AN ELECTROPHYSIOLOGICAL STUDY OF THE PHOTO-EXCITATIVE NEURONES OF *ONCHIDIUM VERRUCULATUM IN SITU*

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INTRODUCTION

The giant neurones and relatively long nerves or connectives of the mollusca have been used extensively in electrophysiological studies, but little work has been done on the physiological behaviour of neurones *in situ*. Hughes & Tauc (1962) have reported investigation on giant cells of *Aplysia* in the whole animal as well as in isolated ganglion preparations. Recently, the gill-withdrawal reflex in *Aplysia* was studied by Pinsker *et al.* (1969), by Kupfermann *et al.* (1970), and by Castellucci *et al.* (1970), and. the swimming behaviour in *Tritonia* by Willows (1967) and by Dorsett, Willows & Hoyle (1969). They studied the influence of peripheral mechanical stimulation on the giant cells but did not analyse the effect of light on whole-animal preparations.

It has been reported that *Onchidium* has many neurones as large as those of *Aplysia* (Hagiwara & Saito, 1959) and some of them are photo-excitative in the isolated ganglion preparation (Hisano, Tateda & Kuwabara, 1972). *Onchidium* is suitable for studying the neuronal pathway in whole-animal preparations because the body size is adequate and many neurones are easily identified due to their large size and pigment.

This paper is concerned particularly with the electrophysiological character of these photo-excitative neurones *in situ*. It was found that *in situ*, impulses from all photo-excitative neurones elicited by light were suppressed, and that only the 'off' spikes originated in these neurones by the stimulation to the stalk and dorsal eyes.

MATERIALS AND METHODS

The sub- and supra-oesophageal ganglion complex of *Onchidium verruculatum* and its nerve roots were used for the experiments. The techniques for isolating the ganglion complex and obtaining intracellular recordings were the same as those in the preceding paper (Hisano *et al.* 1972). For recording the neural responses *in situ* the animal was pinned, dorsal side up, in a fully extended position on a dissecting plate. The sub- and supra-oesophageal ganglion complex and its nerve roots were exposed by dissecting the dorsal surface along the body axis. The connective tissue surrounding the ganglia was removed with forceps under a binocular microscope. The preparation was covered with artificial sea water at 16–18 °C. This procedure will be described as 'whole-animal preparation' in this paper.
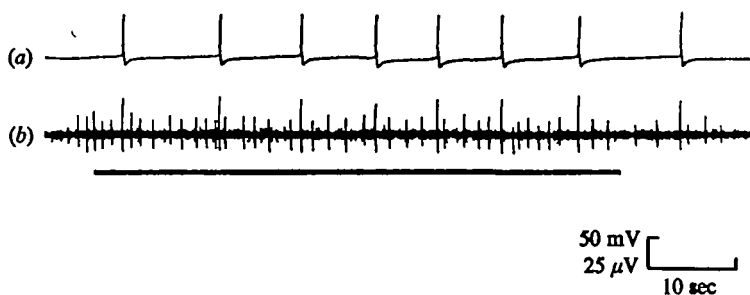
Fig. 1. Simultaneous recording from the cell soma intracellularly and from the nerve root extracellularly. (a) From the cell soma of the neurone, Es-1. (b) from the right posterior pleuro-parietal nerve. Black bar under the record indicates the light stimulation. Voltage calibration of 50 mV is for (a) and 25 μV for (b).

The activity of nerves was recorded extracellularly with a pair of platinum-wire electrodes. The distal end of the nerve was cut in most cases. Intracellular recording in the whole-animal preparation was difficult because of movement of the meso-podium. To reduce this movement the mesopodium, which was located under the sub- and supra-oesophageal ganglia, was cut off carefully without injuring the nerves. In addition, the recording glass microelectrode was hung by a thin silver wire and connected to the input of a cathode-follower amplifier. This method permitted prolonged intracellular recording from the whole animal preparation. The indifferent electrode was placed in the bathing solution. The nerve was placed on a pair of platinum wires for stimulation and was cut distal to this point.

The technique for light stimulation was nearly the same as described in the pre-ceding paper. In addition a small light spot was used for stimulating a single dorsal eye or a single stalk eye in the present experiments.

