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Chemoreception and Mechanoreception in the Gastropod Mollusc *Pleurobranchaea californica*

II. Neuroanatomical and Intracellular Analysis of Afferent Pathways

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Summary. Afferent chemosensory and mechanosensory pathways from peripheral sensory structures (the rhinophore and tentacle) to the cerebropleural ganglion ('brain') of the mollusc *Pleurobranchaea* were investigated using anatomical and electrophysiological methods. In both structures a sensory epithelium is connected by afferent nerves to a peripheral ganglion which sends a nerve (rhinophore or tentacle nerve) to the cerebropleural ganglion.

1. Filling the distal stumps of afferent nerves distal to the rhinophore and tentacle ganglia with cobaltous chloride (centrifugal fills of distal nerves) stained the somata of receptor cells in the sensory epithelium (Fig. 1), suggesting that primary afferent neurons project uninterrupted from the epithelium to the peripheral ganglia.

2. Filling proximal stumps of afferent nerves distal to the rhinophore and tentacle ganglia with cobaltous chloride (centripetal fills of distal nerves) stained mainly fiber tracts that terminated in the peripheral ganglia (Figs. 3, 5) suggesting that primary afferent input is processed mainly in these peripheral ganglia.

3. Filling distal stumps of nerves connecting the peripheral ganglia to the cerebropleural ganglion with cobaltous chloride (centrifugal fills of proximal nerves) stained approximately 100 somata in each peripheral ganglion but stained few axons in distal afferent nerves (Figs. 4, 5), suggesting that the rhinophore and tentacle nerves consist mainly of axons of interneurons arising in peripheral ganglia. Centripetal fills of proximal nerve stumps stained few (10–25) somata in the cerebropleural ganglion.

4. Transganglionic extracellular activity induced by extracellular stimulation of appropriate nerve roots was reversibly reduced in calcium-free sea water, indicating transmission of information across chemical synapses in the peripheral ganglia (Fig. 6).

5. Intracellular recordings were obtained from the somata of 79 interneurons in the tentacle and rhinophore ganglia while delivering sensory stimuli to the corresponding sensory structures. 53 cells showed reliably an action potential response to mechanical and/or chemical stimulation (Figs. 7, 9, 11–13, 15, 16, 18). The majority of cells were bimodal (mechano- and chemosensory), although monomodal (mechanosensory or chemosensory) cells were also encountered. In most cases (47/53) the response was excitatory, but in a few cases (6/53) inhibitory responses were obtained (Fig. 18).

6. Lucifer yellow injections were made for a representative number of such interneurons (Figs. 8, 10, 14, 17). Most of these showed monopolar neurons with a single axon passing from each soma into the rhinophore or tentacle nerve toward the cerebropleural ganglion, although injections of monomodal chemosensory interneurons ($n=2$) revealed a bipolar configuration (Fig. 17).

7. The results collectively suggest that chemosensory and mechanosensory inputs from primary epithelial receptor cells of the rhinophore and tentacle are integrated in the peripheral ganglia and relayed to the central nervous system by a population of sensory interneurons.

Introduction

Gastropod molluscs rely heavily upon their chemical and tactile senses. Chemical stimuli in particular have been used experimentally both as unconditioned and conditioned stimuli in a variety of asso-

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ciative learning paradigms (e.g., Davis et al. 1980; Gelperin 1975; Mpitsos and Davis 1973; Mpitsos and Collins 1975; Mpitsos et al. 1978; Walters et al. 1979). Despite the interest in mechano- and chemoreception in molluscs, however, the nature of the peripheral sensory apparatus and the central integration of these modalities has remained obscure.

With respect to the peripheral sensory receptors, light and scanning electron microscopy have been used to identify a characteristic ciliated cell type in the sensory epithelium of the chief sensory structures of *Pleurobranchaea,* the tentacles and rhinophores (Davis and Matera 1982; Matera and Davis 1982). This cell type is postulated to subserve chemoreception on the basis of its restriction to chemosensitive areas of the body (ibid.). Transmission electron microscopy has shown that the somata of these cells are located in the external epithelial layer, from which they project axons directly to peripheral ganglia at the base of the tentacle and rhinophore (ibid.). The peripheral ganglia are in turn connected directly to the cerebropleural ganglion ('brain') *via* the tentacle and rhinophore nerves.

With respect to the central integration of mechano- and especially chemosensory inputs in gastropods, almost nothing is known. In relatively elementary reflexive circuits, mechanosensory inputs can be relayed directly to the participating central neurons (e.g., Byrne et al. 1974; Kovac and Davis 1980). The preceding paper (Bicker et al. 1982) showed that mechano- and chemostimulation causes centripetal discharge in the tentacle and rhinophore nerves of *Pleurobranchaea,* but in this case the situation is complicated by the interpolation of peripheral ganglia in the tentacle and rhinophore nerves, raising the possibility of peripheral processing of sensory information. Physiological studies of peripheral ganglia in gastropods have not been reported.

In the present study we have investigated these peripheral ganglia, and found that they each contain an elaborately organized neuropile and a large population (>100) of relatively complex integrating interneurons. Evidence is presented that these interneurons process the sensory inputs and relay them to the cerebropleural ganglion. This study thus sheds light on the interneuronal integration of mechano- and chemosensory inputs in molluscs, and provides the first physiological data on molluscan peripheral ganglia. The present study also elucidates the neural composition of a pathway mediating the conditioned stimulus in associative learning paradigms in *Pleurobranchaea* (Davis et al. 1980; Mpitsos and Davis 1973; Mpitsos and Collins 1975; Mpitsos et al. 1978), thus helping to pave the way for cellular analyses of these forms of plasticity.

Materials and Methods

General materials and methods were identical to those described in the companion paper (Bicker et al. 1982).

Preparation. A rhinophore or tentacle was excised from the animal and the major part of the muscular and connective tissue was dissected away. The preparation consisted mainly of the sensory epithelium with its basally attached ganglion and the proximal nerve. The epithelium was spread out and pinned with the ganglion and nerve under seawater to Sylgard. The preparation was kept in a cooled water jacketed chamber at 12° C. In order to facilitate electrode penetrations the outer and inner connective tissue sheath were removed from the ganglion. A stream of the test solution was delivered to the epithelium, usually manually through a plastic tube connected to a syringe, but sometimes using the liquid stimulus delivery system described in the preceding paper (Bicker et al. 1982). Test solutions were kept at a temperature of 12° C. The epithelium was stimulated with 1 ml of test solution delivered at an approximate flow rate of 1 ml/30 s. Different solutions could be applied through tubes which ran parallel to each other through the preparation chamber and terminated at the same distance of 1 cm from the epithelium.

Stimuli. Typically the response of a cell was tested to application of seawater, a chemical, and a mechanical stimulus. Seawater served as a control for artifacts in the cell response which might be attributed to temperature differences or mechanical stimulation due to the flow of the carrier stream. The chemical stimulus was usually a 1 osm solution of soy protein hydrolysate, commercially obtainable as Dr. Bronners Vegetable Seasoning and prepared here as a 4.5% solution in distilled water, which was then diluted 1:9 in seawater. All food stimuli were isosmotic with seawater, and the pH of all food stimuli was 6.5. The mechanical stimulus was a light touch of the epithelium with a fine glass probe. Occasionally the cell response was also tested to 10^{-1} diluted squid and *Corynactis* homogenate which were prepared according to Davis et al. (1980).

In control experiments, intracellular recordings from 4 tentacle and 2 rhinophore ganglia were made after the transection of the ganglia from the sensory epithelium. In cells examined (soma diameter of 50–100 μm) no detectable membrane potential changes were found when chemosensory stimuli were delivered as usual to the sensory epithelium. The chemical stimulation frequently caused contraction of muscle fibers in the preparations. To avoid artifacts in the cell response due to electrode movements only recordings with constant spike amplitudes were evaluated (approximately one fourth of the 320 individual cells penetrated). The spike amplitudes of the reported cells ranged from 40–100 mV and the penetrations were maintained up to 30 min.

Electrophysiological Methods. Conventional glass capillary suction electrodes were used to stimulate and record extracellularly from the cut stumps of the proximal brain nerve and the distal nerve trunks leading into the sensory epithelium. In some experiments isolated ganglia were bathed in artificial seawater of the following composition: (in mmol/l) 420 NaCl, 25 $MgCl_2$, 25 $MgSO_4$, 10 KCl, 10 $CaCl_2$, 5 Tris-HCl buffer, pH 7.5. High Mg^{++}, 0 Ca^{++} seawater was made by replacing

Ca^{++} with Mg^{++} ions in the artificial seawater solution. Intracellular recordings were made using 3 mol/l KCl filled glass microelectrodes having tip resistances ranging from 15 to 30 MΩ. Conventional electrophysiological apparatus was used for the amplification, display and permanent recording of the data.

Anatomical Methods. Neurons were filled with Lucifer Yellow (9% solution in 1 mol/l LiCl), subsequent to electrophysiological characterization. The dye was injected by passing hyperpolarizing current pulses of approximately 50 nA (1 Hz, 50% duty cycle) for 15–30 min. The ganglion was then fixed in phosphate buffered formalin, dehydrated in an ethanol series and cleared in methyl salicylate. Photographs were taken under a compound microscope from the wholemount and tracings were obtained from these color photographs.

