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MULTIPLE POSTSYNAPTIC ACTIONS OF THE GIANT DOPAMINE-CONTAINING NEURONE R.Pe.D.1 OF *LYMNAEA STAGNALIS*

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SUMMARY

(1) Evidence is presented that the giant neurone R.Pe.D.1 is monosynaptically connected to follower cells in the right parietal and visceral ganglia and has excitatory, inhibitory and biphasic postsynaptic actions.

(2) The spike to e.p.s.p. latency is increased to a new fixed value by high calcium saline, but not by saline in which both the Ca^{2+} and Mg^{2+} concentrations have been increased in proportion to one another.

(3) Three distinct classes of p.s.p. latency exist. Short e.p.s.p. and b.p.s.p. latencies of *ca.* 16·0 to 20 ms have been observed, as have longer e.p.s.p. and b.p.s.p. latencies, *ca.* 38–44 ms. An i.p.s.p. with a latency to onset of *ca.* 182 ms occurs on the J cells.

INTRODUCTION

The possibility that a single neurone could have multiple postsynaptic actions was first reported by Strumwasser (1962) and clearly demonstrated by Kandel and collaborators (e.g. Kandel *et al.* 1967; Wachtel & Kandel, 1967), working on the neurone L10, in the abdominal ganglion of *Aplysia*. Later work by Gardner & Kandel (1972) indicated the presence of similar neurones in the buccal ganglia of *Aplysia*. In 1975, Berry & Cottrell described a multi-action neurone in the brain of the sinistral basommatophoran pulmonate *Planorbis corneus*. The soma of this neurone is situated in the left pedal ganglion and contains dopamine (Powell & Cottrell, 1974). It was termed the giant dopamine-containing cell (G.D.C.). The soma of a similar giant dopamine-containing neurone (McCaman, Ono & McCaman, 1979; Cottrell, Abernethy & Barrand, 1979) lies in the right pedal ganglion of the closely related species *Lymnaea stagnalis* (L.) and is termed R.Pe.D.1 (Benjamin & Winlow, 1981). Since *Lymnaea* is a dextral pulmonate and its brain is a mirror image of *Planorbis*, the giant dopamine-containing cells of the two animals are thought to be homologous. Berry & Cottrell (1975, 1979) indicated that the G.D.C. of *Planorbis* is monosynaptically connected to follower cells in the visceral and left parietal ganglia. However, these

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follower cells are not electrophysiologically characterized. Benjamin & Winlow (1981) demonstrated that R.Pe.D.1 of *Lymnaea* has effects on characterized neurones in the visceral and right parietal ganglia, but did not show that these cells were monosynaptically coupled to R.Pe.D.1. In this paper we present evidence that R.Pe.D.1 is indeed monosynaptically coupled to follower cells in these ganglia. Furthermore we describe considerable variations in the latency to onset of the different p.s.p. types. Various aspects of this work have appeared in preliminary form elsewhere (Winlow & Benjamin, 1977; Winlow, 1981).

MATERIALS AND METHODS

Specimens of *Lymnaea stagnalis* (L.), weighing 1–10·4 g but mainly in the 2–6 g range, were obtained from animal suppliers, kept in tap water at room temperature and fed on lettuce. Brains were removed and maintained in Hepes buffered saline as described by Benjamin & Winlow (1981). In addition various high Ca^{2+} salines were used in some experiments. These contained 5× (20 mM) or 10× (40 mM) normal calcium concentrations and were not osmotically corrected. In a further saline both Ca^{2+} and Mg^{2+} concentrations were raised 2·5 times to 10 and 5 mM respectively. When new media were run into the preparation dish, a minimum of 10 min was always allowed for equilibrium before measurements or records were taken.

Individual cells were penetrated with glass microelectrodes filled with either the supernatant from a saturated solution of K_2SO_4 or 1 M tetraethylammonium chloride. Signals were conventionally amplified, displayed and recorded according to the methods of Benjamin & Winlow (1981).

