below the tension recording. (B) Four superimposed oscilloscope tracings triggered on intracellular L cell impulses, to identify its extracellularly recorded impulses in the contralateral DP recording. (C) Eight superimposed traces triggered on intracellular cell 3 impulses, for identification of its somewhat larger DP nerve impulses. Using this technique for identifying extracellular impulses, all the large impulses in the DP recording in A were found to be generated by the L cell or cell 3.

These impulse frequencies cover the range observed in these neurones during local bending (Fig. 7). Fig. 8A shows that the tension caused by L cell impulses increased with impulse frequency over the range from about 3 to 15 Hz; lower frequencies caused no contraction at all and higher frequencies caused no greater contraction than did stimulation at 15 Hz. In fact, in the range of 20–40 Hz, there is a slight decrease in the maximal contraction observed. There is a good correspondence between the observed effective frequency range and the frequencies actually observed in the L cell during the bending reflex; i.e. the muscle was most sensitive to impulses arriving at 5–15 Hz, which was the frequency range actually seen during the reflex. However, as shown in Fig. 8C, there would be some advantage in higher frequencies, because the *rate* of contraction continued to increase up to L cell firing rates of 25 Hz.

The contractions produced by cell 3 differed from those caused by the L cell in three ways. First, the contractions were weaker; the maximal tension caused by cell 3 impulses at any frequency (Fig. 8B) were always 4 to 20 times weaker than the contractions caused by L cell impulses at the same frequency. Secondly, increases in maximal muscle contraction were elicited over a broader frequency range, in this case from 5 to 30 Hz (Fig. 8C). As was true for the L cell, the optimal frequency range for cell 3 activity was the range actually observed in the bending reflex (Fig. 7A, C, E).

