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EXCITATORY, INHIBITORY AND BIPHASIC SYNAPTIC POTENTIALS MEDIATED BY AN IDENTIFIED DOPAMINE-CONTAINING NEURONE

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SUMMARY

1. A giant dopamine-containing cell, situated in the left pedal ganglion of the water snail *Planorbis corneus*, was identified in isolated living preparations of the central nervous system. Spectrophotofluorimetric analysis confirms that the cell contains dopamine, whereas noradrenaline appears to be absent. The cell is unique in being a repeatedly identifiable dopamine-containing neurone.

2. Stimulation of the giant dopamine-containing cell resulted in excitatory, inhibitory or biphasic (depolarizing–hyperpolarizing) synaptic potentials in a number of follower neurones. The duration of the e.p.s.p.s and i.p.s.p.s was 0·3–5 sec; they ranged from barely detectable responses to ones 7 mV in amplitude in different cells. The depolarizing phase of a biphasic synaptic potential (b.p.s.p.) was usually less than 1 mV in amplitude (max. 3 mV) and lasted 40–400 msec. The latency of i.p.s.p.s was long (70–120 msec) compared with that of e.p.s.p.s and b.p.s.p.s (20 msec). Abolition of the depolarizing phase of b.p.s.p.s by tubocurarine left a long-latency (70–120 msec) i.p.s.p. All responses showed summation and marked facilitation.

3. Evidence is presented that the post-synaptic potentials are produced by direct connexions from the giant cell and result from a release of dopamine. Of eight putative transmitter substances tested on these different groups of neurones, only dopamine produced a potential change which in each case was of the same polarity as the post-synaptic potential when this was monophasic. However, generally applied dopamine produced only a hyperpolarization in follower cells showing b.p.s.p.s. This result is probably partly due to rapid desensitization of the receptors mediating the depolarization and also to a masking of the depolarization by the more effective hyperpolarizing response.

4. Ergometrine and 6-hydroxydopamine specifically antagonized the i.p.s.p.s and dopamine receptors mediating inhibition. Neither the e.p.s.p.s nor the excitatory dopamine response were blocked by high concentrations of hexamethonium. Hexamethonium was also ineffective in blocking the depolarizing phase of a b.p.s.p., which was, however, selectively eliminated by tubocurarine.

5. It is suggested that dopamine is the transmitter released from the giant cell and that it can mediate excitatory, inhibitory or biphasic responses in different follower neurones.

INTRODUCTION

Dopamine may be a central transmitter substance in addition to being a precursor of noradrenaline (see reviews by Hornykiewicz, 1966, 1971). General acceptance that noradrenaline is a neurotransmitter resulted from studies of relatively simple peripheral systems in vertebrate animals. There do not, however, appear to be comparable peripheral dopaminergic systems. Advances in elucidating the suggested transmitter role of dopamine are therefore restricted. In many invertebrate nervous systems dopamine occurs in high concentration and is localized in particular neurones (Cottrell & Laverack, 1968; Welsh, 1972). Gastropod molluscs have been studied in most detail, and there is some suggestive evidence of dopaminergic transmission. For example, certain central neurones are very sensitive to applied dopamine (Gerschenfeld, 1964; Walker, Woodruff, Glaizner, Sedden & Kerkut, 1968; Ascher, 1972), and particular i.p.s.p.s may be mediated by dopamine (Ascher, Kehoe & Tauc, 1967; Kerkut, Horn & Walker, 1969; Tauc, 1969; Walker, Ralph, Woodruff & Kerkut, 1971). However, in no case has the presynaptic neurone been identified and shown to contain dopamine.

A large neurone containing a primary catecholamine has been observed in the central nervous system of the water snail *Planorbis corneus* (Marsden & Kerkut, 1970). The cell is usually the largest in the ganglion and it may be termed a giant cell. Recent microspectrofluorimetric and microchemical experiments (see Discussion) have established that this cell contains dopamine, as was suggested by Marsden & Kerkut. The work described in this paper was undertaken to determine whether dopamine might function as a transmitter in this giant neurone. Experiments were designed to locate the neurone and to analyse post-synaptic responses in any follower cells. A brief report of some of the experiments presented in this paper has been published previously (Berry & Cottrell, 1973).

METHODS

Preparation. Experiments were performed on the isolated circumoesophageal ganglia of *Planorbis corneus*. The ganglia were pinned to a plastic sheet at the base of a 5 ml. Perspex chamber and immersed in a continuous flow of physiological solution at 15–22° C (Berry, 1972). The right parietal–pleural connective was cut to allow the visceral and left parietal ganglia to be turned to expose the various surfaces. The overlying connective tissue was partially removed from cells to be studied.

Fluorescence histochemistry. The giant dopamine cell was located by the histofluorescence technique of Falck & Owman (1965) applied to whole mounts and serial sections of the pedal ganglia, and to isolated perikarya placed on a microscope slide.

Electrodes and electrical measurements. For intracellular recording, neurones were impaled with one or two single micro-electrodes or a double-barrelled microelectrode. These were filled with 0·6 M-K_2SO_4 and had a resistance of 5–20 MΩ. Current could be passed into the cells through the second electrode or barrel; a bridge circuit was used when a single electrode served for both stimulation and recording. A 500 MΩ resistor in series with the electrode ensured a constant current. Glass suction electrodes were used for extracellular recording and stimulation. Conventional amplifying and stimulating equipment was used. For accurate measurement of rapid events, photographic records were made of the oscilloscope trace; other records were made on a Brush 220 series pen recorder.

Application of drugs. Possible transmitter substances were applied to neurones by means of a pipette, which contained the drug at a concentration of 10^{-2} M in physiological solution, and had an opening of 15–25 μm. The pipette was lowered until its tip was in close proximity to the perikaryon and the drug was allowed to diffuse on to the neurone and then washed away by continuously flowing physiological solution. The pipette was removed as soon as a response was observed. Drugs were also added to the bath at known concentration.

Possible transmitter substances tested were: dopamine HCl, 5-hydroxytryptamine creatinine sulphate (5-HT) (Koch-Light); L-noradrenaline HCl (Sigma); acetylcholine chloride (ACh), γ-aminobutyric acid (GABA), L-glutamic acid monosodium salt, glycine, histamine acid phosphate (British Drug Houses). Antagonists of dopamine and ACh were: ergometrine maleate, tubocurarine chloride (Burroughs Wellcome); hexamethonium bromide (Koch-Light); 6-hydroxydopamine HBr (Sigma) stabilized by 10^{-3} M L-ascorbic acid (British Drug Houses).

All drugs were dissolved in physiological solution just before use.

RESULTS

Identification of the giant dopamine cell

The giant dopamine cell, situated posterio-ventrally in the left pedal ganglion (Marsden & Kerkut, 1970) was readily located in the appropriate position. The neurone was usually the largest in the ganglion and was relatively constant in position. Fluorescence histochemical studies showed that the cell contained a primary catecholamine; it produced the bright green fluorescence characteristic of dopamine or noradrenaline both in whole mounts and in tissue sections of the pedal ganglia. Another identifiable large cell close by showed the yellow fluorescence characteristic of

5-HT. Most other cells produced no amine-specific fluorescence. The perikaryon and length of attached axon of isolated giant dopamine cells which had been placed on a microscope slide fluoresced bright green, easily distinguishable from the yellow fluorescence of the nearby large cell and from the autofluorescence of cells containing no monoamine which were dissected for comparison.

The axon pathway of the giant cell has been traced by a number of techniques (Berry, Cottrell, Pentreath & Powell, 1974; Pentreath, Berry & Cottrell, 1974) and is shown diagrammatically in Fig. 1.