RESULTS

(I) *Identification of follower cells of R.Pe.D.1*

The electrophysiological properties and synaptic inputs of the follower cells of R.Pe.D.1 have been described by Benjamin & Winlow (1981). In addition to receiving synaptic inputs from R.Pe.D.1 these cells also receive two other wide-acting synaptic inputs whose sources remain unidentified. One of these, input 3, is a very powerful, two-component, compound p.s.p. lasting for many seconds (Fig. 1*a*) and may have either inhibitory or excitatory effects on its follower cells. To be certain of the identity of any given follower cell of R.Pe.D.1 it is only necessary to know the postsynaptic actions of R.Pe.D.1, and of input 3 on that cell. Bursts of spikes in R.Pe.D.1 are able to trigger input 3 (Fig. 1*b*, *c*) and this effect has proved invaluable in our identification of neurones. Repeated bursts of spikes from R.Pe.D.1 over a short time period can cause the temporary cessation of input 3 (Fig. 1*d*). The precise mechanism of these effects is unknown because the sources of input 3 are as yet unidentified.

(II) *Postsynaptic actions of R.Pe.D.1*

As described previously (Benjamin & Winlow, 1981) R.Pe.D.1 makes three types of connexions with its follower cells. It makes excitatory connexions to the giant cells VD2 and VD3 (Fig. 2*b*), most of the cells of A group (gp), the H cells and

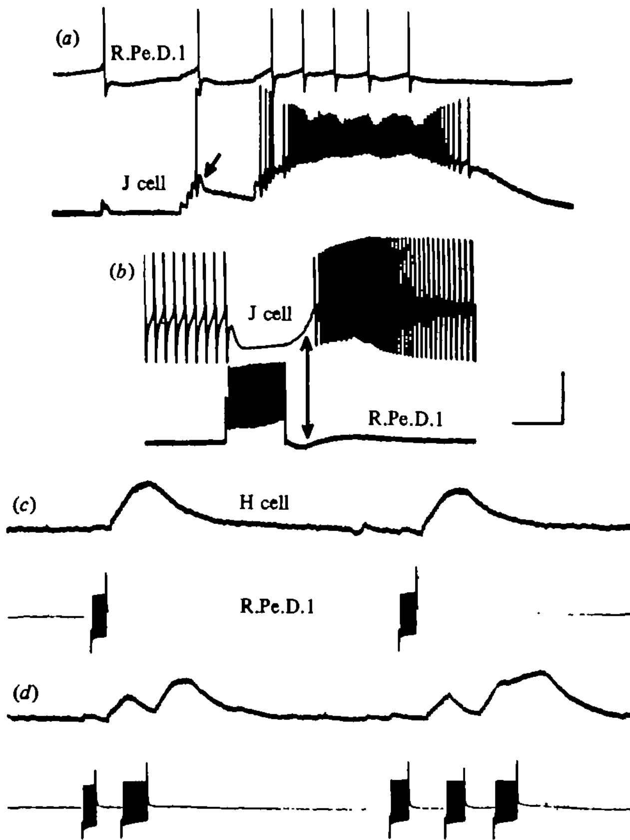

Fig. 1. Interactions between input 3 and R.Pe.D.1. (*a*) Spontaneous discharge of input 3 excites both R.Pe.D.1 and a hyperpolarized J cell. A short input 3 excitatory wave is terminated by a spike in R.Pe.D.1 (arrow) and followed a few seconds later by a full input 3 excitation which leads to a burst of spikes in the J cell. Spikes in R.Pe.D.1 cause partial inhibition of this burst. The partial inhibition could be due to either the inhibitory effect of R.Pe.D.1 on input 3 or the J cell. (*b*) Depolarization of R.Pe.D.1 causes a burst of spikes which inhibits a J cell and a few seconds later input 3 occurs as an excitation in both cells (double headed arrow). (*c* and *d*) Both an H cell and R.Pe.D.1 are hyperpolarized and bursts induced in R.Pe.D.1 trigger depolarizing waves (input 3) in the H cell (*c*). In (*d*), first two (left) and then three (right) bursts were induced in R.Pe.D.1. These caused a temporary cessation of the input 3 depolarizing wave and this leads us to believe that R.Pe.D.1 excites input 3 by post-inhibitory rebound excitation. Voltage calibration. (*a*) Upper, 40 mV; lower, 20 mV; (*b*) upper, 30 mV; lower, 60 mV; (*c*) and (*d*) upper, 40 mV; lower, 100 mV. Time calibration. (*a*) 2 s; (*b*) 4 s; (*c*) and (*d*) 10 s.

the K cells (Fig. 2*a*). It has biphasic actions (excitation followed by inhibition) on all the cells of G gp (Fig. 2*c*), the I cells, and I type cells scattered within A gp. In addition it has inhibitory actions on the J cells (Fig. 2*d*). We believe that the connexions are chemically mediated for the following reasons: (i) the latency to onset of each postsynaptic potential is greater than 17 ms (Figs. 2, 3) whilst the conduction time of the action potential from soma to nerve trunks of the visceral ganglion is not

