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# Secondary hair cells and afferent neurones of the squid statocyst receive both inhibitory and excitatory efferent inputs

R. Williamson
The Marine Biological Association of the U.K., Citadel Hill, Plymouth PL1 2PB, England

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**Summary.** Intracellular recordings were obtained from the hair cells and afferent neurones of the angular acceleration receptor system of the statocyst of the squid, *Alloteuthis subulata*. Electrical stimulation of the efferent fibres in the crista nerve (minor) evoked responses in all of the secondary hair cells recorded from ($n=211$). 48% of the secondary hair cells responded with a small depolarization, 15% with a hyperpolarization, and 37% with a depolarization followed by a hyperpolarization. The depolarizations and hyperpolarizations had mean stimulus to response delays of 6.7 ms and 24 ms, and reversal potentials of about −1 mV and −64 mV, respectively. Both types of potential increased in amplitude, up to a point, when the stimulus shock was increased and facilitation and/or summation effects could be obtained by applying multiple shocks. These data, together with the fact that both responses could be blocked by bath application of cobalt or cadmium, indicate that the secondary hair cells receive both inhibitory and excitatory efferent inputs and that these are probably mediated via chemical synapses. No efferent responses were seen in the primary hair cells but both depolarizing and hyperpolarizing efferent responses were obtained from the afferent neurones.

## Introduction

Although most hair cell based sense organs receive an efferent innervation from the central nervous system, the functions of such systems are only just beginning to be understood. In the vertebrate auditory system the efferents can alter the tuning of the receptors (Nuttall 1986), and in the lateral line organs of fish the efferents can depress the level of afferent input (Flock and Russell 1976). In the vestibular system the situation is less clear with some authors reporting efferent inhibitory actions on the afferent activity (Dieringer et al. 1977), others that the efferents are almost exclusively excitatory (Goldberg and Fernandez 1980), and others that both actions are present (Rossi et al. 1980). Whatever the sources of these differences, e.g. species differences (Kashii et al. 1987), differences in the site of action of the efferents (Highstein and Baker 1985), or differences in the conditions and protocol of the experiments (Bernard et al. 1985), it is clear that the efferents can alter the output from the sense organ.

The cephalopod analogue of the vertebrate semicircular canal system, the statocyst crista system, shows many close parallels with its vertebrate counterpart, in both its general morphology (Stephens and Young 1982; Maddock and Young 1984; Budelmann et al. 1987) and its afferent response characteristics (Williamson and Budelmann 1985). This system also receives a very large efferent innervation (Budelmann et al. 1987) which has been shown to be able to increase or decrease the level of activity from the primary afferents (Williamson 1985). In addition, and unusual for an invertebrate, the systems is made up of both primary receptor hair cells, i.e. hair cells with a centripetal axon, the usual invertebrate type hair cell, and secondary receptor hair cells, i.e. without an axon but contacting an afferent neurone, the vertebrate type hair cells (Budelmann et al. 1987). Recent experiments (Williamson 1988b, 1989a), have shown that it is possible to record intracellularly from the elements of this system and thus the action of the efferents on both types of hair cells and the afferent neurones can be directly examined.

These experiments, therefore, investigate the effects of efferent nerve stimulation upon the cells in the squid angular acceleration receptor system, the crista. The bulk of the work concentrates on the inputs to the secondary hair cells with some recordings from the primary hair cells and the afferent neurones. Despite the complicating factor that at least some of the secondary hair cells are known to be electrically coupled (Williamson 1989a), it is shown that the secondary hair cells and their afferent neurones receive both inhibitory and excitatory efferent inputs. These inputs are probably mediated by chemical synapses and will have a major effect on the operation of the sense organ. Some details of the morphology and physiology of this system have appeared in abstract form (Williamson 1988a, b).

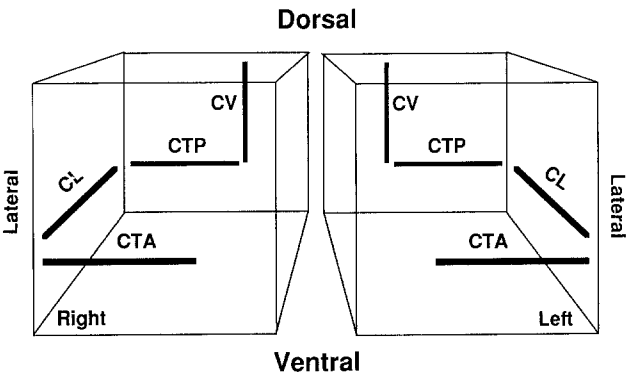


**Fig. 1.** Schematic diagram showing the path of the crista around the inside of the statocyst cavity and its division into 4 sections: anterior transverse crista (*CTA*), longitudinal crista (*CL*), posterior transverse crista (*CTP*), and vertical crista (*CV*). (After Budelmann 1977)

## Materials and methods

Squid, *Alloteuthis subulata,* with mantle lengths in the size range 3–10 cm were used in these experiments. The animals were caught locally and maintained in seawater holding tanks until required. For electrophysiological experiments, the squid was killed by decapitation, without prior anaesthesia, and the statocysts and surrounding cartilage dissected free. This tissue was placed in a small perfusion bath of capacity 5 ml. In squid, the crista epithelium winds around the inside of the statocyst cavity such that it covers three almost orthogonal planes (Fig. 1). The strip is divided in 4 sections, each of which carries an almost totally transparent cupula. These cupulae are fragile, gelatinous structures (cf. Stephens and Young 1982) and were usually destroyed by opening the statocyst, leaving remnants of variable sizes attached to the crista sections. The right or left statocyst was opened such that the anterior transverse crista section and its nerve, nervus cristae minor (Stephens and Young 1982), were left undamaged. The cartilage supporting the crista was fixed to the Silgard base of the perfusion dish so that the hair cells of the anterior transverse section pointed upwards, instead of their normal horizontal direction. This permitted access to cells on both the dorsal and ventral sides of the crista section. The arrangement of the hair cells and afferent neurones in the crista is shown in Fig. 2. Since no detailed electron microscopy study of the elements of the squid statocyst crista is as yet available, especially details of the synaptic interconnections, this diagram is provisional and based on Budelmann (1977), Stephens and Young (1982), and Williamson (1988a, and unpublished). The important features for these experiments are that the row of outermost hair cells on the dorsal side of the crista section are primary hair cells, i.e., having centripetal axons, and the row of outermost hair cells on the ventral side are secondary hair cells, which have no axons, but make contact with afferent neurones (Williamson 1988a).

In order to electrically activate the crista efferents, whose axons also run in the crista nerve, and to antidromically stimulate the dorsal hair cells and afferent neurones, one or more pairs of fine Teflon coated silver wires (diameter 0.25 mm) were inserted into the crista nerve (n. cristae minor) at the point where it enters the statocyst. Intracellular recordings were obtained from the cells using glass microelectrodes filled with 4 *M* potassium acetate having resistances of 80–140 MΩ. In some cases the microelectrode was filled with the fluorescent dye Lucifer Yellow CH (Fluka). Hair cells and afferent neurones were identified and penetrated under visual control. However, to avoid any possible confusion over which row of hair cells was being recorded from, only the outermost row of dorsal or ventral hair cells was used and this was somtimes confirmed by dye injection. This was particularly important where coupling measurements were being made from two neighbouring hair cells. Similarly, the recordings from the afferent neurones were obtained only from the somata of cells lying ventral to the hair cell crista strip. These cells could be clearly identified and there was no possible confusion with hair cells or their axons. In control experiments, microelectrode recordings were made at various extracellular locations within the crista section. No field potentials resulting from the nerve stimulation were observed, indicating that the responses reported here were not due to the pick-up of large extracellular field potentials.