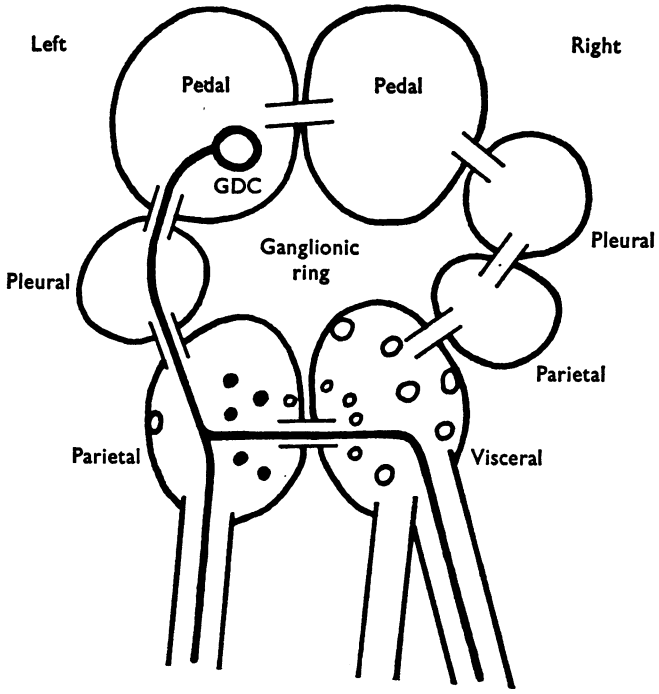

Fig. 1. Diagram of the dorsal surface of the sub-oesophageal ganglia showing the axon distribution of the giant dopamine cell (GDC) and the approximate positions of its follower cells. This cell lies on the ventral surface. Open and filled circles represent follower cells receiving inhibitory and excitatory input respectively from the giant cell. Only the larger visceral and parietal nerve trunks are shown.

Post-synaptic responses to stimulation of the giant dopamine cell

Inhibitory responses

Numerous scattered cells on the dorsal surface of the visceral ganglion were found to show an inhibitory post-synaptic potential (i.p.s.p.) following each spike elicited in the giant dopamine cell (Fig. 2). Recordings have

been made from as many as fifteen different post-synaptic (follower) cells in a single preparation. A few follower cells were also found on the ventral surface. No individual specified follower cell has yet been identified in the visceral ganglion owing to the large number of post-synaptic cells involved and to the very wide variability of cell size and position in different preparations.

I.p.s.p.s following giant-cell spikes ranged up to 7 mV in amplitude and had a duration of 0·3–5 sec. Sometimes individual i.p.s.p.s were not

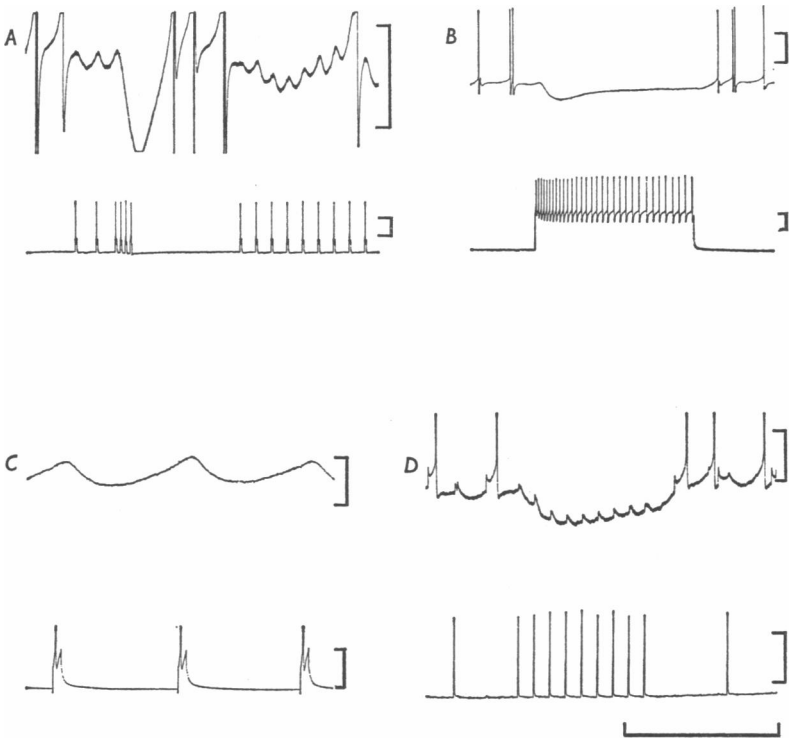

Fig. 2. Inhibitory and biphasic responses of visceral ganglion cells (upper record of each pair) to stimulation of the giant dopamine cell (lower record of each pair). *A* and *B* show facilitation and smooth summation of i.p.s.p.s. *C*: higher speed recording shows the gradual onset and long time course of i.p.s.p.s. Recordings were made from different preparations. In this and some later records (particularly those at high gain) the full amplitude of the action potentials was not recorded by the pen recorder. In *C*, only part of the spike in the giant cell is apparent. *D*: biphasic response of a visceral ganglion cell. These responses were found to be functionally inhibitory whatever the frequency of firing of the giant cell. The visceral ganglion cell was recorded at its resting potential of −60 mV. Voltage calibration, visceral ganglion cells: *A*, *C*, *D*, 10 mV; *B*, 30 mV; giant dopamine cell: *A–C*, 40 mV; *D*, 45 mV. Time scale, *A*, *B*, *D*: 10 sec; *C*: 2 sec.

observed, but a smooth hyperpolarization was seen when the giant dopamine cell was stimulated repetitively at frequencies higher than about 3 Hz (Fig. 2*B*). The i.p.s.p.s showed summation and often marked facilitation (Fig. 2*A*).

Inhibitory responses were occasionally recorded from neurones in the left parietal ganglion. One particular neurone on the left side of the ganglion was identifiable because it was the only neurone in that area to receive inhibitory input from the giant cell.

Biphasic responses

In some cells in the visceral ganglion, a biphasic potential (b.p.s.p.) was observed following stimulation of the giant cell; each hyperpolarization was preceded by a small depolarization of 40–400 msec duration (Fig. 2*D*). The size of the depolarizing phase was increased by artificially hyperpolarizing the cell, which at the same time reduced the size of the hyperpolarizing phase (see e.g. Fig. 8) though it was not usually possible to invert it. The amplitude of the depolarizing phase rarely exceeded 1 or 2 mV and often was barely detectable. Although the first few depolarizations could summate they were never observed to reach threshold even when elicited at high frequency; after 3 or 4 giant-cell spikes, there was facilitation of the larger and more slowly developing inhibitory phase producing an increase in membrane potential. During the course of this study, i.p.s.p.s have been recorded from more than 500 neurones in the visceral ganglion of different preparations. Most i.p.s.p.s were seen to have an initial depolarization if recorded at very high gain, but in only about 50 neurones was the depolarizing phase more than 0·1 mV when recorded at the resting potential of -45 to -65 mV.

Excitatory responses

The responses of follower neurones in the parietal ganglion were predominantly excitatory. In the middle region of the dorsal surface several of the small–medium (30–90 μm) cells receive excitatory input from the giant cell (Fig. 3*A*). There was wide variation in the amplitude of the responses of these cells, excitatory post-synaptic potentials (e.p.s.p.s) ranging from barely detectable responses up to 7 mV. They were increased in amplitude by artificially hyperpolarizing the cell (Fig. 3*B*, *C*) and were usually of long duration (up to 5 sec). They summed and were facilitated even when elicited at relatively low frequencies. Consequently, low-frequency firing of the giant dopamine cell (down to about 0·2 Hz) could alter the firing rate of some follower neurones (Fig. 3*A*).

Excitatory input from the giant dopamine cell has occasionally been recorded from cells on the ventral surface of the parietal ganglion and

from a cluster of very small cells ($< 30\ \mu m$) on the left side of the ganglion. E.p.s.p.s in these small cells had a steeper rising phase than those in the larger cells just mentioned, and were of shorter duration (*ca.* 200 msec). Depolarizing responses of neurones in the visceral ganglion were nearly always part of a biphasic response which was functionally inhibitory, i.e. abolished spontaneous firing without initial excitation (Figs. 2*D*, 9A_1, B_1).