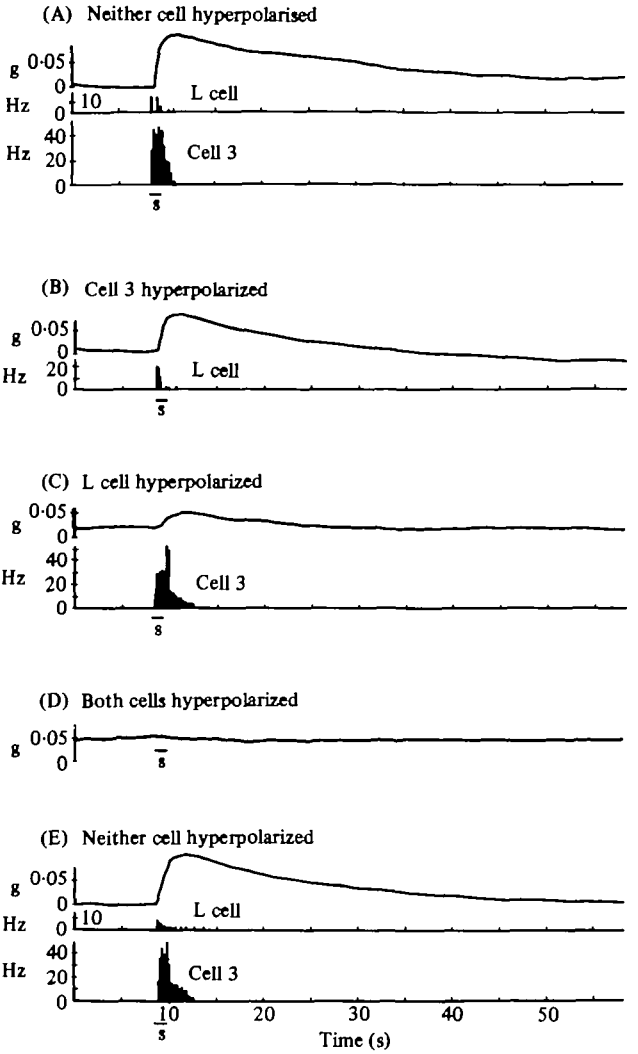

Fig. 7. Contributions of cell 3 and the L cell to tension generated in the dorsal longitudinal muscles during local bending. In all cases, the upper record is a tracing of the dorsal longitudinal muscle tension recording and the graphs below the tension traces show the average impulse frequency of the L cell and cell 3 during successive 200 ms periods. Each bar marked with an 's' indicates the time at which identical 2 s, 5 Hz, 6 V stimulus trains were delivered to the dorsal skin. In (A) no current was passed into either neurone. In (B) cell 3 was sufficiently hyperpolarized that it generated no impulses in response to skin stimulation. Because of a rectifying electrical connexion between the L cell and cell 3 (hyperpolarization passes from cell 3 to the L cell, but not in the opposite direction (Ort *et al.* 1974)), a small amount of depolarizing current was passed into the L cell during cell 3 hyperpolarization so that its response to sensory stimulation was the same as before cell 3 hyperpolarization. In (C) the L cell was hyperpolarized to eliminate its impulses. In (D) both cells were hyperpolarized. In (E) the stimulus train was repeated with neither cell hyperpolarized to show that the reflex pathway was fully as strong as it had been at the start, i.e. in A.

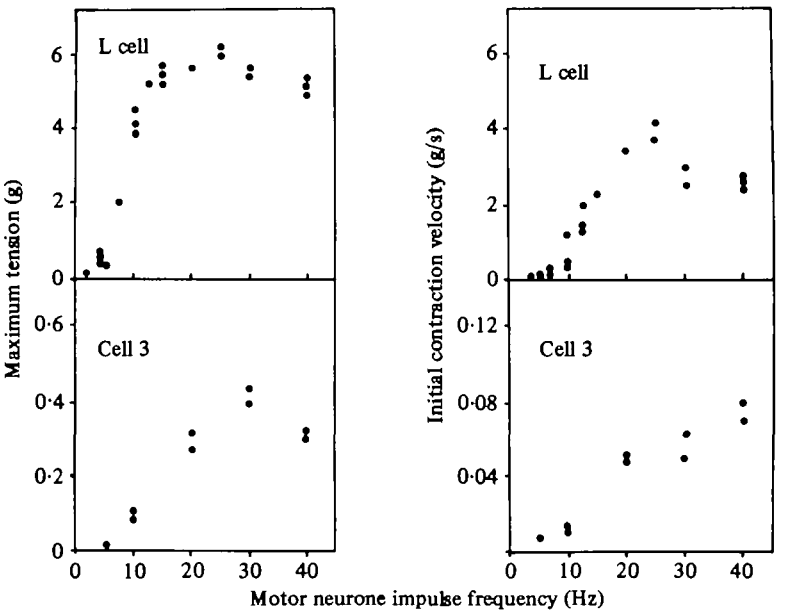

Fig. 8. Comparison of contractions caused by the L cell and cell 3 when stimulated to produce impulses at various frequencies in a single preparation. Graphs on the left: plots of maximal tension as a function of impulse frequency for the L cell and cell 3. Graphs on the right: plots of the initial rate of rise of the tension recording for the L cell and cell 3. During the period of stimulation, the oscilloscope was triggered on the stimulus and the DP recording was monitored to be sure that each intracellular pulse produced a single impulse in the stimulated motor neurone and no impulse in the other. When stimulating the L cell, cell 3 often had to be hyperpolarized to avoid activating it by way of its rectifying connexion to the L cell (Ort *et al.* 1974).

Thirdly, the velocity of contractions caused by cell 3 impulses was also significantly lower than contractions caused by L cell impulses.

These differences in contractions mean that the short, relatively low-frequency train of L cell impulses caused the initial, fast-rising part of the total contraction whereas the longer-lasting, higher-frequency train of cell 3 impulses served to prolong the contraction.

(4) *Activity in other longitudinal muscle motor neurones during local bending*

In response to skin stimulation which elicited local bending, ventral longitudinal motor neurones were strongly inhibited throughout the prolonged period of excitation of the dorsal longitudinal motor neurones (Fig. 9A). Fig. 9(B) shows that the opposite pattern of activation was elicited in the inhibitors of the dorsal and ventral muscles; i.e. the dorsal inhibitor was inhibited and the ventral inhibitor was excited. Because the dorsal and ventral longitudinal muscle inhibitors have a central inhibitory effect on their respective excitors (Ort *et al.* 1974), the observed responses of the inhibitors presumably contributed to the inhibition of the ventral excitor and, by release from inhibition, to the excitatory response of the dorsal excitors.

