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INFLUENCE OF SUBSTRATE ON RETRACTION OF NEURITES FOLLOWING ELECTRICAL ACTIVITY OF LEECH RETZIUS CELLS IN CULTURE

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Summary

1. The aim of these experiments was to determine how electrical stimulation of identified neurones in culture influences their growth on defined substrates. Single Retzius cells isolated from the central nervous system (CNS) of the leech were plated in culture dishes coated with the plant lectin Concanavalin A or with extracellular matrix extract containing leech laminin to promote neurite outgrowth. Stimuli were applied by a fine tungsten microelectrode placed close to the cell surface. The efficacy of electrical stimulation was checked occasionally by recording intracellularly with a microelectrode.

2. After the period of stimulation had ended, there was a short delay before neurones plated on leech laminin retracted their neurites. Of 112 neurones, only 11 failed to respond to stimulation. Neurite retraction in each cell was non-uniform, some processes retracting while others did not. After having retracted, most neurites subsequently showed clear regrowth. The degree of retraction depended on the duration of the stimulus train: whereas a few minutes was sufficient to produce observable effects, prolonged periods of stimulation caused more extensive retraction. Trains of impulses at $4\,s^{-1}$ were equally effective when they were delivered in intermittent bursts or continuously.

3. The time in relation to growth at which stimuli were applied was of critical importance. Neurones stimulated during the phase of rapid outgrowth on leech laminin did not retract their neurites, which continued to elongate during and after stimulation. Neurones that had not retracted during the early phase were stimulated again later, when extension and outgrowth of neurites had ceased or slowed. At this stage stimulation was followed by retraction and subsequent regrowth.

4. Retzius cells plated on a substrate of Concanavalin A instead of leech laminin failed to show any retraction after stimulation.

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5. To investigate the possible role of Ca^{2+}, cells were grown with raised concentrations of Mg^{2+} in the bathing fluid. Raised [Mg^{2+}] did not influence the rate or the extent of neurite outgrowth. It reduced, but did not block, the effects of electrical stimulation. Earlier experiments have shown that growth on Concanavalin A occurs without obvious Ca^{2+} entry following stimulation. Together with the present experiments, the results suggest that Ca^{2+} entry following impulses in cells grown on laminin is responsible for the massive retraction.

Introduction

Electrical activity has a wide range of effects on neuronal function. Striking examples of some of these effects are provided by the fine tuning of connections in the lateral geniculate nucleus and the retraction of geniculate axons in the visual cortex of developing kittens (Wiesel and Hubel, 1963*a*,*b*, 1965; Hubel and Wiesel, 1970; Shatz, 1990), by the distribution of acetylcholine receptors in chronically stimulated denervated muscles (Lømo and Rosenthal, 1972) and by changes in the efficacy of synaptic transmission in the hippocampus (Collingridge and Bliss, 1987). What emerges is that some types of neurones and synapses are more susceptible to activity-dependent modulation than others (Burg and Wu, 1986; Budnik *et al.* 1990). Thus, certain neurones that are actively extending growth cones stop growing or retract following stimulation whereas others do not and certain patterns of connections develop correctly without impulse activity (Stuermer *et al.* 1990) whereas others become disorderly (Stryker and Harris, 1986).

The aim of the present experiments was to assess how the chemical environment of a neurone and its stage of outgrowth influence its responsiveness to maintained electrical activity. For this purpose, we applied defined patterns of stimulation to one identified neurone, the Retzius cell of the leech, isolated in tissue culture. The Retzius cell has been shown to grow rapidly and reliably in defined media on different substrates (Chiquet and Acklin, 1986). Thus, on the plant lectin Concanavalin A, Retzius cells start to grow within an hour; the growth cones are broad and flat, the processes thick, curved, highly branched and fasciculated. The same neurone plated on a substrate containing leech laminin extracted from the CNS also grows rapidly but with a quite different pattern. The processes are thin, straight and branch less frequently (Grumbacher-Reinert, 1989). Of particular interest is the finding that trains of impulses are followed by a marked entry of calcium in processes growing on leech laminin but not in those growing on Concanavalin A (Ross *et al.* 1988). These observations prompted us, first, to assess the effects of electrical activity on leech Retzius cells growing on Concanavalin-A- and laminin-enriched substrates using different frequencies and durations of stimulation and, second, to test whether stimulation applied at various phases of the growth cycle would produce different effects. To this end, we devised a method of stimulation that was non-invasive, did not damage the cell and could be applied repeatedly over several days.

Materials and methods

Cell culture

Ganglia were dissected out of the leech, pinned in a dish and the capsules opened as described previously (Dietzel *et al.* 1986). After mild enzyme treatment with collagenase/dispase ($2\,mg\,ml^{-1}$) for 1 h, individual Retzius cells were removed by suction and plated in Leibowitz 15 medium (L-15) containing 2% foetal calf serum and $0.1\,mg\,ml^{-1}$ gentamycin. Multiwell culture dishes had previously been coated for 2 h with Concanavalin A (Sigma 2010; $2\,mg\,ml^{-1}$) or leech ECM laminin prepared by EDTA extraction of leech ganglion capsules as described by Masuda-Nakagawa *et al.* (1988).

