J. exp. Biol. (1979), 80, 241–250
With 5 figures
Printed in Great Britain

EPITHELIAL CONDUCTION IN SALPS

II. THE ROLE OF NERVOUS AND NON-NERVOUS CONDUCTION SYSTEM INTERACTIONS IN THE CONTROL OF LOCOMOTION

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(*Received* 14 *August* 1978)

SUMMARY

1. The control of locomotion in *Salpa fusiformis* was studied by intracellular recordings from motor neurones and swimming muscles.

2. Regular, synaptically driven, volleys of action potentials were recorded from motor neurones. This pattern of activity was consistent with that expected from the waveform of the compound junctional potentials associated with contraction of the swimming muscles.

3. A second class of brain neurone was identified. These cells were synaptically driven. In some, their firing rate was increased while in others it was decreased by activity in an epithelial conduction system, the Outer Skin Pulse (OSP) system.

4. Cells of the outer epithelium were impaled and OSP's were recorded intracellularly as conventional action potentials. The records from many of these cells showed many depolarising synaptic potentials.

5. Numerous gap junctions were observed throughout the outer epithelial layer and several neuroepithelial synapses were found. The distribution of these synapses coincided with that of the epithelial cells from which synaptic events were recorded.

INTRODUCTION

Salps are transparent pelagic tunicates which swim by jet propulsion, ejecting water from their tubular bodies by contraction of the circular muscle bands of the body wall. Their locomotory behaviour is complex (Fedele, 1923, 1933*a*,*b*). They can swim either forwards or backwards, and the direction and velocity of locomotion can be changed by appropriate stimuli. More recently, Mackie & Bone (1977) studied the electrical correlates of muscular and epithelial activity during salp locomotion. They showed that the muscle bands of the body wall are composed of parallel flattened fibres which are not morphologically or electrically coupled to one another but, as was shown histologically (Bone & Ryan, 1973), are multiply innervated. These muscle

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fibres do not conduct action potentials. Instead, contraction is associated with multiple events which were interpreted as summated neuromuscular junctional potentials. The locomotory rhythm of salps, which originates in the brain, can be modified by activity in some of the epithelial conduction systems present in these animals.

We have extended and confirmed many of these observations and our results, which are chiefly based on intracellular recordings, show that muscular contraction is evoked by regular short bursts of activity in motor neurones and that these produce the summating neuromuscular events described by Mackie & Bone (1977). Furthermore, we have found that activity in an epithelial conduction system can modify the firing pattern of certain brain neurones and at the same time change the locomotory rhythm. Finally, we have obtained good electrophysiological and histological evidence to suggest that nervous activity can activate one of the epithelial conduction systems.

MATERIALS AND METHODS

Blastozooids of *Salpa fusiformis* (Cuvier) were obtained from the plankton in the Rade de Villefranche, France, during the months of March–May 1977 and were kept in a 14 °C cold room in large bowls of sea water. For recording, individual specimens were transferred to a Sylgard-lined Petri dish containing sea water. The animal was opened with a mid-ventral cut and pinned down with the interior of the cylinder uppermost. The inner surface of the animal is lined with the inner or atrial epithelium. This was usually removed to facilitate recording, but great care had to be taken to prevent tissue damage. After the epithelium had been removed, the animal was immobilized with fine insect pins and cactus spines. Under these conditions, rhythmic swimming activity would continue for many hours.

Intracellular recordings were obtained using 3 M-KCl filled 30-40 MΩ electrodes connected to a F.E.T. amplifier. Signals from this, and from a fine polyethylene suction electrode used for extracellular recordings, were displayed on a Tektronix 5103N Storage Oscilloscope or a Gould Brush pen recorder. *En passant* recordings from nerve bundles were obtained using a broken, but fire polished, glass microelectrode connected to a syringe by a plastic tubing adaptor (Clay Adams). Electrical stimuli, supplied by a Grass S48 stimulator, were applied through a second plastic or glass suction electrode. All experiments were conducted at room temperature (20-21 °C).

For electron microscopy, specimens were fixed in 5% glutaraldehyde in cacodylate buffer. After 15 min the animal was opened and the brain was removed with part of the gill. This was then fixed for a further 75 min, rinsed in buffer, post-osmicated in 1% buffered osmium, dehydrated in alcohol and embedded in Epon. Thick sections (0·5 μm) stained with Richardson's stain (Richardson, Jarett & Finke, 1960) were used for light microscopy while thin sections were stained with lead citrate and uranyl acetate and examined in a Philips EM300.