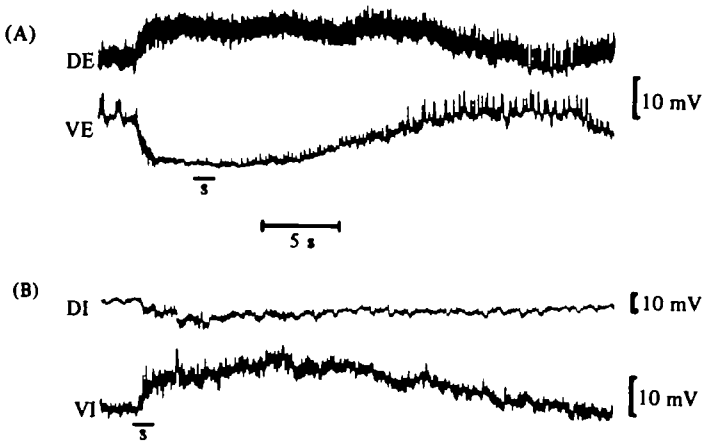

Fig. 9. Intracellular recordings from longitudinal muscle excitors and inhibitors during local bending. The preparation was that shown in Fig. 2A. (A) Intracellular recordings from the dorsal excitor, cell 3 (DE), and the ventral excitor, cell 4 (VE), in response to stimulation of the dorsal skin. (B) Intracellular recordings from the dorsal inhibitor cell 1 (DI) and ventral inhibitor cell 2 (VI) in response to dorsal skin stimulation. In both sets of recordings, the trains of stimuli were delivered during the intervals marked by bars with an 's' below them.

(5) *Synaptic connexions from sensory neurones onto motor neurones*

The T and P mechanoreceptor neurones are known to make monosynaptic excitatory connexions onto the L cell (Nicholls & Purves, 1970). To test for the presence of monosynaptic connexions from these sensory neurones on to the other longitudinal muscle motor neurones, experiments such as that shown in Fig. 10 were performed. Intracellular recordings were made from the L cell and two other motor neurones, in this case a dorsal longitudinal muscle excitor (cell 3) and inhibitor (cell 1), while stimulating the skin electrically at a low frequency (1 Hz, in this case). In order to monitor the T and P cell impulses evoked by these stimuli, a suction electrode was placed on the DP nerve, the only nerve connecting the body wall to the ganglion. At threshold for the T plus P cell response, all neurones began to exhibit synaptic potentials that were at a fixed latency from the T and P cell impulses. The time-locked synaptic potentials were excitatory in the dorsal muscle excitors such as the L cell and cell 3, but inhibitory in the inhibitor, cell 1. Thus, the responses of cell 3 and cell 1 to single sensory cell impulses were the same type seen in response to longer stimulus trains (Fig. 9).

The constancy of the latency to the potential changes in cells 1 and 3 raises the possibility that the connexions from T and P cell on to these motor neurones were monosynaptic. However, the latency to their synaptic potentials were consistently about twice the latency to the L cell synaptic potentials (Fig. 10), which are known to be monosynaptic (Nicholls & Purves, 1970). Also, the addition of a high concentration of Ca^{2+} and Mg^{2+} eliminates the synaptic potentials onto cells 1 and 3 while sparing the synaptic potentials on to the L cell (Fig. 10, middle), thereby showing these connexions to be polysynaptic (Miyazaki & Nicholls, 1976).