The signal from the microelectrode(s) was amplified and, together with the stimulus pulse(s), recorded on an FM tape recorder for subsequent analysis or fed directly to a laboratory computer system. Most signals were computer averaged, typically 10–20 repeats, to improve the signal-to-noise ratio. When measuring reversal potentials, the membrane potential of the cell recorded from was usually changed by passing current from a second microelectrode inserted into the same cell or an adjacent cell; since the hair cells are electrically coupled current injected into an adjacent cell will also change the membrane potential of the cell recorded from. As discussed later, there are possible sources of error in this measurement. A virtual earth system was employed to monitor the injected currents. All experiments were performed at room temperature (21 °C).

Where response latencies were measured, these were taken as the time from the start of the stimulus shock to the time of the response peak. This avoided the difficulty of estimating the start of the response as this was sometimes obscured by the stimulus artefact, and in the case of biphasic responses (e.g. Fig. 5c), could not be determined for the second part of the response. Response decay time constants were measured as the time to decay to 1/e; this assumes a single exponent decay for a first approximation and with no corrections for cell shape or somatic/dendritic effects (Hubbard et al. 1969).

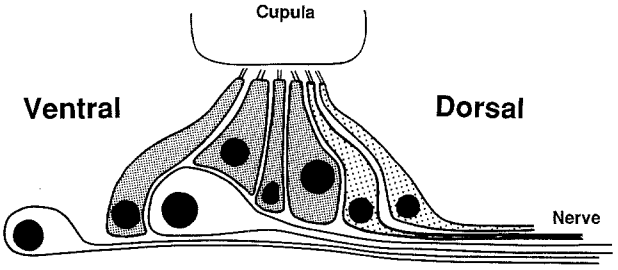


**Fig. 2.** Diagram showing a cross section through the anterior transverse crista section. The heavily stippled cells are the secondary hair cells, the lightly stippled cells are the primary hair cells, and the unstippled cells represent the afferent neurones. The diagram is provisional and based on Budelmann (1977), Stephens and Young (1982), and Williamson (1988a)

## Results

### *Morphology*

Although this investigation has not specifically dealt with the detailed morphology of the squid crista, some points have emerged, particularly from the Lucifer Yellow marking of the cells, which are worth presenting as there is very little other published information available. Firstly, there is a maximum of 6 rows of hair cells in the crista of *Alloteuthis subulata* (see also Williamson 1989a). This differs from the 8 rows of hair cells shown for the cristae of *Octopus vulgaris* (Budelmann et al. 1987) and the 7 rows of hair cells found in the squid *Loligo vulgaris* (Stephens and Young 1982). In Fig. 2, this has been presented as a reduction in the number of primary hair cell rows from 4 and 3, respectively, to 2, but it should be emphasized that this is provisional data from light microscopic examination and has not yet been confirmed by the necessary electron microscopic investigation. Secondly, as in the octopus, the outermost row of dorsal hair cells is clearly made up of primary hair cells with axons extending into the crista nerve (Fig. 3A). The somata of these cells are 3–8 μm in width and up to 30 μm long. Thirdly, the outermost row of ventral hair cells is made up of secondary hair cells which have no centripetal axon. These cells are 10–15 μm in width and some 30 μm long. However, at least some of these cells appear to have processes extending from their bases (Fig. 3B). These processes, some up to 150 μm in length, could extend both along the crista and in a ventral direction into the region occupied by the somata and axons of the primary afferent neurones. None of the Lucifer Yellow fills of the dorsal and ventral hair cells, or the primary afferent neurones showed any signs of dye leakage,

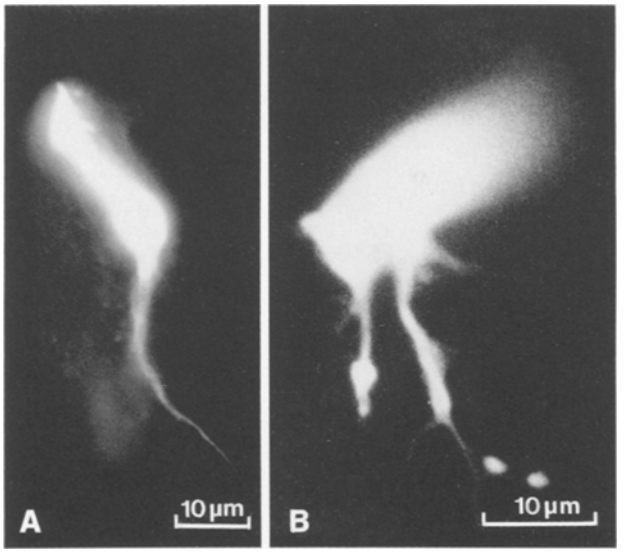


**Fig. 3A, B.** Lucifer Yellow fills of hair cells. **A** Light micrograph of a primary hair cell located on the dorsal side of the anterior transverse crista section. The fluorescence photograph was taken from an in vitro whole mount preparation. Note that the hair cell axon extends off to the right into the crista nerve. **B** Light micrograph of a secondary hair cell located on the ventral side of the anterior transverse crista section. This fluorescence photograph was also taken from an in vitro whole mount preparation. The apical part of the cell is not in focus. Note the processes extending from the base of the hair cell; none of these are axons extending into the crista nerve

or dye coupling with neighbouring cells, despite the fact that it is known that the ventral hair cells are electrotonically coupled (Williamson 1989a).

### *Ventral hair cells*

Microelectrode penetration of these cells was made under direct visual control. Where the cells were marked by iontophoretic injection of the fluorescent dye Lucifer Yellow, the subsequent histology always confirmed them as outermost ventral hair cells with no centripetal axon.

These ventral hair cells had membrane resting potentials of −41 mV to −69 mV, with a mean of $-50\ \text{mV} \pm 7\ \text{mV}$ ($n=211$). The microelectrode recordings from these cells were often noisy in appearance (Fig. 4A), i.e., showing frequent fluctuations in potential of a few millivolts away from a relatively stable baseline. These fluctuations might arise from a number of different sources, for example inherent noise in the cells, or changes in the cell receptor potential due to very small vibrations or from synaptic inputs. In some recordings, spontaneous membrane potential deflections similar in shape and amplitude to synaptic junctional potentials could be observed. These could

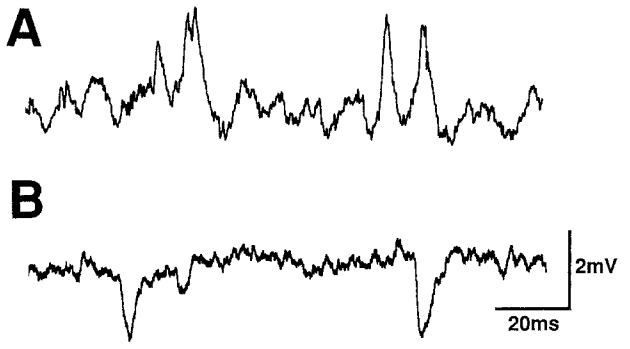


**Fig. 4A, B.** Hair cell spontaneous activity. Intracellular recordings from two different secondary hair cells of the anterior transverse crista section showing (**A**) spontaneous depolarizing fluctuations in the membrane resting potential and (**B**) spontaneous hyperpolarizing fluctuations

be depolarizing (Fig. 4A) or hyperpolarizing (Fig. 4B) potentials of up to 6 mV in amplitude. No single hair cell was found where both depolarizing and hyperpolarizing potentials similar to synaptic junctional potentials could be clearly distinguished. Such potentials could be synaptic inputs from neighbouring hair cells or more likely, efferent inputs. If these are spontaneous junctional potentials from efferent terminals on the hair cells, then electrically stimulating the nerve to the anterior transverse crista section (n. cristae minor), which contains the efferent axons, should activate these synapses.