Cobalt backfills were performed on both proximal and distal main nerve trunks of the peripheral ganglia. The cut nerve stump was brought into contact with 1 mol/l $CoCl_2$ while the rest of the ganglion was immersed in seawater. The preparation was placed for 24 h at 4° C. After the incubation the preparation was rinsed several times and then developed with drops of ammonium sulfide added to the seawater. The ganglion was fixed in phosphate buffered formalin, dehydrated in an ethanol series, and cleared in methyl salicylate. In some preparations a Timm's intensification (Bacon and Altman 1977) was performed on the wholemount after fixing the tissue. Cobalt backfills were also made into the distal nerve roots that innervate the sensory epithelium. After precipitation and fixation, 10 µm sections of the epithelium were Timm's intensified (Tyrer and Bell 1974).

Results

Anatomical Organization of Chief Sensory Structures

Pleurobranchaea bears on its anterior end two pairs of sensory appendages, the rhinophores, which are positioned dorsally, and the tentacles, which are fused to the lateral margins of the oral veil (Matera and Davis 1982). Both appendages are elongated and cone-shaped, with a central lumen created by a longitudinal inward fold. The lumen is lined with a yellow-brown pigmented sensory epithelium composed mainly of a characteristic discociliated cell type believed to mediate chemoreception (Davis and Matera 1982; Matera and Davis 1982). The sensory epithelium is densely innervated by a peripheral ganglion which lies at the base of each tentacle and rhinophore and closely apposed to the most proximal portion of the sensory epithelium. The rhinophore ganglion is slightly larger than the tentacle ganglion and distinguished from the latter by a cluster of whitish cells of unknown function on its surface near its apposition to the sensory epithelium (Fig. 5A, 1). These cells send white fibers into the rhinophore ganglion and nerve, and are most developed in sexually mature specimens.

Numerous fine nerve branches from the sensory epithelium join together into three main nerve roots in each ganglion (Fig. 5), termed roots 1–3. One of these nerve roots, and some side branches of the other two, are not connected to the epithelium but innervate non-sensory areas of the appendage (not shown in Fig. 5). Electron microscope evidence indicates that the main nerve roots contain the axons of primary afferent receptors, which project without interruption to the corresponding peripheral ganglion (Matera and Davis 1982). To test this hypothesis further each of the three roots was cut near the entry into the peripheral ganglion, and backfilled with cobaltous chloride toward the periphery. Eleven such fills were performed for the rhinophore, and nine for the tentacle. Serial paraffin sections of the corresponding sensory epithelium ($n=4$ for the rhinophore, $n=5$ for the tentacle) consistently revealed cobalt stain in the soma layer within the sensory epithelium and nowhere else (Fig. 1). These results are consistent with the hypothesis that axons within the filled nerves are contiguous with the primary afferent cell bodies identified earlier (Matera and Davis 1982) in the peripheral sensory epithelium.

Anatomical Organization of the Peripheral Ganglia

Serial sections through the tentacle and rhinophore ganglia revealed an elaborate organization of neuropile and fiber tracts (Fig. 2). The tentacle ganglion contains an area of neuropile around its center which is surrounded by a cellular rind. Approximately half of the cellular rind on one side is occupied by large somata which are loosely packed, whereas the other half contains smaller somata (diameter 5–10 µm). Inspection of serial sections revealed also fibers which pass through the ganglion from the tentacle nerve into the distal nerve root which supplies non-sensory areas of the appendage.

The organization of the rhinophore ganglion is somewhat different from that of the tentacle ganglion (Fig. 2B). The cellular rind which contains somata of different sizes occupies one major area of the ganglion, located at the side of the ganglion which bears the white cells on its surface. The cross section shows also one giant cell of 100 µm soma diameter at the bottom of the micrograph, which is electrophysiologically reidentifiable (see below). On the other side of the cell rind area the cross section shows thick fiber bundles which pass through the ganglion from the rhinophore nerve to one of the distal nerve roots. The fiber bundle and the cell rind enclose the central neuropilar area

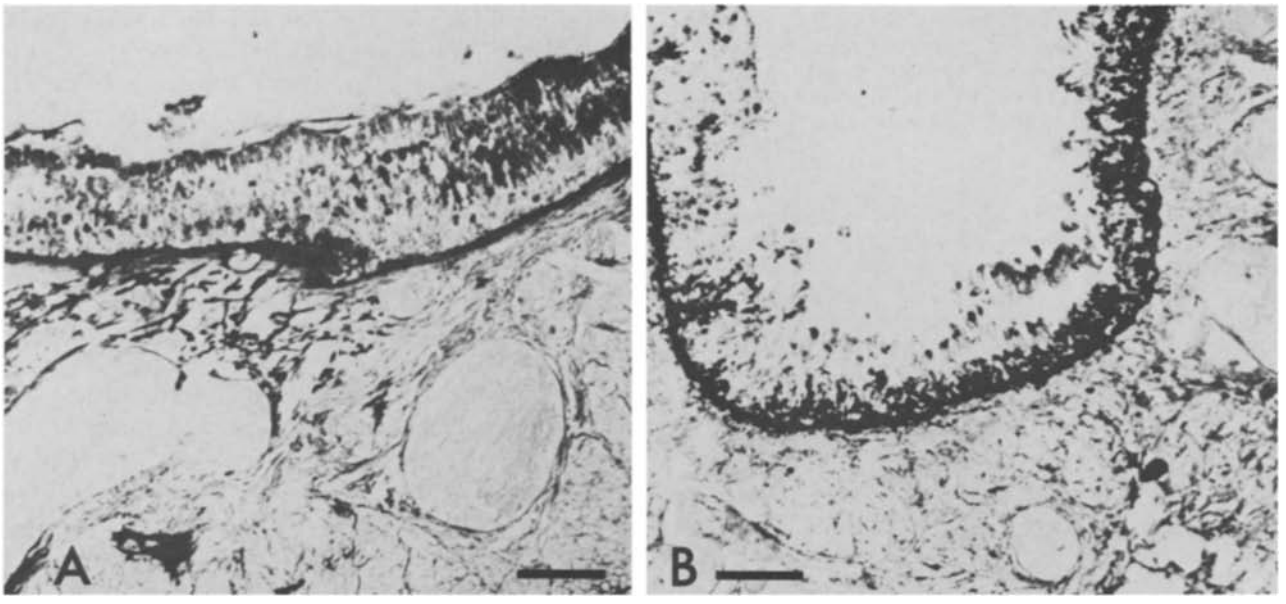

Fig. 1. Cross sections (10 μm, Timm's intensified) of the rhinophore (**A**) and tentacle (**B**) of *Pleurobranchaea* following centrifugal cobaltous chloride backfills of an afferent nerve distal to the corresponding peripheral ganglion. Cobalt is accumulated in the outer band of the sensory epithelium known to contain somata of primary receptors (Matera and Davis 1982). *Bars:* 100 μm

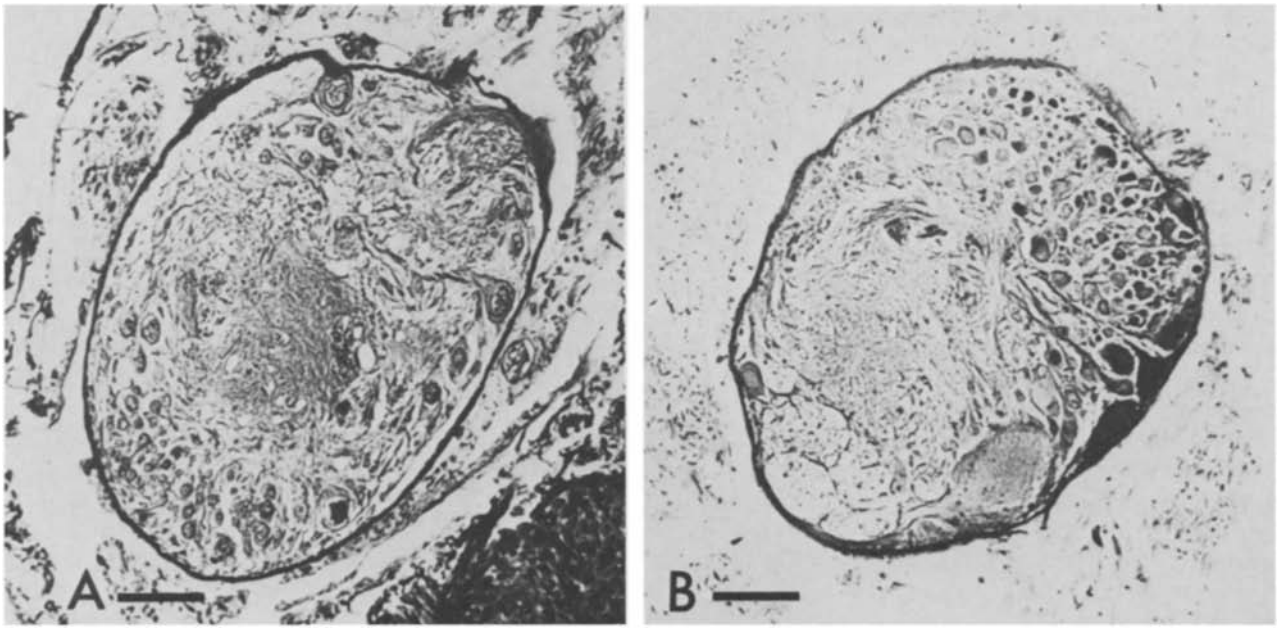

Fig. 2. Cross sections through the tentacle (**A**) and rhinophore (**B**) ganglia showing elaborate and well-defined central neuropilar regions and numerous peripheral somata. 10 μm paraffin sections stained with Mallory's trichrome. *Bars:* 100 μm

of the ganglion. The elaborate neuropilar organization of both tentacle and rhinophore ganglia is consistent with the hypothesis that these peripheral ganglia play a significant integrative role.