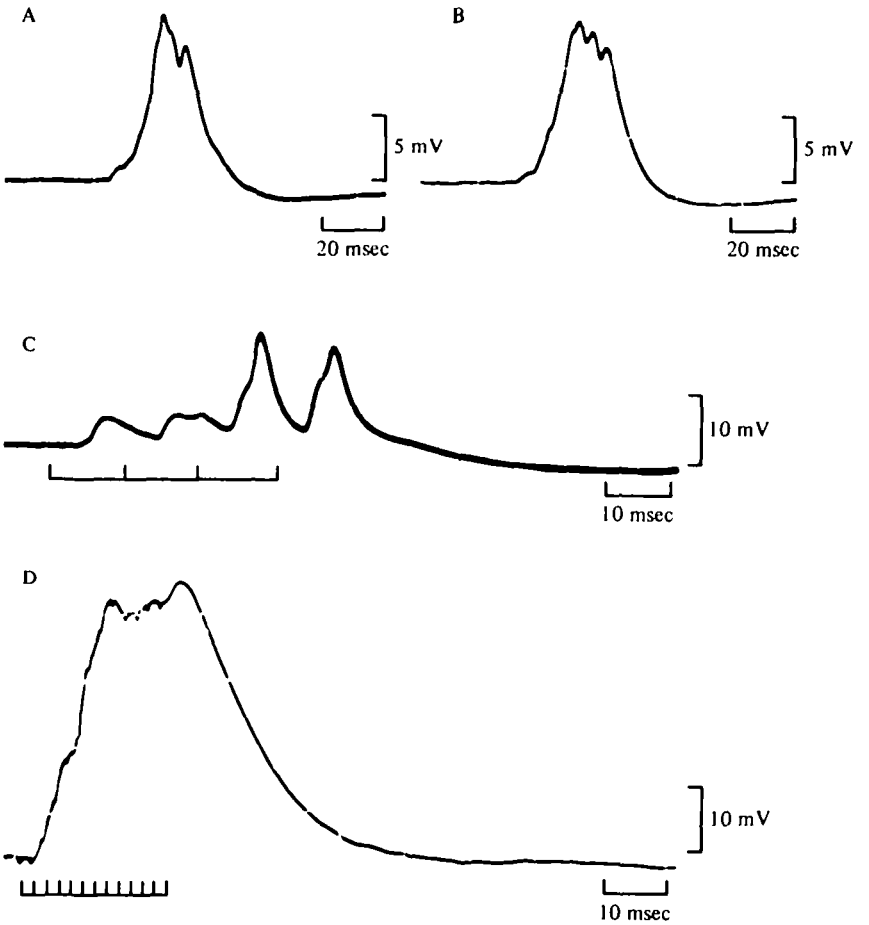

Fig. 1. (A, B) Intracellular records of two compound junctional potentials recorded from a lip muscle band. (C, D). Junctional potentials recorded intracellularly from a lip muscle and evoked by extracellular stimulation of a nerve bundle. Low frequency stimulation (C) evokes non-summating potentials while high frequency simulation (D) evokes summating events. In each record the application of each stimulus is marked on the lower line.

RESULTS

The morphology of the *Salpa fusiformis* blastozooid is well documented (see Berrill, 1950). The animal is basically a cylinder which opens to the exterior through terminal inhalent and exhalent apertures. It is extremely transparent and the seven muscle bands are very obvious. The brain is located above and slightly anterior to the dorsal end of the gill and is easily identified by the large black/brown ocellus on its dorsal surface. Nerves radiate from the brain in a spoke-like array and even the finest branches can be traced throughout the animal. These features make salps attractive preparations for electrophysiological study.

Control of locomotion

The observation that contraction of swimming muscle is accompanied by what appear to be summating neuromuscular junctional potentials (Mackie & Bone, 1977)

implies that the form of the multiple events that accompany each contraction might differ from one contraction to the next, depending on the pattern of activity in the motor neurone. Such changes in shape clearly do occur (Fig. 1 A, B). To confirm that the form of any one compound event is a consequence of the pattern of activity in the motor neurone a lip muscle was impaled, and the nerve bundle leading to it was stimulated extracellularly. Low frequency stimulation evoked non-summating junctional potentials (Fig. 1 C), while summating events which mimic those recorded during normal swimming were evoked by high frequency stimuli (Fig. 1 D).