Single electric shocks, applied to the crista nerve through the stimulating wires, evoked a brief change in the cell membrane potential of all the secondary hair cells examined ($n=211$). This change could be one of 3 types. The first response type, occurring in 48% of the cells, consisted of a small depolarization of the membrane potential (Fig. 5A). The second response type, occurring in 15% of the cells examined, consisted of a small hyperpolarization of the membrane potential (Fig. 5B). The third response type, occurring in 37% of the cells, consisted of a depolarization, followed by a hyperpolarization (Fig. 5C).

If the latencies from the time of stimulation, for single shocks, to the peak evoked depolarizations and/or peak hyperpolarizations are plotted (Fig. 6) it is apparent that the depolarizing response, with a delay of $6.7 \pm 1.8$ ms (mean $\pm$ standard error), has a shorter latency than the hyperpolarizing response, with mean latency of $24 \pm 22$ ms. If the conduction velocities of the efferent axons are similar to those of the afferent neurones (see below for a comparison of response latencies) then these latencies are clearly sufficiently

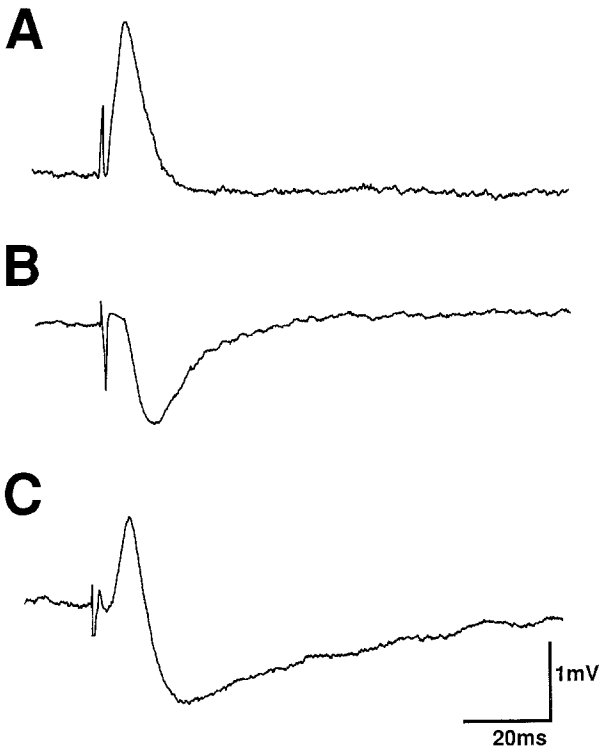


**Fig. 5A–C.** Efferent responses of secondary hair cells. Intracellular recordings from 3 different secondary hair cells showing the 3 different types of response obtained to single shock stimulation of the anterior transverse crista nerve (minor). **A** A depolarizing response; **B** a hyperpolarizing response; and **C** mixed response consisting of a depolarization followed by a hyperpolarization

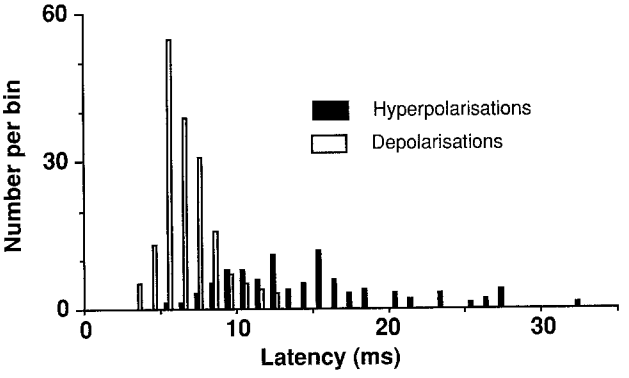


**Fig. 6.** Efferent response latencies. Histograms showing the distributions of stimulus to response latencies for efferent evoked depolarizations and hyperpolarization. Binwidth = 1 ms

long to include the delay of a chemical synapse. It can also be seen in Fig. 6 that the distribution of depolarizing response latencies was much narrower than that of the hyperpolarization. Where both responses occurred together the depolarizing response always preceded the hyperpolarizing response.

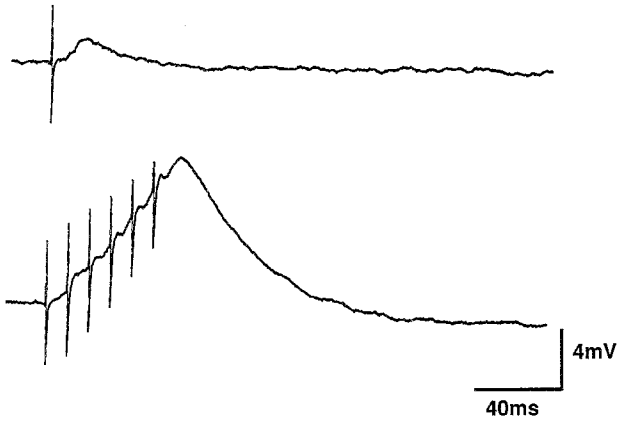


**Fig. 7.** Effect of a train of shocks on the efferent response. Intracellular recordings from a single secondary hair cell showing the increase in the efferent response obtained by multiple stimulus shocks to the crista nerve

An indication of the duration of these evoked responses was obtained by measuring their decay time constants. In order to avoid interactions between depolarizing and hyperpolarizing responses, which may have different characteristics, only overtly monophasic responses were employed. The depolarizing responses had a decay constant of $9.8 \pm 0.7$ ms (mean $\pm$ standard error) ($n = 73$) and the hyperpolarizing responses a value of $23 \pm 4.5$ ms ($n = 26$). The large standard error for the hyperpolarizing responses gives an indication of the large variation in response duration observed.

The amplitude of the response to nerve stimulation could be increased by applying a train of stimuli (Fig. 7). This increased response could be due to synaptic facilitation and/or temporal and spatial summation. An attempt to investigate this was made by applying pairs of efferent shocks of varying delays and calculating the facilitation ratio (e.g. Mallart and Martin 1967). The results obtained from these experiments were not consistent, either for depolarizing or hyperpolarizing responses, with some cells showing facilitation and others not. This may have been due to the responses being a mixture of depolarizing and hyperpolarizing effects, even where only one response was apparent, or perhaps to the summation of responses produced by the electrotonic coupling of the cells.

The amplitude of the response to single shock nerve stimulation could usually be increased by increasing the size of the applied shock (Fig. 8A), however a point was reached where increasing the shock size no longer increased the size of the response. In some cases where a depolarizing evoked response was obtained, an increase in shock size