Stimulation and recording with extracellular tungsten electrodes

Thick-walled glass was pulled to form tubes. Under the microscope, Teflon-insulated tungsten wire (WT-2T Clark Electromedical Instruments) was pushed through the tube. The wire was cut to a length of about 3 mm and sharpened by electrolysis in $1\,mol\,l^{-1}$ potassium nitrite solution until the tip had a diameter of approximately 20 μm. The wire was dipped into nailpolish and held upwards to insulate all but the very tip. Cells stimulated *via* the tungsten wire (Fig. 1A) were

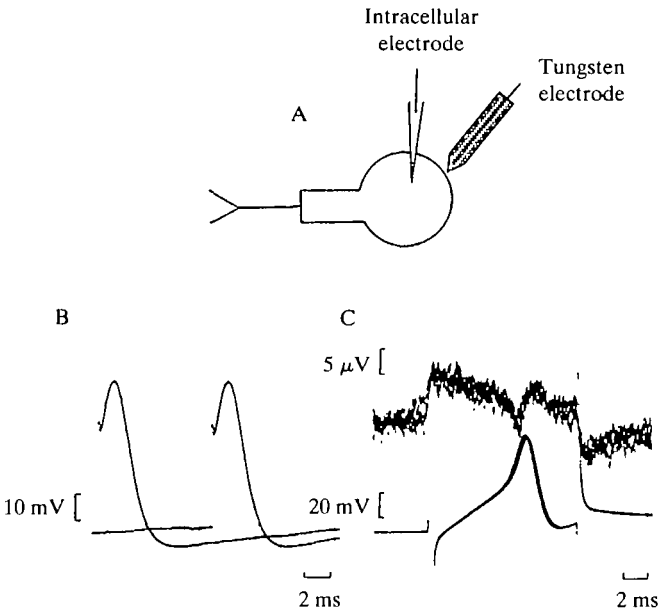

Fig. 1. (A) Drawing of a Retzius cell with a tungsten electrode and an intracellular electrode. (B) Intracellular recording of two action potentials following successive voltage pulses from the tungsten electrode. The resting potential of the Retzius cell was −50 mV. The shape of the action potential is typical for the Retzius cell. (C) Four superimposed recordings of action potentials induced by the intracellular electrode (lower trace) and recorded with the tungsten electrode (upper trace) close to the surface of the neurone.

also impaled with intracellular microelectrodes to monitor the reliability of the stimulation procedure (Fig. 1B). It was also possible to record action potentials with the tungsten electrode following stimulation by the intracellular electrode, as shown in Fig. 1C.

Parameters used for electrical stimulation

Retzius cells survived well for three or more hours of stimulation. During this time, intracellular recordings were made regularly to test for the efficacy of stimulation. After stimulation, cells were washed with fresh medium and kept sterile. Once the technique had been perfected, every cell tested responded with impulses throughout the period of stimulation by the tungsten electrode. In all experiments recordings were made from at least one cell in the culture dish to ensure the effectiveness of stimulation, even though the success rate was such that this test was virtually redundant. The pulse needed to induce action potentials was 3–4.5 V in amplitude with a duration of 0.1 ms. Control experiments were made to test whether the tungsten electrode on its own could affect outgrowth. Subthreshold pulses were applied in bursts at the same frequency as usual. Subthreshold pulses, usually of about 2.5 V, were also applied with the polarity of the stimulus reversed. The maximum rate at which Retzius cells can be stimulated intracellularly or extracellularly for long periods is about $4\,s^{-1}$. Our usual routine of stimulation with bursts consisted of trains at $4\,s^{-1}$ for 6 s delivered every 50 s. More than one action potential was often evoked by each pulse. A single electrical stimulus could induce two, three or even four impulses, particularly at the beginning of stimulation.

Effect of high-Mg^{2+} solution

Isosmolar $MgCl_2$ was added to L-15 medium to achieve a final concentration of $15\,mmol\,l^{-1}$ $MgCl_2$ (high-Mg^{2+}). High-Mg^{2+} solution was applied for at least 30 min before stimulation began. After stimulation, normal L-15 medium was replaced.

Assessment of growth before and after stimulation

Cells were observed by phase contrast microscopy. In the initial experiments, photographs were taken with a Leitz-Orthomat camera before and after stimulation. Outgrowth was followed for up to 3 days. The effects of stimulation were assessed by inspection of the negatives under high magnification. In these experiments, only a rough assessment of outgrowth was made, without detailed measurements. In a second series of experiments, quantitative analysis was carried out on video images using a video analysis system. The retraction and regrowth of processes in these cells were measured precisely using a computer system designed by Dr W. B. Adams.