In order to investigate the afferent projection from the peripheral sensory epithelium into the peripheral ganglia, the three major afferent roots of each ganglion were backfilled with cobaltous chloride toward the ganglia. Thirteen such centripetal fills of all three rhinophore ganglion roots were performed, of which the ganglia of 4 preparations were serially sectioned. The corresponding sample size for the tentacle ganglion was 6, of which 3 ganglia were sectioned serially. The serial sections

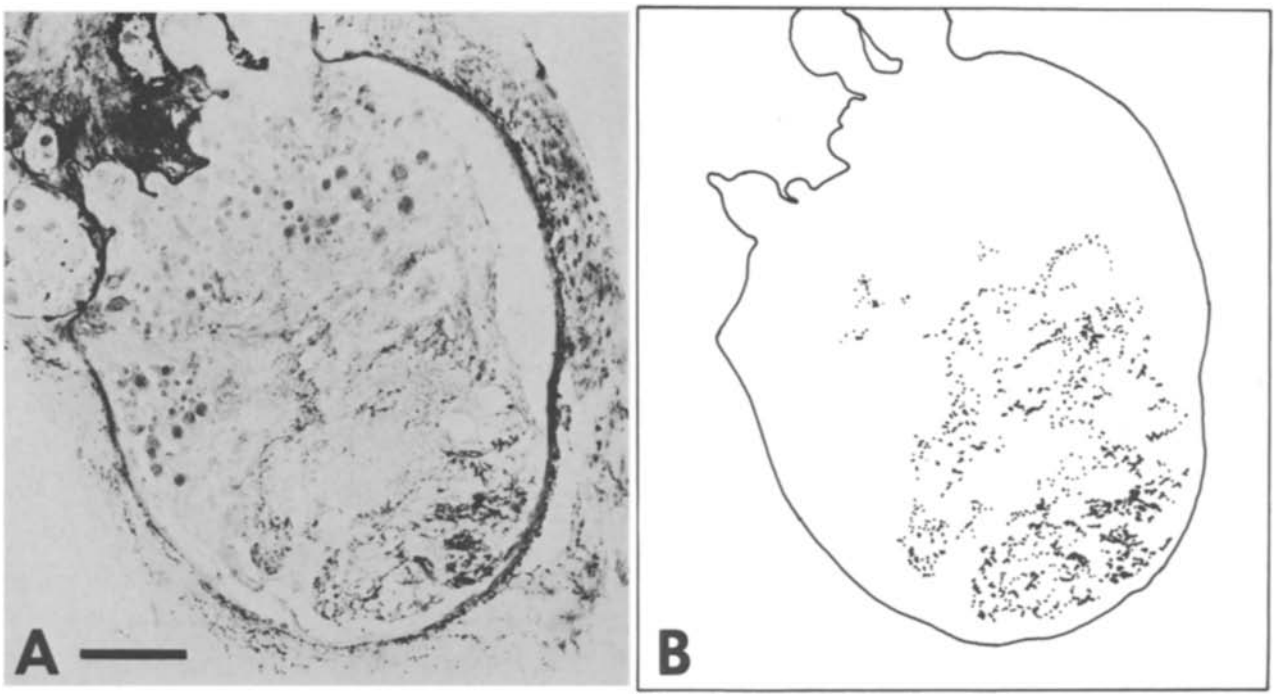

Fig. 3A, B. Cross section (10 μm, Timm's intensified) of a rhinophore ganglion following centripetal cobaltous chloride backfills of the third distal (afferent) root of the ganglion, showing the distribution of cobalt in the neuropile. **A** Photograph of the section; **B** tracing showing the outline of the ganglion and position of cobalt stain. *Bar:* 100 μm

(Fig. 3) revealed that the stain was largely confined to the neuropilar regions of the peripheral ganglia. The majority of somata in the ganglia were not stained by this procedure, and with the exception of one of the three distal roots, no stained axons were detected within the tentacle and rhinophore nerves. Filling the third root, which largely bypasses the sensory epithelium and innervates non-sensory regions of each appendage, regularly stained 10–25 axons in the tentacle and rhinophore nerves, indicating a small population of fibers that pass through the peripheral ganglia without synapsing. The results of backfills into the distal roots varied slightly with the number of side branches that were back injected inevitably during this procedure. Thus, backfills of these distal roots could sometimes stain up to six somata in the peripheral ganglia. These results are, however, consistent with the hypothesis that the majority of primary afferent input to the peripheral ganglia terminates in the neuropilar regions of these ganglia.

In order to investigate the projection from the peripheral ganglia toward the cerobropleural ganglion, the rhinophore and tentacle nerves were backfilled with cobaltous chloride toward the peripheral ganglia ($n=16$ and $n=14$, respectively). This procedure reliably stained approximately 100 somata in both peripheral ganglia (Fig. 4). Only in a few cases could we detect stained axons in two of the three distal roots, but the third root typically contained 10–25 filled axons. This third root was the same which innervates non-sensory regions of the appendages and which, when filled with cobaltous chloride, stained a comparable number (about 10–25) of axons in the tentacle and rhinophore nerves (see above). In the rhinophore ganglion the majority of stained somata were located opposite to the aforementioned through-fibers (Fig. 4A). In the tentacle ganglion, stained somata were separated into two clusters on opposite surfaces of the ganglion (Fig. 4B). The dorsal surface contains a cluster of large neurons (soma diameter up to 80 μm) while the ventral surface contains more somata of smaller size. The geometry of the stained neurons in both the rhinophore and tentacle ganglion varied considerably. Most of the cells showed one main process which extended into the nerve leading to the cerebropleural ganglion. However, cells of bi- or multipolar organization were found in both ganglia.

Figure 5 summarizes the main findings of the foregoing backfill study. The majority of afferent axons terminate in the peripheral ganglia. An exception is the nerve root which contains the fibers

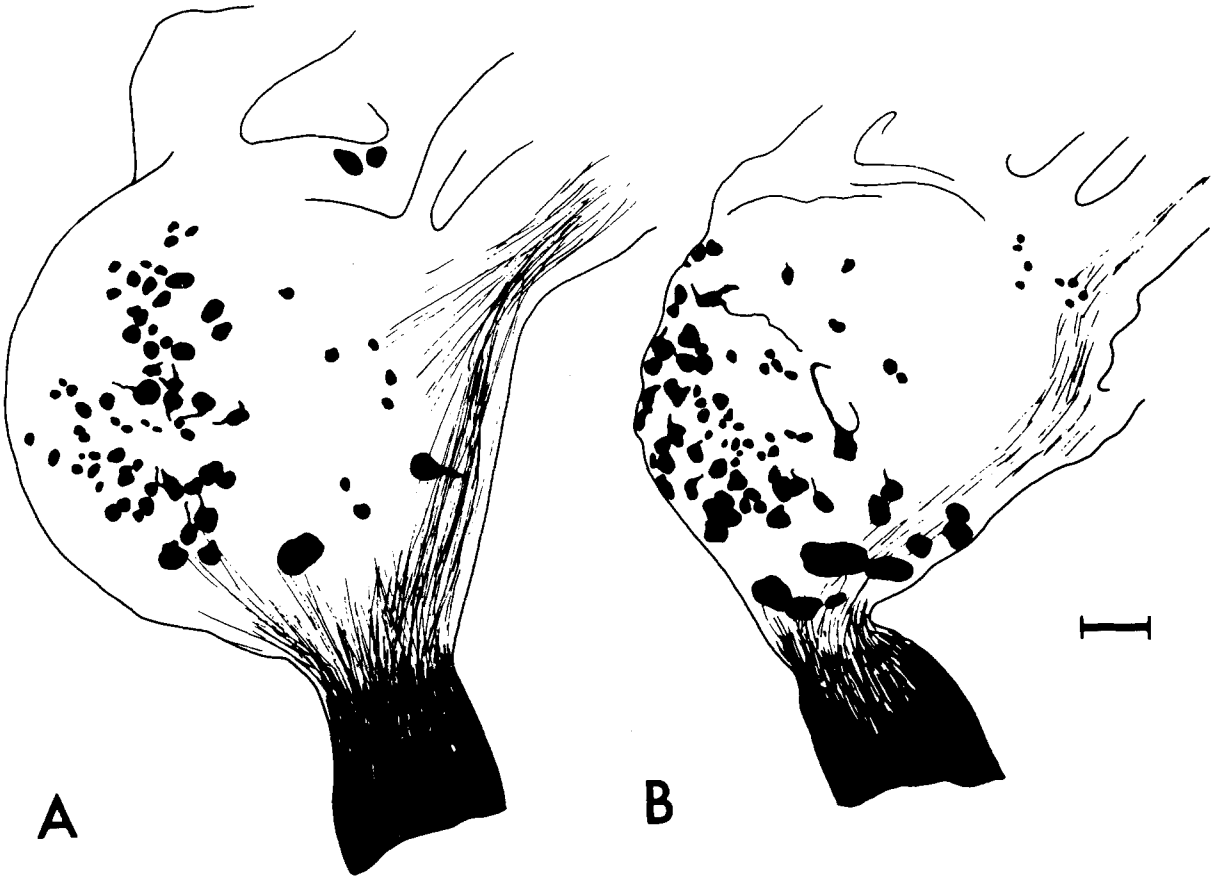

Fig. 4. Tracings of wholemounts (Timm's intensified) of the rhinophore (**A**) and tentacle (**B**) ganglia following centrifugal cobaltous chloride backfill of the rhinophore (**A**) and tentacle (**B**) nerves. Numerous somata are stained, in addition to several axons that pass through the ganglion and into a distal nerve root. *Bar:* 100 μm

passing through the ganglion. Backfills of the rhinophore and tentacle nerves, which connect the peripheral ganglia to the CNS, revealed a large population of stained somata but few projections into the afferent nerve roots. These results are consistent with the hypothesis that the major central projection to the cerebropleural ganglion arises in the peripheral ganglia and is composed of interneurons rather than primary afferent cells.

