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CHEMICAL TRANSMISSION BETWEEN INDIVIDUAL RETZIUS AND SENSORY NEURONES OF THE LEECH IN CULTURE

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SUMMARY

1. Chemical synaptic transmission develops between individual identified neurones dissected from leech ganglia and maintained in culture. Impulses in Retzius cells give rise to hyperpolarizing synaptic potentials in pressure (P) sensory cells. In suitable medium the potentials develop by 3 days and can be observed for more than 3 weeks.

2. The synaptic potentials occur after a synaptic delay, exhibit facilitation and depression and are reversed by hyperpolarization. The blocking effects of reduced calcium and raised magnesium concentrations in the bathing fluid provide additional evidence for the chemical nature of transmission.

3. An increase in chloride conductance is involved in the generation of the synaptic potential in the P cell. With high intracellular Cl in the post-synaptic cell, the synaptic potentials become reversed and amplified. The amplitudes of these reversed responses range from 1 to 20 mV with a falling phase lasting for seconds.

4. Changes in the membrane potential of the presynaptic cell that modify the amplitude and duration of the action potential influence the efficacy of transmission. In addition, impulses in Retzius cells initiated from hyperpolarized values of membrane potential evoke smaller synaptic potentials in the P cells than impulses arising from a depolarized level.

5. With neurones placed directly next to one another in the dish, maintained depolarization of the presynaptic Retzius cell in the absence of conducted action potentials gives rise to slow synaptic potentials in the P cell. In some pairs, the response in the P cell consists of a marked increase in 'noise'.

6. Injection of horseradish peroxidase into the Retzius cell reveals neurites with distinctive varicosities growing over the P cell.

INTRODUCTION

Individual, identified neurones with known properties can be dissected from leech ganglia and maintained in culture for several weeks (Ready & Nicholls, 1979; Fuchs, Nicholls & Ready, 1981). These isolated cells retain their characteristic membrane properties, grow processes and develop electrical connexions when certain types of

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neurones are placed close to one another. However, only in rare instances have chemically mediated synaptic potentials been observed.

One aim of the present experiments has been to establish conditions for rapid and consistent development of chemical transmission between identified cells. Two neurones that proved to be suitable are the Retzius cell, which contains 5-HT and causes mucus secretion (Lent, 1973), and the P sensory neurone, which responds to pressure applied to the skin (Nicholls & Baylor, 1968). These cells can be recognized by their shapes, large sizes and positions in the ganglia; in addition, they are relatively easy to dissect, have distinctive electrical properties and survive well in culture. Moreover, when individual Retzius and P cells are isolated and maintained in appropriate medium, after a few days chemical transmission occurs in one direction: impulses in the Retzius cell evoke hyperpolarizing synaptic potentials in the adjacent P cell in the absence of electrical coupling. These potentials exhibit a number of properties, such as Ca dependence, facilitation and depression, which are seen at chemical synapses within the C.N.S. of the leech and other animals (Muller, 1979).

In contrast to the situation within leech ganglia, where recordings are made from cell bodies at an unknown distance from the synaptic sites, isolated neurones in close contact offer advantages for analysing chemical synaptic transmission. In particular, we have (1) studied the effects of Ca and Mg, as well as facilitation and depression, and (2) used the membrane potential of the P cell to test how release of transmitter from the Retzius cell is modulated by alterations in presynaptic membrane potential.

METHODS

Polylysine and collagen coating of dishes. Identified nerve cells were isolated one by one as previously described (Ready & Nicholls, 1979) and placed on 35 mm diameter tissue culture dishes (Falcon no 3001) or glass cover slips (Van Lab 1 oz. microcover glasses). Dishes and cover glasses had been previously coated with polylysine 1 mg/ml. (1 mg/ml. in 0·1 M-borate buffer, pH 8·2) either alone or over rat tail collagen. Freshly applied polylysine was essential for the cells to stick down. When collagen was used the dishes or cover slips were given a thin coat, dried overnight in a humified 37 °C incubator and covered with a thin layer of polylysine for 2–3 hr. Polylysine solution was then washed off with three to five rinses of filtered double distilled water. The dishes were washed for 1 hr with L15 (Leibowitz 15 medium, Gibco) medium without fetal calf serum (FCS). Exposure to serum-containing medium caused the polylysine coated surface to become much less sticky; therefore the dishes were refilled with normal L15 containing 2% FCS (Fuchs *et al.* 1981) immediately before the cells were added. After 2–12 hr on polylysine the cells became firmly rooted and the dishes could be moved with relative impunity. Cultures were maintained at 20 °C in a humid incubator.

Conditions for development of chemical synapses. In the present experiments leech blood was added to the L15 medium bathing the cells 24–48 hr after plating. Whole blood was collected by dissecting the ventral surface of an animal, cutting either the sinus containing the C.N.S. or a lateral heart tube, and siphoning off the blood with a 27-gauge needle. Usually 0·1–0·2 ml. blood were drawn from one animal and added to 0·5 ml. cold L15. The procedure was repeated to accumulate several mls of blood-L15 mixture which was then centrifuged at 5000 *g* for 20 min, before adding it to regular L15 in a concentration of 1 part in 20. This solution was filtered twice through sterile 0·22 μM Millipore filters and frozen as 2 ml. aliquots. At the appropriate time, the regular L15 bathing the cells was replaced with a 2 ml. aliquot of the newly thawed blood-L15 solution. The final concentration of the leech blood in the medium bathing the cells was approximately 1%. This was in addition to the normal 2% fetal calf serum already present in the L15. New 1% medium was added every 3 days or so.

In earlier experiments of Fuchs *et al.* (1981) a typical culture would be composed of twenty pairs

of cells in a 35 mm tissue culture dish containing 2–3 ml. medium, a very low cell density analogous to a few billiard balls within a swimming pool. Although members of a pair of cells were placed next to each other, each pair could be hundreds of microns from another pair. Thus, cultured leech neurones differed from other types of cell culture in their density and the distance between cells (Gottlieb & Glaser, 1980). Furthermore there were usually only a few fibroblasts and microglial cells and often such cells disappeared after 5–10 days. In the present experiments pairs of cells were arranged close to one another, so that twenty pairs were contained within an area of 0·25 mm^2. Additional clumps of neurones, removed from the anterior packet (sensory cells, anterior pagodas and unidentified cells), were arranged around the perimeter of the identified cells. Although the contribution of such factors as leech blood and close packing is not yet known, under these conditions chemical transmission developed with high frequency in culture.