Van't Hoff's artificial sea water was used for experiments.

RESULTS

Axonal pathway of the photo-excitative neurones

Photo-excitative spikes could be recorded extracellularly from the nerve roots in the isolated ganglion preparation, demonstrating that some photo-excitative neurones send their axons into these nerves. Photo-excitative spikes in the cell soma and those from the nerves were recorded simultaneously. It became clear with this method that there were axon branches of the photo-excitative neurones in the nerves (Fig. 1). Many nerves (except pedal and cerebral nerves) contain the axons of photo-excitative neurones, although it was sometimes difficult to identify the spikes, originating from one neurone. In such a case electric stimuli were applied to the nerves and anti-dromic spikes were recorded at the cell soma of each photo-excitative neurone. Thus, the axonal pathway of the primary photo-excitative neurones (Ep-2, Ep-3, Ep-4) was analysed and the result is summarized in Fig. 2. Because the primary neurone, Ep-1, has not been identified, the axons of the secondary neurone, Es-1, which is innervated by Ep-1, were examined. When light stimuli were applied to the abdominal ganglion alone, where the neurones Ep-1 and Es-1 are found, spikes were recorded from the
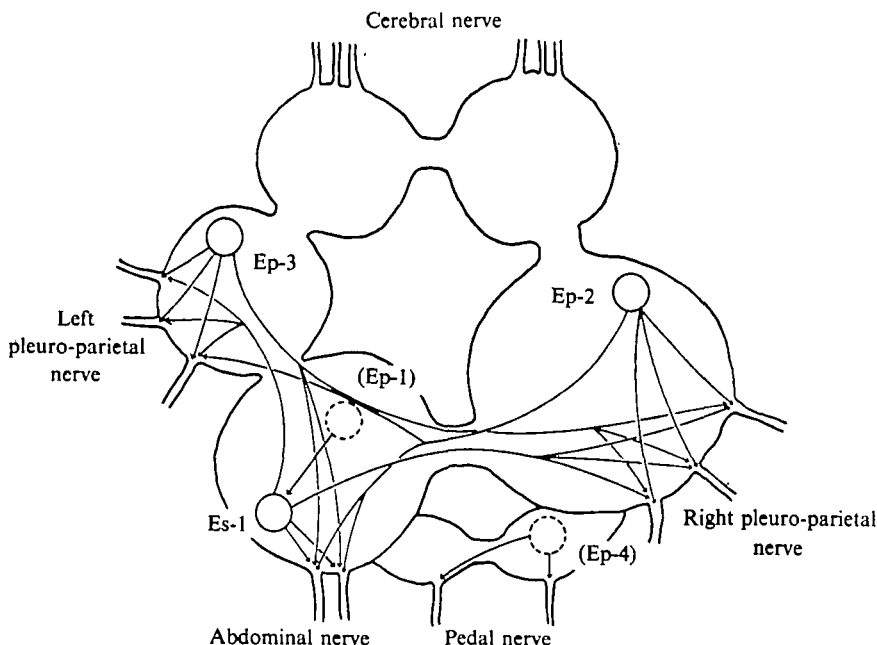
Fig. 2. The axonal pathway of the photo-excitative neurones. See text for detail.

abdominal and pleuro-parietal nerves. These spikes were ascertained to originate from Es-1 through simultaneous recording in the cell soma of Es-1 and in the nerves. The neurone, Ep-4, has not been identified either. The response to light was recorded from the pedal nerves in the isolated pedal ganglion preparation. This response occurred only when the right pedal ganglion was illuminated. There must therefore be a photo-excitative neurone in the right pedal ganglion. This is named Ep-4.

'On' and 'off' responses

Large-amplitude spikes from the photo-excitative neurones could be recorded during illumination in every nerve to which the neurones sent their axons (Fig. 2). The spikes, however, did not occur regularly during light stimuli *in situ*, but occurred phasically immediately after the beginning and cessation of illumination. These responses are called 'on' and 'off' responses, respectively (Fig. 3).