Cobaltous chloride backfills of the tentacle and rhinophore nerves toward the cerebropleural ganglia were also performed ($n = 5$ in each case). These fills were characterized by greater variability than for the peripheral ganglia, and by a relative paucity of stained somata. No more than 15–25 neurons were filled in any preparation. The back injection of the rhinophore nerve in particular stained more fibers than cell somata. Having entered the cerebropleural ganglion the main fiber bundle trifurcates and distributes fibers towards the ventral surface of the ganglion. These findings imply that the majority of centripetal interneurons have their somata in the peripheral rhinophore and tentacle ganglia.

Synaptic Transmission Through the Rhinophore Ganglion

The foregoing anatomical data are supportive of the view that peripheral integration of primary afferent inputs occurs in the tentacle and rhinophore ganglia. As a first step toward testing this hypothesis electrophysiologically, transmission through the peripheral ganglia was examined after ganglia were bathed in normal sea water and then in calcium-free (Mg^{++} substituted) sea water for 15 min. Such experiments were performed on 10 rhinophore ganglia, using the two distal roots that innervate the sensory epithelium. Brief electrical stimulation of an afferent (non-motor) root of the rhinophore ganglion caused a volley of compound action potentials in the rhinophore nerve (Fig. 6A, 1). After bathing the ganglion in Ca^{++} free sea water for

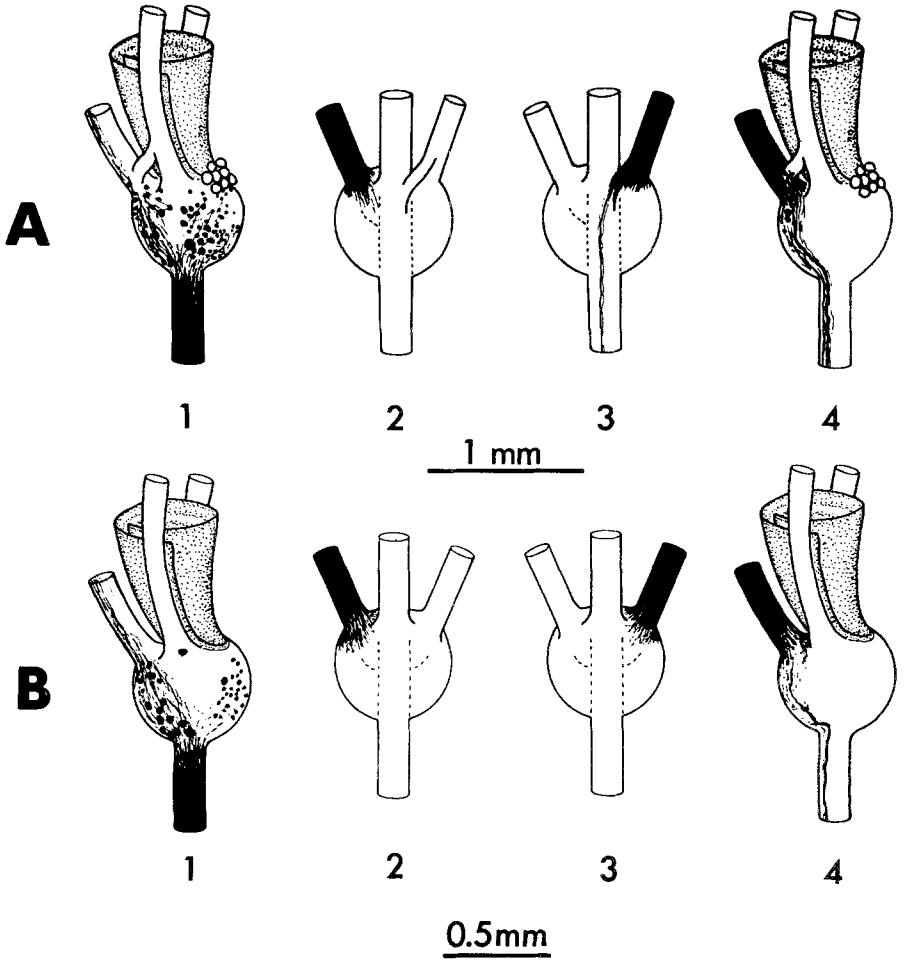

Fig. 5. Schematic summary drawings illustrating the results of all cobaltous chloride backfill experiments performed on the rhinophore (**A**) and tentacle (**B**) ganglion. *1* centrifugal fills of rhinophore (**A**) and tentacle (**B**) nerves; *2* centripetal fills of distal root 1; *3* centripetal fills of distal root 2; *4* centripetal fills of distal root 3. The sensory epithelium is shown (1 and 4) as a folded, conical structure closely apposed to the distal region of the ganglion

15 min, the number of centripetal action potentials was reduced sharply (Fig. 6A, 2). The response was restored by replacing the ganglion in normal sea water (Fig. 6A, 3).

Converse experiments were performed by stimulating the rhinophore nerve and recording the response from distal roots (Fig. 6B). These experiments also revealed some Ca^{++} sensitive pathways, indicative of chemical synaptic transmission through the ganglion in both directions.

Intracellular Exploration of the Tentacle Ganglion

Penetrations of neurons in the tentacle ganglion were made largely from the dorsal side, where the largest somata are located (Figs. 2, 4), although some small somata (<30 μm diameter) on the ventral surface of the ganglion were also penetrated. A total of 31 neurons was successfully sampled, according to criteria outlined in the Materials and Methods, divided into four classes.

Mechanosensory Neurons. Ten neurons generated action potentials in response to mechanical but not chemical stimulation. These neurons showed little or no response to application of sea water (Fig. 7A, 1) or chemical stimulation (Fig. 7A, 2) to the tentacle, but responded with a large (10–20 mV) depolarization and resultant action potentials to mechanical stimulation (Fig. 7A, 3, 4). The threshold for spike generation in these purely mechanosensory neurons ranged from 10–30 mV, and was correlated with somata diameter. Thus for somata 20–50 μm in diameter, thresholds ranged from 10–20 mV, while somata 50–70 μm in diameter exhibited thresholds of 20–30 mV. Since the mechanosensory neurons show no response to the protein hydrolysate stimulus, other stimuli like squid or *Corynactis* homogenate were occasionally tested and no response was found.

A characteristic feature of this cell type was the occurrence of large (up to 20 mV) depolarizing potentials in absence of known stimulation, which

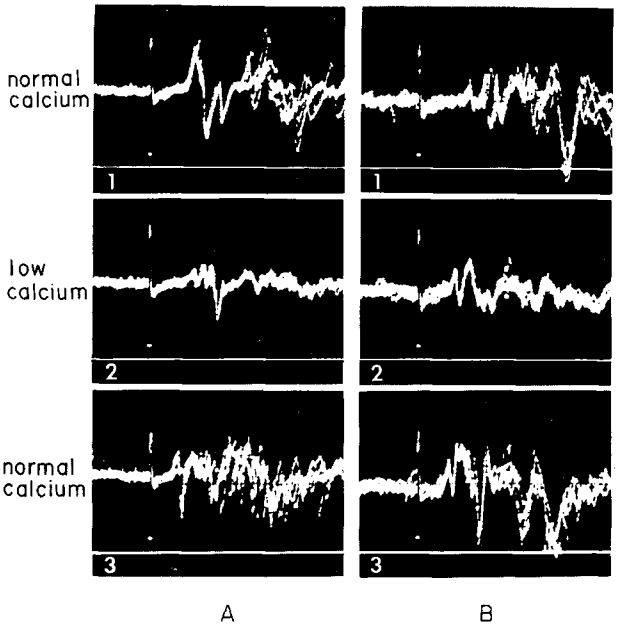

Fig. 6. Extracellular recordings from the rhinophore nerve (**A**) and distal afferent root 2 (**B**) while stimulating root 2 (**A**) or the rhinophore nerve (**B**) with a brief (2 ms) electric shock (stimulus monitor beneath nerve recordings). In each case the activity is shown in normal sea water (1), after a 15 min soak in calcium-free sea water (2), and again in normal sea water (3). Each trace represents 5 superimposed sweeps.

