

# Hair Cell Interactions in the Statocyst of *Hermisenda*

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**ABSTRACT** Hair cells in the statocyst of *Hermisenda crassicornis* respond to mechanical stimulation with a short latency ( $<2$  ms) depolarizing generator potential that is followed by hyperpolarization and inhibition of spike activity. Mechanically evoked hyperpolarization and spike inhibition were abolished by cutting the static nerve, repetitive mechanical stimulation, tetrodotoxin (TTX), and  $\text{Co}^{++}$ . Since none of these procedures markedly altered the generator potential it was concluded that the hyperpolarization is an inhibitory synaptic potential and not a component of the mechanotransduction process. Intracellular recordings from pairs of hair cells in the same statocyst and in statocysts on opposite sides of the brain revealed that hair cells are connected by chemical and/or electrical synapses. All chemical interactions were inhibitory. Hyperpolarization and spike inhibition result from inhibitory interactions between hair cells in the same and in opposite statocysts.

## INTRODUCTION

Gravitational sense, that is, information about the direction of gravity relative to body position, is provided by the otolith organ in higher animals (Ross 1936, and Lowenstein and Roberts, 1949) and by the statocyst in invertebrates. Both organs consist essentially of a cavity lined with mechanosensitive hair cells which contain a fluid and heavy particles. A change in the direction of gravity causes movement of the particles which in turn mechanically excites the hair cells.

Since the statocyst may be considered a simple vestibular apparatus it has attracted considerable interest. The functioning of the statocyst has generally been studied by extracellular recording from the statocyst nerve. In arthropods, mollusks, and ctenophores this approach has provided useful information about the sensitivity of different statocyst organs to various directions of movement (Cohen, 1955, 1960; Wolff, 1970 *a, b*; Wood and Von Baumgarten, 1972). Extracellular nerve recordings, however, furnish no direct information about the transduction process nor about the possibility of interaction between hair cells.

Recently Alkon and Bak (1973) succeeded in recording intracellularly from hair cells in the statocyst of the mollusk, *Hermissenda crassicornis*. They found that the hair cells responded to mechanical stimulation with a depolarizing generator potential that increased with stimulus strength. In 10–15% of the hair cells the generator potential was biphasic, first depolarizing then hyperpolarizing. The hyperpolarization was not a consequence of spike activity, as it followed both generator potentials that were sub- and suprathreshold for spike production. Hence, they suggested that it was either a component of the transduction process, or an inhibitory potential due to synaptic interaction between hair cells.

In the work presented here the origin of the mechanically evoked hyperpolarization is examined. Evidence is presented that the hyperpolarization is an inhibitory synaptic potential, which is caused primarily by interactions between hair cells in the same statocyst, but which may be contributed to by interactions between hair cells in the statocyst on the opposite sides of the brain.

#### METHODS

##### *Preparation*

*Hermissenda crassicornis*, purchased from Dr. Rimmon Fay of the Pacific Bio-Marine Co., Venice, Calif., were maintained in an "Instant Ocean" aquarium at 13°C. The central nervous system of *Hermissenda* was dissected and isolated as previously described by Alkon and Fuortes (1972). When the effects of drugs or ions were studied the isolated nervous system was pinned in a chamber (1 ml volume) filled with continuously flowing (4–8 ml/min) artificial seawater or "Instant Ocean." Artificial seawater had the following composition in millimolar concentration: Na<sup>+</sup>, 479.4; K<sup>+</sup>, 10; Ca<sup>++</sup>, 10; Mg<sup>++</sup>, 53; Cl<sup>-</sup>, 613; HCO<sub>3</sub><sup>-</sup>, 2.4. For some experiments solutions containing Co<sup>++</sup> (5–50 mM) were used. In most cases Co<sup>++</sup> solutions were made up in artificial seawater with appropriate changes in NaCl concentration to maintain isotonicity. Occasionally Co<sup>++</sup> was added directly to "Instant Ocean" in concentrations that did not exceed 10 mM.

##### *Intracellular Recordings*

A connective tissue sheath enveloping the isolated nervous system was partially digested with Pronase (Sigma Chemical Co. St. Louis, Mo.) to facilitate insertion of the microelectrodes. The concentration used varied for each lot of Pronase. Concentrations of the Sigma Type VII Pronase were not used in excess of 4 mg/cm<sup>3</sup>. Higher concentrations caused increased firing of hair cells and cessation of spontaneous movements of the statoconia (*see* Results, Histology).

Intracellular recordings were made with glass micropipettes filled with 4 M potassium acetate (resistances of 60–100 MΩ). The electrode was connected via a silver wire to the input stage of a high impedance amplifier with capacitance neutralization. The bath electrode was a large chlorided silver wire. A Wheatstone bridge circuit was used to pass current through the recording electrode. Current was monitored by re-

ording the potential drop across a  $10^9 \Omega$  resistor in series with the electrode. All experiments were performed at room temperature (approximately 22°C).

### *Stimulation of Hair Cells*

Hair cells were mechanically stimulated by introducing a small displacement to the statocyst. This was accomplished by the method of Alkon and Bak (1973) briefly described as follows. One end of a piezoelectric beam was fixed to a micromanipulator. A 1-inch length of 30 gauge stainless steel hypodermic tubing was attached to the free end of the beam. The tubing, in turn, was fixed by gentle suction to the pleural ganglion, which is adjacent to the statocyst. Pulses (0.6–1 ms in duration) of 30–40 V applied to the piezoelectric beam caused a 10  $\mu\text{m}$  displacement of the statocyst. Such a displacement evokes a maximum hair cell response. Approximately half-maximal stimuli were used in this study.

### *Histological Technique*

The nervous system of *Hermisenda* was dissected out in seawater and fixed in 4% formaldehyde at 4°C for at least 24 h. After fixation the material was dehydrated in acetone and embedded in Epon. Five  $\mu\text{m}$  serial sections of the embedded material stained with toluidine blue, were examined with light microscopy.

Hair cell morphology was also studied using the technique of intracellular dye injection (Stretton and Kravitz, 1968). Micropipettes were filled with a solution of 6% Procion yellow M-4 RAN (Colab Laboratories, Inc. Glenwood, Ill.) in distilled water (resistances of 200–400 M $\Omega$ ). After the cell had been identified by its response to mechanical stimulation, negatively charged dye was ejected from the pipette by passing negative current pulses (10–15 nA and 500 ms duration) at 1/s for approximately 20 min. At the end of this procedure action potentials evoked with depolarizing current confirmed that the electrode was still intracellular. The preparation was kept at room temperature for 3–4 h and then at 4°C for 12 h to allow time for diffusion of the dye along the cell's processes. The preparation was then fixed in formaldehyde, dehydrated in acetone, embedded in Epon, and cut into 10  $\mu\text{m}$  serial sections which were examined under the fluorescence microscope.

## RESULTS

### *Histology*

*Hermisenda* has two spherical statocysts 70–100  $\mu\text{m}$  in diameter. They form a common border with the posterior surface of the optic ganglion along the lateral border of each cerebropleural ganglion (Fig. 1 A). Serial sections, stained with toluidine blue, revealed that the statocyst consists of 12–13 disk-shaped hair cells that are 40–50  $\mu\text{m}$  in diameter and 5–10  $\mu\text{m}$  in thickness. Each hair cell has many motile hairs that project into the lumen of the statocyst which also contains a fluid, statolymph, and a cluster of crystals, statoconia (Fig. 1 B). The individual statoconia, although clustered together, are constantly moving independently of each other. The movements, which are slowed by low temperature and stopped by 10 mM NaCn, presumably

result from the movement of the hairs of the hair cells. The hair cells in *Hermissenda* unlike vertebrate hair cells have axons which join together along the anterior border of the statocyst to form the static nerve. The static nerve leaves the statocyst anterodorsally, and at this point is 7–15  $\mu\text{m}$  in diameter. It lies beneath a connective tissue sheath and travels for a distance of 25–40  $\mu\text{m}$  before entering the ipsilateral cerebropleural ganglion. By iontophoretic intracellular injection of the fluorescent dye, Procion yellow, it is possible to follow individual hair cell axons after they entered the cerebropleural ganglion (Fig. 2). In four out of five marked cells, the axons branched in the neuropile region of the ipsilateral ganglion and sent a branch across the brain to the lateral border of the contralateral cerebropleural ganglion. When hair cells in both statocysts were marked with Procion, their axons were observed to converge in the lateral region of the pleural ganglion neuropile.