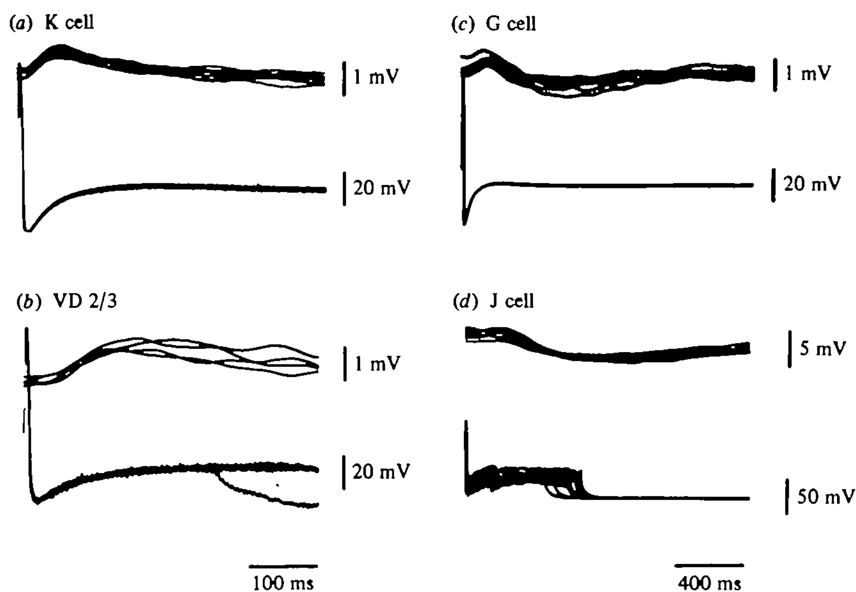

Fig. 2. Examples of the different types of p.s.p. induced by R.Pe.D.1. In (*a*) and (*b*) respectively, examples of e.p.s.p.s. with short and long latencies to onset are shown. In (*c*) and (*d*) respectively biphasic and pure inhibitory p.s.p.s. are demonstrated. In each case the sweep of the oscilloscope was triggered from the rising phase of the R.Pe.D.1 action potential. There are ten superimposed sweeps in (*a*), (*c*) and (*d*) and four sweeps in (*b*). In (*a*) and (*c*) R.Pe.D.1 was depolarized and fired regularly whilst in (*b*) and (*d*) it was induced to fire by application of 1 s depolarizing current pulses at 5 s intervals. Note that the time scale in (*a*) and (*b*) differs from that in (*c*) and (*d*).

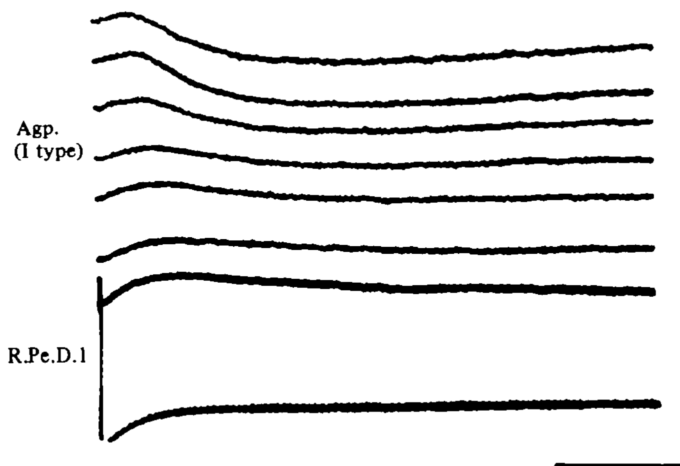

Fig. 3. Sequential hyperpolarization of an A group (I type cell) from top to bottom. Each sweep triggered by the action potential of R.Pe.D.1. Note that with increasing hyperpolarization the depolarizing wave of the b.p.s.p. increases in amplitude whilst the hyperpolarizing wave decreases in amplitude. Voltage calibration. A gp (I type), 40 mV. R.Pe.D.1, 80 mV. Time calibration. 400 ms.

