CENTRAL NEURONAL RESPONSE TO THE ACTIVATION OF OSMORECEPTORS IN THE OSPHRADIUM OF APLYNIA

BY J. STINNAKRE AND L. TAUC

Laboratoire de Neurophysiologie Cellulaire,
Centre d'Études de Physiologie Nerveuse
du C.N.R.S., Paris, France

and Institut de Biologie marine, Arcachon, France*

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The physiology of osphradium (the pallial organ situated near the branchiae in most mollusca with the exception of the higher cephalopoda) is poorly known. Since the work of Copeland (1918) on Busycon canaliculatum, it has been generally accepted that the osphradium serves a chemoreceptive function. This conception has been shared by Henschel (1932), Brock (1936), Brown & Noble (1960) Michelson (1960) and Wolper (1950); all of whom reached this conclusion by means of experiments in which behavioural responses to a variety of stimuli were observed before and after the removal of the osphradium.

A different and more direct approach was attempted by Bailey & Laverack (1963, 1966) on the carnivorous gastropod Buccinum undatum. The osphradium of this animal was put in contact with mussel extracts or with solutions of different amino-acids, and excitatory and inhibitory responses to this stimulation were measured in single cells of the central ganglia. It was concluded from this work that the osphradium is used by these carnivorous gastropods in the detection of their prey. Such an hypothesis does not seem tenable, however, for a non-carnivorous gastropod such as Aplysia. Frings & Frings (1965) have shown that whereas the buccal and tentacular sensory organs were involved in the localization and identification of food (seaweed), the osphradium did not appear to be engaged in this activity; a selective destruction of the osphradium by cauterization did not modify the response of the animal to the presentation of food.

In the present study we have endeavoured, using isolated preparation, to analyse the responses of identified cells of the visceral ganglion to different chemical and physical stimuli applied to the osphradium in Aplysia. Evidence has been found for the presence of receptors which are sensitive to the diminution of osmotic pressure of the medium bathing the osphradium. Excitation of these osmoreceptors produces in an identifiable cell the phenomenon of inhibition of long duration, which phenomenon had been evoked in earlier studies by stimulation of certain nerves afferent to the ganglion, especially by the stimulation of the branchial nerve which joins the visceral ganglion to the osphradium (Tauc, 1958). A preliminary note of the work reported below has already been published (Stinnakre & Tauc, 1966).

*Correspondence to: Laboratoire de Neurophysiologie Cellulaire, 4, avenue Gordon Bennett, Paris 16e France.
MATERIAL AND METHODS

In *Aplysia* the osphradium is connected to the visceral ganglion by the branchial nerve. This nerve has two additional branches: one making contact with the branchial ganglion, the other with the purple gland (see Fig. 1). The osphradium is a sort of yellowish circular depression of 1–3 mm. in diameter situated in the pallial cavity and covered by a ciliated epithelium. According to Merton (1920) sensory cells which are in relation with the underlying osphradial ganglion send prolongations between the epithelial cells and reach the periphery (Fig. 2).

Fig. 1. Dorsal view of visceral ganglion and osphradium (*Aplysia cervina*, from MacFarland, 1909): *a2*, pericardial nerve; *a3*, genial nerve; *a4*, siphonal nerve; *ki.g.*, branchial ganglion; *ki.n.*, distal part of the branchial nerve; *pa.s.b.a.g.*, *pa.s.p.g.*, visceral ganglion; *s2*, branchial nerve; *v.schl.*, connectives; *s4*, nerve of spermatheca; *osph.g.*, osphradial ganglion; *s11*, vulvar nerve; *s2a*, nerve of the purple gland.