#### *The Origin of the Mechanically Evoked Hyperpolarization*

**EFFECTS OF MEMBRANE POTENTIAL** Approximately 5% of the hair cells investigated responded to low intensity mechanical stimulation with a hyperpolarizing response. The latency of the response ranged from 35–60 ms (mean 47). As the stimulus intensity was increased the response of these cells became biphasic, first depolarizing then hyperpolarizing as shown in Fig. 3. More frequently, however, hair cells responded to weak mechanical stimulation with a short latency (<2 ms) depolarizing response (Alkon and Bak, 1973) which, in approximately 15% of the cells, was followed by a hyperpolarization at higher stimulus intensities (Fig. 4). In practically all of the remaining hair cells mechanically evoked hyperpolarization could be demonstrated by depolarizing the cell with extrinsic current. When the hair cell was depolarized by a small amount, mechanical stimulation was followed by a slight hyperpolarization. With larger depolarizations the hyperpolarization was reduced in amplitude, and became coincident with an inhibition of spike activity.

Depolarizing potential changes had similar effects on hair cells that showed a well defined hyperpolarization at resting potential (Fig. 4). Weak depolarizing currents increased the size of the hyperpolarization only slightly and with stronger currents the hyperpolarization decreased but spike activity was transiently inhibited (Fig. 4).

Hyperpolarization of the hair cell with current increased the amplitude of the generator potential and reduced the size of mechanically evoked hyperpolarization (Alkon and Bak, 1973).

**EFFECTS OF REPETITIVE MECHANICAL STIMULATION** Hair cell generator potentials do not adapt to repetitive mechanical stimulation at frequencies up to 10 cps (Alkon and Bak, 1973). The hyperpolarization following the generator potential, however, was found to be very sensitive to repetitive

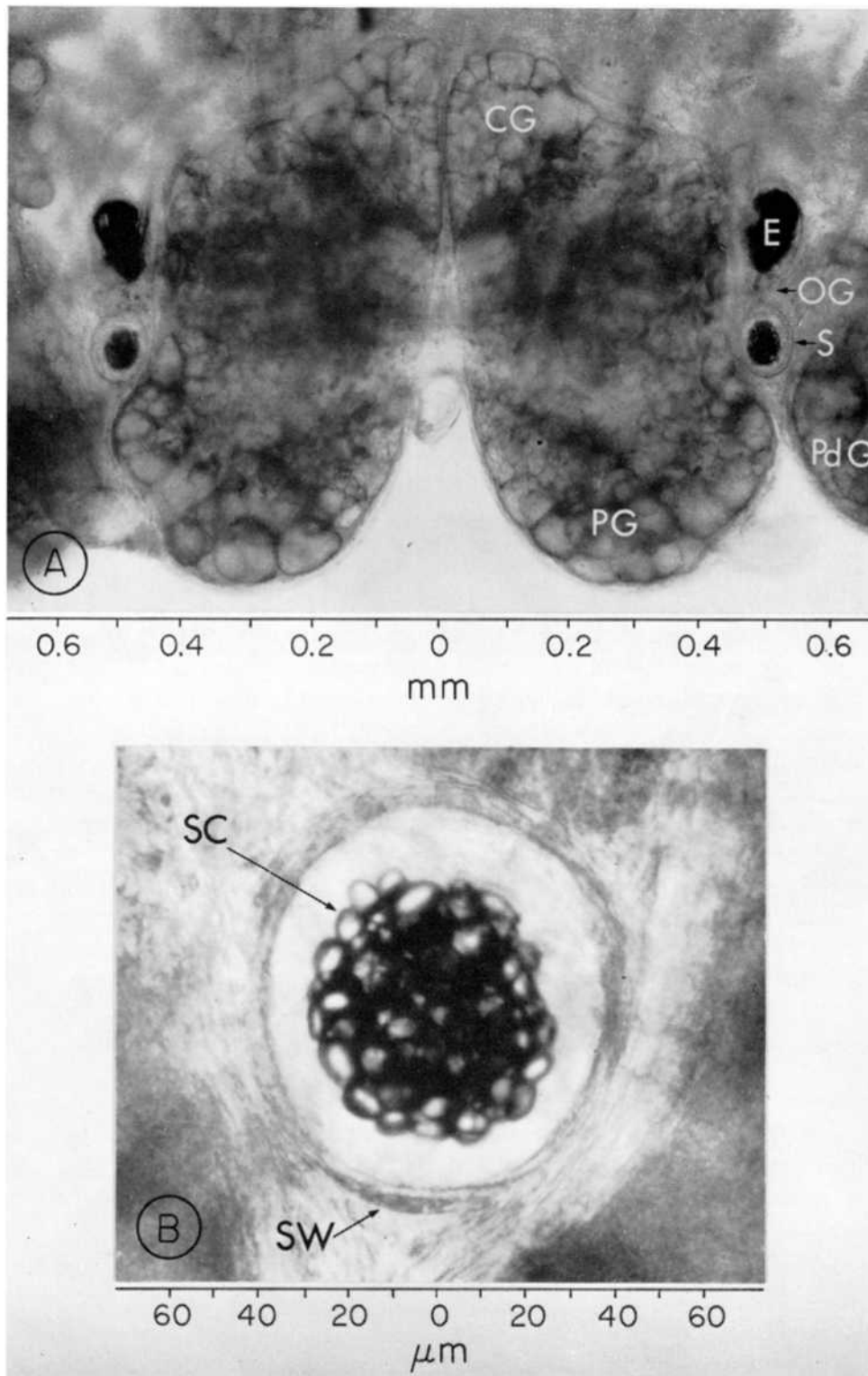


FIGURE 1. *Hermisenda* nervous system. The photomicrographs are of a living preparation that was not fixed, stained, or sectioned. A. The dorsal surface of the *Hermisenda* nervous system. The statocyst (*S*) lies between the cerebropleural ganglion (*CG* and *PG*, respectively) and the pedal ganglion (*PdG*). It is on a line with the eye (*E*) and forms a common border with the posterior aspect of the optic ganglion (*OG*). B. A view of the statocyst at a higher magnification. The statocyst wall (*SW*) is made up of hair cells which enclose a fluid and a cluster of crystals called statoconia (*SC*).