These results imply that the normal pattern of activity in motor neurones is one of regular high-frequency bursts of impulses but that the properties of successive bursts can change. *En passant* recordings from nerve bundles are characterized by regular high frequency volleys of impulses (Fig. 2 A). Each volley is composed of a mixed population of impulses with individual units reappearing regularly throughout each volley. The number and frequency of impulses in a volley can change from one volley to the next.

Similar patterns of activity can also be recorded from cells in the brain. Fig. 2 B, C are examples of activity recorded intracellularly from a cell located at the edge of the brain, adjacent to the point of exit of a nerve bundle. This cell produced regular bursts of 11–24 action potentials every 2–4 s. This pattern of activity closely resembles the pattern with which junctional potentials are recorded from contracting muscle even in the degree of variation observed, and since the muscles did indeed contract during these bursts, it is concluded that the impaled cell was a motor neurone.

The bursts of action potentials in the motor neurones are evoked synaptically. The action potentials are triggered from the peak of a 20–30 mV depolarization which develops from an otherwise stable baseline that shows no sign of oscillatory activity. During the interval between the bursts of action potentials, single or paired synaptically triggered action potentials may be observed (Fig. 2 C). The EPSP's which evoke these 'out of sequence' action potentials are augmented if the cell is artificially hyperpolarized, suggesting that they may be mediated by chemical synapses.

It is not clear whether the EPSP's which evoke the 'out of sequence' action potentials are the same as those responsible for the large depolarizaion which underlies the bursts. Furthermore, it is not clear whether a pair of action potentials was evoked in all motor neurones in the brain simultaneously with those in Fig. 2 C, or whether certain cells can receive independent input and therefore produce action potentials independently of other motor neurones.

It has been shown previously (Mackie & Bone, 1977) that during normal anterior swimming all the body muscles contract simultaneously while those that control the anterior lips contract a few ms earlier and so direct the ejected water posteriorly. The reverse occurs during posterior swimming with the posterior lip muscle contracting first. These observations suggest that the motor neurones which control the main body muscles must be activated synchronously while those that control the lip muscles can be activated slightly earlier. All evidence obtained here confirms this. Analysis of long-term intracellular recordings from swimming muscle, however, clearly shows that in addition to the regular bursts of summating potentials, solitary or small numbers of junctional potentials are recorded but are not associated with an observable con-