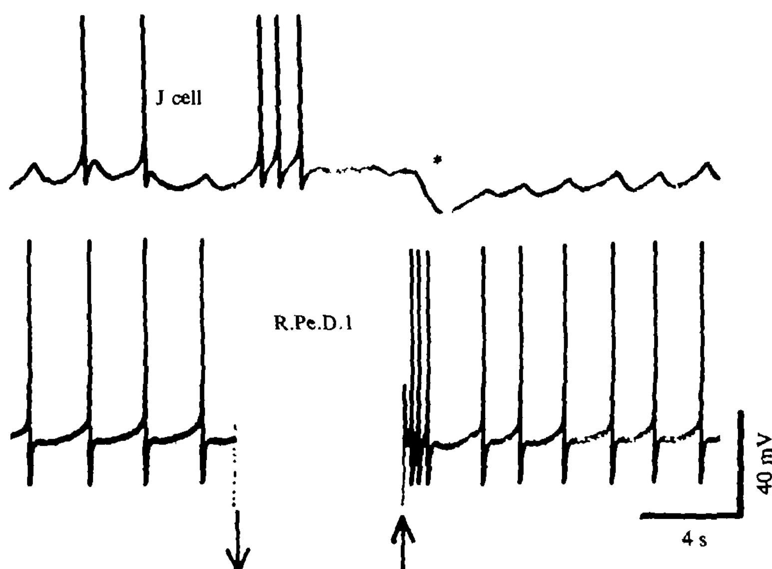

Fig. 4. Smooth summation of J cell i.p.s.p.s. (asterisk) after a short burst in R.Pe.D.1. Both cells were recorded at their natural membrane potentials but R.Pe.D.1 was hyperpolarized between the arrows to produce a burst by post-inhibitory rebound excitation. Note how successive i.p.s.p.s in the J cell increase in amplitude as the cell slowly returns to its natural membrane potential subsequent to the R.Pe.D.1 burst.

greater than 14 ms (Haydon & Winlow, 1981). This latency difference cannot be due to electrically mediated connexions; (ii) high calcium saline increases the amplitude of the postsynaptic potential (e.g. Figs. 5*g* and 6*f*); (iii) injection of hyperpolarizing current into follower cells via the recording electrode increases the amplitude of e.p.s.p.s and decreases the amplitude of i.p.s.p.s and this cannot be explained on the basis of the rectification properties of the membrane. Hyperpolarization of cells receiving b.p.s.p.s causes augmentation of the excitatory phase and diminution of the inhibitory phase (Fig. 3). It was often necessary to hyperpolarize those cells receiving an inhibitory component from R.Pe.D.1 in order to distinguish between pure inhibition and mixed excitation and inhibition, because the excitatory component of b.p.s.p.s was often small when recorded at the normal membrane potential.

(III) *Evidence that R.Pe.D.1 is monosynaptically coupled to its follower cells*

Several criteria have been used to demonstrate that R.Pe.D.1 is monosynaptically connected to its follower cells.

(*a*) *Each presynaptic action potential invariably produces a p.s.p. at constant latency*
A 1:1 relationship between presynaptic spike and p.s.p. at a constant latency suggests that we are recording from monosynaptically connected cells. This relationship is easily demonstrable in all cell types investigated (Figs. 2, 3, 5, 6) at R.Pe.D.1 firing frequencies of 1–2 impulses s^{-1}. At the maximum firing frequency of R.Pe.D.1 (*ca.* 10 spikes s^{-1} for an artificially depolarized cell) 1:1 e.p.s.p.s and b.p.s.p.s are clearly discernible, but it becomes very difficult to identify individual i.p.s.p.s due to smooth summation of these long-lasting events (Fig. 4 and see Benjamin & Winlow, 1981).