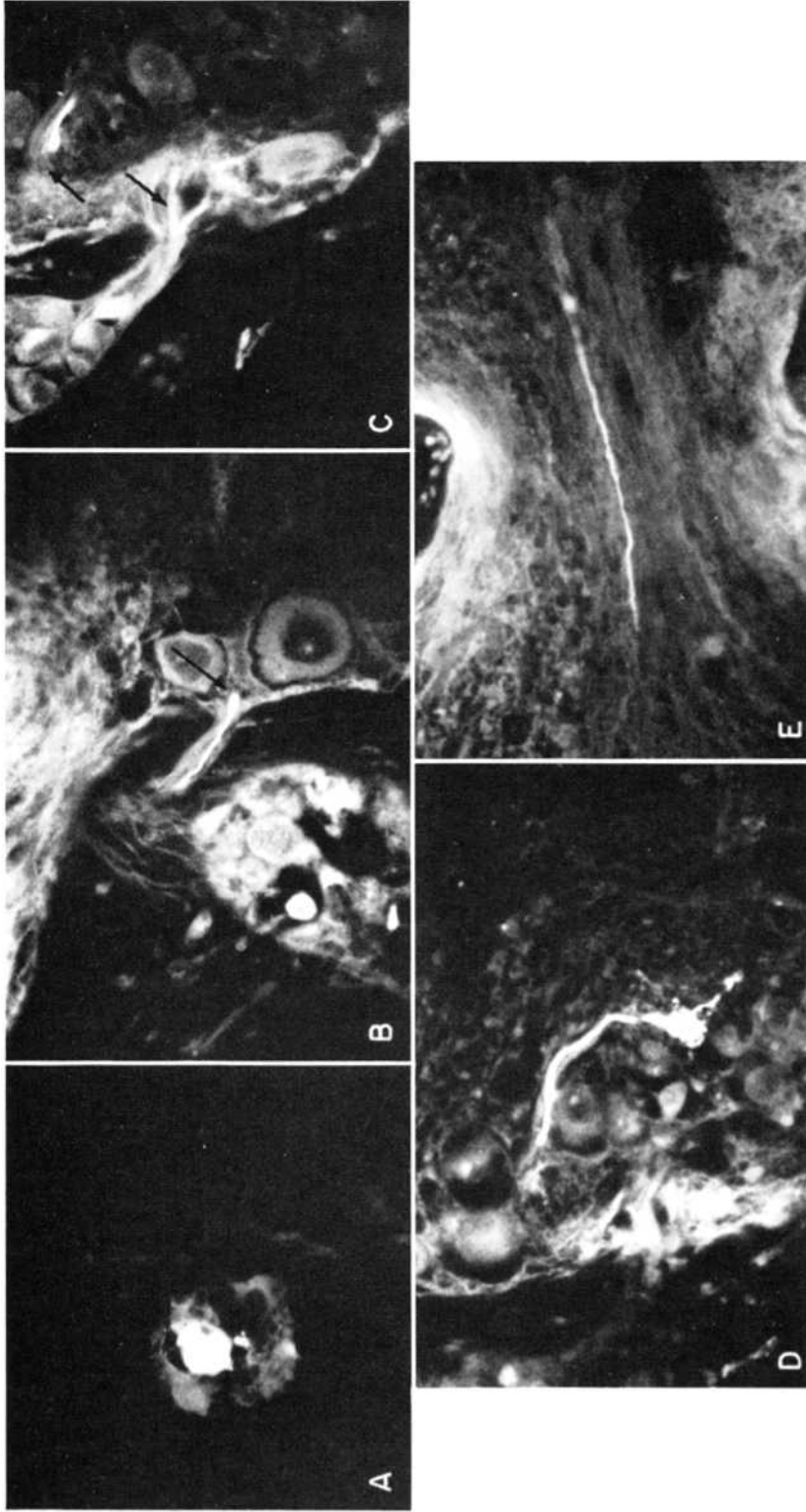


FIGURE 2. Hair cell stained by iontophoretic injection of Procion yellow. Dark-field photomicrographs demonstrate the fluorescent dye within the cell and its processes. A. Partial cross section of cell soma. B. Hair cell axon within the static nerve as it enters the pleural ganglion. C. Upper arrow indicates continuation of hair cell axon within the pleural ganglion. Lower arrow indicates optic nerve made visible by autofluorescence. The axon gives off a spray of fine branches in the ipsilateral pleural ganglion (D). It then crosses (E) to the contralateral pleural neuropile via the connective joining the two pleural ganglia. X212.

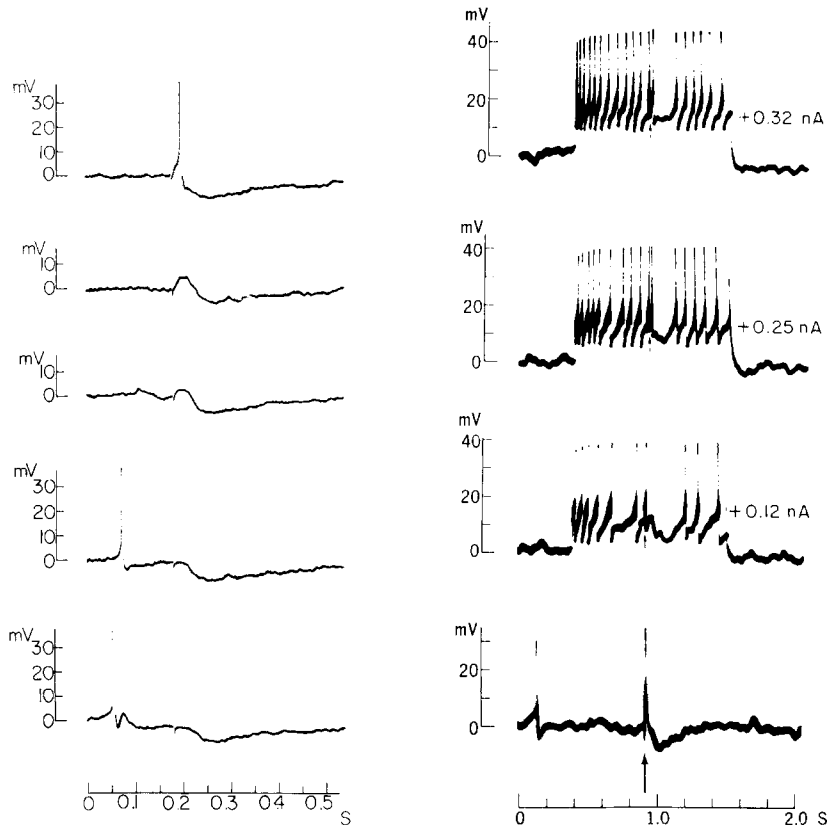


FIGURE 3

FIGURE 4

FIGURE 3. Mechanically evoked hyperpolarization. Weak mechanical stimulation evoked a purely hyperpolarizing response which, with stronger stimulation, was preceded by a short latency depolarizing generator potential. The relative strength of the voltage pulses across the piezoelectric device starting with the bottom trace were 6, 7, 8, 9, and 10.

FIGURE 4. Effect of depolarization on the postgenerator potential hyperpolarization. In the bottom frame mechanical stimulation (indicated by the arrow) evoked a depolarizing generator potential that was followed by a well defined hyperpolarization. The other frames of the figure illustrate the effects of superimposing the mechanical stimulus on a depolarizing pulse. The amplitude of the hyperpolarizing wave was slightly increased by small depolarizations and reduced by larger amounts of depolarization. Note that in the uppermost frame mechanical stimulation evoked an inhibition of spike activity without a discernible hyperpolarization. Numbers refer to the amount of depolarizing current passed.

stimulation even at low frequencies. The hyperpolarization disappeared following a 15 s period of 5 cps mechanical stimulation and recovered after approximately 20 s of rest (Fig. 5). Similarly, the inhibition of spike activity following the generator potential also vanished after a short period of repetitive mechanical stimulation.

**EFFECTS OF CUTTING THE STATIC NERVE** Normal hair cells generate action potentials spontaneously at frequencies that vary from 8 to 200/min (Alkon and Bak, 1973). They also show a synaptically mediated response to light (Alkon, 1973). When the static nerve, which contains the hair cell axons, was cut near its entry point into the cerebropleural ganglion there was no change in resting membrane potential nor in the hair cell input resistance. Cutting the axon consistently abolished the hair cell light response but had a variable effect on spike production. Of 28 hair cells studied in 11 cut nerve preparations, 13 cells generated spikes in response to depolarizing current, and only two of these showed spontaneous spike activity (maximum frequency was 5/min). The effects of mechanical stimulation were studied on 19 of the 28 cells. All 19 cells responded in a normal fashion to a mechanical stimulus, however, in none of the cells was the generator potential followed by a hyperpolarization. Furthermore, nine of the 19 cells were capable of spike generation, yet none of these nine cells showed inhibition of spike activity following the generator potential (Fig. 6).

**EFFECTS OF TETRODOTOXIN** In the absence of spike activity hair cells did not interact synaptically with each other (see below), nor would they be expected to receive synaptic input from other cells. Hence we tested the hypothesis that the mechanically evoked hyperpolarization was a synaptic phenomenon by abolishing spike activity with tetrodotoxin (TTX). Perfusion of the preparation with  $10 \mu\text{g TTX}/\text{cm}^3$  caused a slow hyperpolarization (5–15 mV) and abolished spike generation in approximately 20–30 min. This concentration of TTX had no marked effect on the depolarizing generator potential, yet it abolished the hyperpolarization in three out of three cells (Fig. 7). Of 12 cells that did not act as their own control, i.e. that were not studied first in normal seawater, the hyperpolarizing wave was never observed in the presence of  $10 \mu\text{g TTX}/\text{cm}^3$ . Recovery from the effects of TTX required prolonged washing (2 h or more).

**EFFECTS OF COBALT** Depression of synaptic transmission was attempted by using  $\text{Co}^{++}$ ,<sup>1</sup> which has been shown to be a blocking agent at the frog neuromuscular junction. Cobalt's inhibitory effects on neuromuscular transmission resemble those of  $\text{Mg}^{++}$ , except that it is approximately 20 times more effective than  $\text{Mg}^{++}$  on a molar basis (Weakly, 1973). Cobalt is, therefore, a

<sup>1</sup>The more standard procedures for blocking synaptic transmission such as low  $\text{Ca}^{++}$  or high  $\text{Mg}^{++}$  were not used for the following reasons. Artificial seawater deficient in  $\text{Ca}^{++}$  caused a 30–40% reduction in the amplitude of the hair cell generator potential and increased the spontaneous spike frequency to a point where inactivation of the spike generating mechanism occurred. In order to make artificial seawater containing high  $\text{Mg}^{++}$  (250 mM) isotonic it was necessary to reduce the  $\text{Na}^+$  concentration by approximately 60%. Such drastic changes in the  $\text{Na}^+$  concentration were avoided by using cobalt which was effective at lower concentrations.