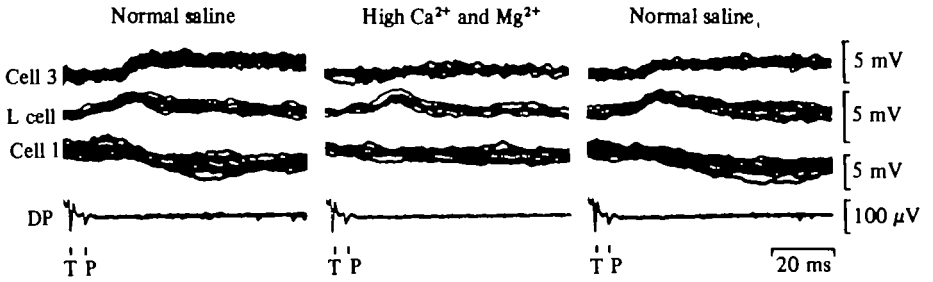

Fig. 10. Synaptic potentials recorded in longitudinal muscle motor neurones in response to T and P cell stimulation. Each panel consists of 5–10 oscilloscope sweeps triggered by the electrical stimulus applied to the dorsal skin every second in a preparation like that shown in Fig. 2(A). The top three trees are intracellular recordings from motor neurones to the dorsal longitudinal muscles (Stuart, 1970; Ort *et al.* 1974): cell 3 is an excitor of the most dorsally located longitudinal fibres, the L cell is an excitor of all longitudinal fibres on one side of the body, and cell 1 is an inhibitor of the dorsal longitudinal fibres from dorsal midline to the lateral edge. The bottom trace is an extracellular recording from the DP nerve, the only nerve left connected to the body wall. The T and P cell impulses were identified in such recordings by matching them with intracellularly recorded impulses from the appropriate cell bodies. The first and third panels were obtained while the preparation was bathed in normal saline; the middle panel shows recordings while the preparation was bathed in a saline with 20 mM-Ca^{2+} and 20 mM-Mg^{2+}. The intensity of the electrical stimulation had to be raised from 7 to 10 V in order to activate the mechanoreceptors in the high Ca^{2+} and Mg^{2+} solution. Twenty minutes elapsed between the recordings used in each panel.

By stimulating the skin near the threshold for T and P cell activation, it was possible to activate the T or P cell individually on occasion. With the example shown in Fig. 10, T cell caused the first excitatory potential in the L cell, whereas the P cell caused the larger second potential. The longer latency for the P cell induced synaptic potential was due to the slower conduction velocity of the P cell and the fact that the major part of the P cell to L cell synaptic potential is chemically mediated, with a 3–5 ms synaptic delay, whereas the T cell to L cell connexion was via a rectifying electrical junction, with essentially no synaptic delay (Nicholls & Purves, 1970). Using the same stimulus conditions, the time-locked synaptic potentials in cells 1 and 3 were found to be caused by the P cell. The latency to these synaptic potentials is 5–10 ms longer than is the P to L cell latency. Since the latency for monosynaptic chemical transmission from leech sensory to motor neurones is 5–7 ms, it is likely that not more than one or two neurones are interposed in the pathway from the P cell to cells 1 and 3.

The connexions from T and P cells onto all the identified longitudinal muscle excitors and inhibitors was investigated, using latency measurements and application of high Ca^{2+} and Mg^{2+} solution to test for monosynaptic connexions. Fig. 11 shows a particularly strong connexion from a T cell on to an L cell. In Fig. 11A intracellular stimulation at 1 Hz sufficient to cause two T cell impulses produces a pair of electrical synaptic potentials. Fig. 11B shows that 10 Hz stimulation of the T cell produced a barrage of large synaptic potentials in the hyperpolarized L cell soma. In the expanded record, these potentials were identified as the monosynaptic T cell synaptic potentials. In addition, there was a prolonged synaptic potential, outlasting the stimulus train by