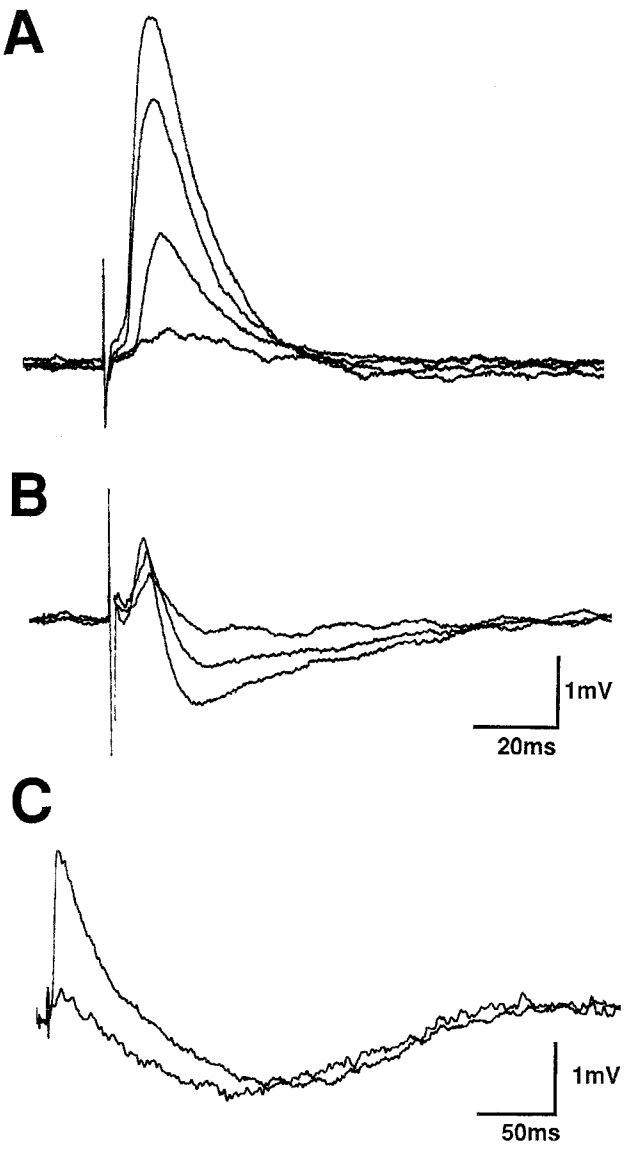


**Fig. 8A–C.** Effect of increasing shock size on the efferent response. Intracellular recordings from 3 different secondary hair cells showing the change in their efferent responses to increasing the size of the electrical stimulation to the crista nerve. **A** Increasing the shock size increases the amplitude of the depolarization. **B** Increasing the shock size increases the amplitude of the depolarization but also brings in a hyperpolarization which then also increases in amplitude. **C** A predominantly hyperpolarizing response is changed to a depolarizing/hyperpolarizing response by increasing the stimulus size. Note the long duration of the hyperpolarization

not only resulted in an increase in the depolarization but also evoked a subsequent hyperpolarization (Fig. 8B), both of which could be increased, up to a point, by increasing the size of the shock. In other cases an increase in shock amplitude could bring in a large depolarizing response to a previously predominantly hyperpolarizing response (Fig. 8C). This last figure also demonstrates the

very long lasting effects that a single shock could evoke; the hyperpolarization in this response has a duration of over 200 ms.

These results clearly imply that both depolarizing and hyperpolarizing inputs can have an effect on the same hair cell, with the actual response evoked being dependent on the precise group of efferent axons in the crista nerve activated by the electrical shock, presumably due to the recruitment of different efferents by different shock parameters. As a further test of this, the effects of two different nerve stimulation protocols on the hair cell response were examined. Firstly, while recording from a single hair cell, the polarity of the shock was reversed. In some cases this resulted in a change in the evoked response, most commonly a depolarizing potential changed to a depolarizing potential followed by a hyperpolarizing potential. Secondly, by inserting a second pair of stimulating wires into the crista nerve, it was found that the two different stimulation sites could produce different responses upon the same hair cell. Again the most common response was a change from a depolarizing potential to a depolarizing plus subsequent hyperpolarizing potential.

If different stimulation parameters can evoke different effects on the same hair cell the question arises as to whether the same stimulus shock has different effects on different hair cells, i.e. is the input to all hair cells identical when a particular subset of efferents is activated. In order to test this recordings were made from a number of hair cells during the presentation of the same stimulus shock. This involved simultaneous recordings from 2, or sometimes 3, secondary hair cells in the same crista section while applying a constant stimulus shock. It was found that the balance of depolarizing and hyperpolarizing potentials in different hair cells could be very different (Fig. 9).

Where cells receive a chemical synaptic input it is often possible to gain information about the ionic species responsible for the conductance changes by measuring the reversal potential of the synaptic response. This proved difficult to perform in this preparation because, as has already been shown (Williamson 1989a), the outermost hair cells on the ventral side of the anterior transverse crista section are electrically coupled along the length of the crista section. One effect of this is to produced very low input resistances in the hair cells, with the result that it can be difficult to pass sufficient current through the high resistance microelectrode to maintain the cell at the new membrane potential. In addition, because of the electrical coupling, the synaptic potentials recorded from

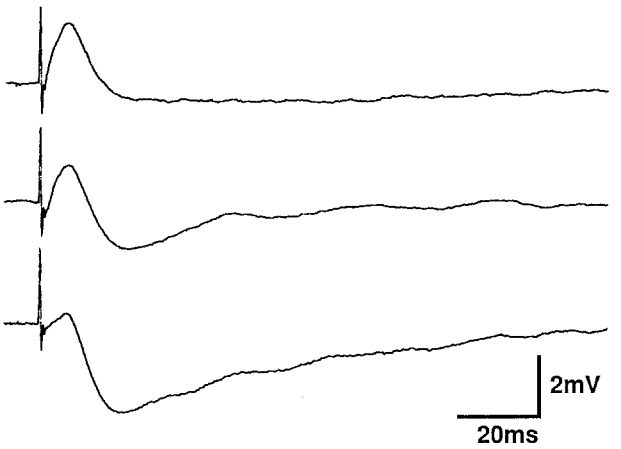


**Fig. 9.** Different efferent responses in different hair cells. Simultaneous intracellular recordings from 3 different secondary hair cells (not immediate neighbours) in the same crista section showing their different responses to a single stimulus shock. Note the different balance of depolarization and hyperpolarization in the different hair cells

a hair cell may not be due to synapses on that cell but may arise from neighbouring cells. This means that, if the synapse is on a neighbouring cell, its membrane potential will be different from that being controlled by the microelectrode. Nevertheless, it has been possible to obtain an indication of these reversal potentials by injecting depolarizing and hyperpolarizing currents into the hair cells, using a second microelectrode, while stimulating their efferent axons. It was found, by extrapolation, that the nerve evoked depolarization could be decreased to zero response at around a membrane potential of $-1 \pm 3$ mV (Fig. 10A) while the nerve evoked hyperpolarization would reverse at a membrane potential of about $-64 \pm 6$ mV (Fig. 10B). The reversal potential of the excitatory response could be interpreted as a non selective cation channel with $Na^+$ and $K^+$ conductances being the most likely ionic candidates. Whereas, $K^+$ and/or $Cl^-$ are likely to be responsible for the inhibitory response.

*Dorsal hair cells*

Microelectrode penetration of these cells was made under direct visual control and in some cases this was confirmed by iontophoretic injection of the fluorescent dye Lucifer Yellow. Cells marked by dye injections always proved to be outermost dorsal hair cells with a clear axon extending into the crista nerve in the subsequent histology (Fig. 3A). Intracellular recordings from these dorsal hair cells ($n=21$) showed that they had membrane resting potentials similar to those of the ventral hair cells.