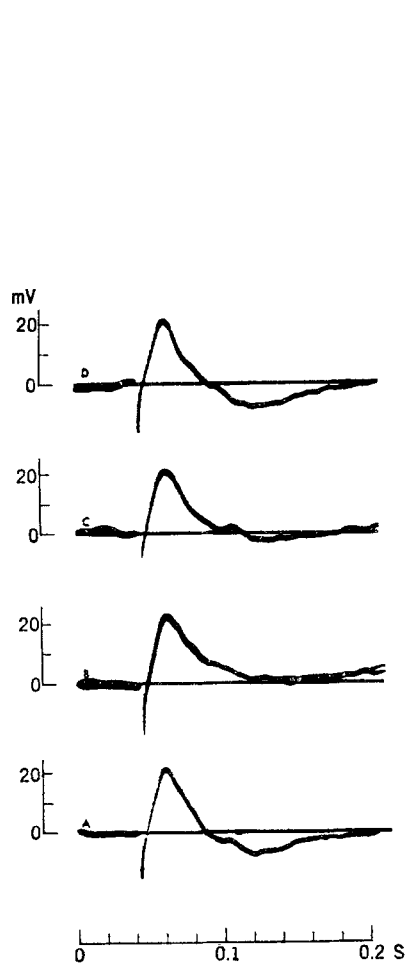


FIGURE 5

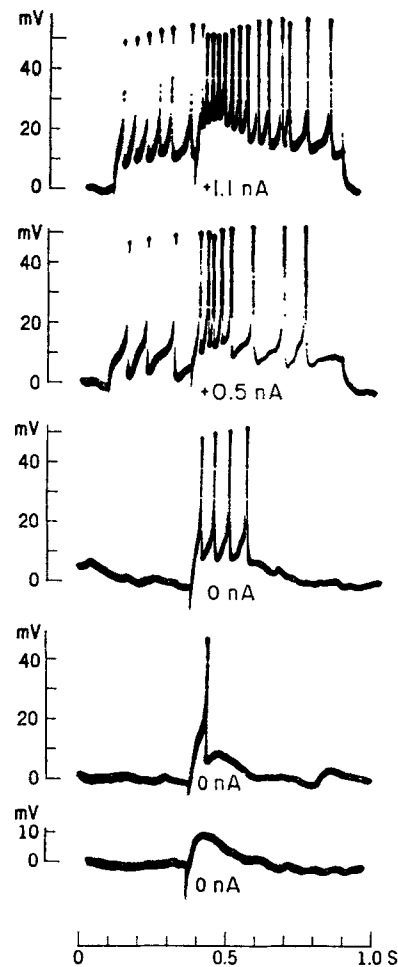


FIGURE 6

FIGURE 5. Effect of repetitive mechanical stimulation on the postgenerator potential hyperpolarization. Frame A was recorded before repetitive stimulation and a well-defined hyperpolarization following the generator potential is apparent. Frame B shows two superimposed traces at the end of a 15 s period of 5 cps mechanical stimulation. Frames C and D show the recovery of the hyperpolarization with rest. Frame C is after 10 s and D after 20 s of no mechanical stimulation. In each frame the mechanical stimulus is indicated by the stimulus artifact. The base line is indicated by the second trace in each frame.

FIGURE 6. Effect of denervation on the hair cell response to mechanical stimulation. All frames are recordings from a cell with its axon cut. The bottom three frames show graded responses to increasing stimuli; voltage pulses to piezoelectric device were 16, 20, and 24. The two uppermost traces show the effect of depolarization on the response (stimulus strength 24). Note that in no case was the generator potential followed by a hyperpolarization or by an inhibition of spike activity. In all frames the stimulus is indicated by the artifact. Numbers refer to amount of current passed in displacing the membrane potential from the resting level.

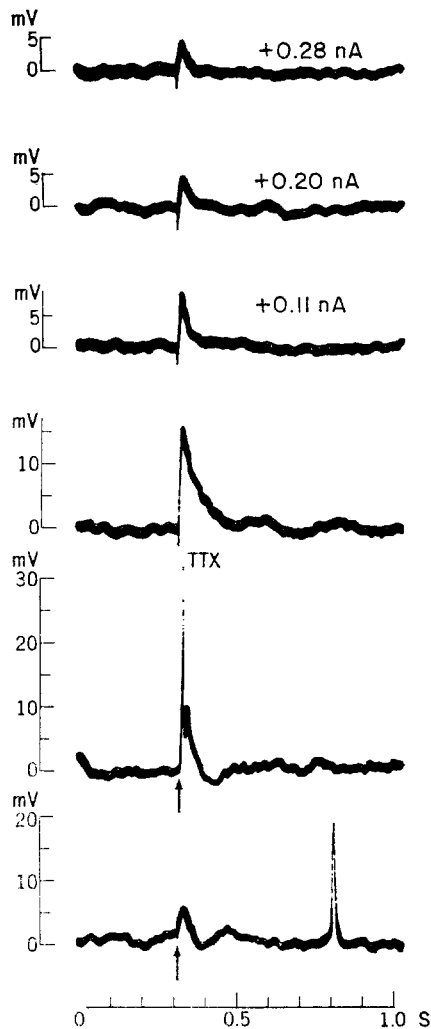


FIGURE 7

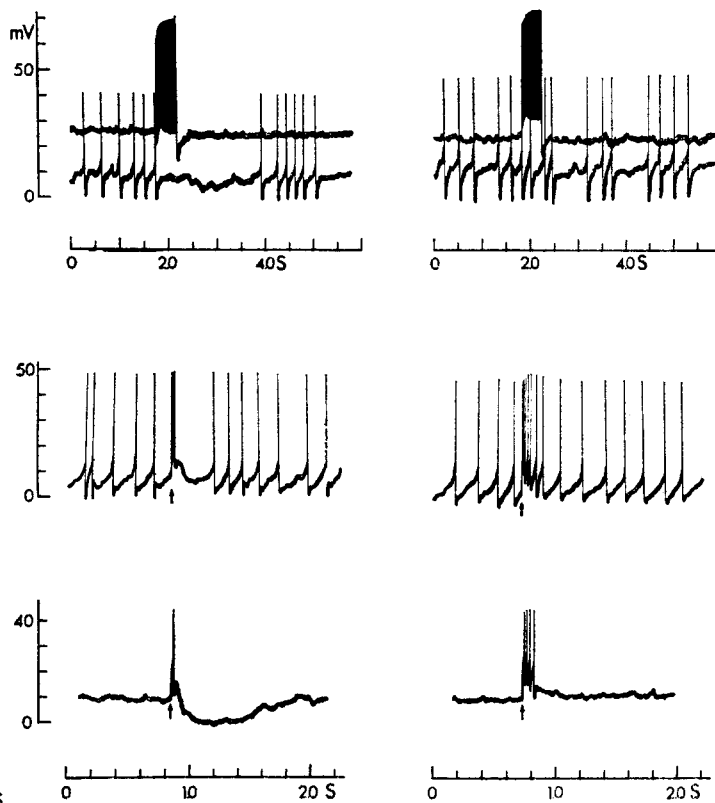


FIGURE 8

FIGURE 7. Effect of tetrodotoxin on the mechanically evoked hyperpolarization. The two bottom frames show control responses to mechanical stimulation (voltages across piezoelectric device 8 and 13). In both cases hyperpolarization followed the generator potential. The traces in the four uppermost frames were recorded after 20 min of perfusion with 10 ng TTX/cm<sup>3</sup> (relative stimulus intensity 24). The postgenerator hyperpolarization was abolished by TTX and as shown in the three upper frames it could not be demonstrated by depolarizing the cell membrane potential. The timing of the mechanical stimulus is indicated by the artifact or the arrows. The fast potential in the lowest frame is a large abortive spike, which on occasion triggered an action potential. The numbers refer to the strength of the depolarizing current steps.

FIGURE 8. Effect of cobalt on mechanically evoked hyperpolarization and spike inhibition. Traces on the left were recorded before and those on the right after perfusion with 10 mM Co<sup>++</sup>. Three separate experiments are illustrated. The bottom two sets of frames show the abolition by Co<sup>++</sup> of mechanically evoked hyperpolarization and spike inhibition. The uppermost set of frames show simultaneous recordings from two hair cells in the same statocyst. A train of spikes evoked by a depolarizing current pulse (0.5 nA) in the upper cell caused inhibition of spike activity in the lower cell. The inhibition was abolished by Co<sup>++</sup>.

particularly suitable synaptic blocker for marine invertebrates, which are somewhat insensitive to changes in  $Mg^{++}$  concentration.

The effects of  $Co^{++}$  (5–50 mM) were studied on a selected population of 21 hair cells. 13 of the cells exhibited mechanically evoked hyperpolarization at their resting membrane potential, and all 21 cells showed spike inhibition following a mechanical stimulus. Perfusion with 5–50 mM  $Co^{++}$  caused the input resistance to increase (mean 17.5% for 10 mM  $Co^{++}$ ) and an initial change in resting potential which was variable in direction and magnitude (up to  $\pm 15$  mV).

Cobalt had pronounced effects on the hair cell response to mechanical stimulation. After 1–2 min of perfusion with 10 mM  $Co^{++}$  the hyperpolarization and the inhibition of spike activity following mechanical stimulation were abolished and the generator potential increased in duration (six out of seven cells) (Fig. 8). These effects of  $Co^{++}$  were reversible. Perfusion with 5 mM  $Co^{++}$  had no effect on either the hyperpolarization or the mechanically evoked inhibition (two out of two cells). Perfusion with 20–50 mM  $Co^{++}$ , in addition to blocking mechanically evoked hyperpolarization and spike inhibition, caused a reversible decrease in the amplitude and eventual abolition of the generator potential (12 out of 12 cells).