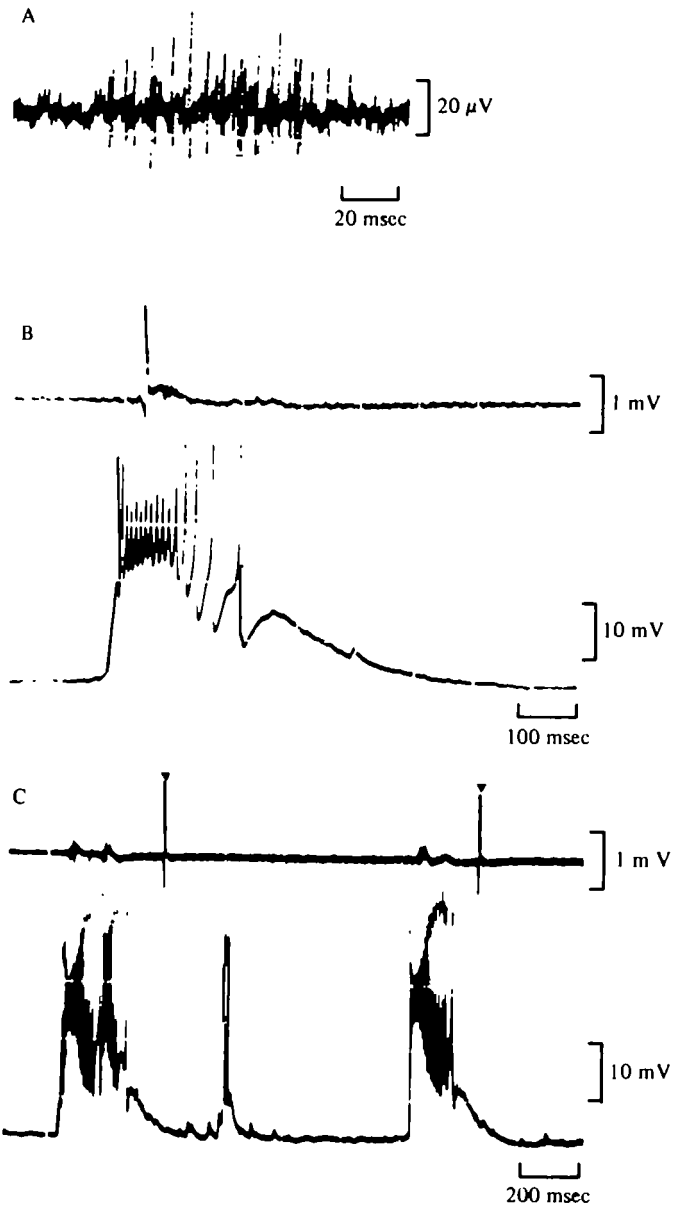

Fig. 2. (A) Extracellular *en passant* recording of a volley of impulses in a nerve bundle near a muscle band. (B, C) Upper trace: extracellular record of electrical activity associated with contraction of a muscle band. Lower trace: simultaneous intracellular record from a motor-neurone in the brain. The two OSP's (dark triangles) in C were evoked by mechanical stimuli from the restraining insect pins. Some of the records have been retouched for clarity.

traction. These JP's may well correlate with the 'out of sequence' action potentials described for motor neurones, but their function remains unclear.

Intracellular records from motor neurones were obtained on only two occasions. Essentially similar records were obtained on other occasions, although impalement was only partial and action potential amplitudes were much lower. Similar records