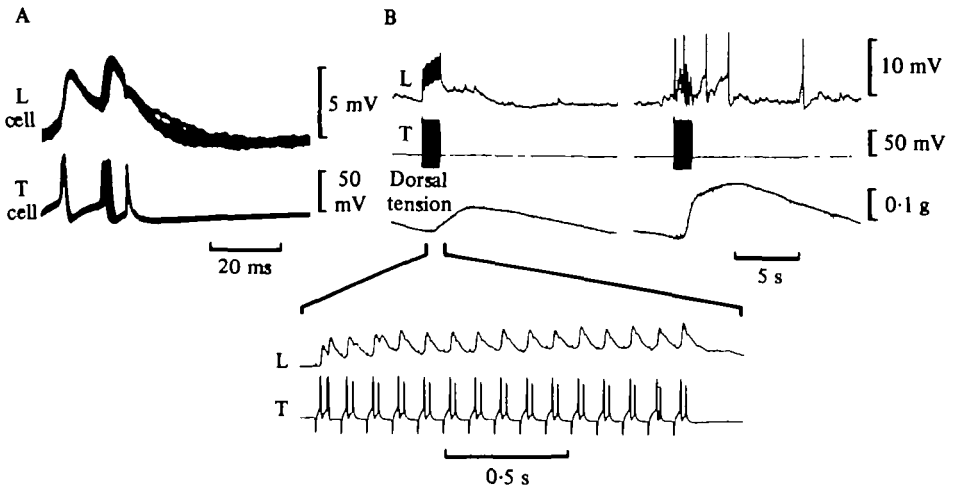

Fig. 11. The contribution to local bending of monosynaptic input from the T cell to the L cell. Preparation as shown in Fig. 2(A). (A) Ten superimposed oscilloscope traces triggered on the start of the stimulus, delivered intracellularly to the dorsal T cell, each of which caused two impulses, showing the monosynaptic electrical potentials on to the L cell. (B) Intracellular stimulation of the T cell while the L cell was sufficiently hyperpolarized to eliminate impulse generation. The expanded recording shows the lack of summation of the synaptic potentials. (C) Identical stimulation of the T cell with no intracellular polarization of the L cell.

about 2 s, from interneuronal input. A small, slowly rising contraction was produced by the T-cell activation of other motor neurones. When the hyperpolarization of the L cell was removed (Fig. 11 C), the L cell produced two impulses during the T cell train and three impulses, at lower frequency, during the post-stimulus period shown. In comparing the tension recording in Fig. 11 (C) with that in Fig. 11 (B), the two L cell impulses which occurred during the T cell stimulus train (C) are seen to cause a fast initial contraction, which reaches its peak before the peak of the slower contraction caused by other motor neurones (B). The impulses after the stimulus train, caused by interneuronal input, causes relatively little additional tension. Functionally, the most important feature of the T to L cell pathway appears to be the monosynaptic, as opposed to the interneuronal, connexion.

The P cell to cell 1 synaptic potential, although not monosynaptic, was often quite large and effective. As shown in the recordings in Fig. 12, the large inhibitory potential caused by the P cell (Fig. 12 A) summates to produce the type of hyperpolarization seen during the bending reflex (Fig. 9 B) when the P cell was made to fire at about 5 Hz near the end of the recording (Fig. 12 B). This potential was not maintained in a high Mg^{2+} and Ca^{2+} solution.

Based upon such multiple recordings from sensory and motor neurones, all the pathways from T and P cells on to the identified longitudinal muscle motor neurones were found to have at least one interneurone; the only monosynaptic connexions found were those from the mechanoreceptors on to the L cell; these connexions are summarized in Fig. 13. In all cases the synaptic potentials from P cells were much larger and more effective in exciting or silencing the motor neurones than were the

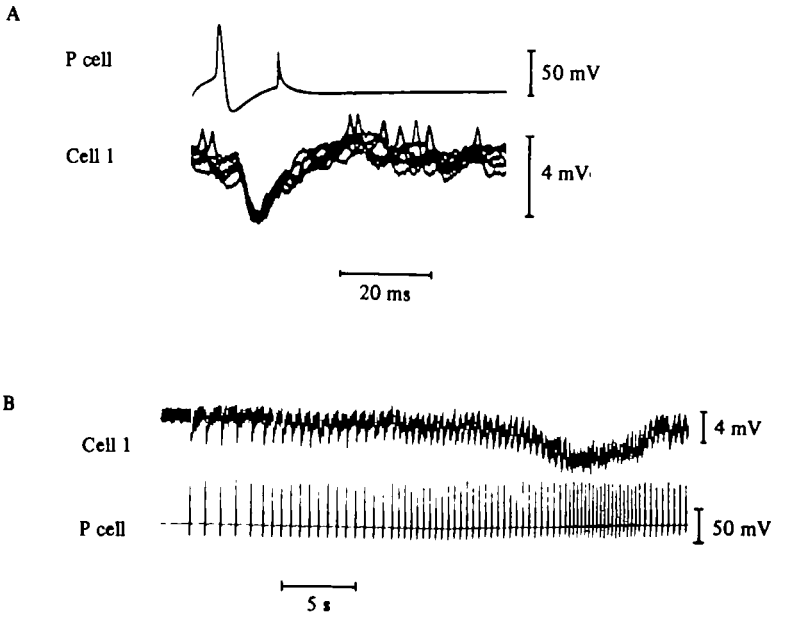

Fig. 12. Synaptic input onto a dorsal inhibitor from the dorsal P cell. (A) Eight superimposed oscilloscope traces triggered on the start of the depolarizing pulse used to produce a single impulse in the P cell. (B) Intracellular recordings from the same two cells, while producing single impulses in the P cell at frequencies from 1 to 5 Hz.