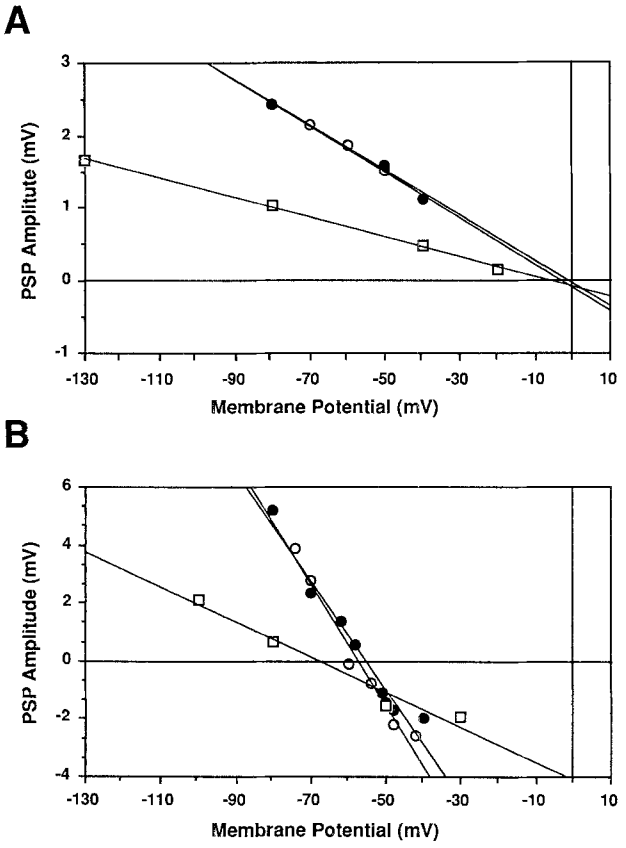


**Fig. 10 A, B.** Response reversal potentials. Graphs showing the change in amplitude of the efferent postsynaptic depolarization (**A**) and hyperpolarization (**B**), obtained by changing the membrane potential of the hair cell. Three different cells are used in each graph. The regression lines have mean intercepts at −1 mV (**A**) and −64 mV (**B**)

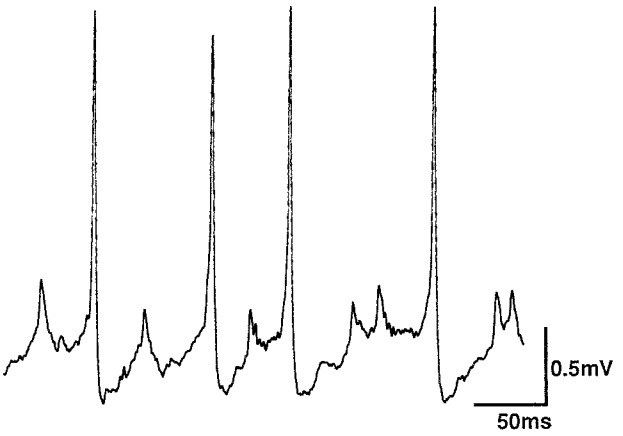


**Fig. 11.** Primary hair cell spontaneous activity. Intracellular recording from a primary hair cell (dorsal hair cell) showing spontaneous spiking activity. Note the small amplitude of the spike. This was identical in size to the antidromic spike evoked by nerve stimulation

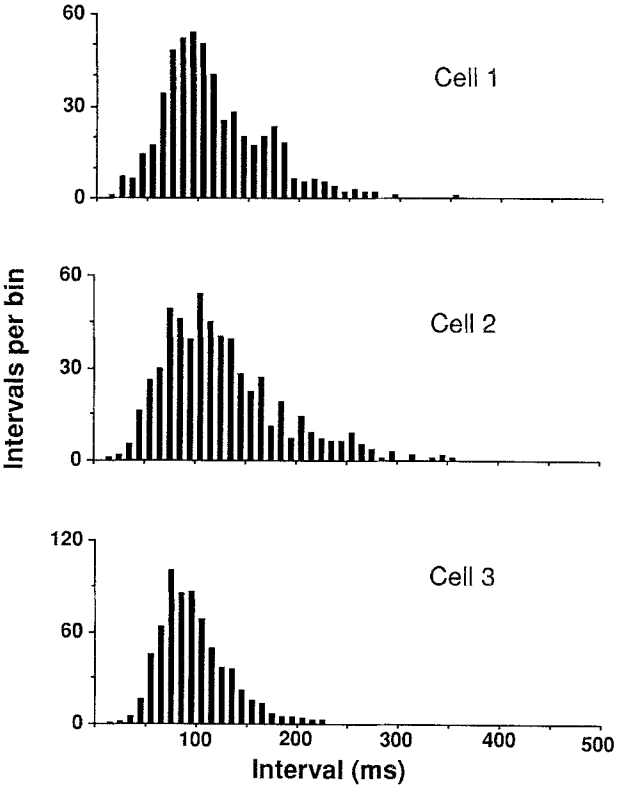


**Fig. 12.** Comparison of spontaneous activity in different primary hair cells. Inter-spike interval histograms of the spontaneous activity, recorded intracellularly, from primary hair cells in different preparations

Some cells showed spontaneous rhythmic depolarizations (Fig. 11) which could be of the same amplitude as the antidromically evoked spike or considerably smaller than this spike. Interval histograms of such spontaneous spike activity (Fig. 12) showed that this was usually of a relatively constant frequency of around 10 impulses/s. This was true even in cells from different preparations (Fig. 12). Where dorsal cells did not show spontaneous spiking activity there was nevertheless a continuous fluctuation of the membrane potential similar to that seen for the ventral hair cells, although transient deflections similar to synaptic junctional potentials were less evident. In contrast to the ventral hair cells, injections of small depolarizing pulses into the dorsal hair cells resulted in the production of spiking activity (Fig. 13), with the frequency of spikes correlated to the amplitude of the current pulse.

Electrical stimulation of the crista nerve (n. cristae minor) resulted in an antidromic potential in the hair cell soma (Fig. 14) appearing with a stimulus to response peak latency of some 2–3 ms. The potential was of constant size and latency for a given cell and would follow one to one high frequency stimulation. The small size of the potential

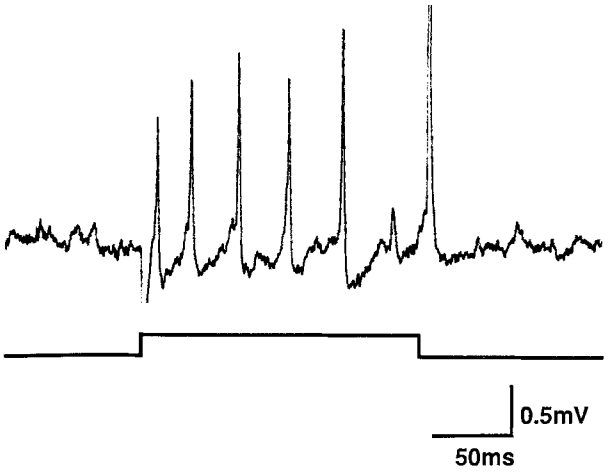


**Fig. 13.** Primary hair cell evoked activity. Intracellular recording from a primary hair cell, which had no spontaneous activity, showing its response to the injection of a small depolarizing current of 0.5 nA through the recording microelectrode. The lower trace shows time course of current injection. The initial stimulus artefact has been retouched to improve picture clarity

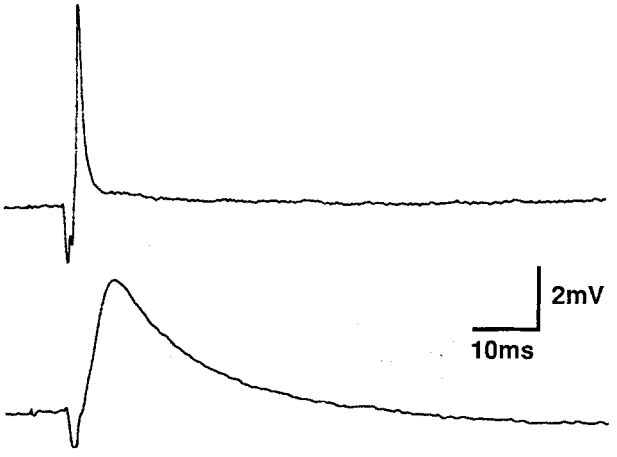


**Fig. 14.** Primary hair cell antidromic spike. Simultaneous intracellular recordings from a primary hair cell (upper trace) and a secondary hair cell (lower trace) showing the relative time courses and latencies of the primary hair cell antidromic spike and the secondary hair cell efferent depolarization. The initial downward deflection in both traces is the stimulus artefact