With extracellular recording it could not be determined from which neurones the 'on' and 'off' spikes originated. To clarify this, it was necessary to record intracellularly from the cell soma *in situ*. Fig. 4 shows the responses of Es-1 and Ep-2 which were elicited *in situ* by illumination of the whole animal. Neither neurone generated spikes during light stimuli, but the 'off' spikes occurred immediately after the cessation of illumination. Other photo-excitative neurones responded similarly. Moreover, there were giant neurones, other than photo-excitative, generating 'off' spikes. These facts indicate that the 'off' response is not restricted to photo-excitative neurones. No giant neurones which were tested intracellularly showed the 'on' response. The relation of the 'on' and 'off' responses to the behaviour of *Onchidium* will be discussed later.

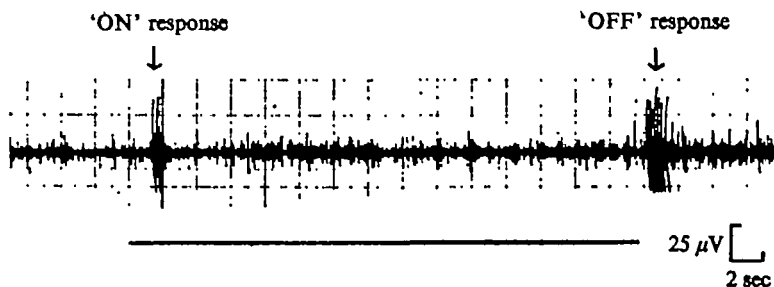'ON' response   'OFF' response

25 μV

2 sec

Fig. 3. 'On' and 'off' spikes recorded from the right pedal nerve in the whole-animal preparation. Light stimulus was applied to the whole animal. Black bar under the record shows light stimulus.

(a)

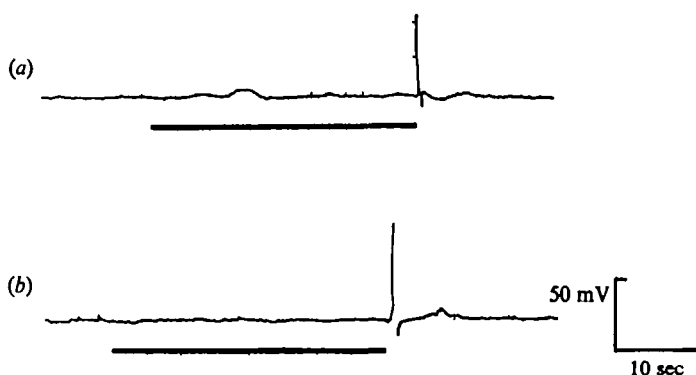(b)   50 mV

10 sec

Fig. 4. Intracellular recordings from Es-1 (a) and Ep-2 (b) *in situ*. Light stimuli were applied to the whole animal. Black bars under the records show light stimuli.

To find the photo-receptor which produces the 'off' response in neurones, light stimuli were applied separately to each photo-receptor, such as stalk eyes, dorsal eyes and photo-excitative neurones (Fig. 5). 'On' and 'off' spikes appeared in the nerves which extended from the sub- and supra-oesophageal ganglia when stalk eyes and or dorsal eyes were stimulated; they did not do so when the ganglia alone were illuminated. The 'on' and 'off' spikes recorded from the nerves *in situ* were therefore the responses of the external photo-receptor, i.e. the stalk eyes and the dorsal eyes. Giant spikes from the photo-excitative neurones began to appear in the nerves during illumination, and 'off' spikes disappeared concurrently when this ganglion complex was isolated and stimulated by light of the same intensity as before (Fig. 6). Thus, *in situ*, the excitation of the inherently photo-excitative neurones was inhibited by some inhibitory input; in most cases they did not generate spikes in response to the light stimuli used here.

Inhibitory input to the photo-excitative neurones in situ

Cutting the nerves

The sub- and supra-oesophageal ganglion nerves remain intact in the whole-animal preparation but not in the isolated ganglion preparation. This difference alters the excitability of inherently photo-excitative neurones for light stimuli. This implie

Fig. 5. Responses recorded from the right pedal nerve in the whole-animal preparation when the light stimuli were applied to each photoreceptor separately. (*a*) Dorsal eye illumination; (*b*), stalk eye illumination; (*c*), ganglion illumination. (*a*) and (*b*) are from the same preparation. Black bars under the records show light stimuli. Voltage calibrations for (*a*), (*b*), and (*c*) are 25 μV, time calibrations for (*a*), (*b*) and (*c*) are 2 sec.