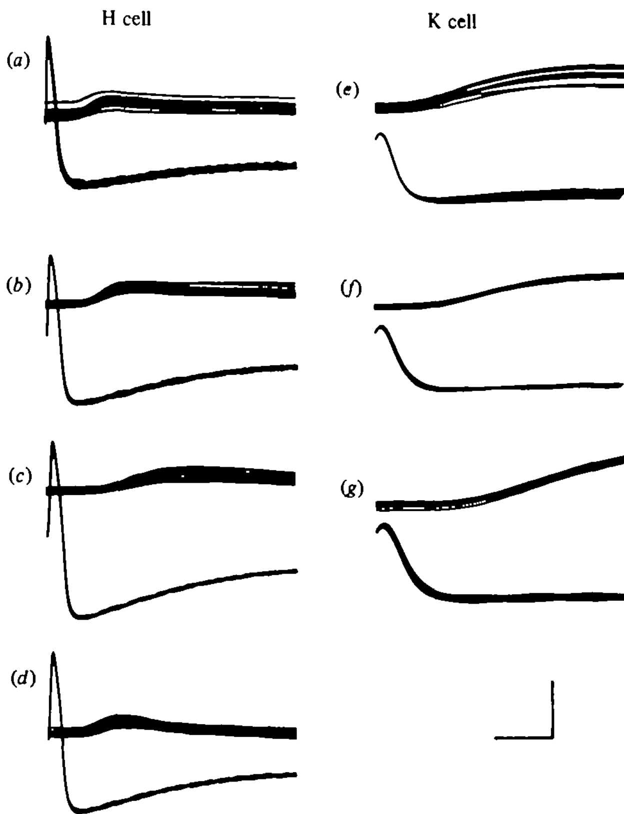

Fig. 5. Lack of blockade of H cell (*a–d*, upper trace) and K cell (*e–g*, upper trace) e.p.s.p.s due to R.Pe.D.1 (lower trace in all cases) by high calcium salines. Both cells were first soaked in normal saline (*a* and *e*), then in 2·5 × calcium/2·5 × magnesium saline (*b* and *f*) and then in 10 × calcium saline (*c* and *g*). A post control in normal saline is shown for the H cell (*d*). Note the increased latency to e.p.s.p. onset in 10 × calcium saline (*c*, *g*). Each pair of traces was triggered on the upsweep of the R.Pe.D.1 action potential and ten superimposed sweeps are shown in each case. Both the H and K cells were hyperpolarized throughout the experiments to prevent spontaneous activity. Voltage calibrations. (*a–d*) Upper, 4 mV; lower, 40 mV; (*e–g*) upper, 4 mV; lower 80 mV. Time calibrations. (*a*), (*b*), (*c*), (*d*) 40 ms. (*e*), (*f*), (*g*) 20 ms.

(*b*) *Effects of high Ca^{2+} saline.* Increased calcium concentrations in the extracellular medium cause a hyperpolarization and a decrease in neuronal excitability (Austin, Yai & Sato, 1967; Berry & Cottrell, 1974). Thus the likelihood that an interneurone, interposed between pre- and postsynaptic cells, would fire is greatly diminished in this saline. We have used both 5 × and 10 × normal calcium levels and on no occasion were we able to block p.s.p.s due to R.Pe.D.1 (Figs. 5, 6 and Table 1). However merely increasing Ca^{2+} levels also enhances transmitter release so that we cannot entirely rule out the presence of an interneurone on the basis of this test alone. Another effect of high calcium saline is to increase the e.p.s.p. latencies to a new constant value (Fig. 5*c*, *g*). Modifications of b.p.s.p. and i.p.s.p. latencies have proved difficult to demonstrate convincingly.

(*c*) *Effects of high calcium/high magnesium saline.* Increasing both calcium and