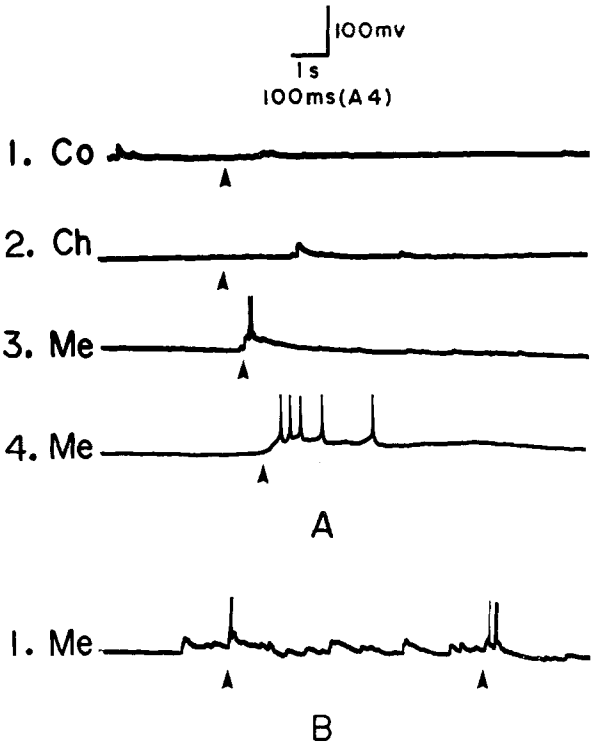

Fig. 7A, B. Intracellular records made from the somata of two mechanosensory neurons in the tentacle ganglion. *Co* application of sea water control; *Ch* application of chemical stimulus; *Me* application of mechanical stimulus. *Arrow* shows approximate time of stimulus arrival at the sensory epithelium. **A** Records from a mechanosensory soma of 50 μm diameter; **B** records from a mechanosensory soma of 70 μm diameter

occasionally gave rise to a solitary action potential. In some experiments the membrane was hyperpolarized, which invariably increased the amplitude of these depolarizing potentials. On this basis we presume these depolarizing potentials were excitatory postsynaptic potentials (EPSP's). The spikes do not rise smoothly from the baseline but always develop from a synaptic prepotential (Fig. 7A, 4). Figure 7B shows EPSP's up to 30 mV between sequential touches in a neuron with 70 μm soma diameter. The receptive field to light tactile stimulation of all mechanosensory neurons found included the entire area of the sensory epithelium. Mechanical stimuli of comparable intensity delivered to the skin areas bordering the epithelium never elicited spikes in the mechanosensory neurons. Repeated tactile stimulation of a single area of epithelium caused a spike train of approximately 20 Hz which adapted within 5 s.

Five mechanosensory neurons in the tentacle ganglion were injected with Lucifer Yellow. A representative example (e.g., Fig. 8) shows a monopolar cell that sends an axon into the tentacle nerve and exhibits a restricted dendritic field near the soma.

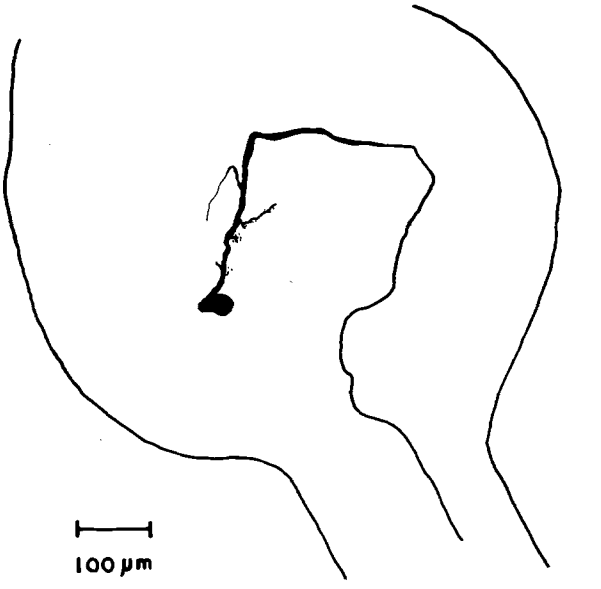

Fig. 8. Tracing of a color photograph of a Lucifer Yellow fill of mechanosensory neuron in a wholemount of the tentacle ganglion. Tentacle nerve is at the bottom

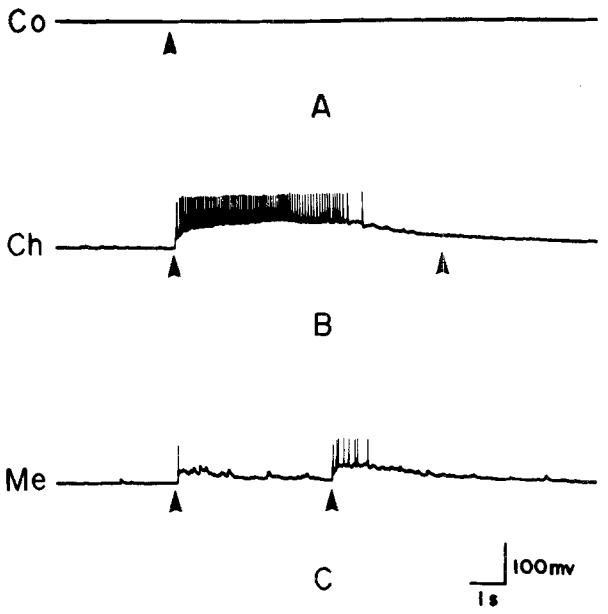

Fig. 9A–C. Intracellular records made from the soma of a bimodal (mechano- and chemosensory) neuron of the tentacle ganglion. Abbreviations as in Fig. 8. **A** Sea water control; **B** application of chemical stimulus; **C** application of mechanical stimulus

Bimodal (Mechano- and Chemosensory) Neurons. Nine neurons in the tentacle ganglion responded with action potentials both to mechanical and chemical stimulation. These cells did not respond when sea water was applied to the tentacle (Fig. 9A). Chemical stimulation (Fig. 9B) resulted in a train of action potentials which usually adapted within 5–10 s. The response of these neurons to mechanical stimuli was indistinguishable from the aforementioned mechanosensory neurons (Fig. 9C). Like the mechanosensory neurons, bimodal neurons exhibited spontaneous EPSP's (Fig. 9B). Spikes in these neurons also rose from a synaptic prepotential, suggesting that the bimodal neurons are also interneurons rather than primary sensory neurons.

Lucifer Yellow injections of three bimodal cells were made, two of which are illustrated in Fig. 10. In both cells shown, an axon could be followed into the tentacle nerve. One cell (Fig. 10B) showed a process extending to the distal part of the ganglion, but this process could not be observed exiting the ganglion. In some experiments the extracellular spike of the penetrated neuron was recorded from the tentacle nerve. These electrophysiological and anatomical findings further support the identification of these neurons as interneurons which project into the cerebropleural ganglion.

Variable-Response Neurons. Three neurons were impaled that were variable both in their spontaneous behavior and their responses to stimuli (Fig. 11). Two of these cells exhibited spontaneous spikes at irregular intervals. A third neuron, held for 20 min, received spontaneously a barrage of EPSP's and IPSP's and responded both to chemical and mechanical stimulation (Fig. 11, a–c). Both chemical and mechanical stimulation caused spike trains which were followed by IPSP's of increased amplitude. After four repetitions of chemical and mechanical stimuli the cell received almost exclusively IPSP's but no longer any EPSP's. Correlated with these changes the cell was no longer responsive to sensory stimulation (Fig. 11, d and e). This state of the cell lasted for 6 min until the penetration was lost.

Unresponsive Neurons. Nine neurons were encountered which showed spontaneous spiking at a constant frequency between 5 and 10 Hz (not shown). Neither mechanical nor various chemical stimuli altered the discharge of these cells.

Intracellular Exploration of the Rhinophore Ganglion

A total of 48 successful penetrations (see Materials and Methods) were made of neurons in the rhinophore ganglion, divided into the following classes.

Giant Neuron. The rhinophore ganglion contains a reidentifiable giant neuron with a soma diameter up to 100 µm. The soma is distinctively orange colored and located proximally near the exit of the rhinophore nerve (Fig. 2B). This cell, penetrated in nine preparations, exhibited a spontaneous fluctuation in excitability, as evidenced by periods of spiking with variable frequency in absence of known stimuli (Fig. 12a and b). Sea water had no effect (Fig. 12c), but both chemical and mechanical stimulation of the sensory epithelium of the rhinophore induced intense discharge (Fig. 12, d and e). Four Lucifer Yellow injections of the cell failed to stain an axon leaving the rhinophore ganglion through the rhinophore nerve (not shown). Backfilling either the distal roots of the rhinophore ganglion or the rhinophore nerve also failed to stain the soma. Extracellular recording from the rhinophore nerve never revealed an action potential associated with induced soma spikes. Therefore all available evidence indicates that the giant neuron is entirely confined to the rhinophore ganglion.