Fig. 6. Comparison of the responses from the right pedal nerve between in the whole animal and in the isolated ganglion preparation. (*a*), response in the whole-animal preparation when light stimulus was applied to the whole animal; (*b*), response in the isolated ganglion preparation. Black bars under the records shows light stimuli. Voltage and time calibrations are for (*a*) and (*b*).

that the inhibitory input to the photo-excitative neurones may come through these nerves. According to this assumption, further experiments were performed.

Each ganglion has several nerve roots which contain both afferent and efferent fibres (Fig. 2). It is impossible to distinguish the inhibitory fibres in nerves histologically. Each nerve was tested electrophysiologically to find whether or not it contained inhibitory fibres (Fig. 7). Although sub- and supra-oesophageal ganglion nerves were
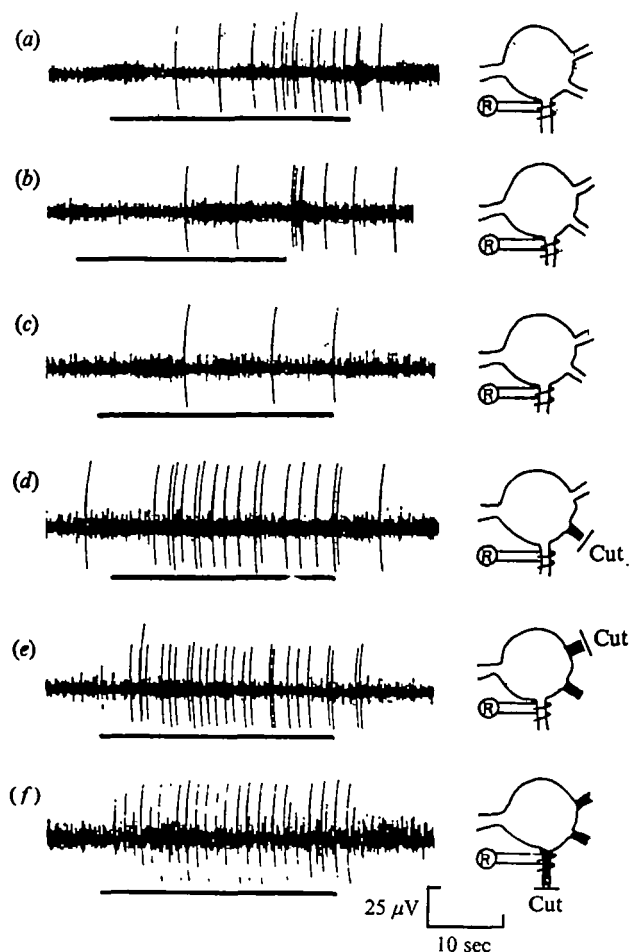
Fig. 7. Records from the right pleuro-parietal nerve in the whole-animal preparation. (a), Whole-animal illumination; (b), whole-animal illumination except ganglion; (c–f) ganglion illumination. (a–c) All the nerves were intact; (d–f) the nerve indicated was cut. Black bar under each record shows light stimulus. Voltage and time calibration is the same for all records. See text for details.