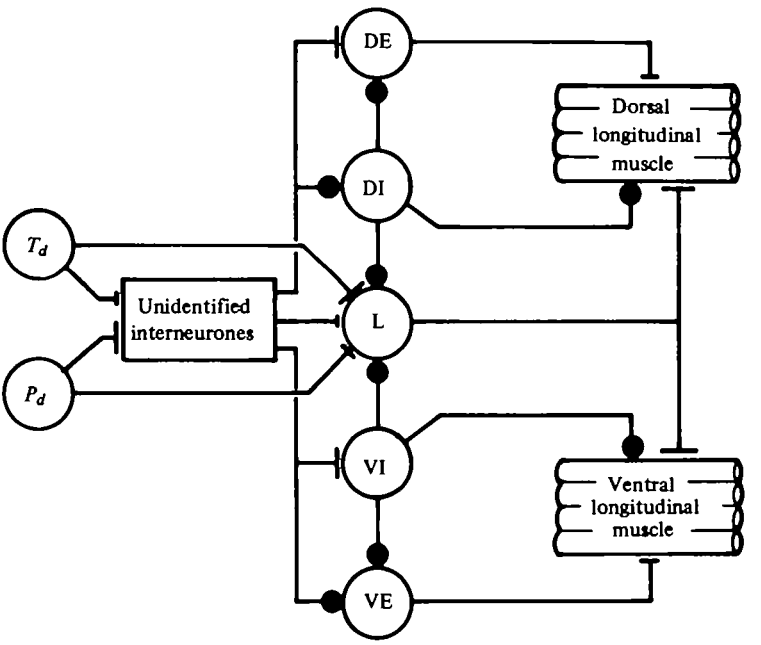

Fig. 13. Summary of neuronal connexions involved in the bending reflex caused by dorsal skin stimulation. DE, Dorsal excitor; VE, ventral excitor; DI, dorsal inhibitor; VI, ventral inhibitor. Round terminals indicate inhibitory connexions whereas T junctions indicate excitatory connexions. The length of the top of each T junction indicates the approximate relative strength of the excitatory connexion.

T cell synaptic potentials. In fact, input from T cells was often difficult to demonstrate, even when the T cells were fired at maximal rates.

DISCUSSION

(1) *Identified sensory and motor neurones are the only sensory and motor neurones causing local bending*

Kupfermann & Weiss (1978), in trying to bring order from a conceptual and semantic morass, proposed three criteria for the establishment of a cell as a command neurone:

(1) The neurone(s) must be active during the behaviour ('involvement test').

(2) Stimulation of the neurone(s) in the same pattern as seen during the behaviour must produce the same behaviour ('sufficiency test').

(3) Removal of the neurone(s), as by hyperpolarization or cell killing (Bowling, Nicholls & Parnas, 1978; Miller & Selverston, 1979), prevents the behaviour ('necessity test').

It should be emphasized that failure of either the sufficiency or necessity test does not rule out a neurone's involvement in a type of behaviour, only that it is not the only neurone involved at a particular hierarchical level. In fact, for most behaviour, it is not possible to apply these criteria even to the sensory and motor neurones because of their large number. However, by selecting a simple behaviour and looking at only a part of the behaviour, it has been possible to show that identified sensory and motor neurones are active in local bending (involvement), that stimulation of them at physiological rates produced the normal local bending (sufficiency), and hyperpolarization of them prevents local bending (necessity).

However, the adequacy of the tests for necessity and sufficiency of the dorsal tactile sensory neurones rests upon the extent to which the experimental manipulations affect only these neurones. Some of the tests for sufficiency involved passing depolarizing current pulses into the neurones (Figs. 3, 4, 7, 8) and the tests for necessity involved passing large, prolonged hyperpolarizing currents (Figs. 5, 7). It is possible that there are other important but unidentified sensory or motor neurones which are linked either electrotonically or via non-spiking transmission to the identified neurones. For this to be true, however, these occult neurones must have activation thresholds identical to those of the identified neurones. In addition they must have small axons whose impulses generate too little current to be detected in extracellular recordings from the DP nerve. Although individually possible, it is unlikely that these conditions exist in every preparation. Therefore, it seems reasonable to conclude that the identified sensory and motor neurones are the only such cells involved in the local bending reflex.