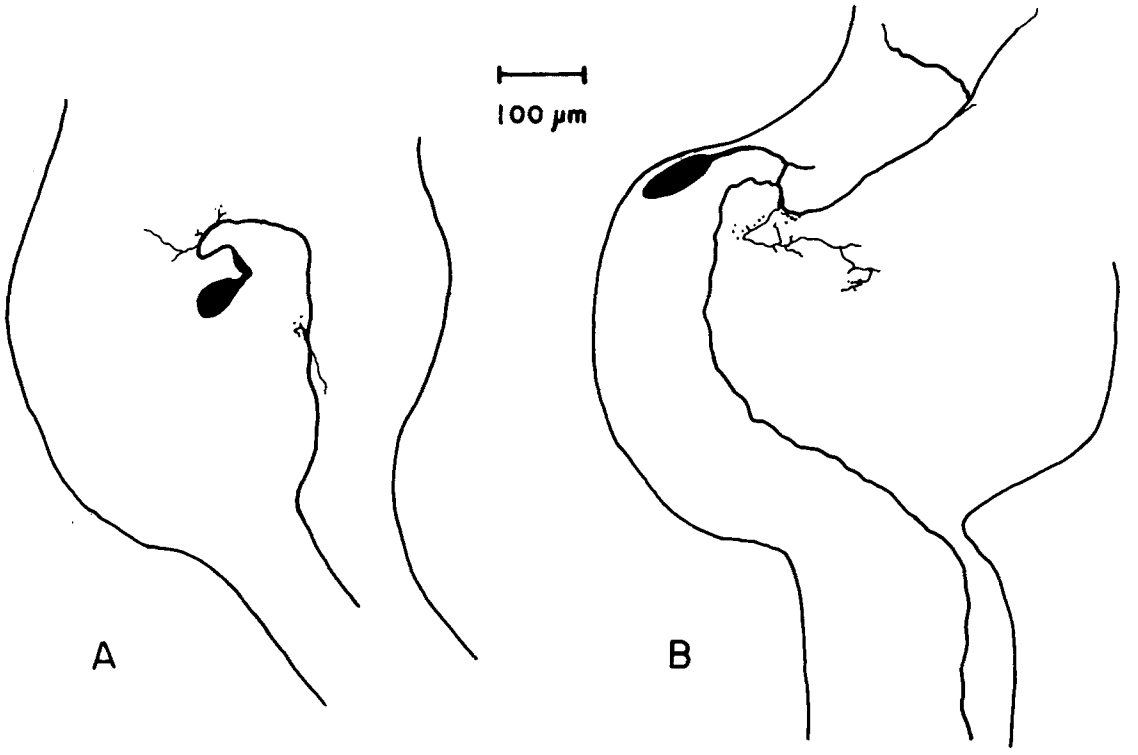

Fig. 10. Tracings of color photographs of Lucifer Yellow fills of bimodal neurons in wholemounts of the tentacle ganglion

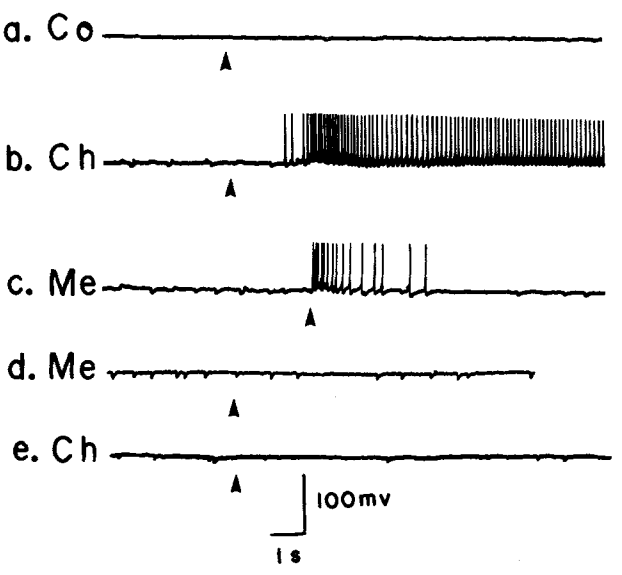

Fig. 11. Intracellular records made from the soma of a variable-response neuron of the tentacle ganglion. Abbreviations as in Fig. 7

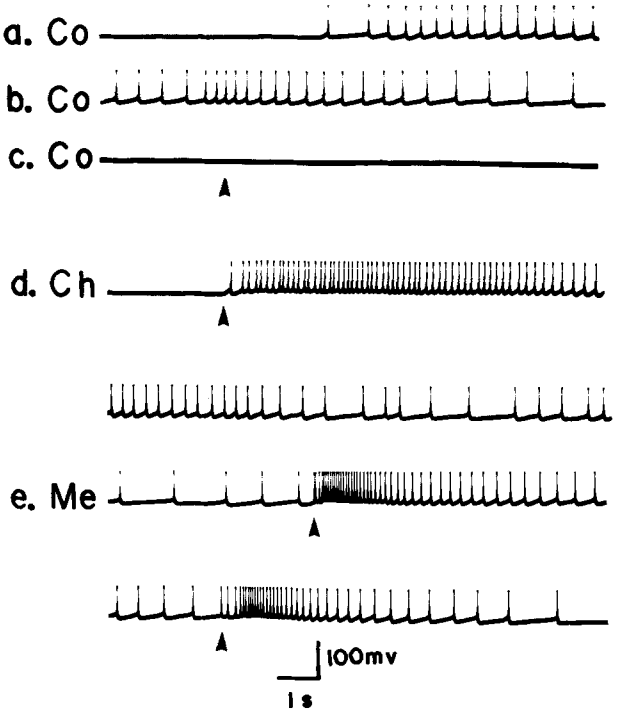

Fig. 12a–e. Intracellular records made from the soma of the 'giant' neuron of the rhinophore ganglion. **a–c** Spontaneous discharge. Sea water was applied to the rhinophore at the arrow (**c**). **d, e** Continuous traces showing responses to chemical stimulus (**d**) and mechanical stimulation (**e**)

Mechanosensory Neurons. Six cells were impaled that behaved exactly like the mechanosensory neurons described for the tentacle ganglion (Fig. 7). Thus these cells showed spontaneous EPSP's (Fig. 13a), lack of responsiveness to chemical stimuli (Fig. 13b), and large (10–20 mV) depo-

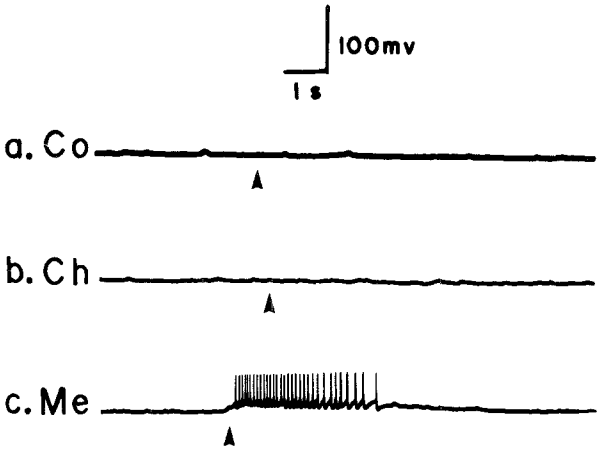

Fig. 13. Intracellular records made from the soma of a mechanosensory neuron of the rhinophore ganglion. Abbreviations as in Fig. 7

larization with superimposed action potentials in response to mechanical stimulation of the rhinophore sensory epithelium (Fig. 13c). Lucifer Yellow injections (Fig. 14) of these cells ($n=3$) showed a monopolar configuration and usually (2/3) an axon exiting the rhinophore nerve. Unlike the characteristic clustering of such cells in the tentacle ganglion, the mechanosensory neurons of the rhinophore ganglion are distributed more evenly opposite to the fibers which pass through the ganglion (Fig. 4A).

Bimodal (Mechano- and Chemosensory) Neurons. Two penetrations were made of rhinophore neurons which responded similarly to bimodal

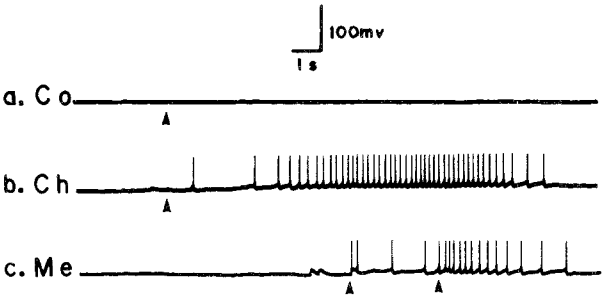

Fig. 15. Intracellular records made from the soma of a bimodal (mechano- and chemosensory) neuron in the rhinophore ganglion. Abbreviations as in Fig. 7

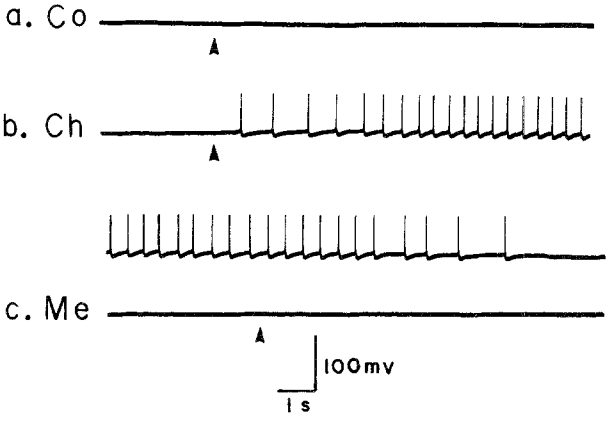

Fig. 16. Intracellular records made from the soma of a chemosensory neuron of the rhinophore ganglion. Abbreviations as in Fig. 7