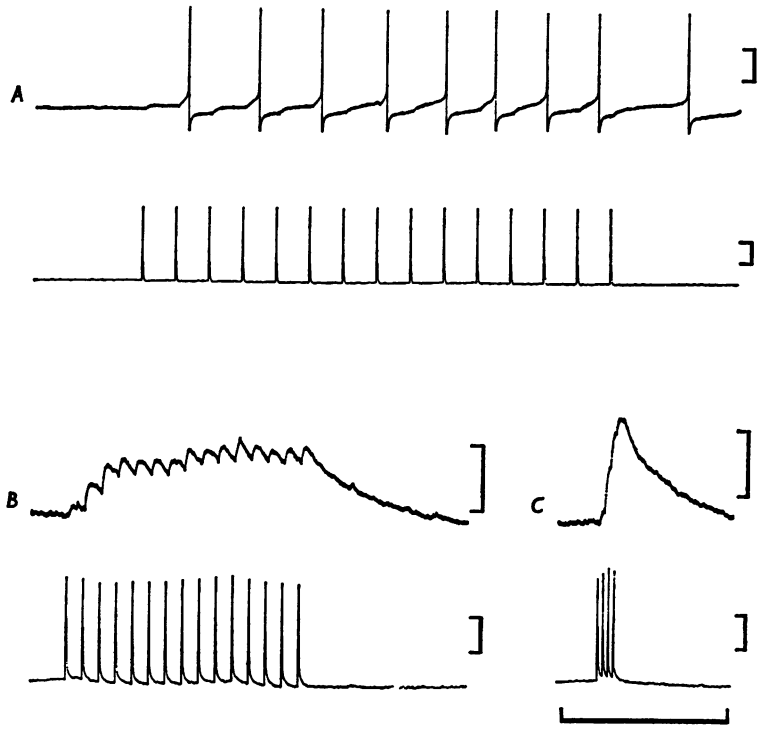

Fig. 3. Excitatory responses of a parietal ganglion cell (upper trace of each pair) observed during stimulation of the giant dopamine cell (lower trace of each pair). *A*: stimulation of the giant cell at 0·5 Hz elicits e.p.s.p.s and spikes in a quiescent follower cell recorded at the resting membrane potential (-60 mV). Firing continued for 25 sec after the last giant-cell spike. *B*, *C*: the follower cell was hyperpolarized by 35 mV; the e.p.s.p.s are increased in amplitude and can be more clearly seen to follow each giant-cell spike. Calibration: 10 sec, 30 mV (presynaptic, i.e. the giant dopamine cell), 15 mV (post-synaptic, i.e. follower neurone).

Are the post-synaptic responses mediated monosynaptically?

Two of the main criteria that have been used as evidence of monosynaptic connexions are a short, constant latency between presynaptic spike and post-synaptic response, and the ability of the post-synaptic

potential to follow each presynaptic spike at a high frequency of firing (e.g. Kandel, Frazier, Waziri & Coggeshall, 1967; Kandel, 1969; Wachtel & Kandel, 1971; Gardner & Kandel, 1972). Unfortunately these criteria could not be applied to the hyperpolarizing responses of the visceral and parietal ganglion cells in *Planorbis*. The i.p.s.p.s developed so slowly that it was difficult to determine the beginning of the response; the time to peak was approximately equal to the decay time. Large i.p.s.p.s appeared to have a shorter latency than smaller more slowly developing i.p.s.p.s even in the same cell, but this may have been due to the fact that the larger response could be seen earlier in the cell body. Because of the long duration of the i.p.s.p.s it was not possible to determine whether they could follow high-frequency stimulation of the giant cell. They showed a smooth summation at frequencies as low as 3 Hz so that it was not possible to determine whether i.p.s.p.s were dropping out.

The criteria of latency and high-frequency response could be applied to the e.p.s.p.s and depolarizing phase of b.p.s.p.s which had more rapid rise times. The responses had short, constant latency (*ca.* 20 msec) and could follow high-frequency stimulation of the giant dopamine cell without loss of transmission. The latency, although longer than the approximately 10 msec that it takes for the giant-cell spike to reach the visceral nerve (Pentreath *et al.* 1974), would seem to be well within the limits expected for a monosynaptic connexion for this type of preparation. Latencies of apparently direct post-synaptic potentials in molluscs may be much longer than those found for other preparations (cf. Wachtel & Kandel, 1967; Kehoe, 1972*c*; see also the Discussion).

Another test for monosynaptic connexions which has been used in *Aplysia* is based on the observation that a high concentration of Ca raises spike threshold (Austin, Yai & Sato, 1967; Gardner, 1971; Gardner & Kandel, 1972). If the recorded post-synaptic responses are produced by an interneurone, high Ca concentrations might be expected to raise the threshold of this hypothetical cell beyond the level of depolarization produced by an e.p.s.p. and so reduce its ability to fire spikes; consequently the recorded post-synaptic potentials would be lost. Extensive experiments have shown that high Ca has a large effect on spike threshold and can very effectively block one-for-one synaptic transmission of action potentials in *Planorbis* (Berry & Cottrell, 1974). It provides a very useful test for monosynaptic connexions in this species.

In experiments on transmission from the giant dopamine cell, the concentration of $CaCl_2$ was increased from 4·5 to 60 mM without osmotic compensation. Fig. 4*A* shows that the giant-cell-elicited i.p.s.p.s were not abolished by high Ca concentrations even over long periods of time. These experiments were performed on a total of twenty-four visceral ganglion

cells which showed discrete i.p.s.p.s, and in each case there was no loss of i.p.s.p.s. There was similarly no effect on e.p.s.p.s or b.p.s.p.s (Fig. 4*B*, *C*).

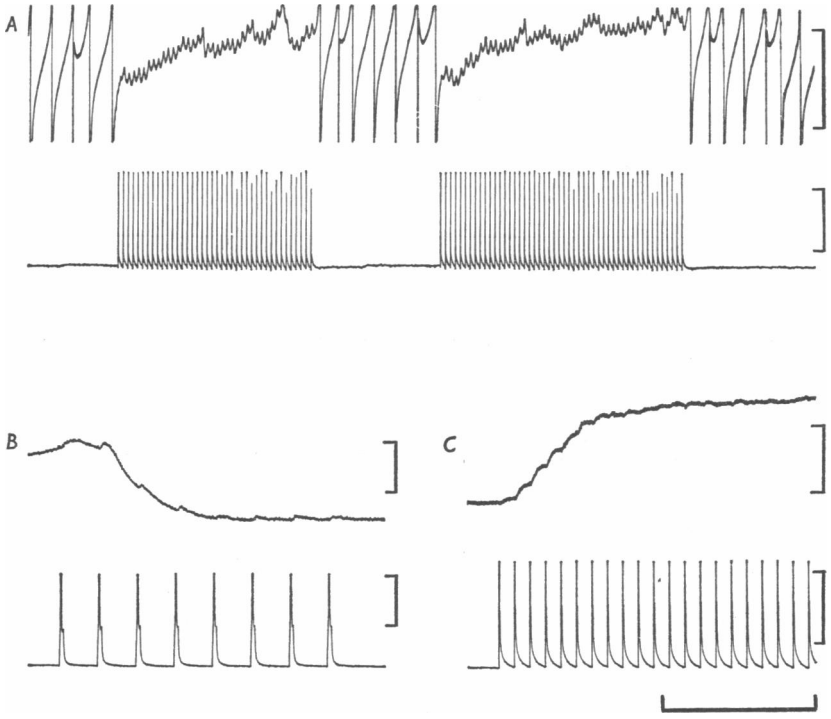

Fig. 4. Post-synaptic responses (upper trace of each pair) to stimulation of the giant dopamine cell (lower trace of each pair) in high Ca solution. *A*: i.p.s.p.s; *B*: b.p.s.p.s in visceral ganglion cells; *C*: e.p.s.p.s in a parietal ganglion cell. Records were made 5–10 min after the addition of physiological solution containing 60 mM-$CaCl_2$ to the bath. Similar records were made for a period of 45 min from the time the high Ca solution was added. Since there was not even the occasional loss of a response in high Ca, this suggests that the potentials are produced monosynaptically. The cell in *A* was depolarized by about 20 mV (to −40 mV) to increase the amplitude of the i.p.s.p.s. Record *B* was made at the resting level (−60 mV). The cell in *C* was also recorded at the resting membrane potential (−65 mV), and in normal medium produced a burst of spikes in response to the same stimulation of the giant dopamine-containing cell. Voltage calibrations: 50 mV (presynaptic), 10 mV (post-synaptic). Time calibration, *A*: 50 sec; *B*: 2 sec; *C*: 10 sec.