(2) *Neuronal connexions responsible for local bending*

Fig. 13 summarizes the current state of knowledge of the neuronal network responsible for the local bending reflex in response to tactile stimulation of the dorsal skin. The L cell is monosynaptically activated by impulses in the T and P cells, with the strongest input coming from the T cell. Activation of the T and P cells produces a

short-lasting burst of impulses in the L cell that in turn causes a relatively large and fast contraction in both the dorsal and ventral longitudinal muscles. With a longer delay the dorsal longitudinal excitors are selectively activated and the ventral longitudinal excitors are inhibited. The longer delay and more prolonged response are via an interneuronal pathway. The inhibition of the ventral excitors may result, at least in part, from the excitation of the ventral inhibitor. Hence, at least part of the 'interneuronal' pathway from the sensory input to the excitors involves the peripheral inhibitors.

Whether these sensory cells are the only contributors to the ventral relaxation phase of the bending reflex has yet to be determined. The threshold for the observed effects on ventral motor neurones and inhibitors is similar to the threshold for dorsal contraction, but the tests for adequacy and sufficiency of this input for ventral relaxation have not yet been performed.

(3) *Relation of local bending to other behaviours in the leech*

The strong connexion of the T cell on to the L cell and the relatively strong polysynaptic connexions of P cells on to the other longitudinal motor neurones suggests a dichotomy in the function of these two classes of sensory cells: T cells should cause a shortening of the whole body segment, whereas P cells should cause the reciprocal local bending reflex. However, in stimulating an animal locally, a localized shortening was never observed; whenever a stimulus was strong enough to produce shortening, it caused shortening of the whole body (Kristan *et al.* 1981). Also, from tension measurements while stimulating T cells alone (Fig. 3), a single T cell was found to elicit only a weak response. These findings minimize the importance of the T- to L-cell response in the normal behaviour of a leech. However, activation of many T cells simultaneously may activate L cells by the monosynaptic pathway. It is possible, for instance, to keep a leech maximally shortened for very long periods by shaking them in one's cupped hand (unpublished observations). However, it is likely that activation of interneurones is also required for maximal shortening. A primary candidate for such an interneurone is the S cell (Gardner-Medwin, Jansen & Taxt, 1973; Frank, Jansen & Rinvik, 1975), a single cell in each ganglion that is electrically coupled to its neighbouring homologues in adjacent ganglia, thereby forming a 'fast conducting system' (Bagnoli, Brunelli & Magni, 1972; Magni & Pellegrino, 1978*a*, *b*). These neurones receive strong T and P cells excitation and in turn excite the L cell through an electrical connexion (Gardner-Medwin *et al.* 1973; Magni & Pellegrino, 1978*a*). However, activation of the S cell alone, by intracellular stimulation, causes almost no behavioural response (unpublished observations), so that other, as yet undiscovered interneurones must contribute significantly to the whole body shortening.

In these studies, increased activation of T and P cells produced an increase in the intensity of the bending reflex, but there were no indications of swimming, shortening or components of curling or whole body bending. The lack of complex behaviours being produced by a single ganglion is consistent with previous observations that a number of acutely isolated interconnected segments or segmental ganglia are required to produce swimming movements (Kristan & Calabrese, 1976). It is possible that interganglionic connexions among interneurones is necessary to produce complex

movements (Friesen, Poon & Stent, 1978; Stent *et al.* 1978; Bagnoli *et al.* 1972; Frank *et al.* 1975). It is of interest, in this regard, that complex behaviour can be seen in isolated segments after they are isolated for about a week (Kristan & Guthrie, 1977).

It is likely that all co-ordinated behaviour in the leech is controlled by a particular group of interneurones. Some types of behaviours, such as swimming and shortening, are incompatible, whereas local bending can be observed during any other behaviour (Kristan *et al.* 1982). It will be of interest, when the local bending interneurones are identified, to see whether they are used exclusively in bending, or, as in the mammalian spinal cord (Lundberg, Malmgren & Schonburg, 1978), whether these interneurones used to produce reciprocal activation and inhibition of groups of motor neurones are themselves influenced by other behaviour or possibly even incorporated into more complex behavioural acts.

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