(1 to 10 mV) in comparison to the membrane resting potential indicates that the axon action potential does not actively invade the cell soma, i.e. the microelectrode recording site. It is not yet known whether the dorsal hair cells are electrically coupled in a manner similar to the outermost ventral hair cells but the fact that the antidromic potential did not change in size when the size of nerve shock was varied implies that the cells are not electrically coupled or that the dorsal hair cell axons have very similar stimulation thresholds and cannot be

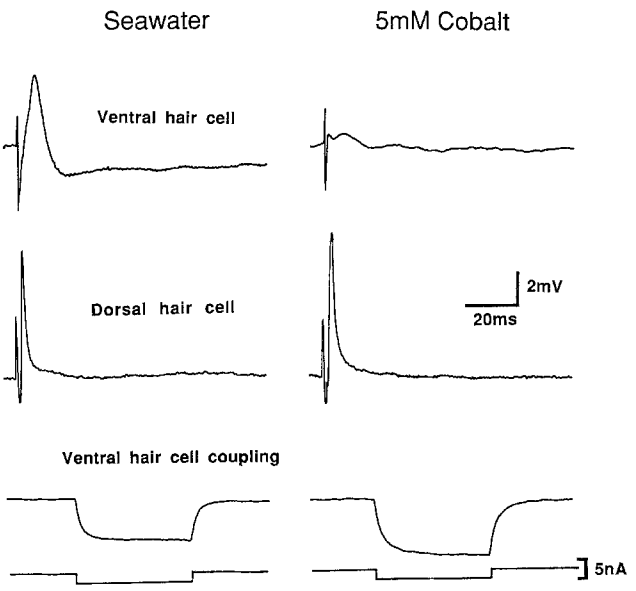


**Fig. 15.** Effect of cobalt on the primary and secondary hair cell responses. Upper traces show that bath application of cobalt blocks the efferent depolarization and hyperpolarization seen in the secondary hair cells. Middle traces show that the antidromic spike in the primary hair cells is not blocked by cobalt. It may even increase in size, indicating that cobalt is not interfering with axon conduction. Lower traces show that the electrotonic coupling between hair cells is not blocked, but enhanced, by the bath application of cobalt. This demonstrates that the reduced secondary hair cell response is not due to a decoupling of the cells by cobalt

separated by small changes in the stimulus parameters.

### *Effect of cadmium and cobalt on the evoked response*

Transmission at chemical synapses can often be blocked by high magnesium, low calcium salines. However marine animals, presumably because of the high calcium and magnesium content of seawater and blood (10 m*M* calcium and 55 m*M* magnesium), are often very resistant to this kind of treatment. Both cadmium (cf. Adams and Gillespie 1988) and cobalt (Detwiler and Alkon 1973; Budelmann and Bleckmann 1988) have been used as alternative blocking agents in marine animals. By gradually increasing the bath concentrations of cadmium and cobalt, in separate experiments, it was found that 1 m*M* cadmium or 5 m*M* cobalt would reduce and eventually abolish both the depolarization and the hyperpolarization evoked by electrical stimulation of the crista nerve (Fig. 15). The cobalt block could be reversed by washing (Fig. 16), but the cadmium block was much more persistent. Since the cobalt did not block the anti-

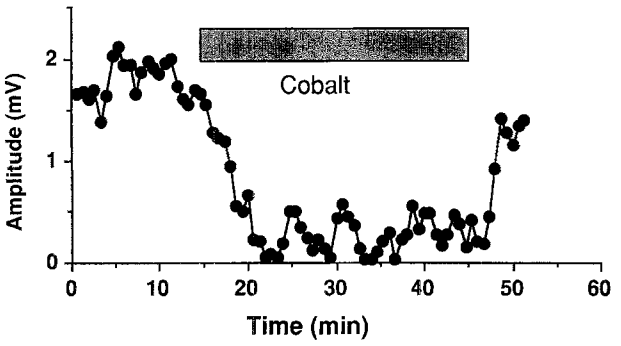


**Fig. 16.** Effect of cobalt on the efferent response. Graph showing the decrease in amplitude of an efferent evoked depolarizing response produced by the bath application of 5 m*M* cobalt

dromic spike in the dorsal hair cells, nor the electrotonic coupling between the ventral hair cells (Fig. 15), the effect is likely to be due to synaptic blockage rather than direct blockage of the efferent axon spike or a reduction in cell coupling. There was little or no change in membrane resting potentials after the application of cobalt or cadmium. The time course of the reduction in the size of the evoked depolarization by bath application of cobalt is shown in Fig. 16. In some experiments, there was an initial increase in the size of the evoked response after cobalt application; this was probably due to a change in the input resistance of the cells (see below). As shown in Fig. 16, and also in Fig. 15, a small residual depolarization often persisted. This could be abolished by prolonged exposure to this concentration of cobalt or by increasing the bath concentration to above 10 m*M* cobalt.

*Coupling of hair cells*

As already mentioned, the electrical coupling of the hair cells causes difficulties both in the manipulation of the cell membrane potentials and in the interpretation of the subsequent results. Clearly it would be useful to be able to uncouple the cells and then examine the efferent synaptic effects. Unfortunately, the agents which have so far been used to uncouple these cells (Williamson 1989a) also cause a block of axon spike transmission and thus block the effects of efferent stimulation. Neither cadmium nor cobalt were found to reduce the electrical coupling between ventral hair cells, indeed there appeared to be a small increase in coupling and also in the size of the dorsal hair cell antidromic spike (Fig. 15), probably due to a cobalt induced increase in cell input resistances.

*The afferent neurones*

As has already been found in the statocyst crista of the octopus (Budelmann et al. 1987), there appear to be two different types of afferent neurones in the squid crista. Afferent neurones with large somata that lie immediately below the hair cells of the crista strip (Fig. 2) and afferent neurones with smaller somata that lie ventral to, and just outside the crista strip hair cells (Fig. 2). The differences between these two types of neurones have not yet been examined. In order to avoid any possible confusion with the ventral hair cells, and because of the difficulties in penetrating the large afferent neurones, only recordings from afferent neurone somata some distance from the crista hair cells have been included. Penetration of these cells was performed under direct visual control and in some cases the cell was marked by iontophoretic injection on the dye Lucifer Yellow. The dye injected cells displayed well filled axons passing into the crista strip and then into the crista nerve. The dye did not appear to spread to any neighbouring cells, implying that there is no dye coupling between these cells.

Intracellular recordings from these cells ($n=17$) showed that they had membrane resting potentials similar to those of the ventral hair cells. However, unlike the dorsal or ventral hair cells these cells had relatively stable membrane potentials showing almost no potential fluctuations. As with the dorsal hair cells, intracellular injections of small depolarizing currents evoked trains of action potentials, similar to those shown in Fig. 13 for dorsal hair cells, whose frequency was related to the magnitude of the injected current.