intact in Fig. 7a–c, the area to which light stimuli were applied was different in each case. The whole animal was illuminated in Fig. 7a, and part of the animal other than the ganglion was stimulated by light in Fig. 7b. Sub- and supra-oesophageal ganglia alone were illuminated in Fig. 7c–f. When stalk eyes and dorsal eyes stimulated, 'off' spikes were recorded from the nerves (Fig. 7a, b). A few giant spikes also appeared in every case in this preparation. This neurone responded slightly to direct ganglion illumination (Fig. 7c). This may be partially due to weak inhibition to the photo-excitative neurone (compare with Fig. 5c). When the right middle pleuro-parietal nerve was cut, many photo-excitative spikes suddenly began to appear (Fig. 7d). When the right anterior and posterior pleuto-parietal nerves were also cut, the number of photo-excitative spikes gradually increased (Fig. 7e, f). The photo-excitative spikes recorded here were ascertained to originate either from
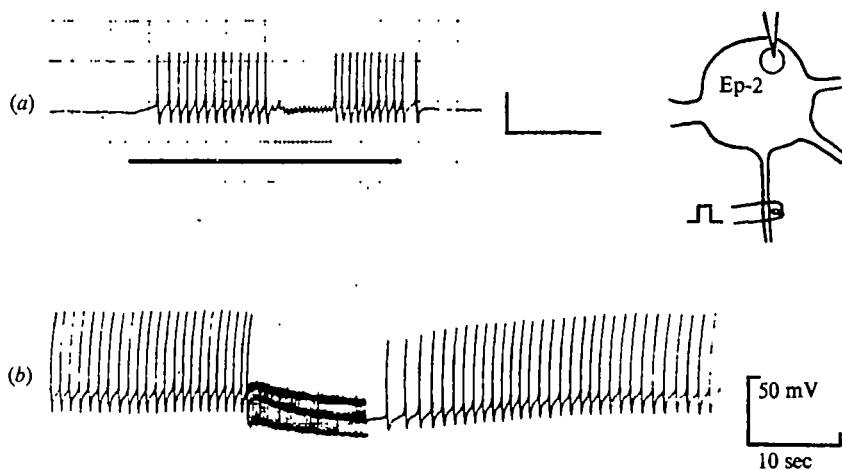
Fig. 8. Inhibition in the neurone, Ep-2, in the isolated ganglion preparation caused by stimulating the right posterior pleuro-parietal nerve electrically. (*a*), Photo-excitative spikes; (*b*), spontaneous discharge. The dots (2·5/sec) in (*a*) and the small spikes (5/sec) in (*b*) indicate the electric stimulation. Black bar under the record (*a*) shows light stimulus. Voltage calibration is 50 mV for (*a*) and (*b*) and time calibration is 10 sec for (*a*) and (*b*).

Es-1 or Es-2. The change in excitability of photo-excitative neurones did not depend upon the order of cutting the nerves but upon the number of the nerves cut. Also, in the case of cutting the left pleuro-parietal nerves, similar results were obtained. When other nerves (pedal, abdominal or cerebral) were cut, however, the phenomenon mentioned above was not observed. These results showed that inherently photo-excitative neurones are inhibited by inhibitory fibres in the pleuro-parietal nerves of both sides; after cutting these nerves, the photo-excitative neurones are no longer inhibited, and photo-excitative spikes appear.

Stimulation of the nerves

Other evidence showing that the pleuro-parietal nerves contain inhibitory fibres has been obtained by electrical stimulation of these nerves (Fig. 8). A microelectrode was inserted into the neurone Ep-2 in the isolated ganglion preparation, and electric stimuli were applied to the nerves during light stimulation. When the right posterior pleuro-parietal nerve was electrically stimulated with square-wave pulses at 2·5/sec or 5/sec, spikes in Ep-2 were inhibited during the stimulation, regardless of whether they were photo-excitative (Fig. 8*a*) or spontaneous discharges (Fig. 8*b*). This indicates that the inhibitory fibre to Ep-2 in that nerve was excited by electric stimulation, and excitation of Ep-2 was suppressed by this inhibitory input. Although the right pleuro-parietal nerve also contains the axon branch of Ep-2 (Fig. 2), an antidromic spike was not recorded by stimulating this nerve before and during the blocking of spikes. The threshold of inhibitory fibres for electrical stimuli must be lower than that of the axon branch of Ep-2.

When the right anterior or middle pleuro-parietal nerve was stimulated, efficacy of inhibitory action to Ep-2 was the same as that of posterior nerve stimulation. Stimulation to the left pleuro-parietal nerves brought about similar inhibition in
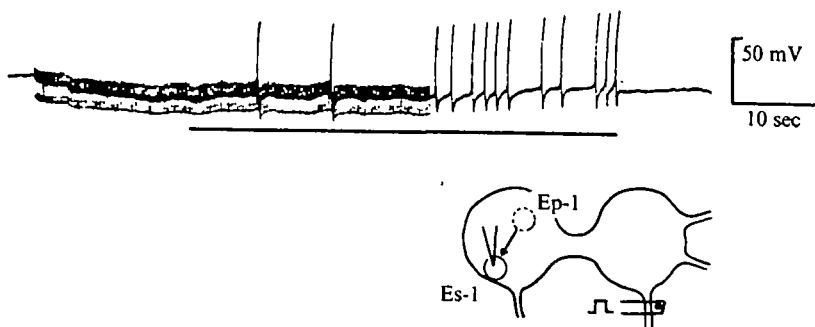
Fig. 9. Inhibition in the neurone Es-1 in the isolated ganglion preparation. Electric square pulses were applied to the right posterior pleuro-parietal nerve at 5/sec. Black bar under the record shows light stimulus.