In summary, mechanically evoked hyperpolarization was abolished by repetitive mechanical stimulation, by cutting the static nerve, by TTX, and by cobalt. These results strongly suggest that the hyperpolarization is a synaptic event. Since the hyperpolarization is initiated by mechanical stimulation it must originate in cells that are mechanosensitive. It was reasonable, therefore, to study the synaptic interaction between hair cells in the same statocyst and between hair cells in statocysts on opposite sides of the brain.

### *Intracyst Interactions*

**TYPES OF INTERACTION** The synaptic interactions between hair cells in the same statocyst were studied by recording simultaneously from pairs of hair cells. Of 75 pairs only 8 did not interact (Table I), the remaining pairs

TABLE I  
DISTRIBUTION OF INTERACTIONS BETWEEN HAIR CELLS

Type of interaction	Number of intracyst pairs	Number of intercyst pairs
No interaction	8	22
Unidirectional inhibition	43	15
Reciprocal inhibition	16	5
Electrical coupling	8	19
Electrical and chemical coupling*	4	0

\* These pairs were also listed under electrical coupling.

were either (a) chemically coupled, (b) electrically coupled, or (c) chemically and electrically coupled.

*Chemically Coupled Cells* 43 pairs of hair cells showed unidirectional inhibition, i.e., stimulation of one cell of a pair resulted in inhibition of the other cell (Fig. 9), but not vice versa. In most cases it was necessary to evoke a short high-frequency train of spikes in the presynaptic cell in order to demonstrate any interaction. Inhibition was never associated with discrete inhibitory postsynaptic potentials (IPSPs) and was most frequently apparent as a cessation of spike activity. In those cases where inhibition was associated with a marked hyperpolarization, latencies could be measured and were found to range from 40 to 90 ms (mean 57).

16 pairs of hair cells showed reciprocal inhibition (Fig. 10). Reciprocal interactions were usually stronger in one direction than the other. The general characteristics of the inhibition were the same as described for unidirectional interactions.

All the inhibitory interactions had features characteristic of chemical synapses. First, inhibition occurred with a long ( $\geq 40$  ms) delay. Second, the interaction was easily fatigued by repetitively evoking a short high-frequency train of action potentials in the presynaptic cell. Third, the amplitude of the inhibitory potential was increased by small depolarizations, and decreased by hyperpolarizing the cell. And fourth, inhibitory interactions were blocked by 10 mM  $\text{Co}^{++}$  in six out of six pairs of cells (Fig. 8).

*Electrically Coupled Cells* Seven pairs of hair cells were electrically coupled, i.e., passage of a steady current, of either polarity, in one cell resulted in a potential change of the same polarity in the other cell (Fig. 11). In this, and all subsequent instances of electrical coupling that are discussed, when one electrode was withdrawn from a cell, and current passed through it, no potential change was recorded by the other electrode. The coupling ratio, defined as the steady voltage change in one cell of the pair divided by the steady voltage change in the actively polarized cell, ranged between 0.05 and 0.22 for hyperpolarizing currents. Coupling ratios were approximately equal for polarizations of either cell of the pair (Fig. 12), and did not depend on the membrane potential of the passive cell. There was no evidence of conspicuous rectification in any of the electrically coupled cells, both hyperpolarizing and depolarizing currents were transmitted from one cell to the other (Fig. 11). Unfortunately it was not possible to compare coupling ratios for depolarizing and hyperpolarizing potential changes, because impulse firing made it impossible to measure reliably the potential changes caused by depolarizing pulses.

The possibility that both micropipettes were in the same cell is ruled out by the observation that spikes recorded by one electrode were not recorded

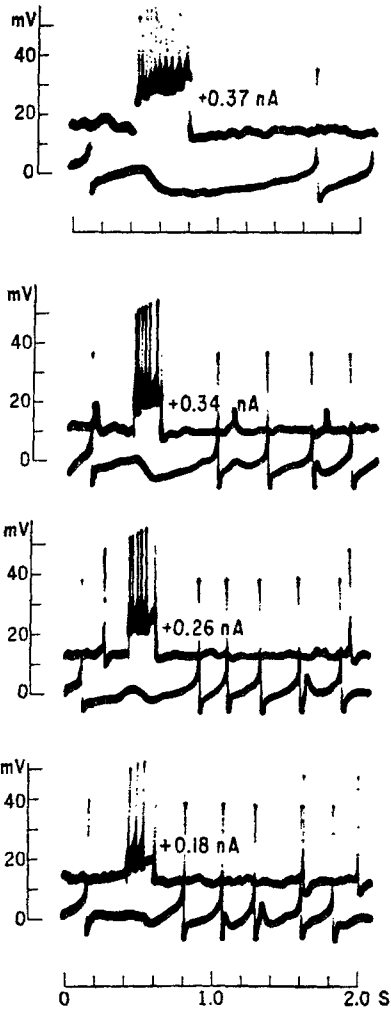


FIGURE 9

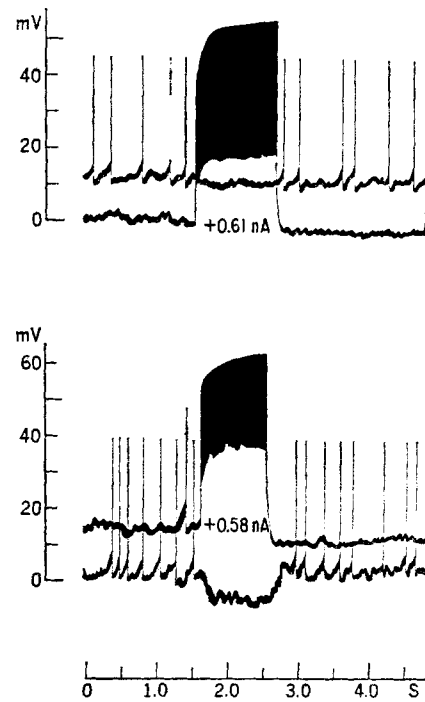


FIGURE 10

FIGURE 9. Inhibitory coupling between hair cells in the same statocyst. Two hair cells were simultaneously impaled. The cell recorded on the upper trace of each frame had an inhibitory effect on the cell recorded on the lower trace. The strength of inhibitory coupling was graded with the intensity of spike activity directly evoked in the upper cell. For the top frame the time base is 100 ms/division. Numbers refer to the strength of the depolarizing current pulse.

FIGURE 10. Reciprocal inhibition between hair cells in the same statocyst. Numbers refer to the strength of the depolarizing current pulses. In each frame the passive cell was slightly depolarized by passage of a steady positive current (0.10 nA upper frame; 0.16 nA lower frame).

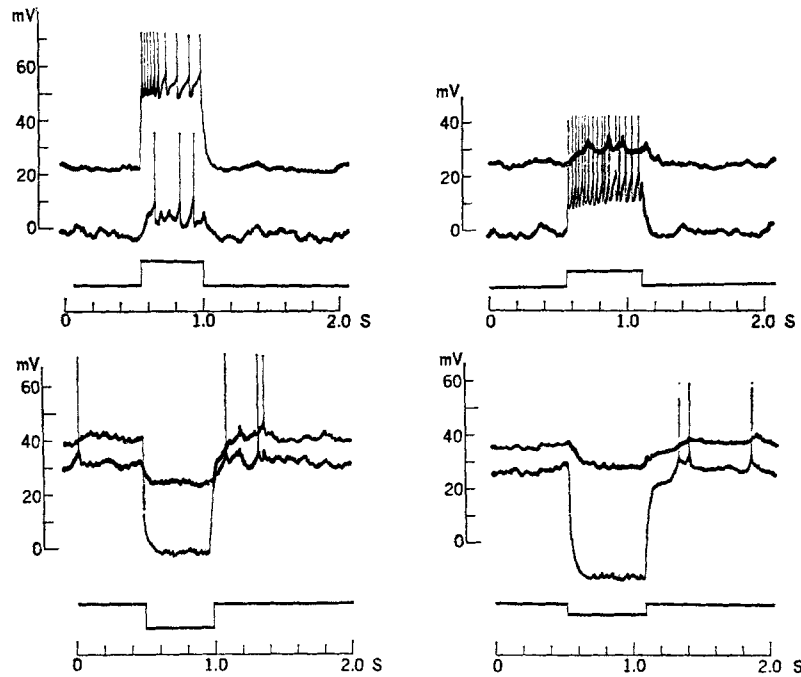


FIGURE 11. Electrical coupling between hair cells in the same statocyst. On the right the cell recorded on the lower trace was directly depolarized (upper frame) and hyperpolarized (lower frame). On the left the cell recorded on the upper trace was directly depolarized and hyperpolarized (upper and lower frames, respectively). In all cases a similar potential change occurred in the other cell of the pair. The bottom trace in each frame is a monitor of the current passed into the directly polarized cell (0.5 nA/division).

one for one by the other electrode, and that mechanical stimulation evoked generator potentials that varied in amplitude as recorded by the two electrodes (Fig. 12).