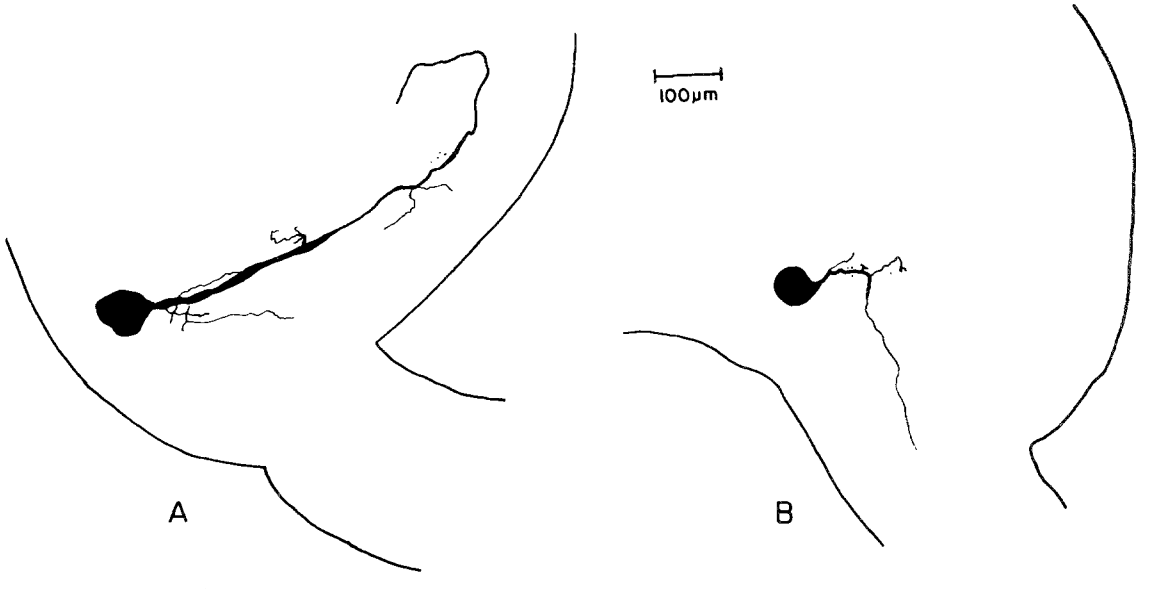

Fig. 14. Tracings of color photographs of Lucifer Yellow fills of mechanosensory neurons in wholemounts of the rhinophore ganglion

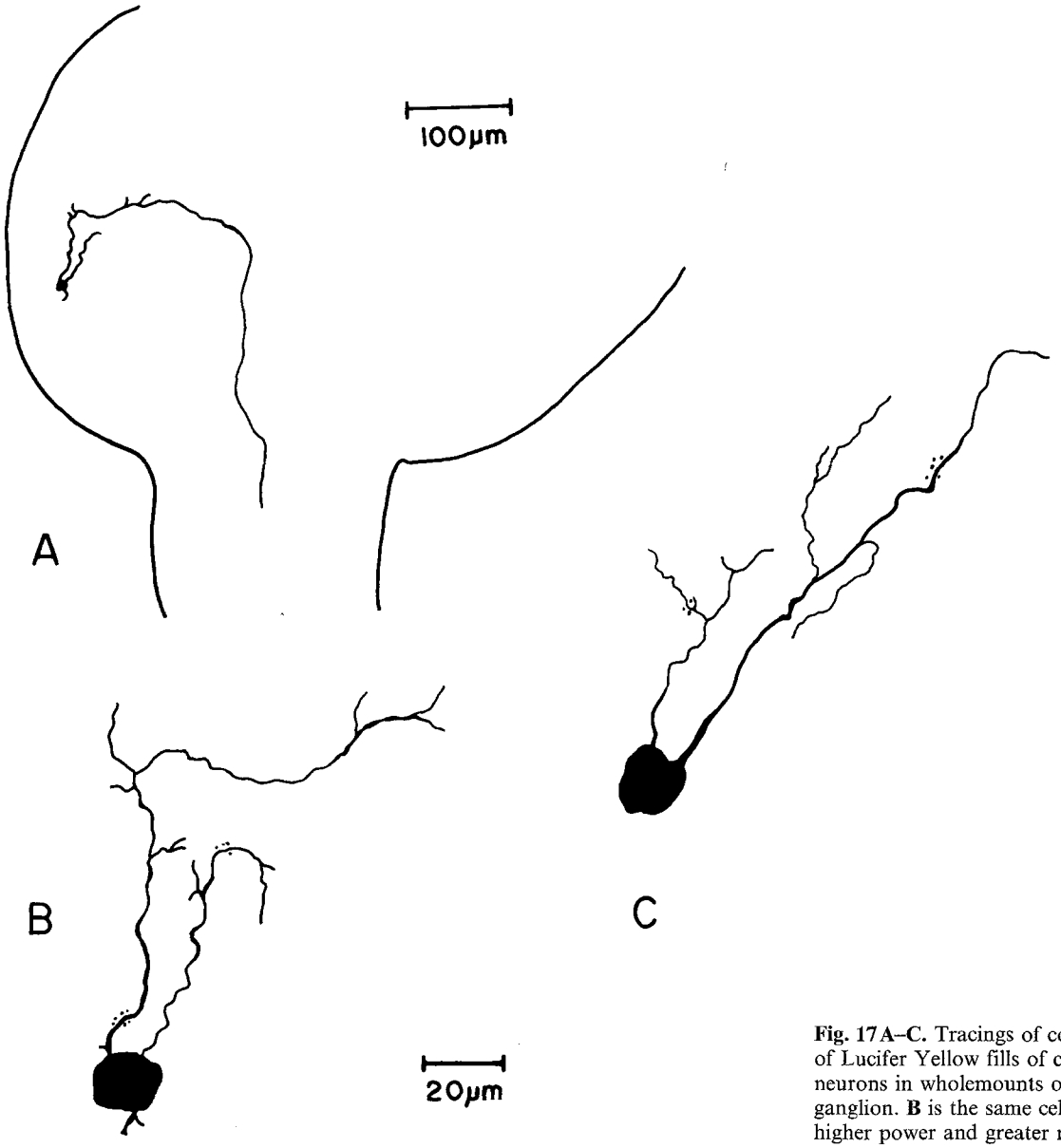

Fig. 17A–C. Tracings of color photographs of Lucifer Yellow fills of chemosensory neurons in wholemounts of the rhinophore ganglion. **B** is the same cell as **A** shown at higher power and greater resolution

neurons of the tentacle ganglion. Thus these cells showed no spontaneous discharge and no response to sea water application (Fig. 15a), but strong responses to chemical (Fig. 15b) and mechanical (Fig. 15c) stimuli. Lucifer Yellow injections of these neurons were not made.

Spontaneously Active Bimodal (Mechano- and Chemosensory) Neurons. In five penetrations cells were encountered that exhibited regular spontaneous discharge, but responded to chemical and mechanical stimuli with a substantial increase in discharge frequency (not shown). A single Lucifer Yellow injection revealed a monopolar cell with no process exiting the ganglion (not shown).

Chemosensory Neurons. Six penetrations were made of neurons that responded to chemical stimuli but not mechanical stimuli (Fig. 16). These cells showed no spontaneous discharge, and no response to application of sea water (Fig. 16, a). Discrete PSP's were absent from these cells. The threshold for spike generation varied within this group of cells. In two of the six cells action potentials were not generated until chemical stimuli had depolarized the neurons by 10–15 mV. In some preparations squid and sea anemone (*Corynactis*) homogenate was used in place of vegetable protein solution, and this was also effective in exciting these cells.

Two Lucifer Yellow injections of chemosensory

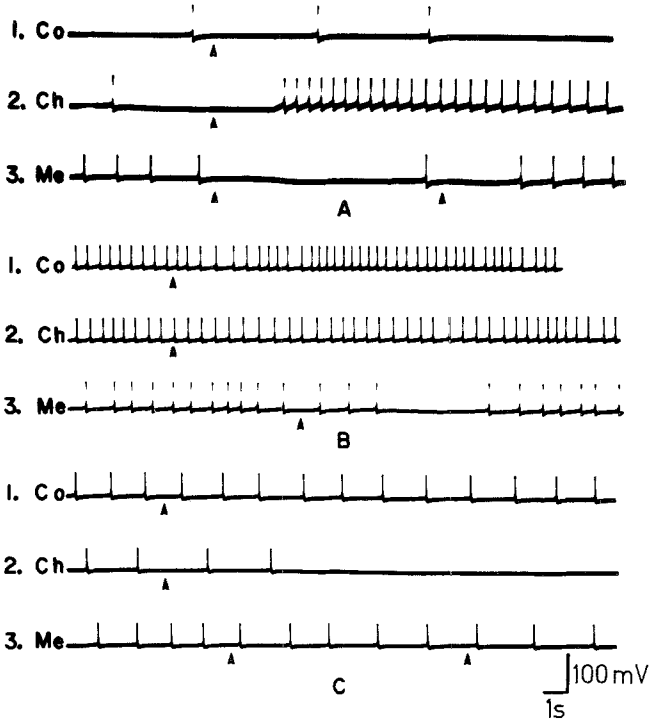

Fig. 18. Intracellular records made from the somata of various rhinophore ganglion cells, illustrating inhibitory responses to stimulation. Abbreviations as in Fig. 7

cells were made (Fig. 17). Both showed a bipolar configuration with two major processes emanating from a small (< 20 μm) soma. One of the two processes exited the rhinophore nerve (Fig. 17A). Cells having the same bipolar configuration are visible also in cobaltous chloride backfills of both the tentacle and rhinophore nerves (Fig. 4A, B), near the center of the respective ganglia. In one cell of this class the extracellular spike could be recorded from the rhinophore nerve, providing additional evidence that this class of cells projects axons into the cerebropleural ganglion.

Variable-Response Neurons. Four neurons were found which showed spontaneous spiking at irregular intervals but no consistent response to sensory stimulation (not shown).

Unresponsive Neurons. As in the tentacle ganglion, several cells (10) were encountered that fired spontaneously but were unresponsive to mechanical or chemical stimuli (not shown). These cells were found in the distal portion of the ganglion. The cobaltous chloride backfills of the rhinophore nerve filled few somata in this region of the ganglion. Thus these neurons may not project to the cerebropleural ganglion.