Ep-2 as stimulation to the right nerve. Therefore Ep-2 might be innervated by almost the same number of inhibitory fibres in the left pleuro-parietal nerve as in the right pleuro-parietal nerve. As mentioned above, stimulation to any nerve other than the left and right pleuro-parietal nerves had no effect on Ep-2 in respect of inhibition. Ep-3 in the left pleuro-parietal ganglion showed almost the same physiological behaviour as Ep-2 in every respect.

The secondary neurone, Es-1 however, responded differently from Ep-2 and Ep-3 (Fig. 9). Electrical stimulation of any amplitude to the posterior pleuro-parietal nerve could not completely block photo-excitative spikes. Conversely, facilitation was often observed during electric stimulation. The neurone Ep-2 can respond to light stimuli directly but the neurone Es-1 cannot. Consequently it is probable that Es-1 has more complex neuronal pathways in respect to the photoresponse than Ep-2 or Ep-3. At least, it can be stated from the results in Fig. 9 that inhibitory fibres in the right posterior pleuro-parietal nerve innervate Es-1 or Ep-1. It is not clear which neurone is innervated by them. Stimulation of other pleuro-parietal nerves brought about a similar phenomenon in Es-1 as stimulation of the right pleuro-parietal nerve. The mechanism of this inhibition will be discussed later.

Behavioural observations

Onchidium responds to a sudden increase and decrease in light intensity as well as to tactile stimulation. It exhibits reactions such as cessation of locomotion, withdrawal of stalk eyes and contraction of the mantle. Light reactions occur rarely, when compared to shadow reactions. It has been said that dorsal eyes and stalk eyes play a role in these reactions. This was indicated electrophysiologically in the present work. The 'on' response and 'off' response, recorded in many nerves in the whole-animal preparation, might be related to these reactions. However, an individual from which both stalk eyes and dorsal eyes were removed could still respond to shadow stimuli. The 'blinded' animals were used as material for finding the unknown photo-receptor mediating the shadow reaction. Through the method of putting a small region of the animal body in shadow during illumination of the whole animal, it was found that the whole of the labial palps and the peripheral region of the mantle were photosensitive and mediated the shadow reaction. Although photo-sensitive elements in
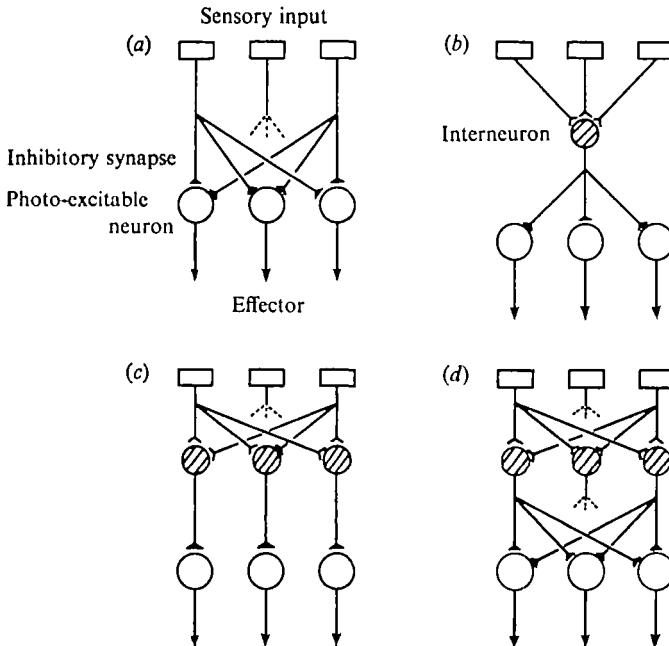
Fig. 10. Schematic diagrams indicating the mechanism of inhibition to the photo-excitative neurones (see text for details).