Recording techniques. The methods for recording electrically have been described elsewhere (Fuchs *et al.* 1981). Micro-electrodes were made from Haer ultratip thin walled capillary tubing (Frederick Haer & Co., Brunswick, ME) filled with either 4 M-KCl. To enhance chemical synaptic interactions external Ca was increased from 1·8 to 10 mM. Increases in the concentrations of Ca and Mg were made by adding appropriate amounts of sterile 1 M-chloride salts in filtered distilled water to the L15. Extracellular chloride was replaced with methylsulphate (Na methylsulphate: Pfaltz & Bauer Co., Stamford, CT) in a modified leech Ringer solution.

All experiments were made at room temperature (21–22 °C).

RESULTS

Neurite outgrowth from Retzius and P cells. When individual Retzius and P cells were placed immediately adjacent to one another on the bottom of the dish, the two cells appeared with transmitted light or phase contrast to be in contact (Pl. 1) but it was not possible to discern the presence or extent of neurites running between the cells. Accordingly, Retzius or P cells were injected with horseradish peroxidase in preparations where chemical synaptic transmission had been established. Pl. 1 (*A* and *B*) shows a pair of cells cultured for 19 days, viewed first with phase microscopy (Pl. 1 *A*) and then in bright field after filling the Retzius cell with horseradish peroxidase (Pl. 1 *B*). Numerous small processes contacted the soma of the post-synaptic P cell. By varying the focus it was observed that many of the neurites from the Retzius cell appeared on the underside of the P cell, in contact with the bottom of the dish or on neurites growing out of the P cell, with relatively few sprouts on the upper surface. Characteristic swellings or varicosities occurred along the neurites in contact with the soma of the P cell. By as early as 3 days (the first stage at which we have seen chemical transmission) a profuse outgrowth with varicosities was already present (Pl. 2). This same pair of cells showed synaptic potentials in the P cell following impulses in the Retzius cell. Pairs of cells positioned at a distance from one another and connected only by sprouts, were also able to develop chemical transmission without soma to soma contact.

When the post-synaptic P cell was stained with horseradish peroxidase, its neurites showed a similar pattern of outgrowth with numerous processes contacting the Retzius cell on its underside (Pl. 1 *C*, *D*). Varicosities were present even though there was no sign of chemical transmission from the P cell to the Retzius cell (see below). The actual sites of transmitter release remain to be established by electron microscopy and detailed physiology.

Properties of chemical interactions between Retzius and P cells. Under the improved culture conditions chemical transmission was regularly established from Retzius cells to P cells. Occasionally some fraction, or even all the cells in a dish would fail to stick

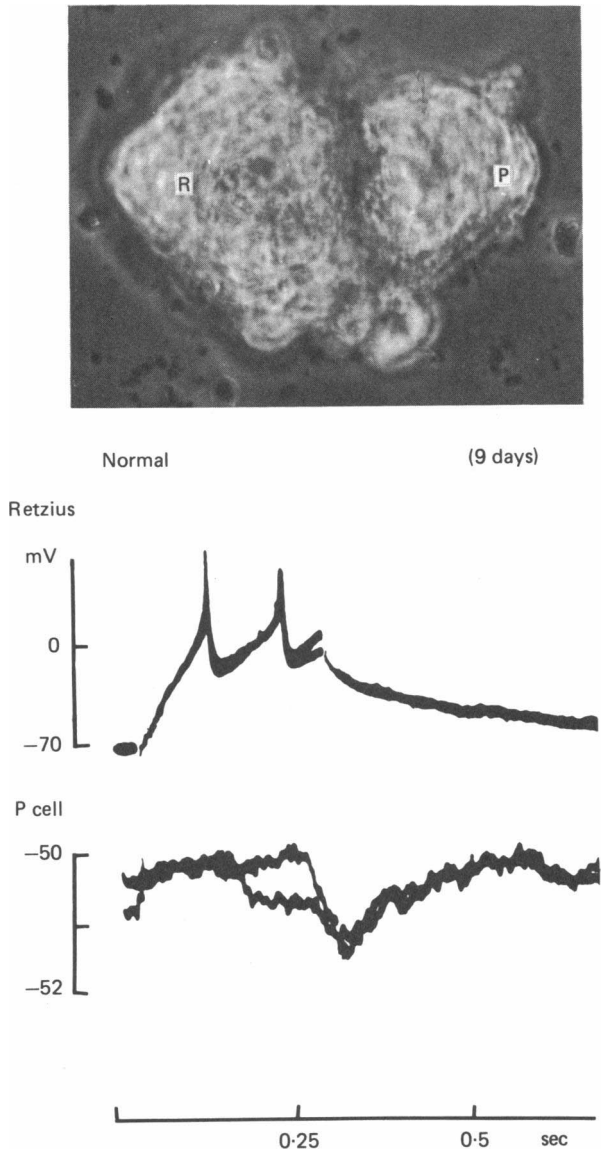

Fig. 1. I.p.s.p.s evoked in P cell by impulses in Retzius cell (9 day culture). The electrodes were filled with 4 M-KAc, and the medium contained 10 mM-Ca.

down and live; but when the cells did survive, transmission was observed between about 60% of the closely apposed pairs of Retzius and P cells. A total of more than 100 pairs of cells was recorded from.

An example of the synaptic potential in a P cell recorded with a KAc electrode is shown in Fig. 1. Each impulse in the Retzius cell evoked a hyperpolarization of 1–2 mV in the P sensory neurone. Such P cell responses were small and difficult to reverse with hyperpolarization.

In contrast, intracellular chloride injection was remarkably effective in producing a rapid reversal and amplification of the P cell response (Fig. 2). Almost immediately after inserting a KCl filled micro-electrode into the P cell, the responses elicited by