The data presented below were gathered from *Aplysia depilans* and *A. fasciata* from the French coast and *A. californica* from the California coast. The dissection of the osphradium and of the accompanying parts of the nervous system was performed in sea water. The final isolated preparation included the following components: (1) the osphradium, accompanied by a large piece of surrounding skin; (2) the branchial nerve; (3) the visceral ganglion. In order to limit the sensitive zone to the osphradium, all visible nerves branching from the main trunk were severed; particularly, we cut the nerve which joints the branchial ganglion to the branchial nerve. This was an important condition because the branchial ganglion exerts a relatively constant inhibitory influence on R15 (Fig. 4).
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Fig. 2. Transverse section of the osphradium and the osphradial ganglion (*Aplysia punctata*, from Merton, 1920). *cts*, cuticle; *gls*, ganglion cell; *drz*, glandular cell; *kbz*, conjunctive cell; *epz*, epithelial cell; *mf*, muscle fibre; *fiz*, ciliary cell; *se*, sensory ending; *sx*, sensory cell.

Fig. 3. Schematic diagram representing the external chamber with its two compartments *A* and *B* and the recording and polarizing systems.
The preparation was placed in an experimental chamber of two watertight divisions $A$ and $B$ (Fig. 3). The visceral ganglion was attached to the paraffin-covered floor of division $A$. The branchial nerve was passed through a slot in the adjustable divider separating the two divisions of the chamber and the skin surrounding the osphradium was gently stretched on the divider. Division $B$ is a movable compartment and the wall of this compartment which faces the divider between $A$ and $B$ contains a circular opening having a rubber border. Thus, it was possible to press the division $B$ against the skin surrounding the osphradium, sealing the latter to the divider. In this way the external surface of the osphradium faced the division $B$ through the rubber ring and the internal surface faced the division $A$ through the slot of the divider. In order to prevent a leakage or a contamination of liquid, this slot and the junctions of the adjustable parts of the chamber were coated with petroleum jelly. Consequently, the liquid contained in compartment $B$ was unable to pass into compartment $A$ and mix with the liquid (sea water) which bathed the visceral ganglion, the branchial nerve and the internal part of the osphradium.

When extracellular recordings were made, chloridized silver electrodes were used. Capillary microelectrodes filled with KCl or K$_2$SO$_4$ were used for intracellular recording. A second intracellular electrode was frequently used to pre-set the membrane potential to any desired level.

The recording system was conventional (Fig. 3), including a high-impedance amplifier (unitary gain), an oscilloscope (Tektronix 502) and an oscillograph (Brush Mk 280).

The experiments were performed at the ambient temperature (20–25° C.).

RESULTS

When extracellular recordings were taken of the activity of the branchial nerve, no modifications were detected in this activity as a function of stimulation of the osphradium. These negative results might be explained by the presence of excessive background activity of a limited number of fibres.

In order to obtain a more sensitive measure, intracellular recordings of individual identifiable cells of the visceral ganglion were made. Of the many neurones tested, one showed clear modifications of activity following the stimulation of the osphradium. The cell body of this neurone is situated in the right caudal quadrant of the dorsal part of the visceral ganglion, slightly to the left of the point of origin of the branchial nerve. Axonal branches of it can be found in the pericardial nerve and in the nerve going to the spermatheca ($a_3 s_4$ in Fig. 1). Its diameter varies between 300 and 400 microns, and it can be distinguished from the neighbouring neurones by its peculiar grey-orange pigmentation. This neurone has been variously labelled by previous authors: 'Br', by Arvanitaki & Chalazonitis (1958); parabolic burster (BR), by Strumwasser (1965), 'Oberon' by us (1966) and, most recently, R15 by Frazier et al. (1967). Having regard to the relatively thorough nature of the identification of the visceral ganglion cells made by these latter authors, we have decided to accept their nomenclature in preference to that of the earlier authors.

The rather particular pattern of spontaneous activity shown by R15 has been frequently described (Arvanitaki & Chalazonitis, 1958; Strumwasser, 1965; Alving,
Central neuronal response to the activation of osmoreceptors. It consists of repeated trains of spikes (so-called bursts) separated by prominent hyperpolarizing waves. The rate of firing during the burst shows a parabolic pattern (the spikes first increasing in frequency, reaching a maximum rate, and then decreasing in frequency). The interburst hyperpolarization gives way to a slowly developing depolarization which culminates in the next parabolic burst of spikes. The endogeneous origin of the spontaneous activity pattern shown by this cell has been rather convincingly established (Strumwasser, 1965; Alving, 1968).