these areas have not been analysed, it is unquestionable that photo-sensitive regions of the labial palps and mantle, as well as stalk eyes and dorsal eyes, are participating in the shadow reaction of *Onchidium*, since any individuals from which the labial palps and the peripheral region of mantle were removed (in addition to stalk eyes and dorsal eyes) were unable to show the shadow reaction. It seems that *in situ* the photo-sensitive neurones of *Onchidium* do not respond to sudden changes in light intensity.

DISCUSSION

The photo-excitative neurones in the sub- and supra-oesophageal ganglia may be thought of as possible photo-receptors of *Onchidium*. It is known that invertebrates possess photo-sensitive neurones which do not seem to be normally associated with visual functions and are located within the nervous system. The caudal photo-receptor of the sixth abdominal ganglion can be substituted for the compound eye in regulating the diurnal activity cycle of crayfish (Chapple, 1960). Also, the pallial nerve in the lamellibranch mollusc *Spisula* responds to light stimuli and mediates the shadow response of siphon retraction (Kennedy, 1961). The radial nerve in the echinoid *Diadema* is also photo-sensitive and mediates the shadow reaction of spines and podia (Yoshida & Millott, 1959). No one, however, has given any description of the role of photo-sensitive neurones in *Aplysia*. This may be partially due to the difficulty in determining whether or not photo-sensitive neurones *in situ* function as photo-receptors.

In the case of *Onchidium* the experiments for determining the function of photo-sensitive neurones as photo-receptors in the whole-animal preparation were easily

performed, compared to the case of *Aplysia*. Electrophysiological data on the whole-animal preparation imply that the inherently photo-excitative neurones cannot function as photo-receptors, as do stalk and dorsal eyes, since in most cases photo-excitative neurones were silent when they were stimulated by light but only mediated 'off' spikes originating from dorsal eyes and stalk eyes. This fact became clear by demonstration of inhibitory input to the inherently photo-excitative neurones through the left and right pleuro-parietal nerves, i.e. by cutting the inhibitory fibres and by stimulating the inhibitory fibres electrically.

The diagram in Fig. 10 illustrates four possible mechanisms of this inhibition. There are no ganglia on the peripheral side of the left or right pleuro-parietal nerves. We assume the inhibition comes from some sensory input. (i) Direct inhibitory action of the sensory inputs is illustrated in Fig. 10*a*. (ii) A common interneurone is assumed to exist between the sensory inputs and the photo-excitative neurones in Fig. 10*b*. (iii) In Fig. 10*c* each photo-excitative neurone receives input from one interneurone. (iv) A combination of Fig. 10*b* and *c* is illustrated in Fig. 10*d*. Any dissection of stalk eyes, dorsal eyes, labial palps, and the photo-sensitive region of the mantle had no effect on the excitability of the photo-excitative neurones in a whole-animal preparation. The only condition that diminished the inhibitory inputs during illumination was cutting the pleuro-parietal nerves. This may imply that the inhibitory sensory inputs do not come from photo-receptors but from receptors of other modality. The existence of interneurones which connect the sensory input to the photo-excitative neurones has not been confirmed. The difference in inhibitory mechanism between primary and secondary photo-excitative neurones has not been analysed in the present work. Further experiments will be necessary to make these points clear.

Hagiwara & Kusano (1961) reported presynaptic inhibition in giant neurones of *Onchidium* by applying electrical stimulation to nerve bundles. The authors said nothing about the location of the neurones used; some of them may be photo-sensitive, since similar results on the presynaptic inhibition in the primary photo-excitative neurones have been obtained by stimulating the nerves in the present work.

In *Aplysia*, giant neurones have recently been studied in terms of the central nervous system (Tauc & Hughes, 1963; Hughes & Tauc, 1962, 1963). The neuronal pathways and their function have become considerably more clear. On the other hand, in *Onchidium* there has been little work on the physiology of the central nervous system. To confirm the function of the photo-excitative neurones on *Onchidium*, studies on the neurones in terms of the central nervous system are required.