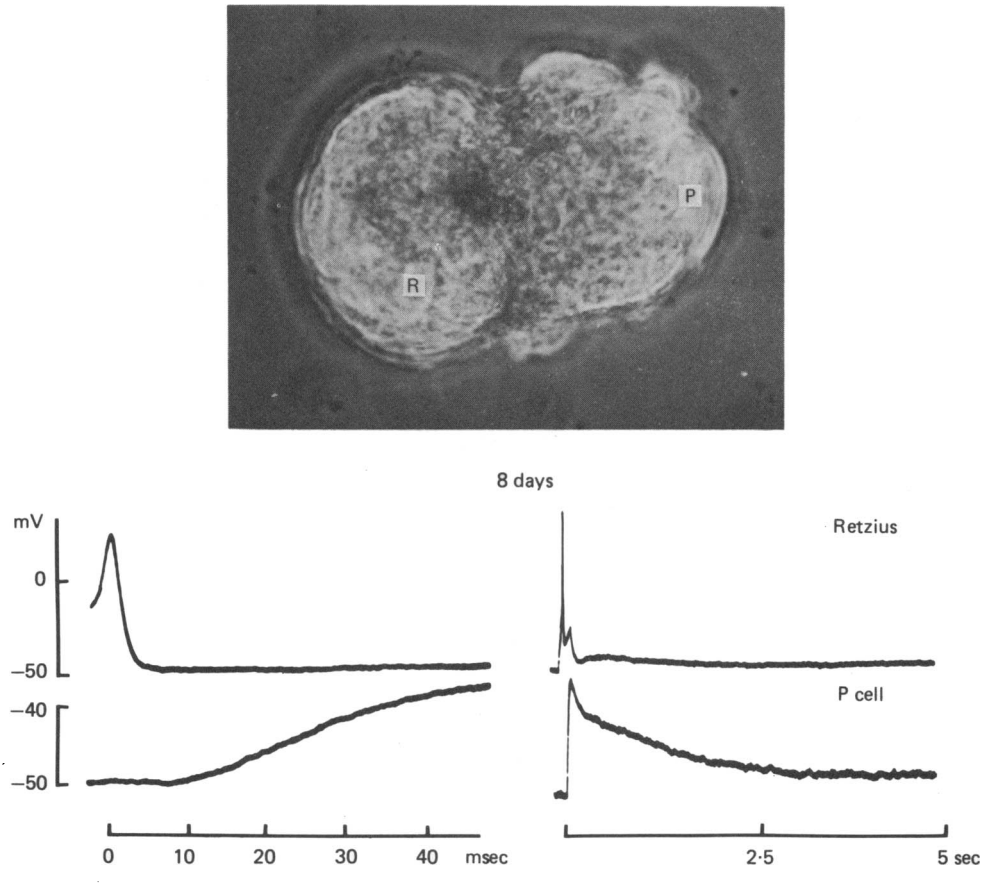

Fig. 2. Time course of a reversed i.p.s.p. evoked in P cell by impulse in the Retzius cell (R, 8 day culture). In this and all following records the electrode in the P cell contained 3 M-KCl to reverse and amplify the synaptic potentials, and the external Ca concentration was 10 mM; no electrical coupling was observed.

action potentials in the Retzius cell became depolarizing. Constant current pulses applied to the P cell indicated a decrease in resistance during the synaptic potential. These results suggest that the potentials in the P cell arise at least in part as a result of an increase in Cl conductance. In all the subsequent experiments described in this paper, recordings were made with intracellular KCl electrodes in the P cell and raised external Ca in the culture medium (10 mM) to reverse and amplify the synaptic potentials.

The amplitude of the synaptic potential depended on the quality of the penetration, the membrane potential of the P cell, and the properties of the interactions between that pair of cells. Although typical responses were 1–5 mV in amplitude, under

appropriate conditions with raised external Ca, potentials as large as 20 mV were recorded. In several P cells the reversed potentials were able to initiate impulses.

Time course of synaptic potentials in P cells. Several lines of evidence indicated the chemical nature of transmission from Retzius to P cells. In addition to the absence of electrical coupling (see below) and the reversal experiments mentioned above, the synaptic potentials arose after a discrete delay and were of long duration (Fig. 2). Thus, the synaptic potentials in P cells arose after a delay of approximately 5 msec (range 3–8 msec) measured from the peak of the Retzius cell action potential to the foot of the rising phase of the P response. The time to peak of the potential was 50 msec when the P cell was at its normal resting potential of −60 mV.

The synaptic potentials declined over periods of several seconds. As seen in Fig. 2, an initial fast phase with a time constant of decay of 60–100 msec was followed by a second slower phase with a time constant of 1–2 sec. Two such distinct phases of decay could arise from several sources: for example, transmitter might be released from the Retzius cell at a number of spatially disparate sites. On the other hand, transmitter released from a single site might act on populations of receptors with different kinetics. In addition, however, the passive electrical properties of the P cell membrane contribute to the two phases of decay. A constant depolarizing current pulse delivered to a cultured P cell causes a voltage change which rises to a peak and then declines to a maintained value. Such delayed rectification (which occurs in normal as well as cultured P cells) superimposed onto a slowly decaying synaptic potential would on its own contribute to a rapid decline followed by a slower phase.

Release of transmitter from Retzius cells. As at conventional chemical synapses *in situ* and in culture, transmission from Retzius to P cells was influenced by the concentrations of calcium and magnesium in the bathing fluid. Increasing Ca from 1·8 to 10 mM caused an approximately 5-fold augmentation of the reversed synaptic potential recorded in the P cell. Conversely, increasing magnesium from 1·8 to 18 mM abolished reversibly all synaptic potentials. Presumably these effects depended on altered transmitter release (Katz & Miledi, 1965). The efficacy of transmission also depended upon the frequency of firing. Fig. 3*A* shows that brief trains gave rise to marked facilitation. Similar effects were observed using two shocks at variable intervals from 50 to 200 msec. Depression of synaptic transmission could also be seen, particularly with prolonged firing at 0·5–1 Hz (Fig. 3*B*).

An advantage for studying synaptic transmission provided by cultured cells is their close physical proximity, which could permit intracellular recordings from the cell body to be electrically close to the site of transmitter release. Evidence for such proximity was suggested by a number of experiments. For example, small changes of membrane potential in the soma of a Retzius cell produced marked alterations in the efficacy of transmitter release following an action potential, as shown in Fig. 4. Here the mean amplitude of the post-synaptic response was plotted as a function of the membrane potential from which the Retzius cell impulse had been elicited. Steady depolarization of the Retzius cell by 5 mV through current injection could double synaptic potentials evoked by impulses. Presumably these changes in the membrane potential of the cell body affect processes occurring in the terminals as at certain synapses *in situ* (see Thompson & Stent, 1976; Nicholls & Wallace, 1978). Insets in Fig. 4 reveal that the peak amplitude of the action potential arising from a hyperpolarized level was not decreased.