Does dopamine mediate the post-synaptic responses?

Inhibitory and biphasic responses

Possible transmitters. As far as we are aware, the number of candidates for transmitter function in molluscs (and other animals) is limited at the

present time to about a dozen substances. Of these, the best studied is ACh (Wachtel & Kandel, 1971; Gardner & Kandel, 1972; Kehoe, 1972*a*, *b*, *c*). There is also evidence for a transmitter role of dopamine (Ascher *et al.* 1967; Kerkut *et al.* 1969; Walker *et al.* 1971), 5-HT (Cottrell, 1970; Gerschenfeld & Paupardin-Tritsch, 1973; Cottrell & Macon, 1974), and glutamate (Gerschenfeld, 1973; Szczepaniak & Cottrell, 1973). In a systematic study of visceral ganglion cells receiving inhibitory input from the giant dopamine cell, the effects were observed of these four substances and also of noradrenaline, glycine, GABA and histamine. The drugs were

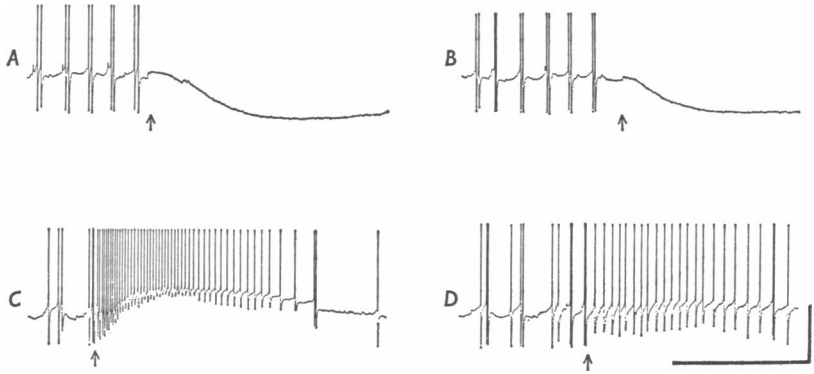

Fig. 5. Responses of a visceral ganglion cell, which received inhibitory input from the giant dopamine cell, to possible transmitter substances applied from a pipette (arrow). *A*: dopamine; *B*, glutamate; *C*: ACh; *D*: 5-HT. Calibration: 10 sec, 20 mV.

individually applied to the cell from a pipette. The results are summarized in Table 1*A*. Only dopamine and glutamate produced a hyperpolarization in each follower cell (Fig. 5*A*, *B*). Examples of depolarizing responses to ACh and 5-HT are shown in Fig. 5*C*, *D*.

Effects of dopamine and ACh antagonists. To determine whether dopamine or glutamate is the more likely inhibitory transmitter substance released on to the visceral ganglion cells from the giant dopamine cell, a number of dopamine-blocking drugs were tested. ACh antagonists were tested for effects on the depolarizing phase of b.p.s.p.s (ACh depolarized each follower cell, Fig. 5*C*).

Ergometrine is a potent antagonist of dopamine in snail neurones (Walker *et al.* 1968) but may produce a conductance change (Ascher, 1972) and thus be non-specific. In the present experiments, ergometrine was tested on 24 preparations. A typical result is shown in Fig. 6. Addition of ergometrine (10^{-6}–10^{-5} M) to the bath resulted in a hyperpolarization and a reduction or abolition of spontaneous firing in the follower cells after a few minutes; there was an accompanying small reduction in the amplitude

of i.p.s.p.s from the giant cell (summed rather than unitary i.p.s.p.s were generally studied because it was easier to measure their amplitude accurately). After 5–10 min, i.p.s.p.s from the giant dopamine cell were reduced from 15–25 mV to 1–5 mV or more usually completely abolished. The response to applied dopamine was also reduced or abolished whereas the

TABLE 1. *A*: responses of visceral ganglion cells receiving inhibitory input from the giant dopamine-containing cell to possible transmitters applied from a pipette. Results taken from forty different preparations. *B*: summary of the effects of blocking drugs on i.p.s.p.s and b.p.s.p.s elicited in visceral ganglion cells by stimulation of the giant cell

A

Possible transmitter	Number of cells tested	Response: Hyperpolarized	Response: Depolarized	Response: No response
Dopamine	100	100	—	—
Acetylcholine	50	—	50	—
L-Glutamic acid	80	80	—	—
5-HT	50	—	38	12
Noradrenaline	35	28	2	5
γ-Aminobutyric acid	30	5	14	11
Histamine	30	—	19	11
Glycine	25	—	—	25

B

Blocking drug	Effect on inhibitory transmission from the giant dopamine cell and the inhibitory response to dopamine
Ergometrine (10^{-6} M–10^{-5} M) 6-Hydroxydopamine (2×10^{-3} M)	Specific abolition of i.p.s.p.s and blockade of dopamine receptors (little effect on most i.p.s.p.s from other, unknown sources or on hyperpolarizing response to glutamate. No effect on depolarizing responses to ACh or 5-HT)
Tubocurarine (10^{-4} M)	No effect on i.p.s.p. or hyperpolarizing dopamine response. Selectively abolishes depolarizing phase of b.p.s.p. No effect on hyperpolarizing phase or hyperpolarizing response to dopamine at 10^{-3} M
Hexamethonium (10^{-4} M)	No effect on i.p.s.p.s, b.p.s.p.s or the hyperpolarizing response to dopamine (slight reduction of depolarizing phase of b.p.s.p. at 10^{-3} M)

glutamate response was usually unaffected or occasionally (five preparations) converted into a biphasic response (Fig. 6). Partial or sometimes full recovery of i.p.s.p.s and dopamine response occurred after washing for at least 1 hr.