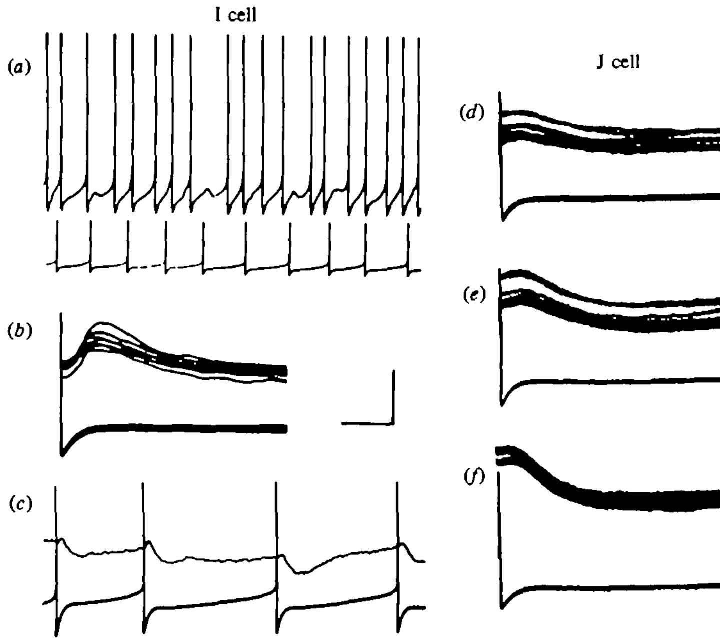

Fig. 6. Lack of blockade of an I cell b.p.s.p. (*a–c*, upper trace) and a J cell i.p.s.p. (*d–f*, upper trace) due to R.Pe.D.1 spikes (lower trace in each case) by high calcium saline. Both cells were first soaked in normal saline (*a* and *d*), then 2·5 × calcium/2·5 × magnesium saline (*b* and *e*) and finally in 10 × calcium saline (*c* and *f*). It proved difficult to record ten superimposed sweeps of a b.p.s.p. in all three media due to the fluctuations of membrane potential in the follower cell. In (*a*) the I cell is at normal membrane potential, but is slightly hyperpolarized in (*b*) and (*c*). In (*b, d, e, f*) each pair of traces was triggered from the upsweep of the R.Pe.D.1 action potential and ten superimposed sweeps are shown in each case. Voltage calibrations: (*a*) upper, 20 mV; lower, 100 mV; (*b*) and (*c*) upper, 4 mV; lower, 40 mV; (*d*)–(*f*) upper, 10 mV; lower, 40 mV. Time calibrations: (*a*) and (*c*) 2 s. (*b, d, e, f*) 400 ms.

Table 1. *The number of attempts at p.s.p. blockade by high calcium salines for each cell type thought to be monosynaptically connected to R.Pe.D.1*

		No. of attempts at p.s.p. blockade		
		High Ca^{2+}/no Mg^{2+}		
p.s.p. type	Cell type	5 × Ca^{2+}	10 × Ca^{2+}	High Ca^{2+}/ High Mg^{2+}
	VD2/3	1	3	3
	A gp	4	7	6
e.p.s.p.s	H cells	4	4	3
	K cells	8	3	4
	A gp	—	4	5
b.p.s.p.s	G gp	—	3	6
	I cells	3	4	6
i.p.s.p.s	J cells	4	4	4

In no case were we able to block a p.s.p. and this suggests that R.Pe.D.1 makes monosynaptic connexions with these cells.

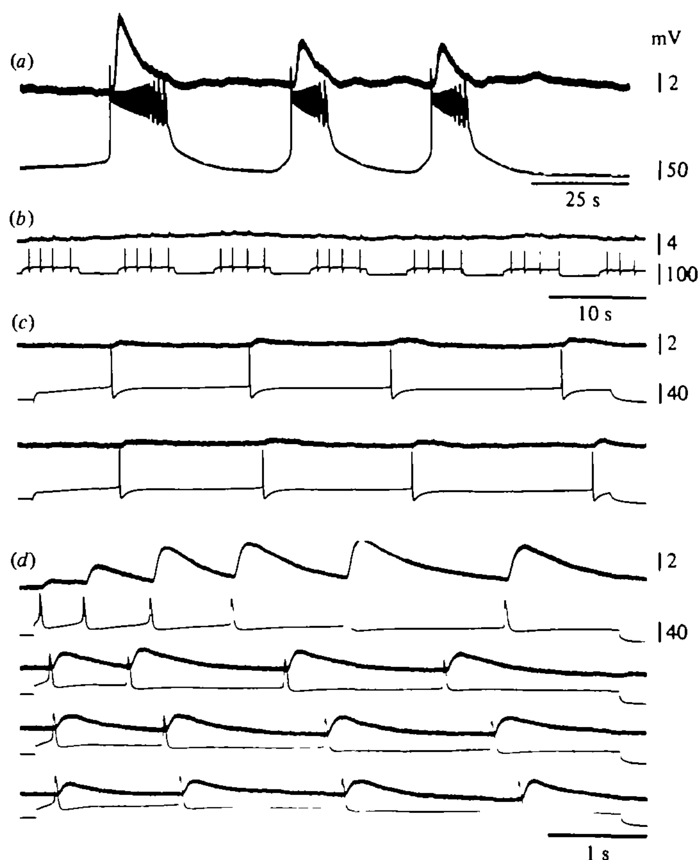

Fig. 7. Effects of T.E.A. injection into R.Pe.D.1 (lower trace in all cases) on the e.p.s.p. of VD2/3 (upper trace in all cases). In the experiment shown in (*a*), T.E.A. was injected into R.Pe.D.1 with a constant depolarizing current of 2 nA for 2 h. Multiphasic spikes developed in R.Pe.D.1. Each apparent burst of action potentials shown here is a single spontaneous discharge recorded from R.Pe.D.1 a few minutes after T.E.A. injection ceased. Each peak in the discharge elicited an e.p.s.p. in VD2/3. Another experiment is illustrated in (*b*–*d*), and in this case T.E.A. was injected into R.Pe.D.1 with 2 nA depolarizing current pulses applied for 2 h as shown in (*b*). In (*c*) two consecutive pre-injection pulses are illustrated and the e.p.s.p.s elicited by R.Pe.D.1 spikes may be compared with post-injection pulses illustrated in (*d*). (*d*) The upper pair of traces shows the first pulse given 5 min subsequent to the cessation of T.E.A. injection. The lower three traces were from consecutive pulses after the e.p.s.p.s had settled to an approximately constant amplitude.