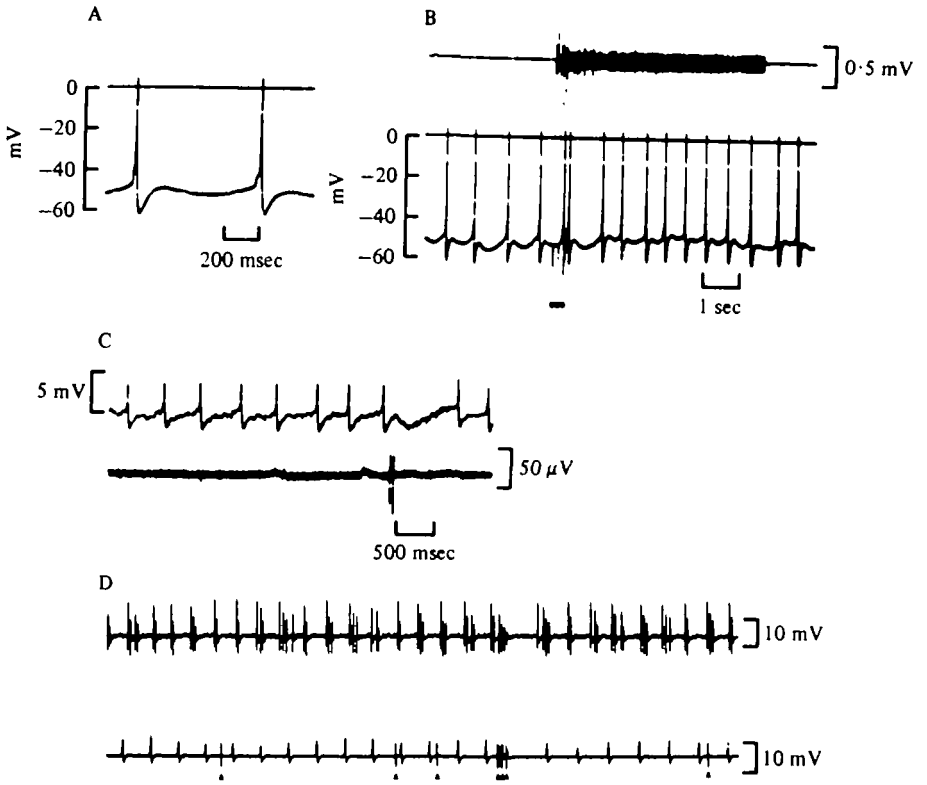

Fig. 3. (A) Intracellular record of action potentials in a brain cell. Note the small prepotential that precedes each action potential. (B) Lower trace: part of a series of action potentials recorded from the same cell as those in A. Upper trace: extracellular record of a burst of OSP's evoked by three electrical stimuli (dots). Note the change in the firing pattern of the brain cell during the OSP burst. (C). Upper trace: intracellular record from a partially impaled brain cell. Lower trace: extracellular record of two OSP's evoked by mechanical stimulation of the epithelium. (D) Lower trace: intracellular recording from a partially impaled brain cell. Upper trace: simultaneous extracellular record of electrical activity in a lip muscle. OSP's recorded by the intracellular electrode are marked with a dark triangle.

were also obtained by Mackie & Bone (1977) from an isolated brain preparation although, once again, the resting potential was low and the recordings were interpreted as partial or proximity impalements.

A second type of brain cell was impaled more regularly. These cells which were distributed seemingly at random around the brain fired with a regular rhythm of single action potentials at frequencies of 0·5-2·0/s, but the firing pattern was never sufficiently regular for the cells to be termed pacemakers. Indeed, in many instances small pre-potentials appeared to trigger the action potentials suggesting that the cells are synaptically driven (Fig. 3A).

Frequently the firing pattern of many of these cells could be modified by stimuli which evoked action potentials in cells of the outer epithelial layer (outer skin pulses or OSP's). Such stimuli sometimes increased the firing rate of the cells while at other times, in different cells, they decreased it. The record presented in Fig. 3B was obtained from a cell with a resting potential of -51 mV which fired regularly with an interspike interval of approximately 800 ms. Three electrical stimuli were then applied

to the animal at a point approximately 1 cm from the brain. These stimuli evoked a 5·5 s burst of skin pulses which, from analysis of their waveform and conduction field, were later identified as OSP's. During the burst of OSP's, the cell depolarized by 4 mV and the interspike interval decreased to an average of 550 ms ($n = 7$). At the end of the burst the cell repolarized and the firing rate returned to its previous level.

Another effect of OSP's is illustrated in Fig. 3C. This cell was only partially impaled, but while the resting potential was low a clear firing pattern was evident. When two OSP's were evoked by mechanical stimulation of the epithelium, a brief hyperpolarization and interruption of the firing rhythm occurred. Stimuli which evoked only a single OSP had no effect on the firing pattern of this cell. Similar results are also presented in Fig. 3D although here the pattern of muscle contraction recorded extracellularly from a lip muscle is also displayed. Clearly, a single OSP has little observable effect on either the firing thythm of the neurone or the locomotory rhythm, while a burst of OSP's interrupts both the firing pattern of the cell and the locomotory rhythm. Mackie (unpublished) has noted that in chains of young salps, single OSP's can effectively modify locomotion, suggesting perhaps that the degree of sensitivity to such events changes as the animal matures.

We have not proved whether the above-mentioned effects of OSP's are indeed the action of OSP's on the brain or some other coincidental effect of the applied stimuli. Since the effect of the stimuli can outlast the duration of that stimulus but not the duration of the evoked OSP burst (Fig. 3B), however, it can be assumed that the observed effects are being mediated by the OSP system itself.

The OSP system and its neural input

The OSP system has been extensively studied at the extracellular level, but only a few momentary intracellular records have been obtained (Mackie & Bone, 1977), probably because the epithelium is too thin to support impalement. We have now obtained stable intracellular recordings from cells of the OSP system adjacent to the brain; more specifically, from cells located between the nerve bundles which radiate from the brain. These cells have resting potentials of up to −80 mV and they could be impaled for up to 15 min. OSP's evoked by electrical or mechanical stimulation are recorded as conventional (90 mV, 20 ms) overshooting action potentials (Fig. 4A). The actual classification of these impulses as OSPs was made by determining their conduction field and comparing it with those of the several skin pulse systems described by Mackie & Bone (1977). When the OSP system was stimulated mechanically, it tended to fire repetitively (Fig. 4A) and during the resulting burst the amplitude and duration of the action potentials increased slightly. This effect was not as dramatic as in the OSP bursts recorded from embryonic tissue (Anderson, 1979).