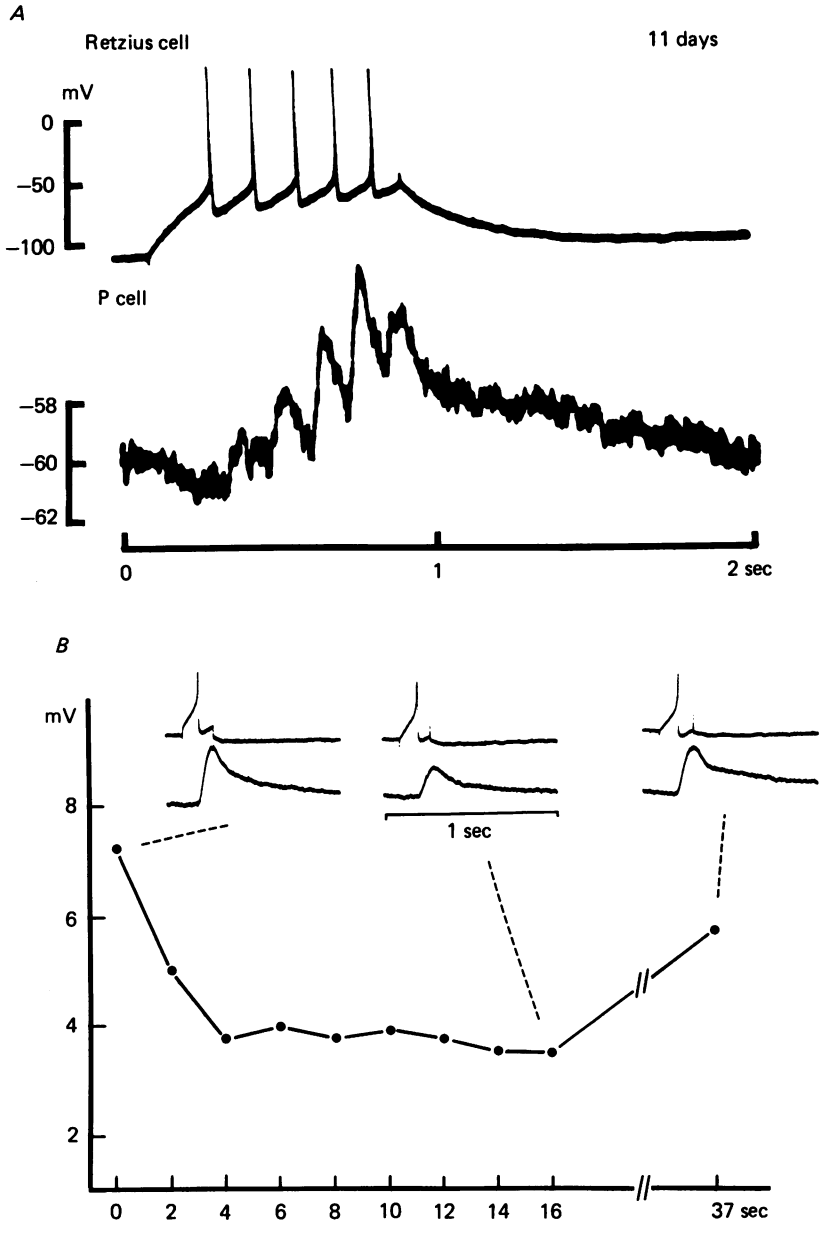

Fig. 3. *A*, stimulation of a Retzius cell with a long depolarizing pulse resulting in a series of action potentials at approximately 10 Hz and causing progressive facilitation of responses in the P cell (11 days). *B*, peak amplitude of the post-synaptic response in the P cell plotted as a function of time during a series of action potentials in the Retzius cell at 0·5 Hz. After nine sequential responses, stimulation was halted for 17 sec after which the next response showed partial recovery from the previous depression. Resting potentials were −52 and −54 mV in Retzius and P cell respectively (19 days). Inset records show responses indicated.

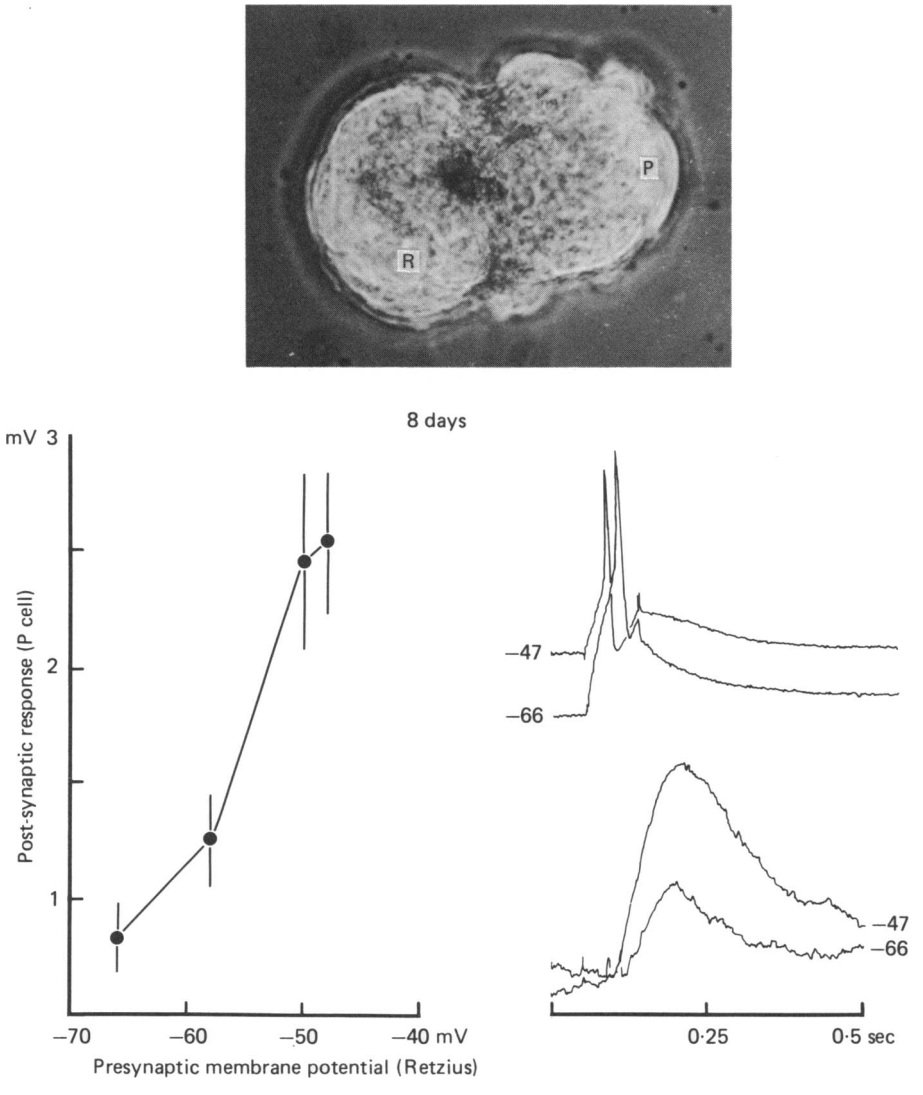

Fig. 4. Mean amplitude of synaptic potentials in a P cell as a function of presynaptic membrane potential of the Retzius cell at which impulse originated. Bars represent S.E. of mean. As at certain leech synapses *in situ* (Nicholls & Wallace, 1978) presynaptic hyperpolarization reduces the efficacy of release. Records on right show that the impulses in the Retzius cell did not become reduced in amplitude under these conditions (cells not coupled electrically).