To determine the specificity of action on synaptic potentials, the effect of ergometrine was observed on compound i.p.s.p.s elicited by stimulation of a parietal or visceral nerve. (The stimulus voltage was increased until

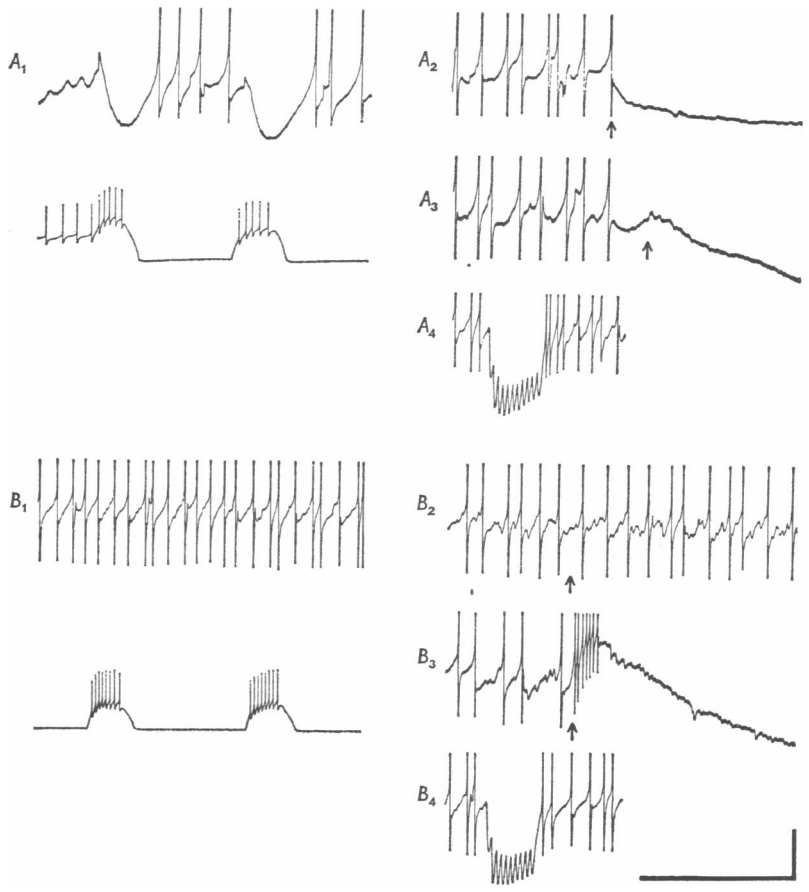

Fig. 6. Effects of ergometrine on the inhibitory response of a visceral ganglion cell to stimulation of the giant dopamine cell, and to applied dopamine and glutamate. *A*: control responses; *B*: effect of $2{\cdot}5 \times 10^{-6}$ M ergometrine on the responses. A_1: i.p.s.p.s produced in a visceral ganglion cell (upper) by stimulation of the giant cell (lower). A_{2-4}: response of the same visceral ganglion cell to dopamine (A_2), glutamate (A_3) and repetitive stimulation of a parietal nerve (A_4). B_1: ergometrine abolishes the inhibitory input from the giant cell. It also abolishes the response to dopamine (B_2) and, although in this case it affects the glutamate response by making it biphasic in nature (B_3), it does not abolish the inhibition. Ergometrine has little effect on the response to stimulation of the parietal nerve (B_4). Dopamine and glutamate were applied from a pipette (arrows). The visceral ganglion cell was artificially depolarized by about 10 mV in B_{1-4}. Note the spontaneous inhibitory input in the presence of ergometrine, presumably mediated by a transmitter other than dopamine. Calibration: 10 sec, 110 mV (presynaptic), 10 mV (post-synaptic).

a maximum response was obtained, and the amplitude of the i.p.s.p.s during each train of stimuli remained constant provided each train was separated by about 20 sec). The effect of ergometrine was variable, reducing the potentials by a half in some cells and producing no measurable effect in others (Fig. 6). It seems likely that much of the inhibitory input resulting from nerve stimulation is due to a transmitter other than dopamine, possibly glutamate. The response to glutamate was not abolished by high ergometrine concentrations (10^{-4} M), which were similarly ineffective on i.p.s.p.s from nerve stimulation. The results indicate that dopamine rather than glutamate is the transmitter from the giant dopamine-containing cell, and that the cells receive inhibitory input not mediated by dopamine from other sources.

6-Hydroxydopamine (6-OHDA) produces a selective destruction of certain catecholamine neurones in many different vertebrates (Tranzer, 1971). It appears to have a similar action on the dopamine cell (Berry, Pentreath, Turner & Cottrell, 1974). When 6-OHDA was tested on i.p.s.p.s from the giant dopamine cell, a post-synaptic effect was also observed. There was a reduction of i.p.s.p. amplitude at a concentration of from 4×10^{-4} to 1×10^{-3} M and a total abolition of response at about 2×10^{-3} M. The effect, which was reversible up to about 1 hr exposure to the drug, was found to be due to a blockade of dopamine receptors (Fig. 7). After washing for 10–60 min, there was a gradual recovery of i.p.s.p.s and concomitant recovery of the response to dopamine. There was little or no effect of 6-OHDA on the glutamate response (Fig. 7*C*) or the depolarizing ACh and 5-HT responses. A check on the specificity of the responses was made by observing the action on i.p.s.p.s produced by stimulating a nerve trunk (Fig. 7). These results again suggested that dopamine rather than glutamate is released by the giant dopamine cell, though the follower cells also have input presumably mediated by a different transmitter. Control tests showed the absence of an effect of ascorbic acid, used to stabilize the 6-OHDA. Recovery from 6-OHDA (usually within about 20 min) was much quicker than recovery from ergometrine.

Tubocurarine was found by Ascher (1972) to block the excitatory effect of dopamine on *Aplysia* neurones without affecting the inhibitory response: for example it abolished the depolarizing phase of a biphasic response but not the hyperpolarizing phase. Tubocurarine might therefore be expected to have a similar effect on biphasic synaptic potentials from the giant dopamine cell found in some visceral ganglion cells. Experiments of the type illustrated in Fig. 8 showed that tubocurarine (10^{-4} M) did in fact abolish the depolarizing phase of a b.p.s.p. without affecting the larger inhibition.

Hexamethonium was shown by Ascher to block the depolarizing

response to iontophoretically applied ACh without affecting the depolarizing response to dopamine in *Aplysia* neurones. When tested on cells showing b.p.s.p.s, high concentrations of hexamethonium (10^{-4} M) had no effect on the depolarizing phase of the b.p.s.p. but eliminated the depolarization produced by ACh. At higher concentrations (10^{-3} M), hexamethonium did usually produce some small reduction of the depolarizing phase (Fig. 9);

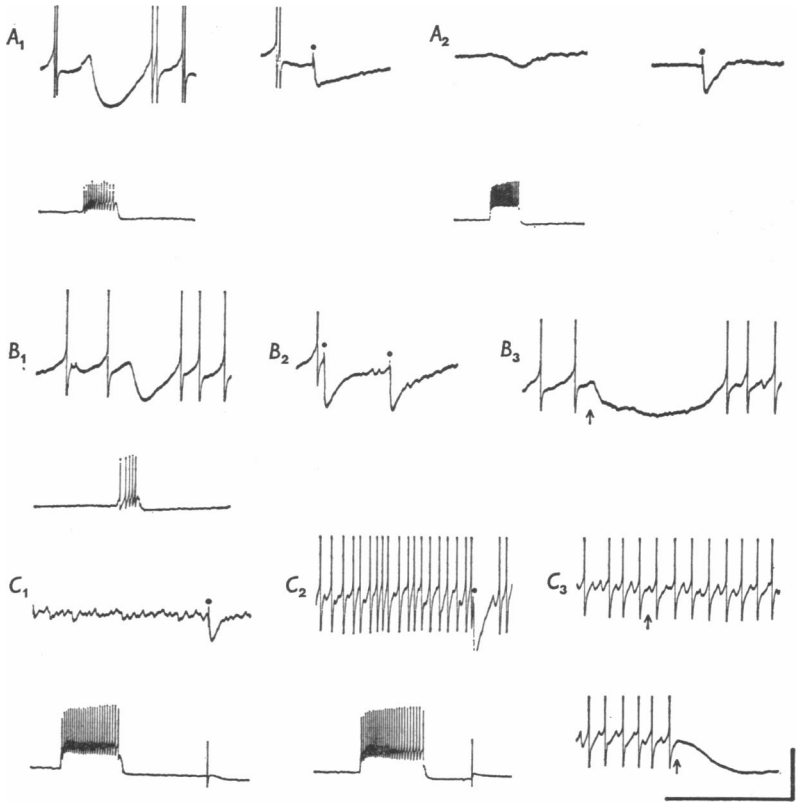

Fig. 7. For legend see opposite page.

however, in no case was there a reduction of more than one half after exposure to this very high concentration of hexamethonium for 30 min. The response of this first phase of the b.p.s.p. to tubocurarine and hexamethonium excludes a cholinergic or electrical transmission mechanism. Table 1*B* summarizes the effects of blocking drugs on i.p.s.p.s and b.p.s.p.s produced by stimulation of the giant dopamine cell.