Fig. 4. Intracellular records of spontaneous activity of R15: (A) in which, differing from the usual preparation, contact was maintained between the branchial ganglion and the rest of the preparation; (B) after contact between the branchial ganglion and the branchial nerve was severed; (C) after cutting the branchial nerve between the visceral ganglion and the osphradium; an arrow indicates the time of the section. On the three records can be seen spontaneous inhibitions of long duration (graphic records).

But this 'typical' activity of R15 was observed in the isolated ganglion. In the preparation described above, it is seen only if the connexion to the branchial ganglion is severed (Fig. 4). When the connexion is intact, complex variations in the membrane potential of R15 are observed and the spike activity is rather irregular (fig. 4A). On the other hand, when the branchial nerve is cut beyond the osphradium, the burst activity is clearly seen (Fig. 4B). The cutting of the branchial nerve between the visceral ganglion and the osphradium produces only a slight decrease of the bursting rate and a suppression of some small postsynaptic potentials (Fig. 4C).

As mentioned above, input to this cell is known to include a peculiar type of inhibition, called inhibition of long duration (Tauc, 1958, 1959, 1960, 1968). The electrical stimulation of any nerve afferent to the visceral ganglion, and especially of the branchial nerve, produces a short depolarization followed by a slowly developing, long-lasting hyperpolarization, the duration of which depends upon stimulus intensity and can attain several minutes. This type of inhibition differs from the classical postsynaptic inhibition, and does not result from a repetition of classical IPSPs (Tauc, 1958). The pharmacological properties of this cell resemble those of the cell group designated under the name of CILDA cells (Gerschenfeld & Tauc, 1964).
Our initial investigations of the influence of the osphradium on R15 were devoted to applying a range of substances (to the external surface of the osphradium) which has previously been tested in other similar preparations. Most of these substances were ineffective stimuli in our experiments.

**Stimuli found to be ineffective**

- Sea water which previously contained a number of fresh seaweeds being the basic food of *Aplysia* (Frings & Frings, 1965).
- Sea water which previously contained a number of copulating *Aplysia*. Shown by Wolper (1950) on *Paludina* to serve as a sexual stimulus.
- Sea water solutions containing substances which were found to be effective stimuli in *Paludina*: vaniline, cumarine.
- Sea water with increased KCl content (no 'on' effect; see later).
- Sea water with increased oxygen or CO2.
- Sea water lacking oxygen.

**Stimuli giving inconsistent results**

Extracts of sexual organs of *Aplysia* which provoked some modifications of activity in R15, which modifications were too inconsistent to permit a definitive conclusion concerning the effectiveness of the stimulus.

**Effective stimuli**

- Quinine sulphate (Wolper, 1950).
- Procaine and yocaine (anaesthetics exerting their action at relatively low concentrations \((10^{-3} - 10^{-4})\)).
- Diluted sea water.

This last agent (diluted sea water), due to its possible physiological significance, was selected from the above for further studies.

**ACTION OF DILUTED SEA WATER**

When diluted sea water was placed in contact with the osphradium the spontaneous spike activity in R15 was reduced. Sea water diluted to 50% rapidly and totally suppressed the action potentials, and the cell remained silent for the entire duration of the application of the stimulus (7 min.). Forty-five seconds after return to normal sea

![Fig. 5. Blocking of spike activity in R15 during application to the osphradium of sea water diluted to 75% (0.75). At \(N\) (after approximately 7 min.) the solution was changed to normal concentration. Upper and lower traces represent identical recording reproduced with different amplifications.](image)
water the normal level of spike activity was resumed. A similar effect was observed with sea water diluted to 75% of the normal (75% sea water; 25% distilled water) (see Fig. 5).

Although dilution to 50 and 75% is rather dramatic, this initial result suggested that the osphradium is sensitive to the concentration of sea water, and that perhaps it performs the role of an osmoreceptor. To verify this hypothesis, it was necessary to demonstrate: (1) that the sensitivity of the organ to this stimulus corresponded to stimulus variations which the animal might encounter in normal life; (2) that the effect is due to purely osmotic variation, and not to an alteration in ionic content of the solution bathing the osphradium; (3) that the stimulus really acts at the level of the osphradium.