In other experiments, the amount of transmitter released depended on the amplitude of the action potential in the Retzius cell arising at a constant resting potential. As in the animal, the strength of the stimulating pulse, the level of the steady membrane potential and the rate of firing all influence the peak amplitude and the duration of action potentials in the Retzius cell. For example, after applying a small depolarizing pulse to a Retzius cell, its active response may be only a few

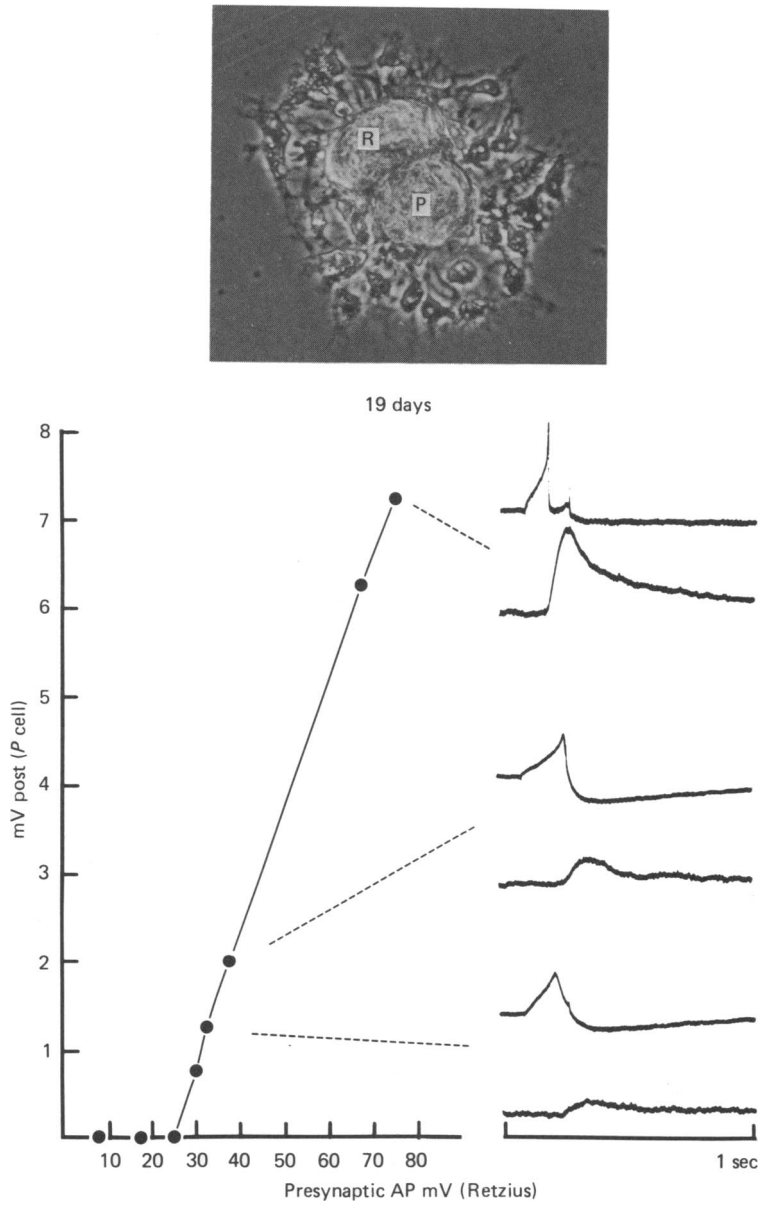

Fig. 5. Dependence of synaptic potential on amplitude of presynaptic impulse in Retzius cell (19 days). Stronger stimuli produced larger action potentials (as for cells *in situ*) which gave rise to larger synaptic potentials (cells not coupled electrically). The membrane potentials of Retzius and P cells were held at −52 mV and −54 mV, respectively. Same cells as in Fig. 4.

millivolts in size while a stronger pulse gives rise to a full overshooting potential. A series of these graded action potentials elicited by different stimulus strengths and their responses in one pair of cells is shown in Fig. 5. The large overshooting impulses produced by a strong stimulus gave rise to larger synaptic responses in the P cell than the smaller action potentials. However even small active presynaptic responses did elicit synaptic potentials. The striking feature of this Figure is the correlation of the

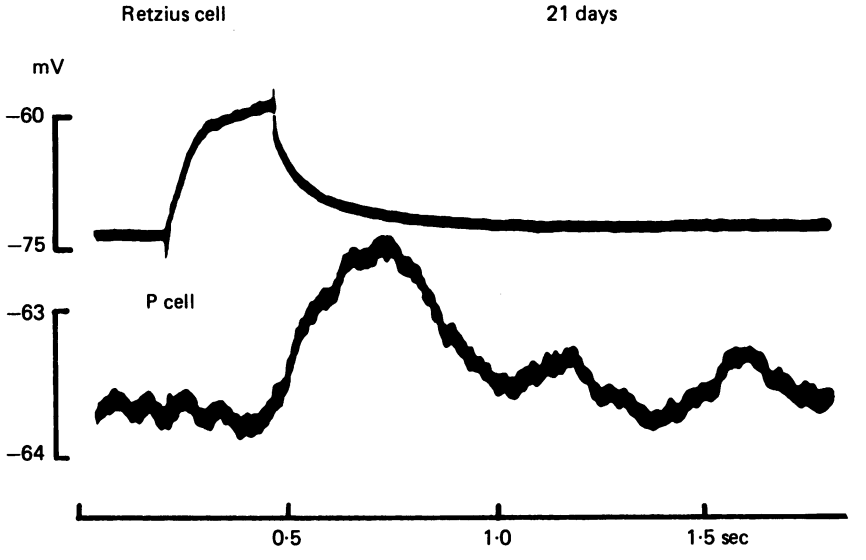

Fig. 6. Tonic release of transmitter by Retzius cell produced by depolarization without impulses. In this 21 day old culture the Retzius cell did not initiate action potentials. Note the delayed release. These cells were not electrically coupled.

impulse size recorded in the Retzius cell body with the amount of transmitter it released as measured by comparing synaptic potential amplitudes in the P cell. The exact relationship is probably not revealed by plotting peak amplitudes of the presynaptic spikes since their time courses are also altered by different current pulses. As shown in Fig. 5, the smaller presynaptic spikes rose more slowly and were of longer duration than the full action potentials.