In many records from these OSP cells, large numbers of small (2–4 mV) summating EPSP-like events were observed (Fig. 4B). Despite reaching 10 mV or more in amplitude, these summating events never triggered action potentials. These EPSP-like events were recorded from only a proportion of the impaled cells; most of our records were completely devoid of any electrical activity other than evoked OSP's.

The morphological relationship between the OSP epithelium and the brain is illustrated in Fig. 5A. The brain is circular in cross section and has a diameter of 200–

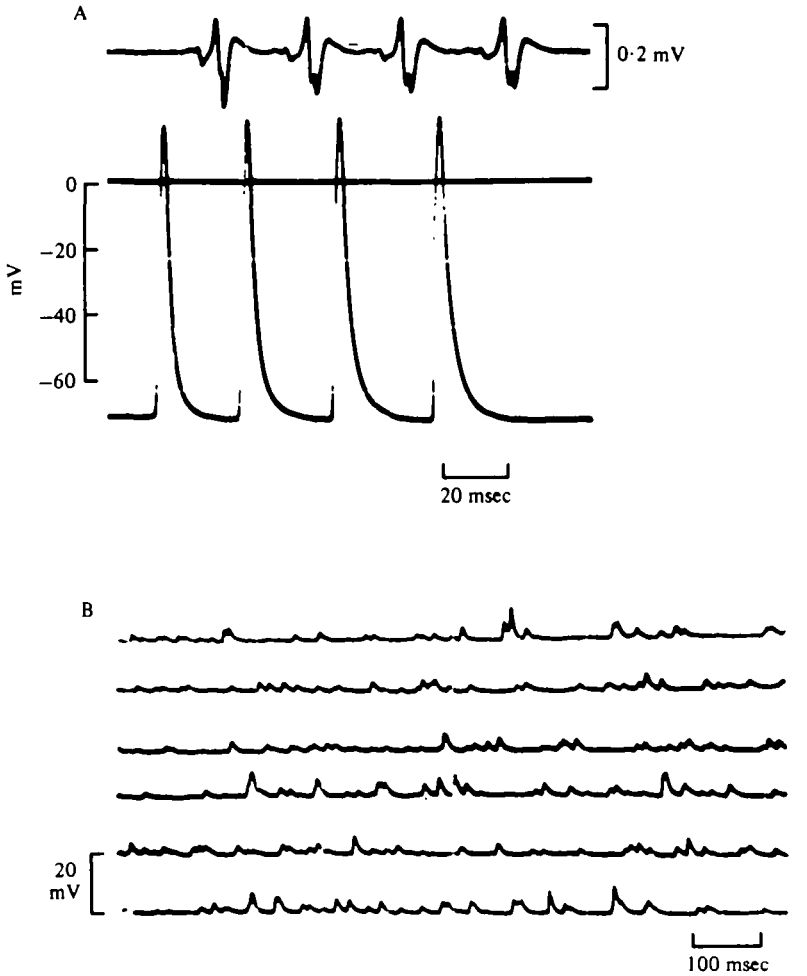

Fig. 4. (A) Upper trace: extracellular record of four OSP's that were evoked by mechanical stimulation of the epithelium of the scalp. Lower trace: the same OSP's recorded intracellularly from an epithelial cell adjacent to the brain.

250 μm. The neuronal soma are distributed around the periphery while the centre is occupied by an extensive neuropile. The outer epithelium covers the dorsal surface of the brain. At this point, the epithelium is 2·5–5 μm thick and is composed of a single layer of cells which often contain a large vacuole. The cells frequently overlap one another and the boundaries between them are often very convoluted. Well developed gap junctions (Fig. 5 B) occur throughout the epithelium, and several may be found between any two cells particularly in the convoluted areas. The outer margins of the epithelial cells are connected by what appear to be tight junctions.

Over the dorsal surface of the brain the gap between the epithelium and the brain is of the order of 0·5–2 μm wide. At the equator of the brain the epithelium separates away and extends circumferentially around the animal, becoming substantially thinner as it does so. This thinning of the epithelium explains the difficulty of impaling OSP cells in areas away from the brain.

The nerve bundles which radiate from the brain also exit at the equator and run parallel to the outer epithelium on its ventral surface. At several points along a given nerve bundle, well defined neuroepithelial synapses occur (Fig. 5 C). These synapses are characterized by 50–60 nm vesicles and by electron-dense membrane specializations. Since there is no evidence that axons terminate at these points, it is assumed that these synapses are of the *en passant* type. The fact that only a proportion of the epithelial cells is innervated correlates well with what is known about the physiological properties of these synapses, since EPSP's were recorded from only a proportion of the impaled OSP cells around the brain.