(1) Threshold of action

In all preparations (Mediterranean Aplysia excepted), clear blocking effects of the activity of R15 were observed with sea water diluted to 90% of the normal. At this
concentration, however, the cell readily adapts and the original activity level recovers after a few minutes. Dilution to 95% in some preparations has blocking effects; however, in others it only changes the interval between bursts. Again this effect is only temporary. The threshold for this blocking action varies between species: in *Aplysia californica* 95% sea water has clear blocking effects, whereas with the Mediterranean *Aplysia* a dilution to 85% is necessary to produce an equivalent effect. Clear variations of activity can thus be observed with a diminution of concentration of sea water of 5%; that is, a change of salinity of 1.6 g/l.

An example of the inhibitory effect exerted on R15 by exposure of the osphradium to diluted sea water can be seen in Fig. 6, for which experiment an *Aplysia californica* was used. 95% sea water caused the cessation of spike activity in R15 for about 5 min.; 90%, for 6½ min. (not reported in Fig. 6) and 85% for about 10 min. The varying dilutions were presented in the order in which the results are listed, each diluted solution separated by a 10-15 min. exposure to normal sea water. The recovery times seen in this preparation are longer than those typically seen in *A. depilans* or *A. fasciata*.

(2) Action of ionic concentration

The ionic content of diluted sea water is different from that of the normal sea water. To be sure that the observed variations in the activity of R15 were not a result of a variation in ionic concentration rather than of that of the osmotic pressure of the

![Fig. 7. Comparison between the action of sea water (A) diluted to 75% by distilled water and (B) diluted by isotonic sucrose solution on the same preparation. Note the absence of effect of the sucrose-compensated sea water.](image-url)
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diluted solutions, but the decrease in activity was considerably smaller and of shorter duration than that due to corresponding non-compensated sea water.

It therefore seems clear that the observed variation of the activity of R15 in response to a diluted sea-water solution is not due to a variation in ionic concentration but rather to variations in the osmotic pressure of the saline solution which makes contact with the osphradium. Surprisingly enough, when the osphradium is put in contact with a hyper-concentrated sea-water solution (i.e. more concentrated than normal by evaporation) no effect on the activity of R15 could be detected. Even when the concentration was doubled there was only a slight and transitory inhibitory effect. However, in all cases, when hyper-concentrated solution was replaced by normal sea water, there was a profound inhibition of spike activity, similar to that observed when normal sea water was replaced by diluted sea water (Fig. 8). The same results were observed when the osmotic pressure was increased by other means, for instance by the addition of sucrose or KCl. It seems, thus, that the osmotic receptors of the osphradium are selectively sensitive to a diminution of osmotic pressure.

(3) Localization of the action

It is evident that the inhibitory effect observed at the level of R15 results from nervous activity initiated in the region of the osphradium. When the branchial nerve is cut, no effect is observed on the activity of R15 when the concentration of the solution bathing the osphradium is changed. Thus no artifact appears capable of explaining the effects observed. Likewise, a direct application of diluted sea water to the visceral ganglion does not produce a similar change in activity of R15. Such direct
application of the diluted solution to the ganglion can cause a very slowly developing small depolarization which does not affect the spontaneous activity until the ganglion has been exposed to the solution for many minutes (Fig. 9).

![Fig. 9. Spike activity of R15: (A) when diluted sea water (0.8o) was put in contact with the osphradium in the whole preparation; (B) ditto, but after the branchial nerve had been cut; (C) when diluted sea water (0.8o) was substituted for the normal sea water bathing the visceral ganglion.](image)

(4) Analysis of the inhibitory response

The depression in spike activity in R15 caused by osphradial stimulation is due to a hyperpolarization of the cell which can be observed even if the cell is not spontaneously active during the application of the stimulus. In occasional preparations, R15 was inactive spontaneously, or was made inactive by artificially hyperpolarizing the cell to a potential below the spike threshold. In spite of the fact that there was no on-going spike activity, osmotic activation of the osphradium produced a hyperpolarizing wave which appeared with the same latency and had the same duration as the inhibition previously described in the spontaneously active preparations. The amplitude and duration of this hyperpolarizing wave depends upon the degree of dilution of the sea water, and again osmotically compensated sea water was without effect (Fig. 10).