Comparison of dopamine and noradrenaline responses. Although available evidence indicates that the giant dopamine cell does not contain noradrenaline, the possible presence of small quantities of this amine cannot be

excluded with certainty. In most cases it produced a hyperpolarization which was blocked by the dopamine antagonists ergometrine and 6-OHDA. If noradrenaline is the transmitter, one might expect that noradrenaline would be as potent, if not more potent than dopamine in producing responses in the follower cells. In twelve preparations, bath application of 10^{-6} M dopamine produced a hyperpolarization which reduced or abolished spontaneous firing in the visceral ganglion cells. In no case was the same concentration of noradrenaline observed to have any effect; concentrations of 10^{-5} M or higher were necessary to produce a measurable response. Thus dopamine was at least ten times more potent than noradrenaline on these cells.

Excitatory responses

Possible transmitters. The post-synaptic neurones located on the dorsal surface of the parietal ganglion, illustrated in Fig. 1, were depolarized and excited by dopamine (Fig. 10*A*). There was wide variation in the strength of the response which was usually a gradual depolarization, very much weaker than the response to an equivalent dose of ACh (Fig. 10*B*).

Legend to Fig. 7.

Fig. 7. Effects of 6-OHDA on the inhibitory response of visceral ganglion cells to stimulation of the giant dopamine cell, and to applied dopamine and glutamate. A_1: control responses of a visceral ganglion cell (upper) to stimulation of the giant dopamine cell (lower) and to a single stimulus to a visceral nerve (stimulus artifacts dotted). A_2: responses in the presence of 6-OHDA, 4×10^{-4} M. The hyperpolarization produced by stimulating the giant cell is greatly reduced, whereas there is little effect on the amplitude of the i.p.s.p. produced by stimulating the visceral nerve (though it is reduced in duration). Records *B* and *C* are from a different preparation. *B*: control responses; *C*: responses during exposure to 6-OHDA, 2×10^{-3} M. B_{1-3}: response of a visceral ganglion cell to stimulation of the giant dopamine cell (B_1), stimulation of a visceral nerve (B_2), and to dopamine (arrow) applied with a pipette (B_3). C_1, C_2: response of the visceral ganglion cell to stimulation of the giant cell and of the visceral nerve at the resting potential (C_1) and during artificial depolarization of about 20 mV (C_2). There is no response to stimulation of the giant cell even though the depolarization has increased the amplitude of the response to stimulation of the visceral nerve above the control value. The response to dopamine is abolished (C_3, upper) but glutamate produces a large hyperpolarization (C_3, lower). A brief application of dopamine was given in B_3, whereas perfusion of the bathing medium was turned off during the application of dopamine in C_3 and the pipette left in position for several seconds to expose the cell to a high concentration of dopamine. Note the spontaneous inhibitory input in the presence of 6-OHDA. Calibration, *A*: 10 sec, 130 mV (presynaptic), 15 mV (post-synaptic); *B*, *C*: 10 sec, 80 mV (presynaptic), 15 mV (post-synaptic).

Intervals of several minutes were needed between applications of dopamine for recovery of sensitivity. Glutamate can probably be eliminated as the excitatory transmitter because of its inhibitory action or absence of effect (Fig. 10*C*), but ACh (Fig. 10*B*) and often 5-HT produced depolarizing responses. Blocking drugs were used to try to distinguish between dopamine and ACh as the possible transmitter.

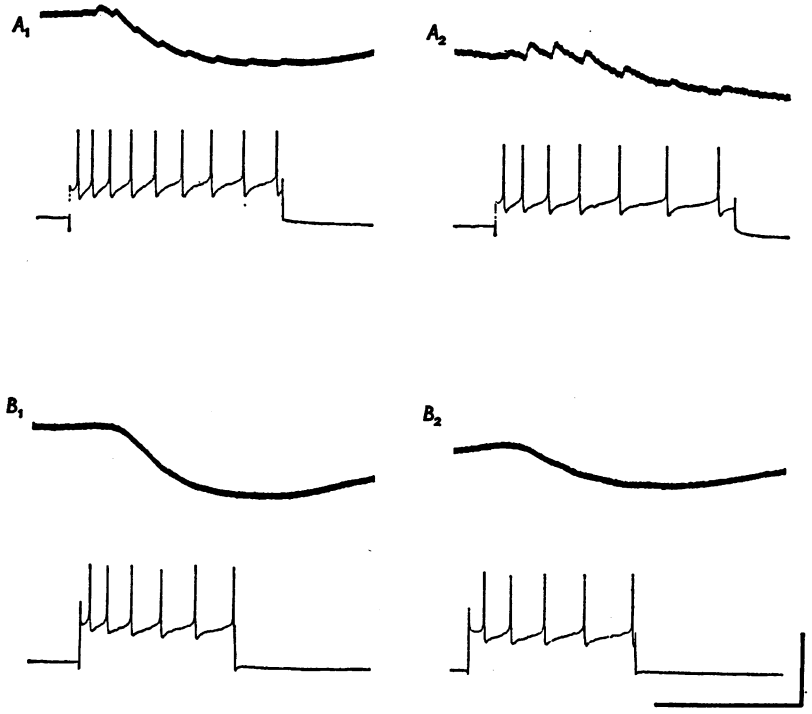

Fig. 8. Effect of tubocurarine on b.p.s.p.s produced in a visceral ganglion cell (upper trace of each pair) by stimulation of the giant dopamine cell (lower trace of each pair). *A*: control; *B*: tubocurarine, 10^{-4} M. Tubocurarine blocks the depolarizing phase without affecting the hyperpolarizing phase of b.p.s.p.s at concentrations as high as 10^{-3} M. The visceral ganglion cell was recorded at the resting membrane potential (− 65 mV) in A_1 and B_1, and was hyperpolarized by 20 mV in A_2 and B_2 in order to increase the amplitude of the depolarizing phase. Full recovery occurred after 30 min of washing. Calibration: 2 sec, 80 mV (presynaptic), 10 mV (post-synaptic).

Effects of dopamine and ACh antagonists. Tubocurarine abolishes excitatory responses to dopamine and ACh in *Aplysia* neurones (Ascher, 1972). In *Planorbis*, the effects of tubocurarine on e.p.s.p.s produced by stimulating the giant dopamine cell were somewhat variable. In some cases, tubocurarine had little effect (Fig. 11*A*) but in others it produced a considerable reduction in e.p.s.p. amplitude. Higher concentrations (10^{-3} M)