If the release sites are electrically near the soma, it should be possible to evoke transmitter release by depolarization in the absence of an action potential. Depolarization without action potentials in cultured Retzius cells was achieved by depolarizing slowly or, especially in cells older than 15 days, failure of impulse initiation. In such cells, even large current pulses sometimes produced only small active responses. Under such conditions Retzius cells were capable of releasing transmitter in response to steady depolarization of the cell body without impulses, as shown in Fig. 6. The responses arose after a long delay and persisted for some seconds. In other pairs of cells, prolonged depolarization of the Retzius cell gave rise to a gradually increasing frequency of discrete depolarizing fluctuations in the P cell. No action potentials or oscillatory events in the Retzius cell corresponded to these responses in the post-synaptic neurone (Fig. 7). Presumably the pre-synaptic depolarization

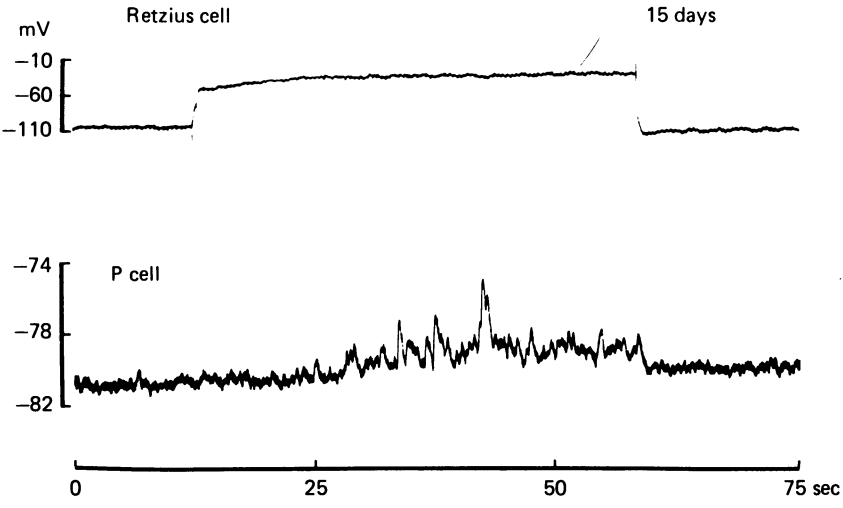

Fig. 7. Steady depolarization of Retzius cell and concomitant increase in fluctuations of membrane potential in the P cell (15 days).

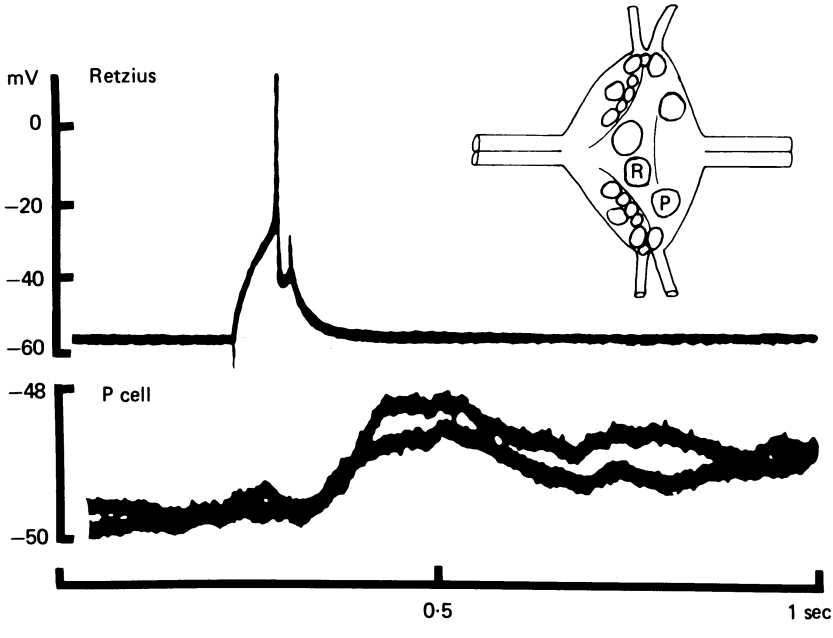

Fig. 8. Recording from Retzius and P cells *in situ*, in the presence of 10 mM-Ca and low Cl (70 mM instead of 140 mM) using KCl micro-electrodes. These ionic concentration changes accentuate the appearance of interactions such as those seen in culture. Only occasional Retzius/P pairs showed such clear synaptic potentials.

enhanced the probability of transmitter release, the increased frequency of events in the post-synaptic P cell resulting from the effects of quanta and multiples of quanta.

Direction of chemical transmission. An invariable feature of the chemical synapse between Retzius and P cells in culture was that transmission was unidirectional: there was no evidence of transmitter release from P cells onto Retzius cells. Thus, in more than 100 pairs of cells, impulses in P cells never evoked chemical synaptic potentials in Retzius cells. Interestingly, a search made in normal ganglia following these experiments has revealed a synaptic action of Retzius cells on P cells similar in many respects to that observed in culture (Fig. 8). Such responses *in situ* were depolarizing when the chloride gradient favoured outward Cl movement (low external chloride and use of a KCl micro-electrode) and had a slow onset and time course. They were, however, highly variable from ganglion to ganglion and often difficult to record. In addition, it proved difficult to provide conclusive evidence for or against mono-synapticity (see Discussion).

Other electrical and chemical connexions formed in culture. In earlier experiments (Ready & Nicholls, 1979; Fuchs *et al.* 1981), Retzius cells became coupled electrically to Retzius cells, but not to P cells. Since using the improved culture conditions described in Methods, we have observed weak electrical coupling in approximately 5% of the pairs of Retzius and P cells. Such coupling, when it did occur, was seen only with large voltage changes. This was in marked contrast to the strong coupling found between Retzius cells in culture. For those cells the frequency of occurrence was high (greater than 50%) and the coupling ratio could reach a value of 1 to 1 compared to 20 to 1 for Retzius to P coupling.