In order to get information concerning the mechanism of the inhibition we have measured the modification of membrane resistance during the application of diluted sea water to the osphradium. The method consisted of passing square current pulses through the membrane using a second intracellular microelectrode, and measuring the amplitude of the electrotonic potential thus produced. It is well known that the amplitude of such an electrotonic potential is proportional to the resistance of the membrane. To avoid a possible intervention of the anomalous rectification (Tauc &
Kandel, 1964), which is especially pronounced in this cell, the measurement of resistance has been performed by compensating slow voltage changes by appropriate trans-membrane current. In these conditions it appears that application of diluted sea water to the osphradium is followed by a clear diminution of the membrane resistance of R15, which follows the same time course as the polarization changes previously described (Fig. 11). The resistance changes show the same adaptation to

Fig. 10. Diagram representing the variation of membrane potential of R15, which remained silent after some hours of experiment, as a function of time during the application to the osphradium of diluted sea water of different concentrations. The time of return to normal sea water is marked by an arrow. There was no modification of polarization during application of sea water compensated by sucrose.

Fig. 11. Relative changes of membrane resistance in R15 (silent by hyperpolarization) as a function of time during application to the osphradium of different dilutions of sea water. Arrows indicate the time of return to normal concentration.
persistent stimuli and vary in the same way with stimulus intensity as does the polarization of the membrane. Once more, *Aplysia californica* showed more prominent effects, e.g. for a sea water diluted to 75% the maximum conductance change was 40% in *A. californica*, whereas it was only 32% in *A. fasciata*.

It appears thus that the hyperpolarization observed in R15 in response to exposure of the osphradium to diluted sea water results from a mechanism which involves the action of a chemical transmitter which produces conductance modifications of the membrane. This hyperpolarization is similar to inhibition of long duration previously described (Tauc, 1958, 1968) except that the small initial excitatory phase is not present.

**DISCUSSION**

The results point to the existence in *Aplysia* of a sensory system adapted to the perception of the diminution of osmotic pressure in the surrounding medium. So far, clear effects of such activation have been observed only in one central cell but it is not excluded that other central neurones are affected by such action. There is no doubt that the sensory osmoreceptor cells are situated at the level of the osphradium. The activation of the osmoreceptors produces a hyperpolarization in R15 whose delay is very difficult to determine. To avoid mechanical stimulation of the osphradial cells (which have a transient inhibitory effect on R15) the solution surrounding the osphradium could not be changed immediately but only progressively and the total operation required about 30 sec. This is approximatively the latency of the first manifestation in R15 of the effect of perfusion. It is thus difficult to analyse a possible intervention of interneurone which would be situated in between the sensory cells and R15. The efforts which we have made to find such interneurones in the osphradial ganglion by using intracellular electrodes have been without success. These unsuccessful penetrations, however, always produced large hyperpolarizations in R15.

It is known (Tauc, 1968) that interneurones which can produce inhibition of long duration in R15 are present in the visceral ganglion and it is possible that such interneurones are in fact placed in the sensory chain. It appears, indeed, that the hyperpolarization produced by osphradial action and the inhibition of long duration involve the same mechanisms. Inhibition of long duration is the only phenomenon, so far known, which is able to produce such considerable conductance changes in the cell body. In fact the conductance changes which appeared in Fig. 9 which have been produced in already hyperpolarized cell are smaller than conductance changes which could be observed in cells held at a normal polarization level. Hyperpolarization brings the cell to a potential level at which the membrane conductance is already considerably diminished due to anomalous rectification. As a result the relative conductance change produced by inhibition of long duration is considerably diminished. Anomalous rectification combines with the conductance increase caused by the transmitter action, thus causing an extremely marked variation in resistance.