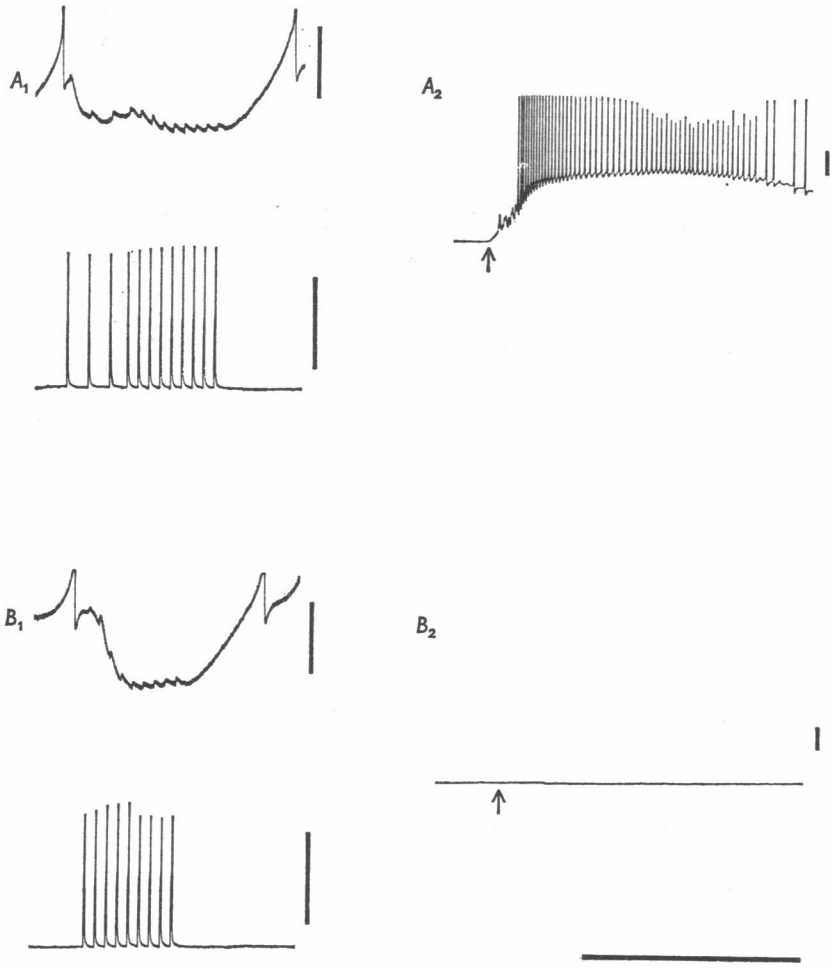

Fig. 9. Lack of effect of hexamethonium on the depolarizing phase of b.p.s.p.s produced in a visceral ganglion cell by stimulation of the giant dopamine cell. *A*: control. *B*: hexamethonium (10^{-3} M). A_1: biphasic response of visceral ganglion cell (upper) to stimulation of the giant cell (lower). A_2: response of the visceral ganglion cell to ACh (arrow) applied with a pipette. B_1: after 20 min in hexamethonium the depolarizing phase is little affected. B_2: the response to ACh (arrow) is abolished. The results suggest that the depolarizing phase of the b.p.s.p. is not cholinergic. In A_1 and B_1 the visceral ganglion cell was recorded at the resting potential (-65 mV); in A_2 and B_2 the cell was hyperpolarized by about 20 mV to prevent spontaneous firing. Calibration: 10 sec, 50 mV (presynaptic), 15 mV (post-synaptic).

were necessary than those used to block the depolarizing phase of a b.p.s.p. (10^{-4} M). These high concentrations abolished the response to ACh but often reduced the response to dopamine. Because tubocurarine could produce similar effects on the response to dopamine and ACh, it was not possible to obtain unambiguous evidence of the more likely candidate for the transmitter.

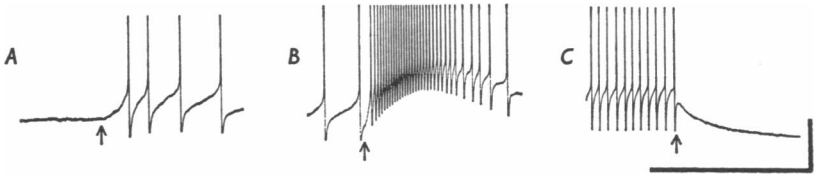

Fig. 10. Responses of parietal ganglion cells receiving excitatory input from the giant dopamine cell to dopamine, ACh and glutamate applied from a pipette (arrows). *A*: dopamine; *B*: ACh; *C*: glutamate. Dopamine produces a gradual depolarizing response which results in a much lower frequency of firing than the response to ACh, and the cell takes several minutes for recovery of sensitivity. Records *A* and *B* are from the same parietal ganglion neurone; glutamate had no effect on this cell, but its inhibitory action on a different neurone which was excited by the giant cell is shown in *C*. Glutamate is therefore unlikely to be the excitatory transmitter from the giant cell. Calibration, *A*, *B*: 10 sec, 15 mV; C: 10 sec, 18 mV.

Ergometrine was found by Ascher (1972) to have no effect on the excitatory response to dopamine in *Aplysia* neurones. In *Planorbis* a block of the excitatory response of the parietal ganglion cells to dopamine and to stimulation of the giant dopamine cell (Fig. 11*B*) was obtained with fairly high concentrations of ergometrine (10^{-5} M) which had no effect on the response to ACh. This implicates dopamine as the transmitter but, in view of the possibility of non-specific actions of high concentrations of ergometrine, is not conclusive.

Hexamethonium blocks excitatory ACh responses but not dopamine responses in *Aplysia* (Ascher, 1972). Very high concentrations of hexamethonium (2×10^{-3} M) did not block the excitatory response to stimulation of the giant dopamine cell or to applied dopamine but completely abolished the response to ACh (Fig. 12). Hexamethonium also abolished spontaneous (presumably cholinergic) e.p.s.p.s, and greatly reduced the compound e.p.s.p. produced by stimulating a nerve trunk. The evidence therefore favours dopamine rather than ACh as the excitatory transmitter from the giant dopamine-containing cell.

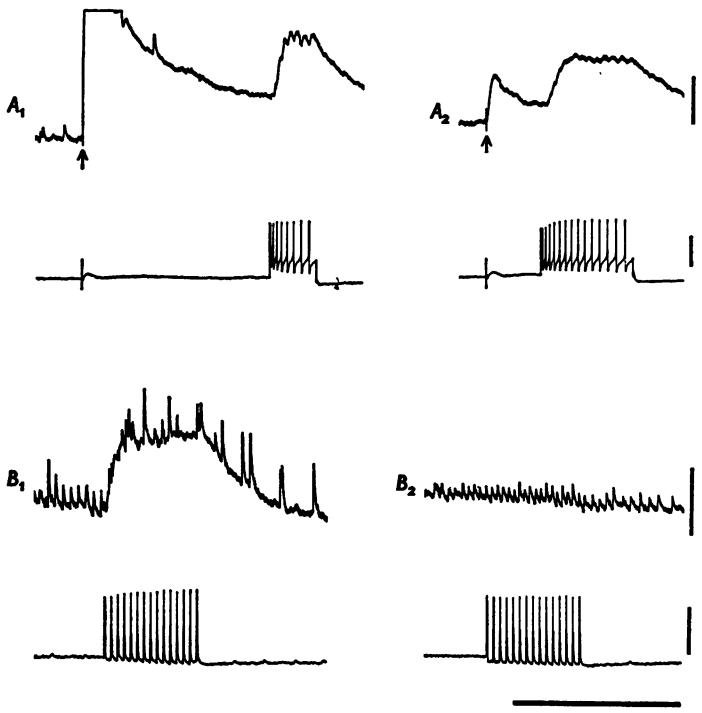

Fig. 11. Effect of tubocurarine and ergometrine on the excitatory response of parietal ganglion cells to stimulation of the giant dopamine-containing cell. *A*: tubocurarine (10^{-3} M). *B*: ergometrine (2×10^{-5} M). A_1: control response of parietal ganglion cell (upper) to stimulation of a parietal nerve (arrow) and repetitive firing of the giant cell (lower). The stimulus to the parietal nerve produced a large compound e.p.s.p. A_2: after 20 min exposure to tubocurarine the response to giant-cell activation was only slightly affected, whereas there was a very large reduction in the e.p.s.p. resulting from stimulation of the parietal nerve. The results suggest that much of the input is mediated by ACh but that transmission from the giant cell has a different mediator. However, the variability of the response in different preparations and the fact that tubocurarine could reduce the response to dopamine prevent this type of experiment from giving unequivocal evidence that dopamine is the transmitter. B_1: control response of a parietal ganglion cell (upper) to stimulation of the giant cell. B_2: 20 min after the addition of ergometrine, the response to the giant cell stimulation is abolished. The continued presence of spontaneous e.p.s.p.s shows that the effect is specific. The response to ACh was unaffected by ergometrine which abolished the dopamine response. The evidence is again suggestive of dopamine rather than ACh transmission from the giant cell. The parietal ganglion cell in *A* and *B* was hyperpolarized by 30 mV (resting potential, −60 mV). Calibration: 10 sec, 50 mV (presynaptic), 10 mV (post-synaptic).