Retzius cells were also found to cause chemical synaptic potentials in cells other than the P cell. These included nociceptive (N), and touch (T) sensory cells, as well as other Retzius cells (see Fuchs *et al.* 1981). These responses were similar to those seen in cultured P cells and could be reversed by Cl injection.

DISCUSSION

Development of chemical synaptic transmission in culture. Our initial aim in these studies has been to describe the properties of transmission between isolated identified cells in culture. The main emphasis has been on the physiological properties of transmission once it has been established rather than on the nature of the structures involved or the stages of development. Sprouts and synaptic potentials have been seen at 3 days. We have not however recorded from earlier stages to determine how soon transmission can start in culture. By 2 weeks, the neurites of Retzius cells are more extensive and elaborate, without obvious changes in synaptic efficacy. It remains to be determined whether with age the quantum content and such processes as facilitation and depression become modified.

Several lines of evidence show that transmission from the Retzius to the P cell is mediated chemically; thus the synaptic potentials occur in the absence of electrical coupling (Fig. 2), arise after a delay, have prolonged time courses and can be reversed. Moreover, certain characteristics of transmission resemble those of conventional inhibitory synapses in the C.N.S. of the leech and elsewhere: Cl injection reverses the

response and, with repeated stimulation at appropriate frequencies, facilitation and depression occur. In other respects the synaptic potentials recorded in cultured P cells show properties not observed hitherto at known synapses within the leech C.N.S., including a long delay as well as slow rising and falling phases. These differences may reflect properties of the transmitter used by Retzius cells. In the ganglion, Retzius cells have been shown to contain 5-HT by (1) synthesis from precursors (Sargent, 1977); (2) specific histofluorescence (Ehinger, Falck & Myhrberg, 1968); (3) staining by neutral red (Stuart, Hudspeth & Hall, 1974) and by (4) the observation of dense-core vesicles within Retzius varicosities (Rude, Coggeshall & Van Orden, 1969). Similarly, Henderson has shown recently that Retzius cells in culture maintain their ability to synthesize, store and release 5-HT (Henderson, 1981). At other synapses where 5-HT has been implicated, synaptic potentials with slow kinetics have been observed (Kehoe & Marty, 1980). Those potentials have been shown to arise from either prolonged increases or decreases in conductances, often after a long delay. At present however, it is not known whether 5-HT is the transmitter released by the Retzius cell to produce the responses on isolated P cells. The synapses previously studied in detail in the leech ganglion such as the N sensory cell to L motor neurone synapse and those in the heart circuit are known not to use 5-HT and their synaptic potentials are brief and of short latency. In contrast, there are similarities in the time course and delay of the Retzius to P cell interactions seen *in situ* to the responses seen between these cells in culture.

Although the kinetics of the synaptic potentials in P cells could be attributed to the action of 5-HT and the channels it affects, such slowed potentials might result, as well, from the nature of the 'synapse' formed in culture. Release of transmitter from the Retzius cell might occur without specialized junctions or from scattered release sites at various distances; in addition, the post-synaptic receptors might be distributed over a large surface rather than organized as distinct junctional clusters. Slowed transmission could therefore occur between the two cells in culture as a consequence of the absence of a discrete, morphologically well defined 'synapse', as at developing neuromuscular junctions of the rat (Dennis, Ziskind-Conhaim & Harris, 1981). Indeed similar considerations may apply within the C.N.S. of the leech where (1) no unequivocally monosynaptic connexions made by Retzius cells have yet been found by recording physiologically (the records obtained in Fig. 8 have not been proved to be monosynaptic) and (2) no synaptic complexes comparable to those of T, P, and N sensory terminals have yet been observed by electron microscopy of Retzius cells injected with horseradish peroxidase (K. J. Muller, personal communication). In culture, the search by electron microscopy for the sites of transmitter release and receptor distribution is relatively simplified with two isolated cells having short processes orientated in well defined planes.

Whatever factors determine synapse formation in culture, it is likely that the pattern of connexions will be different from that determined by processes operating during development *in vivo*. At first glance the 'connexions' reported here might seem specific in the sense that impulses in P cells did not evoke synaptic potentials in Retzius cells. Clearly, however, such observations do not rule out the possibility of transmitter release by P cells in culture or the presence of P cell terminals closely apposed to the Retzius cell membrane. It may simply be that cultured Retzius cells

do not have receptors for the P cell transmitter. Indeed, in the ganglion, P cells make monosynaptic inhibitory connexions on Retzius cells (L. P. Henderson, unpublished). It seems likely that, as for electrical connexions, culture conditions may influence the degree of selectivity. In earlier studies Retzius and P cells never became electrically coupled (Fuchs *et al.* 1981), while in the present experiments such coupling was seen occasionally.

Control of transmitter release from the Retzius cell. Regardless of the structural relationship of Retzius and P cells in culture, the P cell membrane potential can serve as a sensitive assay for studies on the control of transmitter release. In particular, the experimental results suggest that for two cultured cells in close apposition the degree of attenuation of an electrical signal from cell body to release sites must be small. Knowing the dimensions of the Retzius cell and its input impedance, one can estimate the extent to which signals arising in the soma are attenuated as they spread along neurites (Jack, Noble & Tsien, 1975). Even for processes 50 μm long and 1 μm in diameter the attenuation would be negligible because of the high membrane resistance of isolated Retzius cells (Fuchs *et al.* 1981). Similar considerations would apply even if the membrane were inhomogeneous, the processes having lower resistance membranes. Thus, voltage changes induced and recorded by an electrode in the cell body may well be very similar to those occurring at the release sites (I. Parnas & I. Segev, unpublished).

At the giant synapse of the squid, the amount of transmitter released depends on the amplitude and duration of the presynaptic depolarization. There is a clear resemblance between the relationship shown in Fig. 5 of this paper and that obtained by Katz & Miledi (1967) using tetrodotoxin to block progressively the presynaptic impulses in the squid: in both preparations smaller impulses gave rise to smaller synaptic potentials, in a graded manner. A complication in the present experiments is that both Ca and Na are involved in the generation of the action potential in Retzius cells (Kleinhaus & Pritchard, 1975) and that its time course becomes altered with smaller amplitudes. The smaller, slower action potentials could involve relatively greater Ca fluxes than fast overshooting spikes. Hence the simple plot of pulse amplitude in Fig. 5 may not describe accurately the transfer function from Retzius cell to P cell, and this in turn could account for the slope being less steep than that found at the squid synapse.