The following factors point to a physiological sensory role of this response of the osphradium to diluted sea water:

1. The threshold effects clearly appeared for modifications of a few per cent of sea water concentration; there is no doubt that with statistical techniques this threshold sensitivity could be observed with an even weaker stimulus.
(2) The effect is perfectly reversible irrespective of the intensity of the applied stimulus.

(3) The sensitivity of the sensory system seems to correspond to conditions which the animals currently meet in their natural environment. *Aplysia* lives close to the shore and can often encounter sea water diluted by the rains or by the water coming from the rivers.

(4) The response is specific to a decrease of sea water concentration; the osmotic nature of this sensitivity was made evident by the results obtained with the iso-osmotic control solution containing saccharose which is known not to be absorbed by the cells.

(5) There is a clear adaptation, especially for osmotic stimulation in the physiological range. The level at which this adaptation takes place is unknown. When inhibition of long duration is produced by nerve stimulation, the efficacy of the stimulation decreases with repetition (Tauc, 1968). It is possible that in the case of osmotic activation the adaptation takes place at the ganglionic level.

A peripheral localization of an osmoreceptor might be useful for an animal which, like *Aplysia*, completely lacks a mechanism of regulation of the internal osmotic pressure (Bethe, 1930; Van Weel, 1957).

Recently Jahan-Parvar *et al.* (1968) have observed some variations in the activity of neurosecretory cells of the visceral ganglion of *Aplysia* in response to stimulation of the osphradium with hypo- and hyper-concentrated sea water. The only cell in which we have observed a marked and consistent response to such stimulation is R15, and the cells in which we have observed ambiguous responses are not those studied by these authors. A slight variation in the preparation used by these authors could possibly explain the difference in the results obtained in the two studies. For example, the preparation of Jahan-Parvar *et al.* was not apparently strictly limited to the osphradium, and probably included the branchial ganglion: or, if their solutions reached both faces of the osphradium, they would directly stimulate axons which were not necessarily in the sensory chain. This latter possibility could also explain their observation of antidromic responses to osphradial stimulation. We certainly cannot exclude the possibility, however, of the existence of other cells which are responsive to osmotic stimulation of the osphradium.

The fact that Bailey and Laverack (1966) did not obtain a response to osmotic stimulation of the osphradium in *Aplysia* might be explained by the undoubtedly restricted number of neurones which are affected by this action. On the other hand it is possible that a chemoreceptive role of the osphradium escaped our detection either because we did not apply a correct stimulus, or because we did not find the corresponding central cells. The lack of evidence of a chemoreceptive function of the osphradium is consistent with the limited development of this organ in *Aplysia*. The reduction of the importance of the osphradium might be the result of a migration of the chemoreceptors towards the buccal region. In *Aplysia*, this region is more heavily endowed with sensory organs than it is in other gastropods.

The presence of osmoreceptors in *Aplysia* represents a rare example in the animal kingdom of a precise localization of the osmotic reception. However, the way in which this animal uses the information coming from the osphradium is completely unknown, as is the function of R15.
SUMMARY

1. The function of the osphradium in *Aplysia* has been studied using an isolated preparation including the osphradium, the branchial nerve and the visceral ganglion. The experimental chamber was constructed in such a way that all contact was avoided between the solution bathing the internal face of the osphradium and that bathing its external face.

2. A dilution of the sea water bathing the external face of the osphradium provoked a marked inhibition (ILD) in an identifiable neurone (R15) in the visceral ganglion, which inhibition is accompanied by a diminution in membrane resistance. The dilution of the sea water was the only stimulus found to have a clear and constant effect, and a dilution of only 5% was sufficient to produce a discernible inhibition in R15.

3. Hyper-concentrated solutions were without effect. However, following exposure to a hyper-concentrated solution the return to normal sea water caused an inhibition in R15.

4. The effect of diluted sea water was eliminated when sucrose was added to the diluted water in order to compensate for the changes in osmotic pressure due to the dilution.

5. It was concluded that the inhibition observed in R15 is the result of excitation of osmoreceptors situated in the osphradium. These receptors, which are quite sensitive to a diminution in osmotic pressure, do not appear to be excited by even a marked increase in osmotic pressure.

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MACFARLAND (1909). See Hoffmann, above.