Inhibitory Effects of Stimulation on Rhinophore Ganglion Cells

All neurons described so far exhibited excitatory responses to mechanical and/or chemical stimuli. Of the 79 neurons reported in this study, six showed inhibitory responses to sensory stimulation, all located in the rhinophore ganglion. Figure 18A, for example, shows a neuron that was unaffected by sea water (Fig. 18A, 1), excited by chemical stimuli (Fig. 18A, 2), but inhibited by mechanical stimuli (Fig. 18A, 3). The same test was applied to the purely chemosensory neurons studied earlier without the inhibitory effect. One cell of this type was found.

Figure 18B shows a second neuron which fired spontaneously. Chemical stimulation caused little or no effect in this cell (Fig. 18B, 2), but subsequent mechanical stimulation was strongly inhibitory (Fig. 18B, 3). The latency of the inhibitory effect was comparatively long (3 s). Three cells showing this type of response were located.

Finally Fig. 18C shows a third type of cell, in which chemical stimulation strongly inhibited spontaneous discharge, sometimes for periods of up to 20 s (Fig. 18C, 3), but mechanical stimulation was without effect (Fig. 18C, 3). Two cells of this response type were encountered.

Discussion

Central Integration of Mechano- and Chemosensory Information in Gastropods

Studies of the central integration of mechanical and chemical stimuli in gastropods have been rare. Mechanoreceptive cells with centrally-located somata have been described in *Aplysia* (Byrne et al. 1974; Cobbs and Pinsker 1978). Getting (1976) described a population of about 100 neurons in *Tritonia* termed S-cells, which respond phasically to tactile stimuli and tonically to noxious chemical stimuli, and provided evidence that these cells mediate reflexive withdrawal and escape swimming. Central mechanosensory neurons responding to oral stimulation in *Tritonia* have also been described (Audesirk 1979). More recently, a class of primary mechanoreceptor neurons has been found in the cerebral ganglion of *Tritonia* which also receive chemosensory inputs via chemical synapses (Audesirk and Audesirk 1980a, b). These "complex mechanoreceptors" fire bursts of spikes during escape swimming but are relatively refractory following swim episodes.

All of the above investigations deal with primary afferent cells. Studies of integration of chemo-

sensory inputs by interneurons in molluscs are apparently confined to the investigations of type A and B cells in *Aplysia* (Fredman and Jahan-Parwar 1975; Jahan-Parwar 1972; Jahan-Parwar and Fredman 1976, 1978a, b). These cerebral neurons appear to integrate stimuli from food and sex attractants, and may be implicated in the control of locomotion (Jahan-Parwar and Fredman 1978b, 1979), although their exact function remains unknown.

Studies of Gastropod Peripheral Ganglia

With respect to peripheral or accessory ganglia in gastropods, available data are likewise scanty. The existence of peripheral ganglia associated with sense organs has been known for nearly a century (Garnault 1887; Thiele 1887) but with the exception of mainly morphological investigations of the tentacle ganglion of certain gastropods (Chase and Kamil 1981; Hanström 1925, 1926, 1928) these ganglia have not been studied. In their exhaustive 1965 review of the invertebrate literature Bullock and Horridge wrote that

> Too little is known to warrant special remark concerning most of the accessory ganglia in gastropods. It is little more than assumption that some of them, like the osphradial, are merely clusters of primary cell bodies, without relay or independent reflex function, whereas others, perhaps the genital ganglia, may be local concentrations of a semi-autonomous plexus, largely motor.

Anatomical Evidence for Peripheral Integration

The present investigation thus furnishes what appear to be among the first data on peripheral chemo- and mechanosensory integration by interneurons in gastropods, and also provides the first information on the neural function of gastropod peripheral ganglia. In contrast to earlier assumptions about accessory ganglia in gastropods, the present study indicates that the rhinophore and tentacle ganglia of *Pleurobranchaea* play neither a primary sensory nor exclusively motor role, but rather serve mainly as peripheral integrating and relay stations for sensory inputs arising from the corresponding sensory structures. The evidence for this position is both anatomical and physiological. With respect to anatomical evidence, previous electron microscope studies (Davis and Matera 1982; Matera and Davis 1982) have identified a population of sensory neurons in the peripheral sensory epithelium of the rhinophore and tentacle, and suggested that these cells are primary afferent receptors. In the present studies, cobaltous chloride backfills of the distal afferent nerves toward the periphery stained these sensory cells, indicating that they project without interruption to the peripheral ganglia. Cobaltous chloride backfills of the same distal afferent nerves toward the peripheral ganglia stain but few somata in these ganglia, and stain only a few axons that pass through the ganglia toward the central nervous system. Instead, most stained afferent fibers terminate in the neuropile of the peripheral ganglia. In contrast to the paucity of somata filled by these procedures in peripheral ganglia, cobaltous chloride back fills of the nerves that connect the peripheral ganglia with the central nervous system (CNS) fill numerous (about 100) somata in the peripheral ganglia, but few axons in the afferent nerves distal to the ganglia, and few somata in the central ganglia. We interpret these collective anatomical data to mean that primary afferent neurons in the rhinophores and tentacles project directly to the peripheral ganglia, where they terminate in the neuropile on sensory interneurons that in turn project to the CNS.

The anatomical data therefore indicate that the rhinophore and tentacle ganglia contain few if any primary afferent cell bodies, but rather serve as peripheral integrating stations for afferent inputs from the major sensory structures, namely the rhinophores and tentacles. Available data enable a precise if simplistic calculation of the convergence ratio from primary receptors to interneurons. Primary receptors are found in the peripheral sensory epithelium of the rhinophore at a density of 2,000 to 5,000 per square millimeter (Davis and Matera 1982; Matera and Davis 1982), while a lower estimate of the number of integrating interneurons in the rhinophore ganglion is approximately 100 (Fig. 4). The area of the rhinophore's sensory epithelium is about 0.5 cm^2, and therefore an estimated 100,000 to 250,000 primary receptors converge on an estimated 100 interneurons, yielding a mean convergence ratio of 1,000–2,500. To this simplistic interpretation a cautionary note must be added. Namely, electron microscopy of the rhinophore and tentacle nerves reveal hundreds of small axons (<1 μm in diameter) whose function is unknown (Matera and Davis, unpublished data). The presence of these axons is neither accounted nor explained in the above interpretation, although two possibilities may be recognized. First, these axons may represent multiple branchings of single neurons, which is not uncommon in molluscs. Second, these axons may comprise a centrifugal efferent conducting pathway (see below).

Physiological Evidence for Peripheral Integration

The hypothesis that the peripheral ganglia serve as integrating and relay stations is supported by two types of physiological data, extracellular and intracellular. Extracellular studies of the peripheral ganglia in normal and calcium-free sea water reveal a large Ca^{++} sensitive transmission component in both directions through the ganglia, and a smaller Ca^{++} insensitive component. The existence of a centripetal Ca^{++} sensitive component is consistent with synaptic integration of primary afferent input within the peripheral ganglion. The smaller Ca^{++} insensitive component is consistent with the presence of a small population of axons passing through the peripheral ganglia, as revealed by cobaltous chloride backfills. Both centrifugal components may represent an efferent pathway involved in the motor control of the distal sensory appendages.

Intracellular studies provide the most direct evidence for peripheral integration within the rhinophore and tentacle ganglia. Approximately 320 neurons have been sampled in the peripheral ganglia, of which 79 are included in the present study, on the basis of high resting and action potentials (signifying healthy cells) and recording durations sufficient for complete characterization of the neurons. On the basis of their physiological responses, three major categories of neurons have been recognized, mechanosensory, chemosensory and bimodal (both mechano- and chemosensory). Three lines of evidence indicate that these neurons are interneurons. First, soma spikes were typically accompanied 1:1 by extracellular spikes in the rhinophore or tentacle nerve, and antidromic spikes were also frequently elicited. Correlated with these physiological data, Lucifer Yellow injections showed that 9/13 injected cells sent an axon into the nerve that connects with the CNS (rhinophore or tentacle nerve). In contrast, injected neurons never appeared to send an axon into distal (afferent) nerves, although one possible instance of a distal branch was observed (Fig. 10B).

Second, EPSP's and IPSP's were regularly recorded from the somata in the peripheral ganglia. The nerve connecting these ganglia to the CNS (rhinophore or tentacle) was severed, and hence these PSP's were not caused by action potentials originating in the CNS. This evidence is by itself weak, since primary afferent neurons can also exhibit PSP's (e.g., Audesirk and Audesirk 1980a, b).

Third, in the case of mechanoreceptors, at least, single neurons in the peripheral ganglia are activated by the entire surface area of peripheral sensory field. Thus, these neurons are either interneurons, or sensory neurons with receptive fields much larger than any precedented case.

Order of Putative Peripheral Interneurons

The anatomical and physiological data therefore collectively indicate that neurons studied here in peripheral ganglia are interneurons. The order of these putative interneurons, however, cannot be ascertained from the present experiments. The bulk of our data are consistent with a direct, monosynaptic connection between the primary receptors on the periphery and the interneurons in the peripheral ganglia. Thus, most interneurons studied were simply and directly excited by mechanical stimuli, chemical (food) stimuli, or both, consistent with the possibility that they are first order interneurons. In contrast, a small number of cells (6/79) were excited by one stimulus modality, but inhibited by another (Fig. 18). Such complex integrative behavior could in principle be performed by a first order interneuron, but it seems simpler to postulate that such neurons are higher order, and that their activity is reflective of integrated inputs from other neurons within the peripheral ganglion and potentially also from primary afferent receptors. The latency between the inhibitory effect upon the neuron and the stimulus onset is in the order of seconds, consistent with the intervention of higher order pathways. Resolution of this issue, and the nature of intramodal sensory discrimination in this system, can be ascertained only by further experiments.

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