*Electrically and Chemically Coupled Cells* Four pairs of cells were both chemically and weakly electrically coupled. Direct hyperpolarization of one cell caused a steady, passive, hyperpolarization of the other cell. The coupling ratios for the four pairs of cells varied from 0.05 to 0.08. That such pairs were also chemically coupled was demonstrated by the observation that trains of spikes in one cell caused inhibition of the other cell.

**EFFECTS OF CUTTING THE NERVE** In several preparations the static nerve was cut near its entry point to the cerebropleural ganglion. This abolished the synaptic interaction of the hair cell with the visual pathway and abolished spike generation in 46% of the recorded cells. Of nine pairs of hair cells studied under these conditions, none showed signs of chemical or electrical synaptic interaction. In three of the nine pairs, at least one cell (four

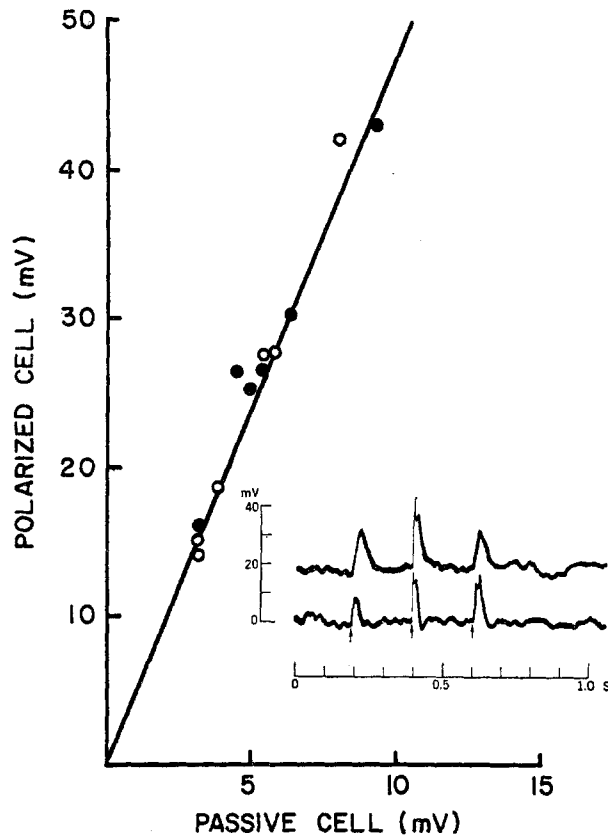


FIGURE 12. Graph showing that the coupling ratio (slope of the straight line) is independent of which cell in an electrically coupled pair is actively polarized (open circles, first cell polarized; solid circles, second cell polarized). The ordinate is the hyperpolarizing potential change directly evoked in the polarized cell and the abscissa is the corresponding potential change in the other cell of the pair. The insert shows the response to mechanical stimulation of a pair of electrically coupled cells. Note that the response recorded is not always larger for one cell than the other which indicates that the two electrodes are not in the same cell. The mechanical stimulus is indicated by the arrows.

out of six) was able to generate spikes, yet showed no signs of interaction. The probability that none of four pairs would show interaction is 0.025. These results suggest that the hair cell synaptic zone is in the axon distal to the spike trigger zone.

**LOCATION OF INTERACTING CELLS** For 66 of the 72 pairs of cells it was possible to accurately position the electrodes on the statocyst. Since the electrode could be best seen when it was placed along the dorsal-ventral equator of the statocyst, most of the localized pairs were in that plane. The results are summarized in Fig. 13. 90% of the pairs that were chemically

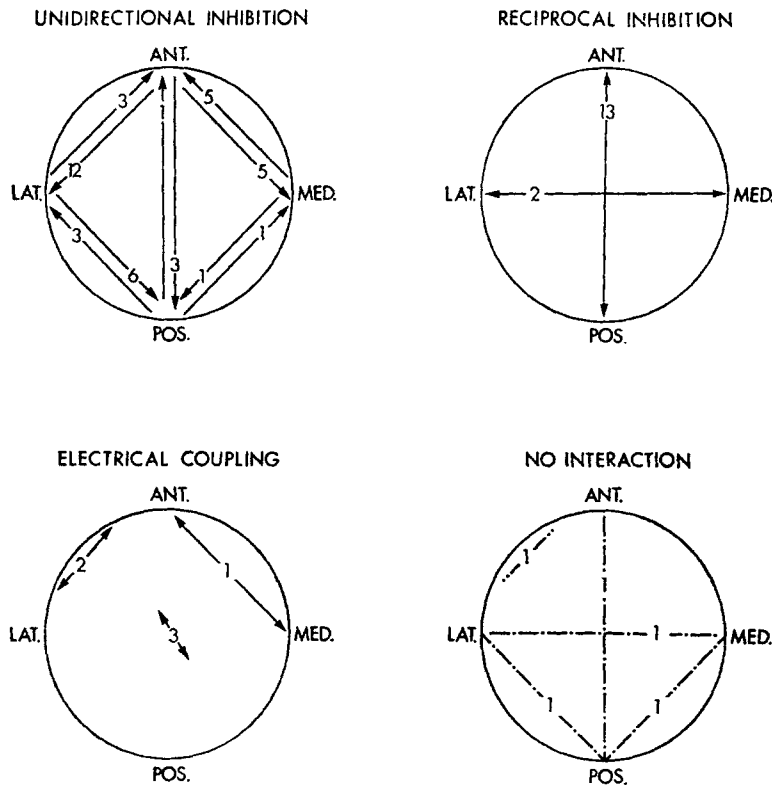


FIGURE 13. A summary diagram showing the location of pairs of hair cells that were unidirectionally inhibitory, reciprocally inhibitory, electrically coupled, and not coupled. The location of cells in a pair is represented by the ends of each line. Arrows point to the cell receiving inhibition in the upper two diagrams. Note that 36 out of 40 pairs of cells showing unidirectional inhibition were located  $90^\circ$  to each other and that 15 out of 15 pairs showing reciprocal inhibition were  $180^\circ$  to each other.

coupled in one direction were at  $90^\circ$  to each other and all the pairs that were chemically coupled in both directions were at  $180^\circ$  to each other. The electrically-coupled cells were usually, but not always, near each other at the dorsal or ventral apex of the sphere.

#### *Intercyst Interactions*

**TYPE OF INTERACTIONS** Simultaneous recordings were made from hair cells in each of the two statocysts on 61 occasions (Table I). Of these 61 pairs 22 did not interact and the remaining pairs were either chemically, or electrically coupled.

**Chemically Coupled Cells** 20 pairs of hair cells were chemically coupled across the brain. The coupling was always inhibitory and occurred in either one direction (Fig. 14) or reciprocally. The characteristics of the coupling



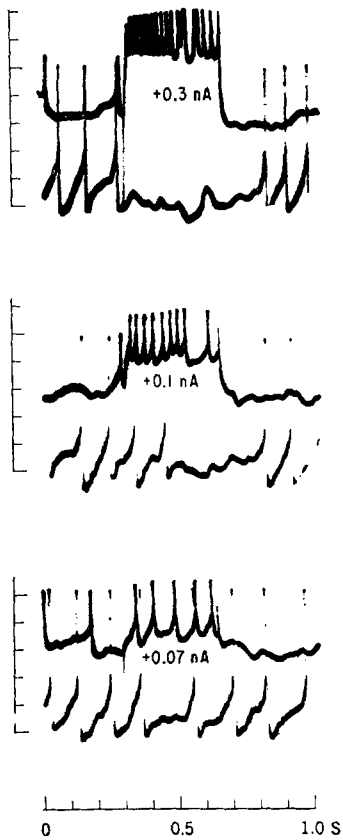


FIGURE 14

FIGURE 14. Unidirectional inhibition across the brain. A hair cell in each statocyst was simultaneously impaled. Spike activity in the cell recorded on the upper trace caused inhibition of the cell recorded on the lower trace. The strength of inhibition depended on the intensity of spike activity evoked in the upper cell. The vertical sensitivity for the upper trace in each frame was 10 mV/division and for the lower trace 5 mV/division. There was no inhibitory interaction in the opposite direction.