Transmitter release by Retzius cells in culture can also be modulated by alterations in the steady level of the membrane potential of the presynaptic cell, as at synapses between the neurones of various other invertebrate preparations (Burrows, 1979). Records such as those of Fig. 6 and 7 provide clear evidence for tonic release by isolated cells. In addition, maintained depolarization or hyperpolarization of the presynaptic cell body has a marked effect on the amount of transmitter liberated by an impulse. Unlike the situation at synapses in leech and *Aplysia* ganglia (Nicholls & Wallace, 1978; Waziri, 1977), in cultured neurones it can be shown that reduction in transmitter release occurs under conditions where the peak amplitude of the action potential recorded in the cell body, close to the terminals, is not reduced. The cultured cell system presents the possibility of investigating with precision the presynaptic impulse and the ionic currents responsible for releasing transmitter, as well as providing direct access to post-synaptic sites.

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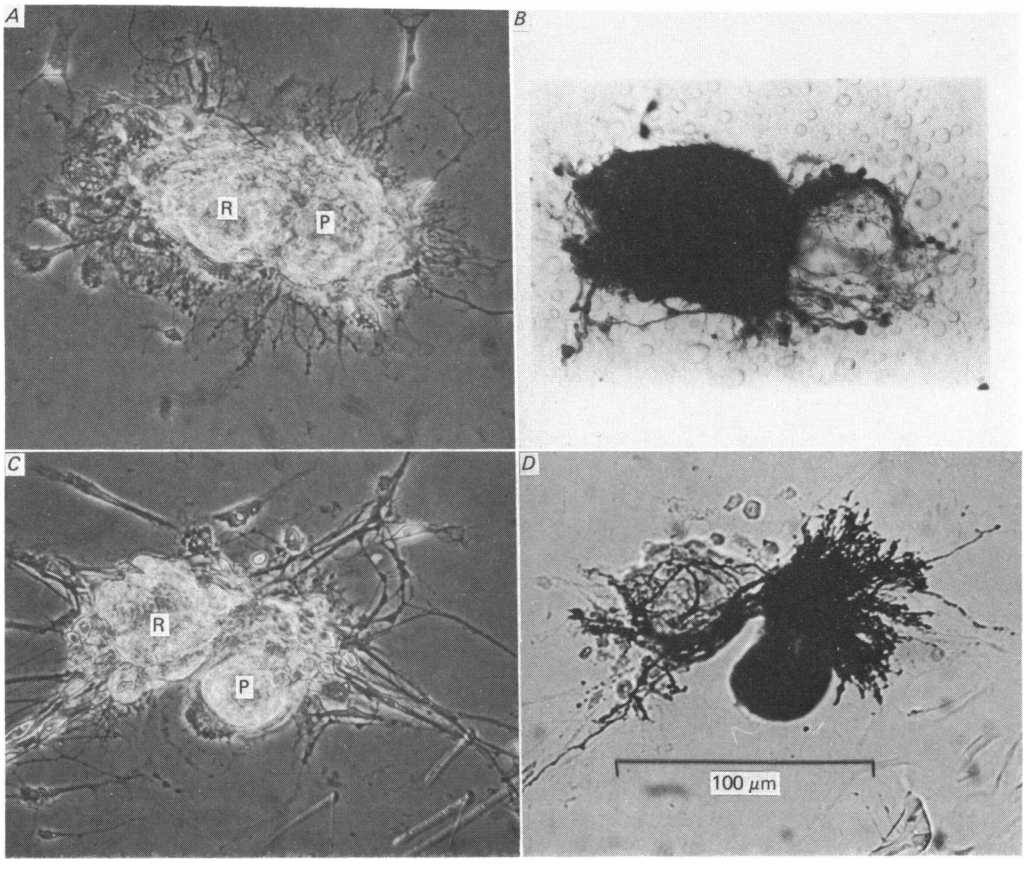
EXPLANATION OF PLATES

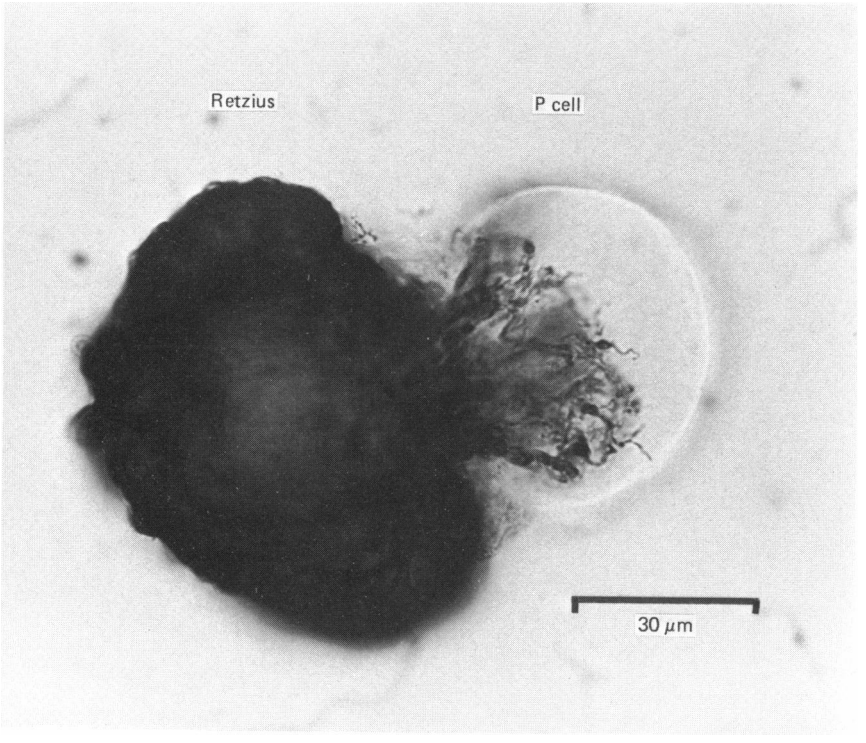
PLATE 1

A, *B*, Retzius (R) and P cell in culture for 19 days seen with phase contrast and after filling the Retzius cell with horseradish peroxidase (HRP) 1 hr beforehand. *C*, *D*, similar pair of cells cultured for 15 days with P cell filled. Distinct varicosities occur on both Retzius and P cell processes. The HRP filled cell body is distorted by the injection procedure.

PLATE 2

Retzius cell filled with horseradish peroxidase showing sprouts and varicosities on P cell body at 3 days. In this pair of cells chemical transmission had already been established: there was no electrical coupling.


A
R
P
B
C
R
P
D
100 μm


Retzius
P cell
30 μm