Electrical stimulation of the crista nerve produced small depolarizations of constant latency and amplitude in the afferent neurone somata. These antidromic spikes were of very small amplitude, again indicating that the axon spike does not actively invade the cell soma, and appeared with a latency of about 3 to 4 ms. No spontaneous spike activity was seen in any of the afferent neurones penetrated. In some cases, the antidromic spike in the afferent neurone soma was followed by a subsequent depolarization and hyperpolarization (Fig. 17). As can be seen these had latencies and time courses similar to those of the depolarizing and hyperpolarizing potentials seen in the ventral hair cells. In other afferent neurones, however, the antidromic spike was followed by a hyperpolarization and a long duration small depolarization. In one cell (Fig. 18) it was possible by reducing the amplitude of the nerve shock, to obtain the hyper-

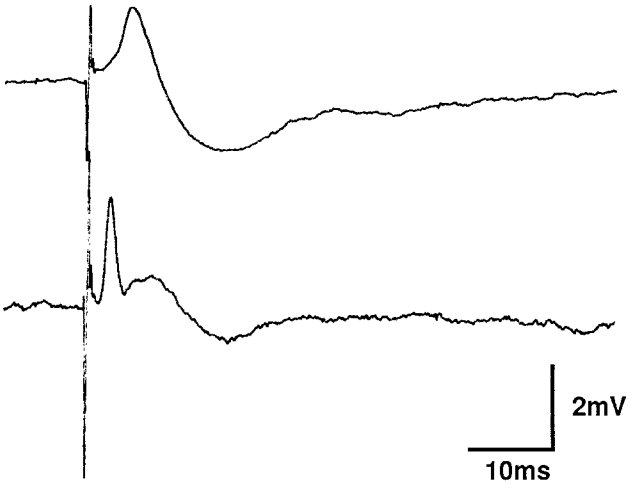


**Fig. 17.** Comparison of secondary hair cell and afferent neurone responses. Simultaneous intracellular recordings from a secondary hair cell (upper trace) and an afferent neurone (lower trace) in the anterior transverse crista section showing their responses to crista nerve (minor) stimulation. The afferent neurone displays a small, short latency antidromic spike after the stimulus artefact, followed by a depolarization and hyperpolarization. The time course of these responses are similar to the efferent responses in the secondary hair cells

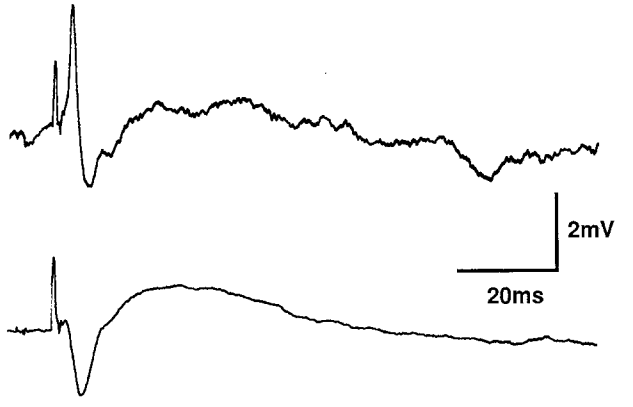


**Fig. 18.** Unusual efferent response in an afferent neurone. Upper trace is a single sweep recording from an afferent neurone showing the short latency antidromic spike, after the stimulus artefact, followed by the efferent response. In the lower trace, an average of 20 sweeps, the stimulus shock amplitude has been reduced such that the neurone no longer shows an antidromic spike. This reveals an initial hyperpolarization followed by a slower time course depolarization

polarization without the antidromic spike. These results imply that the afferent neurones also receive efferent inputs of both depolarizing and hyperpolarizing types.

## Discussion

### *Ventral hair cells*

These experiments have shown that the secondary hair cells of the squid statocyst crista receive depolarizing and hyperpolarizing efferent inputs. The long stimulus to response latencies of both types of input to the ventral hair cells, compared to the much shorter latencies for antidromic potentials in the dorsal hair cells and afferent neurones, imply that these effects are mediated via chemical synapses. This is supported by the fact that, at least in some cases, facilitation of the response was observed by applying pairs of stimulation pulses. The only other likely possibility is that the inputs are via electrotonic synapses and the different delays are due to different axon conduction velocities for the afferents and both populations of efferents. Although different conduction velocities may well be present, the fact that both types of efferent inputs on the ventral hair cells were blocked by bath application of cobalt, without blocking electrical coupling between the ventral hair cells or the antidromic potentials in the dorsal hair cells, makes it unlikely that these effects are due to efferent electrotonic synapses on the hair cells.

Although both depolarizing and hyperpolarizing evoked potentials were observed in individual hair cells, this cannot be taken as unequivocal evidence that single hair cells receive both types of synapses. The difficulty here is that the ventral hair cells are electrically coupled (Williamson 1989a) and so the synapses could be on neighbouring cells. Clearly this could be tested by uncoupling the cells whilst stimulating the efferents. Unfortunately, the uncoupling agents so far tested on this preparation (Williamson 1989a) also block the antidromic spike in the dorsal hair cells and so are likely to block the efferent action potentials as well. However, the relatively low space constant (about 50 μm) of the electrotonic coupling, together with the large proportion of hair cells showing both types of responses, implies that if the two types of synaptic inputs are on different hair cells, then these hair cells must be interspersed with each other in an almost alternating pattern. Given the electrotonic coupling and therefore the spread of any input, such an arrangement would seem unnecessarily complex and ineffective. In addition, there is morphological evidence from the octopus crista showing that individual hair cells receive two types of efferent synapses (Budelmann et al. 1987).

The differences in latencies between the depolarizing and hyperpolarizing evoked potentials could be due to differences in the conduction velocities of the two populations of efferents or to differences in the kinetics of their synaptic transmission. There is no evidence for a bimodal distribution of efferent axon diameters in octopus (Budelmann et al. 1987), but there is evidence that there

are two different efferent transmitters. Morphological and histochemical studies have identified both acetylcholine (ACh) (Auerbach and Budelmann 1986) and catecholamines (Budelmann and Bonn 1982) in the efferent terminals. In addition, recent recordings from the crista afferents in octopus have shown that ACh has an inhibitory action on the crista afferent activity that can be blocked by the ACh antagonist, gallamine, and that some catecholamines have an excitatory action on the afferent activity which can be blocked by the relevant antagonists (Williamson 1989b).

Another possibility to be considered is that the two types of efferent responses might have been due to differences in the cell membrane potentials in different cells around the reversal potential of a single synaptic input, i.e. some cells having resting potentials more positive than the reversal potential and so showing a depolarization, and other cells having resting potentials more negative than the reversal potential and so showing a hyperpolarization to the same synaptic input. However, this was seen not to be the case because the two types of synaptic responses had different reversal potentials, both responses were often seen in the same cell, and the responses had different latencies.

It is unlikely that all of the efferent fibres in the anterior transverse crista were activated by single shocks to any one pair of stimulating wires but this was nevertheless sufficient to produce an efferent response in all of the secondary hair cells examined. This indicates the very widespread innervation of the hair cells by the efferents, although electrotonic coupling will also play a role here. Since simultaneous recordings from several hair cells revealed that the balance of efferent inputs to different cells could be different, each hair cell is not innervated by every efferent fibre.

The source of the 'noisy' baselines in the recording from hair cells has not yet been identified. Although some of these small membrane potential deflections were very similar in their rise times and decay constants to synaptic junctional potentials, others seemed too frequent and of an unlikely shape to be synaptic. In addition, they were also seen in dorsal hair cells that did not show an evoked efferent response to nerve stimulation, but not in primary afferent neurones that did show evoked junctional potentials. It seems likely therefore, that some of these fluctuations were the result of changes in the hair cell receptor potential. This could be due to external vibrations reaching the preparation, despite vibration isolation precautions, or perhaps due to Brownian movement whose effects may be more evident in hair cells where the kinocilia are not bound to the cupula (Harris 1968). Nevertheless, some of the fluctuations are likely to have been synaptic in origin. The fact that they were not often identified in these experiments is probably due to the low input resistances of these cells (1–10 MΩ). Such conditions would require close apposition of the microelectrode tip to the synapse in order to observe the spontaneous junctional potentials.