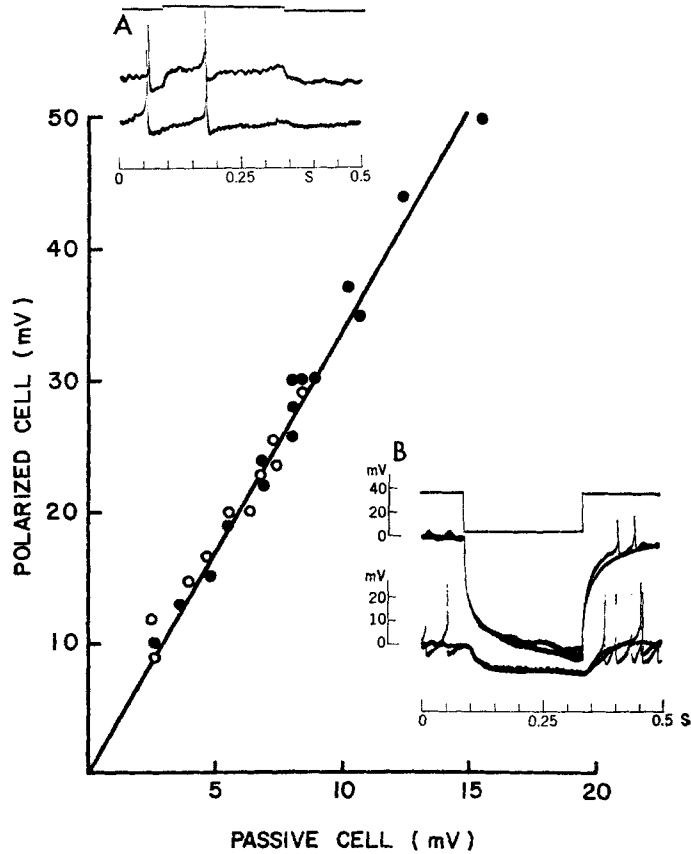


FIGURE 15

FIGURE 15. Electrical coupling across the brain. The graph shows that the coupling ratio is independent of which cell of a pair is actively polarized (open circles, first cell polarized; solid circles, second cell polarized). Insert A—Simultaneous recording from two electrically coupled hair cells showing that one electrode does not always record a spike before the other electrode. The first spike in each trace is a spontaneous spike which was recorded first by the lower cell. The second spike is a directly evoked spike which was recorded first by the upper cell. Insert B shows that electrical coupling is independent of changes in the membrane potential of the passive cell. Three superimposed traces in which the same amount of current was passed into the upper cell and membrane potential of the bottom cell was displaced from resting potential by + and -10 mV. The upper trace in each insert is a current monitor (0.5 nA/division).

were the same as described for the intracyst chemical interactions. Inhibition was generally weak, easily fatigued, and not associated with discrete synaptic potentials.

**Electrically Coupled Cells** Another common interaction between hair cells across the brain was electrical (19 out of 61 pairs). Coupling ratios for hyperpolarizing voltage changes were measured in 18 pairs and ranged from 0.02 to 0.42. Coupling ratios were the same for polarizations of either cell of a pair and were not influenced by changes in the membrane potential of the passive cell (Fig. 15).

In the two cases in which the coupling ratio was greater than 0.40 identical spike discharges were recorded by both electrodes (Fig. 16). An evoked spike was always recorded first by one electrode regardless of through which electrode the stimulating current pulse was passed. It seems possible therefore, that in these cases the two electrodes were in the axon and soma of the same cell. In the other electrically-coupled cells, all of which had coupling ratios less than 0.30, different trains of spikes were recorded by the two electrodes. Impulses, directly evoked in one cell, produced either small potential changes or full spikes in the other cell (Fig. 17).

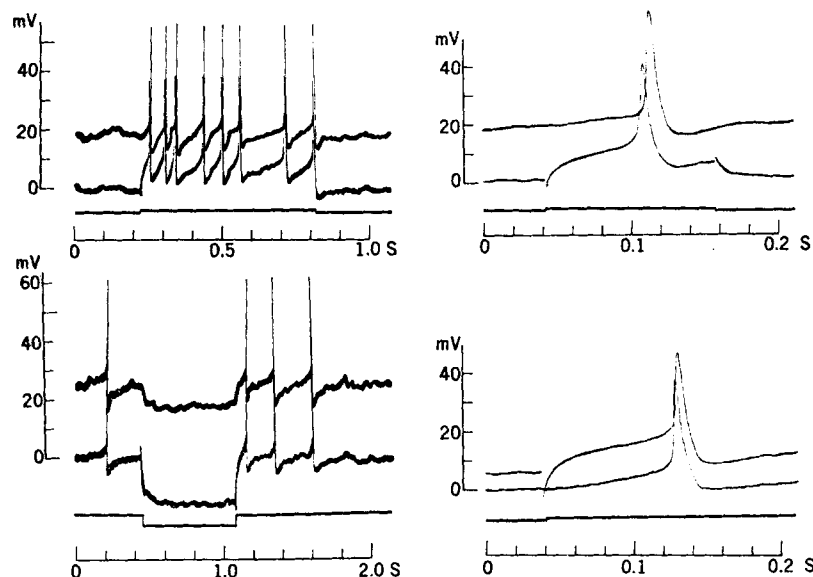


Figure 16. Possible simultaneous axon and soma recordings in a hair cell. The left side of the figure shows that the cells were closely electrically coupled for depolarizing and hyperpolarizing potential changes (coupling ratio 0.42). Note that the spikes were recorded one for one. The right side of the figure shows that the electrode recorded on the lower trace always recorded a spike before the other electrode, regardless of through which electrode the evoking current pulse was passed. The bottom trace in each frame monitors current (0.5 nA/division).

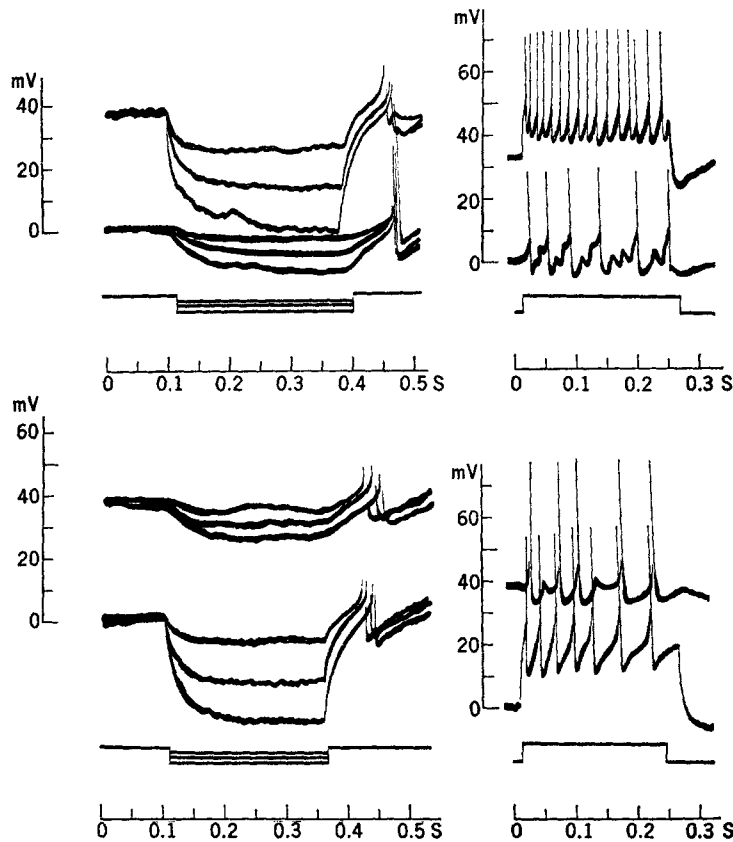


FIGURE 17. Electrical coupling between hair cells across the brain. Cells were coupled for both depolarizing and hyperpolarizing potential changes. In the upper frames the cell recorded on the top trace is actively polarized and in the lower frame the cell recorded on the bottom trace is polarized. Note that the spikes are not recorded one for one by the two electrodes. Current monitor 0.5 nA/division.

**LOCATION OF INTERACTING CELLS** In 38 of the 61 pairs the position of both electrodes was identified on their respective statocysts. No systematic relationship was evident between the type of coupling and the location of the cells.

#### DISCUSSION

##### *Mechanically Evoked Hyperpolarization*

Our results indicate that the hyperpolarization following the generator potential arises synaptically and is not a direct consequence of the transduction process, as is the generator potential. This conclusion is based on the following evidence.

(a) Sectioning the hair cell axon, which surgically prevents the soma

from receiving axonal synaptic input, abolishes the hyperpolarization but does not grossly alter the generator potential.

(b) Block of spike activity (and hence most likely of synaptic transmission) by TTX abolishes the hyperpolarization but not the generator potential.

(c) In the presence of concentrations of  $\text{Co}^{++}$  that block the inhibitory synaptic interactions between hair cells, the generator potential persists, but the hyperpolarization is abolished.

(d) Repetitive mechanical stimulation causes the hyperpolarization to disappear, but not the generator potential.

The results indicate that the hyperpolarization and the inhibition of spike activity following a mechanical stimulus are different expressions of the same phenomenon. They are commutable by changing the membrane potential, and are both abolished by cutting the hair cell axon, application of  $\text{Co}^{++}$ , and repetitive stimulation. With large decreases in membrane potential, i.e. depolarization, the hyperpolarizing wave was not appreciably increased in amplitude, and spike inhibition was either associated with a small or an undetectable hyperpolarization. The failure of the hyperpolarization to grow with reductions in membrane potential, as do other synaptic potentials, may be because hair cells show marked rectification with depolarization. A reduction in input resistance would reduce the amplitude of a passive synaptic potential. The finding that it is possible to have spike inhibition while recording a negligible hyperpolarization at the soma suggests that the soma electrode records only a fraction of the hyperpolarization responsible for the inhibition. This implies that the hyperpolarization is large near the axon spike trigger zone and that the soma electrode records a decremented, inhibitory potential that originates at the axon synaptic zone.