magnesium concentrations by a factor of 2·5 decreases neuronal excitability and ameliorates the increased transmitter release and increased latency to onset of e.p.s.p.s caused by increasing calcium alone (Fig. 5). Using this saline, we were again unable to produce synaptic blockade of R.Pe.D.1 p.s.p.s (Figs. 5*b*, *f*, 6*b*, *e* and Table 1).

(*d*) *Presynaptic injection of Tetraethylammonium* (*T.E.A.*). We have met with varying degrees of success when attempting to inject T.E.A. into R.Pe.D.1. The main difficulty is that although T.E.A. increases the duration of the presynaptic spike it also causes multiphasic action potentials to occur (Fig. 7*a* and see Berry & Pentreath, 1975) making analysis difficult. In one preparation, however, the duration of presynaptic spikes was greatly enhanced, without the appearance of multiphasic action

potentials, and the e.p.s.p.s recorded in VD2/3 increased in both duration and amplitude (Fig. 7*b–d*). Furthermore, only one p.s.p. per action potential was recorded. This provides further evidence that R.Pe.D.1 is monosynaptically connected to VD2/3. Unfortunately it has proved extremely difficult to record from the smaller follower cells of R.Pe.D.1 (A gp, G gp, H, I, J, and K cells) for periods of several hours as is necessary when we inject T.E.A. into R.Pe.D.1. Attempts at re-entering previously penetrated small cells after a period of T.E.A. injection into R.Pe.D.1 also proved most unsatisfactory because the postsynaptic cells were usually damaged by multiple impalements.

(IV) *Differential latencies of R.Pe.D.1 induced p.s.p.s*

As will be seen from Fig. 2 and Table 2 the latency to onset of a p.s.p. induced by R.Pe.D.1 varied considerably from one cell type to the next. The e.p.s.p.s on A gp, H and K cells and the A gp b.p.s.p.s appear to have similar latencies to onset (*ca.* 17–19·5 ms) whilst those found on VD2/3 are considerably greater and compare with the excitatory phases of the biphasic p.s.p.s on G and I cells (*ca.* 38–44 ms). The i.p.s.p.s on the J cells have a mean latency to onset of 182 ms (Fig. 2*d*) and this appears to be comparable with the inhibitory phase of the G and I cell b.p.s.p.s (Figs. 2*c*, 6*b*). Thus at least two types of excitatory receptor and one type of inhibitory receptor to dopamine appear to occur on follower cells of R.Pe.D.1.

Table 2. *Observation of* 1:1 *p.s.p.s and latencies in follower cells of R.Pe.D.*1

p.s.p. type	Cell type	(*N*)	Mean latency ± S.D.	(*n*)
e.p.s.p.s	VD2/3	9	41·3 ± 6·3	(4)
	A gp	34	17·1 ± 4·9	(12)
	H cells	22	18·7 ± 4·9	(11)
	K cells	27	16·3 ± 4·0	(12)
b.p.s.p.s	A gp (I type)	6	19·5 ± 6·6	(6)
	G gp	13	44·0 ± 9·7	(8)
	I cells	12	38·8 ± 2·0	(4)
i.p.s.p.s	J cells	27	182·4 ± 44·0	(17)

(*N*) indicates the number of times that 1:1 p.s.p.s have been recorded between R.Pe.D.1 and any given follower cell. The mean p.s.p. latency is also shown and here (*n*) indicates the number of preparations in which p.s.p. latency was accurately determined from superimposed oscilloscope sweeps. The p.s.p. latencies shown for each cell type were tested against those for all other cell types by means of the Student's *t* test. The latencies to onset for e.p.s.p.s on cells of A gp, H cells and K cells and for b.p.s.p.s on A gp were not significantly different from one another. They were however significantly different (P value < 0·001 in most cases) from the latencies to onset of e.p.s.p.s on VD2/3 and b.p.s.p.s on G gp and I cells (which were not significantly different from one another). The J cell i.p.s.p. latencies were significantly different from all other p.s.p. latencies (P value < 0·001 in all cases).