The photo-excitative neurones seem to have the following disadvantages in functioning as photo-receptors: (1) constant inhibitory inputs to the neurones *in situ*; (2) very high threshold to light stimuli; (3) deep location covered by visceral organs in the intact body. The photo-excitative neurones, however, may play a role regulating the activity of *Onchidium*. Experiments directed toward the function of the photo-sensitive neurones in *Onchidium* are in progress.

SUMMARY

1. The distribution of the axons of the photo-excitative neurones in *Onchidium verruculatum* has been traced by intracellular stimulation of the soma and extra-cellular stimulation of the axon. They send axon branches mainly into the pleuro-parietal and abdominal nerves in both sides.

2. In the whole-animal preparation, photo-excitative spikes could be recorded from neither the soma nor the nerves of inherently photo-excitative neurones during light stimulation. 'On' and 'off' spikes were initiated only immediately after the beginning and the cessation of illumination of a whole animal.

3. 'Off' spikes originated from dorsal eyes and stalk eyes to which shadow stimuli were applied. Those spikes were not the direct response of photo-excitative neurones to light.

4. The excitation of the inherently photo-excitative neurones *in situ* was suppressed by inhibitory inputs coming through the right and left pleuro-parietal nerves. Cutting one (or some) of the pleuro-parietal nerves was the only condition that diminished the inhibitory inputs to the photo-excitative neurones in the present work. Adequate electrical stimulation of the pleuro-parietal nerves inhibited spikes of photo-excitative neurones due to photo-excitator or spontaneous discharge.

REFERENCES

CASTELLUCCI, V., PINSKER, H., KUPFERMANN, I. & KANDEL, E. (1970). Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia. Science, N.Y.* **167**, 1745–8.

CHAPPLE, W. D. (1960). Light and the movement of crayfish. Masters Thesis, Syracuse University (cited by D. Kennedy (1963). *J. gen. Physiol.* **46**, 551).

DORSETT, D. A., WILLOWS, A. O. D. & HOYLE, G. (1969). Centrally generated nerve impulse sequences determining swimming behaviour in *Tritonia. Nature, Lond.* **224**, 711–12.

HAGIWARA, S. & SAITO, N. (1959). Voltage–current relations in nerve cell membrane of *Onchidium verruculatum. J. Physiol., Lond.* **148**, 161–79.

HAGIWARA, S. & KUSANO, K. (1961). Synaptic inhibition in giant nerve cell of *Onchidium verruculatum. J. Neurophysiol.* **24**, 166–75.

HISANO, N., TATEDA, H. & KUWABARA, M. (1972). Photosensitive neurones in the marine pulmonate mollusc, *Onchidium verruculatum. J. exp. Biol.* **57**, 651–660.

HUGHES, G. M. & TAUC, L. (1962). Aspects of the organization of central nervous pathways in *Aplysia depilans. J. exp. Biol.* **39**, 45–69.

HUGHES, G. M. & TAUC, L. (1963). An electrophysiological study of the anatomical relations of two giant nerve cells in *Aplysia depilans. J. exp. Biol.* **40**, 469–86.

KENNEDY, D. (1961). Neural photo-reception in a lamellibranch mollusc. *J. gen. Physiol.* **44**, 277–99.

KUPFERMANN, I., CASTELLUCCI, V., PINSKER, H. & KANDEL, E. (1970). Neuronal correlates of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia. Science, N.Y.* **167**, 1743–5.

PINSKER, H., KUPFERMANN, I., CASTELLUCCI, V. & KANDEL, E. (1969). Habituation and dishabituation of the gill-withdrawal reflex in *Aplysia. Science, N.Y.* **167**, 1740–2.

TAUC, L. & HUGHES, G. M. (1963). Modes of initiation and propagation of spikes in the branching axons of molluscan central neurons. *J. gen. Physiol.* **46**, 533–49.

WILLOWS, A. O. D. (1967). Behavioral acts elicited by stimulation of single, identifiable brain cells. *Science, N.Y.* **157**, 570–4.

YOSHIDA, M. & MILLOTT, N. (1959). Light-sensitive nerve in an echinoid. *Experientia* **15**, 13–14.