With regard to the morphologies of the ventral hair cells it is worth highlighting the long processes seen in some Lucifer Yellow fills of ventral hair cells. Such long processes have not been identified previously either in vertebrate secondary hair cells or in cephalopod secondary hair cells, although some short processes, up to 8 μm in length, have been seen in secondary hair cells of the octopus macula (Budelmann and Thies 1977). Their function is unknown but clearly these could be responsible for the observation that electrical coupling along the entire row of outermost ventral hair cells is not abolished when a single cell in the row is damaged (Williamson 1989a). Thus the electrical contact might be via the long processes which span the space produced by the damaged cell. It remains to be seen whether these processes are associated with gap junction type structures.

*Dorsal hair cells*

These recordings represent the first single unit recordings of dorsal hair cell activity from cephalopods. Previous attempts to record their activity from the crista nerve (Williamson and Budelmann 1985, and unpublished) have been unsuccessful, probably due to the small size of their axons and hence the difficulty in obtaining a good signal-to-noise ratio. The similarity of activity levels obtained in different preparations is somewhat surprising as this would be expected to be dependent on the exact position of the cupula remnant in relation to the hair cell kinocilia and this would be unlikely to be identical in each of the 3 preparations shown in Fig. 12. It may be that regardless of the cupula position, the receptor potential adapts over a period of time to a set level and thus produces a relatively constant afferent firing frequency. Another possibility is that these discharges were due to depolarizations of the cells caused by microelectrode penetration damage. However, the cells which showed spontaneous levels of activity did not have significantly lower membrane resting potentials from those that showed no activity. The steady level of activity in these cells is of course reminiscent of the steady

discharge seen in some vertebrate vestibular afferents (Goldberg and Fernandez 1977).

The dorsal hair cells showed very little response to stimulation of the efferent fibres. Although it is possible that any efferent effects were synchronous with, and hence masked by, the antidromic spike this would imply that the dorsal cell efferents have a much higher conduction velocity than the ventral cell efferents or that the synaptic transmission is faster, perhaps electrotonic. It seems more probably that the dorsal hair cells receive fewer efferent inputs, at least on the cell soma where they would be detected in these recordings. Although Budelmann et al. (1987) state that the primary hair cells in octopus receive an efferent innervation they do not quantify this or compare it with the level of efferents to the secondary hair cells. One other possibility is that the efferents to the dorsal hair cells do not run in the minor crista nerve and are thus not activated by the electrical stimulation. Stephens and Young (1982) identified a small branch of the major crista nerve in *Loligo* which, additionally, innervates the anterior transverse crista section. However, there is no information on its target cells and this branch has not been identified in *Alloteuthis*.

It is not yet known whether the dorsal hair cells are also electrotonically coupled along a hair cell row and the small below threshold spikes seen in some of the hair cells (e.g. Fig. 14) could be interpreted as the electrotonic spread of above threshold spikes in neighbouring cells. However, against this view is the fact that the antidromic spike in the dorsal hair cell did not vary in size with the size of the nerve shock. This, taken together with the higher input resistances of these cells, as indicated by the very small currents needed to depolarize the cells above spike threshold, suggests that the level of coupling, if any, is much less than that found in the ventral hair cells.

*Afferent neurones*

Although recordings were obtained from only a few afferent neurone somata it is clear that these cells do receive an efferent innervation, both depolarizing and hyperpolarizing. However, there appears to be some difference between the innervation of these cells and that of the ventral hair cells. In all of the ventral cells where both depolarizing and hyperpolarizing efferent responses were obtained the depolarizing response always preceded the hyperpolarizing response. However, in at least one afferent neurone a fast hyperpolarization was followed by a slow, long duration depolarization (Fig. 18). The latencies of these responses are within the spread of times obtained for the ventral hair cell responses (Fig. 6) and other afferent neurones did show bipolar responses similar to those found in the ventral hair cells. Nevertheless, this is the only cell in which the hyperpolarization preceded the depolarization.

As with the dorsal hair cells, the constant size of the antidromic spike in response to varying the size of the nerve shock, and the higher cell input resistance, implies that the afferent neurones are not electrotonically coupled. It should be remembered however, that these recordings were made from the cell somata, probably some distance away from the spike initiating zone, and so a low level of coupling at the initial segment, where it would be most effective, might not be detected in the cell soma.

*Comparison with other hair cell systems*

Efferent systems have been found in the statocysts of a number of other molluscs, notably gastropods (Wolff 1970). The hair cells of these systems receive mainly inhibitory efferent inputs (Detwiler and Alkon 1973) although excitatory inputs have also been reported (Janse et al. 1988). However, there are notable differences between the gastropod and the cephalopod statocysts. Firstly, the gastropod statocyst hair cells are primary hair cells, the type of squid hair cells that did not show a response to efferent stimulation. Secondly, the statocyst is less complex than that of the squid, usually having no large array of polarized hair cells or a cupula structure. In addition the gastropod hair cell transduction mechanism may be different from that of cephalopods and vertebrates in that the cilia are reported to be motile (Stommel et al. 1980).

However, cephalopod statocysts are by no means uniform in structure, with the statocyst of *Nautilus* more like that of a gastropod than of a coleoid cephalopod (Young 1965). Even the octopus and squid differ in the arrangement and number of crista sections (Budelmann 1977). In this respect, it should be noted that stimulation of the efferents to the octopus crista resulted in a decrease in activity of 77% of the afferent neurones from the secondary hair cells and an increase in activity in only 16% (Williamson 1985), whereas the majority of the squid secondary hair cells showed an excitatory response to efferent stimulation. This could be due to the different recording sites, one from the hair cells and the other from the afferent neurones, or perhaps the longer time constant of the inhibitory response being more ef-

fective for trains of stimuli, as were used in the octopus experiments, or it may be a real species difference.

The small amount of data from vertebrate hair cell recordings clearly establishes an efferent inhibitory input onto the cells of the fish lateral line (Flock and Russell 1976) and the frog sacculus (Ashmore and Russel 1982). However, recordings from turtle cochlear hair cells (Art et al. 1984) show that the response to efferent stimulation consists of an initial depolarization followed by a slower hyperpolarization. In addition, recent work on isolated hair cells from the frog crista (Norris et al. 1988) has shown that the proposed efferent transmitter acetylcholine, can have both suppressive and facilitatory effects.

*Functional aspects*

These experiments have shown that there are inhibitory and excitatory efferent synapses onto the squid statocyst cells. Such a system can provide the animal with direct and independent control of both the hair cell receptor potential and the level of activity of the afferent neurones. Thus, not only can the gain of the overall system be increased or decreased, but that of the individual elements can also be altered. This permits an extension of the dynamic range of the system by allowing adjustments to the hair cells and afferent neurones, so that the cells are maintained within their operating ranges and maintained at their maximum sensitivities.

In circumstances where the efferents operate to block a particular afferent input, as has been proposed for the efferent feed-forward mechanism (e.g. Klinke and Galley 1974), this could be achieved by inhibiting the hair cells or the afferent neurones. Further, where the afferent discharge should nevertheless be maintained during a movement, perhaps to maintain trophic or baseline levels of activity, this could be achieved by inhibiting the hair cells but simultaneously exciting the afferent neurones. Alternatively, where an afferent input is to be transiently blocked, perhaps during an escape response, this could be done by inhibiting the afferent neurone but exciting the hair cell. Since the hair cells appear to have longer time constants than the afferent neurones, this would minimize the delay in re-establishing normal level of sensory input after the inhibition.

Another possibility is that one or both of the efferent transmitters could affect the level of electrotonic coupling between the hair cells. This would alter the response characteristics of the system by changing the hair cell input resistances and time constants, and hence the sensitivity and frequency response of the system. This hypothesis is supported by the finding that one of the efferent transmitters may be dopamine (Williamson 1989b). Dopamine is known to be involved in the control of electrotonic coupling in the receptor cells of the vertebrate retina (Teranishi et al. 1983).

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