DISCUSSION

Is R.Pe.D.1 monosynaptically coupled to its follower cells?

The various criteria for monosynaptic connexions between neurones are fully discussed by Berry & Pentreath (1976) and by Kandel (1976). The evidence presented here strongly suggests that R.Pe.D.1 makes monosynaptic connexions with its various follower cells. First, there is a 1:1 relationship between presynaptic spikes and p.s.p.s

at all frequencies of discharge of R.Pe.D.1. Admittedly the maximal discharge frequency of R.Pe.D.1 is low (*ca.* 10 spikes s^{-1}) and at these frequencies the long-latency, long-lasting i.p.s.p.s tend to smoothly summate. Second, for any given follower cell there is a constant latency between each presynaptic action potential and each p.s.p. Third, our attempts to block synaptic transmission with the divalent cations, Ca^{2+} and Mg^{2+} failed in all cases (Table 1). Unfortunately, our experiments with T.E.A. were inconclusive, except in the case of VD2/3 (Fig. 7). Thus none of the evidence we have presented negates the suggestion that R.Pe.D.1 and its follower cells are directly and chemically connected and we feel justified in stating that R.Pe.D.1 makes monosynaptic connexions to its follower cells.

Alteration of spike/p.s.p. latency by raised external [Ca^{2+}]

Raising the calcium concentration in the bathing medium raises the firing threshold for neurones (Austin *et al.* 1967; Berry & Cottrell, 1974). If we consider a polysynaptic chain of neurones this raised threshold will tend to abolish spikes in the interneurones intercalated between a presynaptic cell and its follower cells. The overall effect would be: (i) a total loss of p.s.p.s, or (ii) the p.s.p.s would not follow 1:1 with the presynaptic spike, and (iii) the p.s.p. latency to onset would be variable from one spike to the next. Monosynaptically connected cells should be unaffected by this treatment and several authors have used raised external [Ca^{2+}] to demonstrate monosynaptic connexions between neurones (e.g. Gardner, 1971; Garnder & Kandel, 1972; Cottrell & Macon, 1974; Berry & Cottrell, 1975).

One interesting finding of this present study is that raising the concentration of Ca^{2+} (but not both Ca^{2+} and Mg^{2+}) in the bathing medium will alter the spike/p.s.p. latency from one fixed latency to another greater fixed latency. We are unable to explain this phenomenon at present, but since the longer spike/p.s.p. latency is constant in high calcium saline this does not detract from the hypothesis that R.Pe.D.1 is monosynaptically connected to its follower cells.

Differential p.s.p. latencies on different follower cells of R.Pe.D.1

Large differences in p.s.p. latencies to onset are indicated in Table 2 and similar latency differences between the G.D.C. and its follower cells have been reported in *Planorbis* (Berry & Cottrell, 1975). We do not believe that the differential latencies can be accounted for by differences in path length or conduction velocity of R.Pe.D.1 since Haydon & Winlow (1981) have shown extracellularly recorded axon spikes of R.Pe.D.1 in the nerve trunks of the visceral and right parietal ganglion to occur 11–14 ms after the intracellular spike recorded in the soma. A conservative estimate of the transit time of the presynaptic spike to its axon terminals would therefore be of the order of 8–10 ms. We suggest that the wide differences in p.s.p. latencies induced by R.Pe.D.1 are due to differences in 'the kinetics of the postsynaptic actions of the transmitter rather than conduction velocity or delay in release of transmitter' (Berry & Pentreath, 1976). We speculate that second messengers such as cyclic nucleotides may mediate the very long latency i.p.s.p.s we have observed.

How is R.Pe.D.1 connected to input 3?

In Fig. 1 we demonstrated that bursts of spikes in R.Pe.D.1 could initiate the activity of input 3 after a delay of several seconds. Additional bursts of spikes in R.Pe.D.1 were then able to cause a temporary cessation of input 3. These results are consistent with the view that R.Pe.D.1 inhibits the sources of input 3 and produces its excitatory effect by post-inhibitory rebound excitation. This effect has been shown to occur in cat hippocampal neurones (Kandel & Spencer, 1961) and similar actions have been demonstrated in *Aplysia* by Kandel, Frazier & Wachtel (1969). It is not known whether R.Pe.D.1 is monosynaptically coupled to the source neurones of input 3 since their locations are as yet unidentified.

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