It is concluded that the hyperpolarization is a synaptic potential that results from inhibitory synaptic input from other mechanically excited hair cells. It follows from this conclusion that the method of mechanical stimulation used excites hair cells in addition to the one impaled. It also explains the recording of long latency mechanically evoked hyperpolarization without a preceding generator potential. In such a circumstance the stimulus is subthreshold for activating the impaled cell but suprathreshold for other hair cells that inhibit the recorded cell.

#### *Directional Sensitivity*

Some mechanoreceptors respond to a mechanical stimulus with either an increase or decrease in membrane potential (Ilyinsky, 1965, 1966; Nishi and Sato, 1968; Harris et al., 1970). Since the different responses are evoked by stimuli that would be expected to displace the receptive membrane in opposite directions they may be taken to be a sign of directional sensitivity. The ques-

tion of directional selectivity has been most investigated in vertebrate hair cells. Morphological studies have shown that the sensory bundle or cilia of vertebrate hair cells are arranged in a stereotyped orderly fashion (Lowenstein and Wersäll, 1959; Wersäll and Flock, 1965; Flock, 1971 *a*). On the basis of the morphological polarization of the cilia and extracellular recording of either microphonic responses or higher order neuron discharges, the hypothesis has been advanced that movement of the hairs in one direction causes depolarization and movement in the opposite direction causes hyperpolarization (Jielot et al., 1952; Kuiper, 1956; Trincker, 1957; Flock, 1965, 1971 *a*). Direct confirmation by intracellular recordings, has been reported for the hair cells in the lateral-line organs of *Necturus* (Harris et al., 1970), and *Lota lota* (Flock, 1971 *b*). The possibility of directional sensitivity in invertebrate hair cells is more doubtful. Extracellular recordings from the static nerve of some pulmonate gastropods have demonstrated that the receptor elements of the statocyst respond to tilting in multiple directions (Wolff, 1970 *a*). This is consistent with structural studies on hair cells from molluskan statocysts which have shown that the sensory bundle is not morphologically polarized and that the cilia are randomly oriented (Barber and Dilly, 1969; Wolff, 1969). The present work offers no evidence that *Hermisenda* hair cells are directionally sensitive. Of the several hundred hair cells that have been recorded from, a short latency (<2 ms) hyperpolarization in response to mechanical stimulation was never observed. If the hair cells were directionally sensitive it is unlikely that a depolarizing generator potential would always have been recorded, particularly since the method of stimulation would move the hairs of some cells in one direction and those of other cells in another direction. The long latency (45 ms) mechanically evoked hyperpolarizing response has been shown to be an inhibitory synaptic potential. While it may, with additional neuronal processing, provide information about the direction of the stimulus, it is not evidence of directional sensitivity of the receptor membrane.

#### *Intercyst Hair Cell Interactions*

Hair cells in statocysts on opposite sides of the brain interacted chemically or electrically. The chemically mediated interactions were always inhibitory and had the same characteristics as intracyst inhibition (see below). Cells that were electrically coupled across the brain had coupling ratios that varied from 0.02 to 0.29 (excluding the two cases in which the electrodes were suspected of being in the soma and axon of the same cell).

A length constant ( $\lambda$ ) for the hair cell axon may be estimated if we assume that in the case where the coupling ratio was 0.42 we were recording from the axon and soma of the same cell. Under these circumstances the coupling ratio would be a measure of the amount of attenuation due to electrotonic

decay and would equal  $e^{-x/\lambda}$  where  $x$  was the distance between recording sites. The upper and lower limit for  $\lambda$  is then 1.72 and 0.92 mm for separations of 1500–800  $\mu\text{m}$  between statocysts.

#### *Intracyst Hair Cell Interactions*

Hair cells in the same statocyst are chemically and/or electrically coupled. The chemical interactions are always inhibitory and are sufficient to account for the post-generator potential hyperpolarization. Inhibitory coupling (as revealed by electrical stimulation) and mechanically evoked hyperpolarization have the following similarities: (a) comparable latencies, (b) destruction by cutting the static nerve and by the same concentration of  $\text{Co}^{++}$ , and (c) weakness and easily induced fatigue. A major difference is that postgenerator potential hyperpolarization can be evoked in some cells with weak mechanical stimulation, which would be expected to generate only a few spikes. Instead, the demonstration of inhibitory coupling required high frequency trains of spikes. This discrepancy may be explained by considering that impulses from several cells, excited by mechanical stimulation, may converge on the impaled cell.

Chemically coupled cells can be inhibitory in either one or both directions. The finding that inhibition between hair cells is reciprocal at  $180^\circ$  may indicate the importance of the animal not confusing activation of diametrically opposed cells. That inhibition is predominantly unidirectional at  $90^\circ$  could be useful for discriminating direction of rotation. Since no movement will be a perfect rotation, the statoconia would be expected to fall predominantly against the hair cells of one part of the cyst first and subsequently strike others around the cyst as the rotation of the animal is completed. If the inhibition between two or more hair cells has a direction opposite to that of the rotation, e.g. clockwise, it might exaggerate the difference between the signal of the hair cell stimulated first and one stimulated subsequently. When the inhibition has the same direction as that of the rotation, it would tend to minimize such a difference. The necessary information, then, to signal one direction of rotation could be the difference between signals in one set of hair cells, and, for the opposite direction, the difference between signals in a second set of hair cells.

Cells in the same statocyst were also electrically coupled. The strength of coupling varied greatly with coupling ratios ranging from 0.05 to 0.23. Electrical coupling would permit cells to respond synchronously to either a sensory stimulus or a synaptic input. The location of electrically coupled hair cells, near each other at the dorsal or ventral apex of the statocyst, suggests that at these poles they may be arranged in a functional syncytium. The dorsal-ventral position information is certainly important to the animal. The ability of *Hermissenda* to discriminate when it is on its foot from when it is

floating on its back would be based primarily on a difference in the firing rates of dorsal versus ventral hair cells. Electrical coupling of the hair cells at these poles would tend to accentuate the predominant signal at each pole; i.e., spike activity at the mechanically stimulated pole and inhibition at the opposite pole. Thus the interactions in the statocyst may serve to facilitate a few simple discriminations concerning the direction of gravity relative to body position.

#### SUMMARY

(a) Hair cells in the statocyst of *Hermisenda crassicornis* respond to mechanical stimulation with either a long latency (45 ms) hyperpolarization or a short latency (<2 ms) depolarizing generator potential that is followed in some cases by a hyperpolarization and in all cases by an inhibition of spike activity. The present work is an investigation of the origin of the mechanically evoked hyperpolarization and spike inhibition.

(b) Mechanically evoked hyperpolarization and spike inhibition were abolished by repetitive mechanical stimulation, by cutting the static nerve, by  $10 \mu\text{g}/\text{cm}^3$  TTX, and by 10 mM cobalt. Since none of these procedures significantly altered the depolarizing generator potential, the results strongly suggested that the hyperpolarization and spike inhibition were synaptic events. Since the hyperpolarization and spike inhibition were initiated by mechanical stimulation they must originate, therefore, in mechanosensitive cells.

(c) Simultaneous intracellular recordings were obtained from pairs of hair cells within the same statocyst. By passing intracellular currents using a bridge circuit, four types of interactions were observed: unidirectional inhibition; reciprocal inhibition; electrical coupling; inhibition and electrical coupling.

(d) 90% of intracyst pairs that showed unidirectional inhibition were at  $90^\circ$  with respect to each other; and 100% of the pairs which showed reciprocal inhibition were at  $180^\circ$  to each other.

(e) The inhibition between hair cells, like the mechanically evoked hyperpolarization and spike inhibition, had a latency  $\geq 40$  ms, was weak and easily fatigued, and was abolished by axotomy and 10 mM cobalt.

(f) Simultaneous intracellular recordings were made from hair cells in each of the two statocysts. Of 61 pairs of hair cells, 22 did not interact and the remaining pairs were either chemically or electrically coupled.

(g) The data suggested that the interactions in the statocyst may serve to facilitate a few simple discriminations concerning the direction of gravity relative to body position.

We thank M. G. F. Fuortes for his valuable advice and discussion. We are also grateful to L. von Sallmann and P. Grimes for their help with the photomicrography, to H. G. Wagner for the use of

his laboratory and equipment during this work, and to J. N. Weakly for sharing with us his information concerning  $Co^{++}$ .

Received for publication 25 April 1973.

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