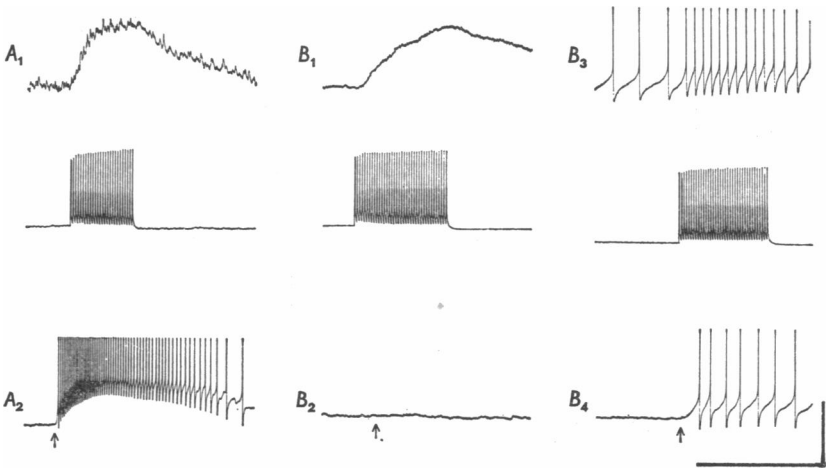

Fig. 12. Hexamethonium and the excitatory input from the giant dopamine-containing cell to parietal ganglion cells. *A*: control. *B*: 15–25 min after the addition of hexamethonium (2×10^{-3} M) to the bath. A_1: response of a parietal ganglion cell (upper, hyperpolarized from -60 to -90 mV) to repetitive stimulation of the giant cell (lower). A_2: response of the parietal ganglion cell at the resting level to ACh applied from a pipette (arrow). B_1: the response to giant-cell stimulation is not abolished by hexamethonium though the time to peak is somewhat prolonged. Note the reduction of spontaneous synaptic potentials which are probably cholinergic. The parietal ganglion cell was hyperpolarized to -90 mV, as in A_1. B_2: the response to ACh (arrow) is abolished. B_3: response to giant dopamine cell stimulation of the parietal ganglion cell recorded at the resting potential. The excitatory input is still very effective even in the presence of such high hexamethonium concentrations. B_4: the usual gradual depolarizing response to dopamine applied with a pipette (arrow). Excitatory input from the giant dopamine cell is therefore unlikely to be cholinergic. The upper parts of action potentials in the parietal ganglion cell were not recorded. Calibration: 10 sec, 65 mV (presynaptic), 15 mV (post-synaptic A_1, B_1), 30 mV (post-synaptic A_2, B_{2-4}).

DISCUSSION

The giant dopamine-containing neurone produces three types of post-synaptic potentials in different follower neurones. The potentials appear to be produced by direct connexions from the giant cell and to result from release of dopamine. There is now good evidence that the giant cell contains dopamine; it is more difficult to provide unequivocal data that the connexions are direct and that dopamine is released to mediate the different post-synaptic potentials observed. Each of these points is discussed below.

Presence of dopamine in the giant cell. Recently, Powell & Cottrell (1974) have made a spectrofluorimetric analysis of individually dissected

cells which reveals dopamine at a level of $5{\cdot}4 \pm 0{\cdot}6$ p-mole/cell ($n = 8$), but no noradrenaline (minimum detectable amount was approximately 1·3 p-mole/cell). These observations are consistent with microspectrofluorimetric studies by B. Falck (personal communication) which strongly suggest the presence of dopamine rather than noradrenaline in the giant cell.

Monosynaptic nature of the connexions. Morphological autoradiographic studies (Pentreath *et al.* 1974) involving injection of tritiated leucine into the giant-cell perikaryon, show that the latter could make direct synaptic contacts with the various follower cells; these experiments reveal that the giant cell has a large axon which branches extensively in the neuropile of the visceral and left parietal ganglia where the follower cells are situated. The e.p.s.p.s and depolarizing phase of b.p.s.p.s had a short, constant latency (*ca.* 20 msec) and were observed to follow each giant-cell spike without loss up to the maximum frequency above which smooth summation occurred. This indicates that the responses are mediated monosynaptically (Kandel *et al.* 1967; Kandel, 1969; Wachtel & Kandel, 1971; Gardner & Kandel, 1972).

It was not possible to observe whether i.p.s.p.s in the follower cells could follow high-frequency stimulation of the giant cell or to measure their latency accurately. However, it was obvious that the latency of 70–120 msec for the i.p.s.p.s was extremely long. There is a number of possible explanations for this delay: (1) synapses may be located a long way from the cell body, and synaptic potentials will therefore not be seen in the soma until the intervening membrane has been sufficiently charged by the synaptic current (there is some evidence for this view, for example i.p.s.p.s from the giant cell were difficult to invert compared with i.p.s.p.s from other sources). Ascher (1972) found that dopamine receptors may be nearly 1 mm from the perikarya of *Aplysia* neurones; (2) Kehoe (1972*c*) observed that in a two-component unitary cholinergic i.p.s.p. in *Aplysia* the approximate latency (90–140 msec) of the second component was twenty times that of the initial component; this illustrates that the latency of a response is not necessarily determined principally by conduction velocity of the spike in the presynaptic neurone. Such rather negative evidence has been presented to show that the apparently extreme values of latency of the inhibitory responses do not necessarily preclude direct connexions. It may be significant that most apparently simple i.p.s.p.s seemed to have a very small depolarizing phase when recorded at high gain, suggesting that the i.p.s.p.s are the late phase of a two-component response.

The best evidence of monosynaptic connexions came from experiments with high Ca. In view of its potency in blocking normal one-for-one

transmission of action potentials (Berry & Cottrell, 1974) the presence of interposed synapses is extremely unlikely.

Dopamine as the transmitter. Of the substances tested, only dopamine produced a potential change that in each case was of the same polarity as the synaptic input where this was monophasic. It was not possible to produce a biphasic potential change by bath application of dopamine which produced only a hyperpolarization. However, Ascher (1972) found that cells giving biphasic responses to iontophoresed dopamine in *Aplysia* often gave only hyperpolarizing responses to perfused dopamine. Where ACh or glutamate produced similar responses to dopamine, the use of dopamine and ACh antagonists showed dopamine to be the more likely candidate for the transmitter from the giant dopamine cell.

The effects of blocking drugs on the post-synaptic response to stimulation of the giant cell are similar to Ascher's results from dopamine-iontophoresis studies. For example, tubocurarine partially blocked e.p.s.p.s and completely abolished the depolarizing phase of b.p.s.p.s, whereas hexamethonium was ineffective; there was abolition of i.p.s.p.s by ergometrine which, however, also had an effect on e.p.s.p.s at high concentration. The discovery of the specific antagonistic effects of 6-OHDA on the dopamine, and post-synaptic, responses was particularly useful in confirming the results of experiments with ergometrine. It has already been noted that 6-OHDA may block some peripheral α-adrenergic receptors in vertebrates (Furness, 1971; Haeusler, 1971).

The results of this study suggest that the system comprising the giant cell and the observed follower neurones in *Planorbis* is a favourable model for analysing neuronal dopamine, particularly its possible role as a transmitter substance. The available data indicate that dopamine is released from the endings of the giant dopamine cell and that it can mediate different effects on different post-synaptic cells. However, further experiments are required to establish these points beyond doubt. In particular, it is important to compare the nature of the ionic mechanisms responsible for the dopamine effects and the post-synaptic responses, and to detect dopamine release into the medium following giant-cell stimulation.

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