DISCUSSION

We have shown in this paper that the earlier view (Mackie & Bone, 1977) of salp muscle fibres as non-propagating fibres which are driven by volleys of impulses in motor neurones is correct and that the motor neurones are synaptically driven and not intrinsically oscillatory. We have also described a second population of neurones which display a marked sensitivity to activity in the OSP system. These cells are driven synaptically and fire at a frequency which, although not identical to that of swimming, is very similar. Furthermore, when the firing pattern of these cells is interrupted by OSP's (Fig. 3 D) the manner by which it is interrupted is very similar to that by which the locomotory rhythm is interrupted. For these two reasons it is not unreasonable to assume that these neurones may play some part in generating the swimming rhythm of the animal, although their exact role is uncertain.

The nature of the circuits responsible for generating the swimming rhythm is not known. Clearly, the output must be regular and capable of depolarizing the motor neurones adequately. Furthermore, some provision to account for the accurate timing of the contraction of different muscle bands must be present. It is not certain, however, whether the various requirements are met by a single circuit, or whether there are separate circuits for forward and reverse swimming. Mackie & Bone (1977) showed that anterior and reverse swimming differ in several parameters; namely, the sequence of muscle band contraction, the frequency of locomotion and the force of individual swim beats. It is conceivable that such variations in overall output could be met by a single neuronal circuit, but equally possible is the presence of two separate circuits which are activated by the appropriate stimuli. Whichever the case, transition from forward to reverse swimming could well be accompanied by several changes in the activities of the neurones which compose the circuits. Such changes might include increases or decreases in firing frequency. For this reason, the variable effects of OSP'S on brain cells of the second class is not unexpected, particularly since we have shown that OSP's can interrupt locomotion (Fig. 3 D) and Mackie & Bone (1977) found that they could reverse the direction of locomotion of an intact salp by evoking OSP's.

The site of the interactions between the OSP system and the brain neurones is uncertain. The manner by which the outer epithelium envelops the brain (Fig. 5 A) presents the possibility that the interaction is central. However, since the epithelium can be removed from the surface of the brain without affecting the ability of OSP's to modify brain cell activity, a more likely possibility is that the interaction is peripheral. In *Oikopleura* paired sensory structures on the trunk are connected with axons of

central cells. These axons are also joined by gap junctions to the epithelial cells that form a skin pulse system in this animal (Bone & Ryan, 1979). It is very likely that in *Salpa fusiformis* a similar relationship occurs and that the OSP system acts similarly as an extension of the sensory field of peripheral receptors.

Perhaps the most novel observations reported here are those of the neuro-epithelial synapses located around the brain. Morphologically these synapses appear to be efferent (i.e. from neurone to epithelial cell) and this is confirmed by intracellular recordings of synaptic events from OSP cells from the same location. While we have not been able to demonstrate propagated synaptically evoked OSP's, it is not unreasonable to assume that these synapses do function in the generation of OSP's.

Kater, Rued & Murphy (1978) have recently shown that salivary glands of the mollusc *Helisoma* conduct action potentials which can be triggered synaptically. However, the OSP system appears to function as a means of communication rather than an effector system, so the function of any synaptically evoked OSP's is uncertain, particularly since they would presumably re-enter the brain through the axons of nearby sensory cells.

All our studies were conducted on solitary blastozooids, and in nature these exist in long chains connected by specialized junctions, and the swimming activities of the individuals in a chain show high levels of co-ordination. It is possible that the role of the neuro-epithelial synapses may be found in the co-ordination of the activities of blastozooids in such chains, rather than in the activity of isolated individuals, and it would be interesting to extend this study to encompass events within chains.

This study was carried out in the Station Zoologique, Villefranche-sur-mer, France. The authors are very indebted to the Director, P. Bougis, and his staff for their hospitality and assistance.

One of us (G. O. M.) visited France under the Scientific Exchange Program between Canada and France. Other travel and operating costs were met by funding from the National Research Council of Canada.

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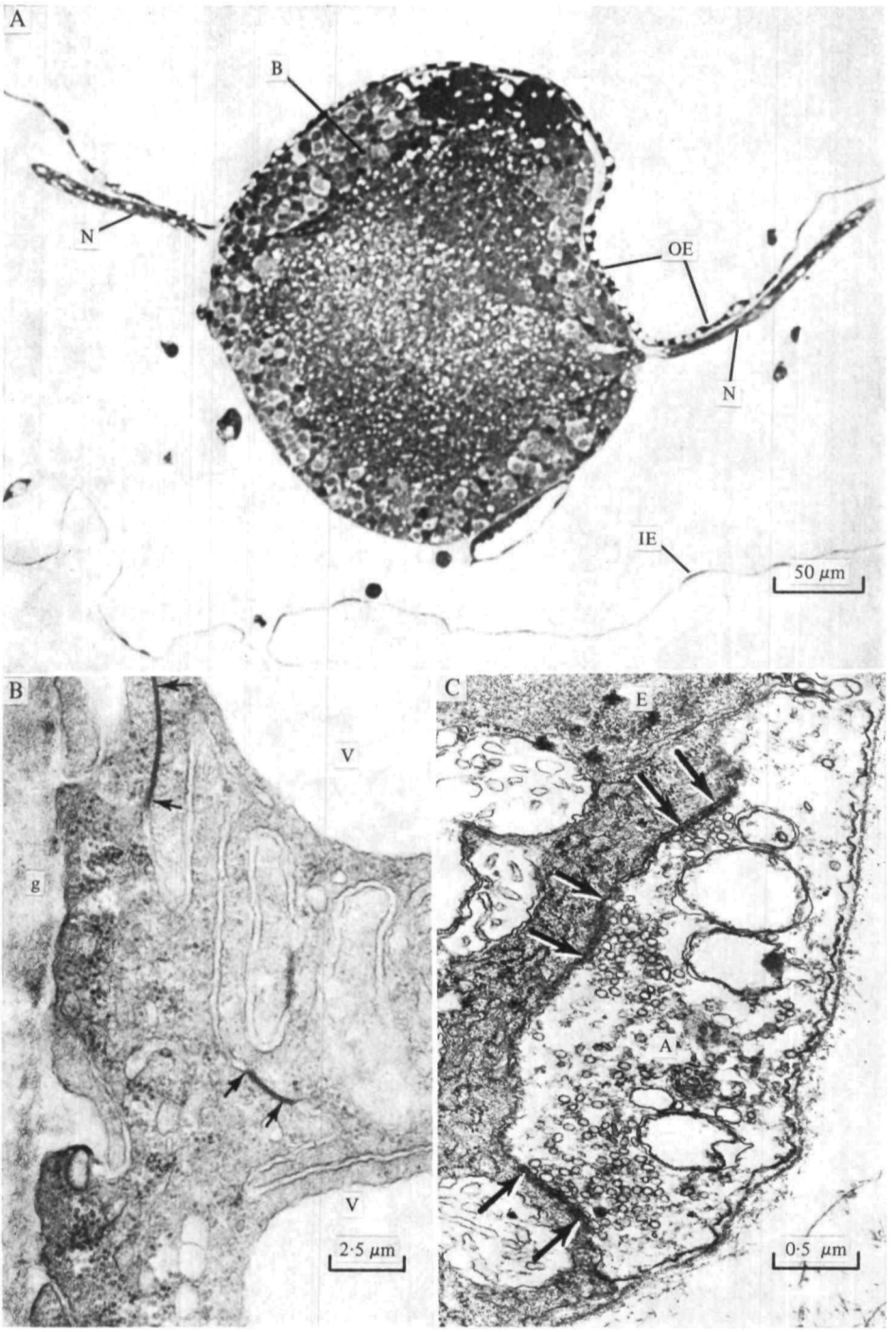

Fig. 5. (A) Low power micrograph of a transverse section through the brain of *Salpa fusiformis* showing the organization of the brain and the relationship between the brain (B) and the inner (IE) and outer (OE) epithelial layers. N, nerve bundle. (B). An electromicrograph of part of the epithelium around the brain illustrating the convoluted nature of the cell boundaries and the numerous gap junctions (arrows) that connect the cells. g, Cleft between brain and epithelium; V, vacuole. (C) Synapses (arrows) between an axon (A) and a cell (E) of the outer epithelial layer close to the brain.