Results

Retraction of neurites following electrical activity in Retzius cells growing on leech laminin-enriched substrate

Fig. 2 shows a typical example of the withdrawal of processes induced in a Retzius cell by bursts of action potentials. This cell had been plated on leech laminin substrate 2 days beforehand and had grown extensively. Three consistent features of the retraction are apparent in Fig. 2. First, retraction was not uniform for different processes of the same Retzius cell growing on leech laminin. Thus, comparing Fig. 2A with Fig. 2B some processes (one of which is marked by a circle) have withdrawn entirely, others have only partially retracted (square) and

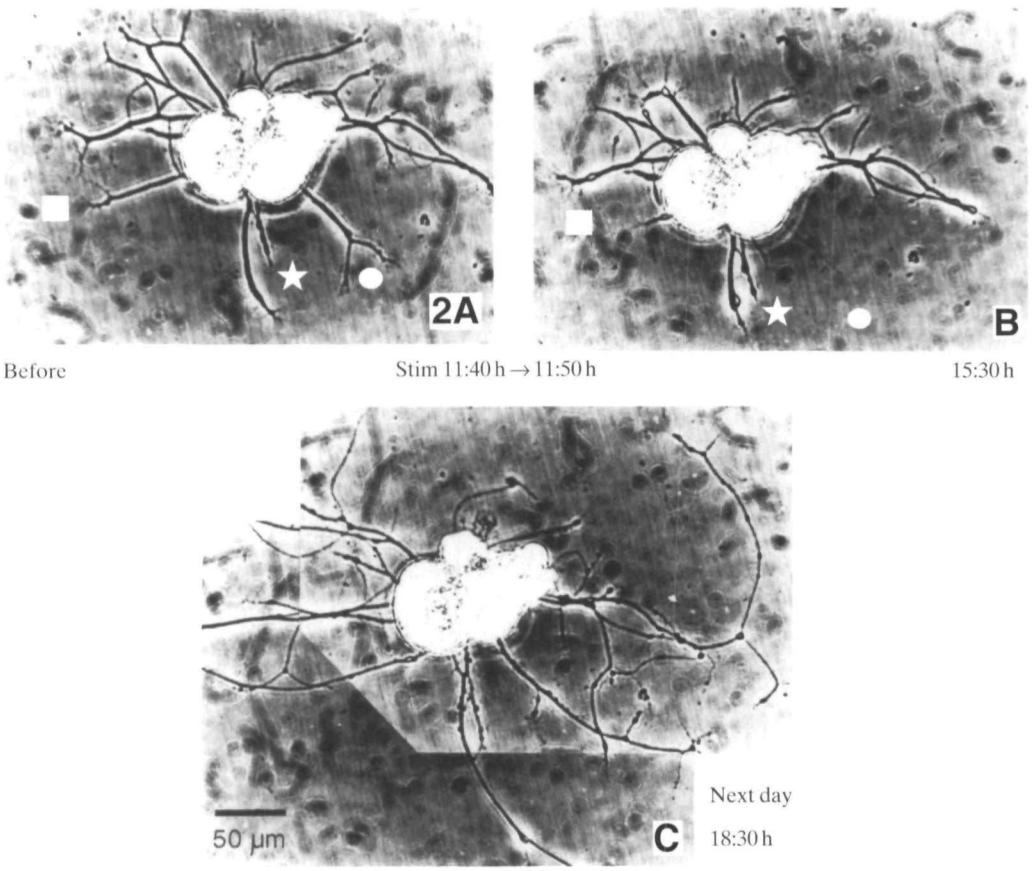

Fig. 2. A Retzius cell after 2 days in culture plated on leech laminin. (A) Before stimulation. The cell was stimulated every 50 s in bursts lasting for 6 s. The frequency during the burst was 4 pulses s^{-1}. (B) 3.5 h after stimulation, neurites retracted to variable degrees. Some retracted completely (circle), some partially (square) and one not at all (star). (C) After 30 h of rest without stimulation, the cell processes had regrown.

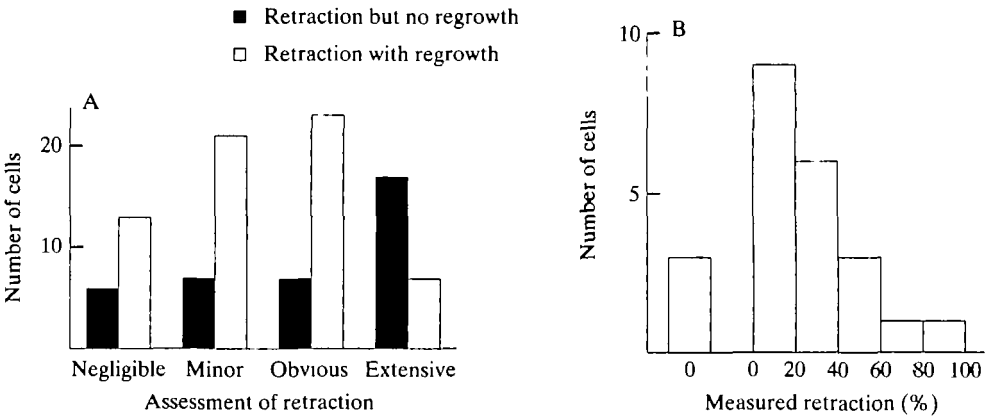

Fig. 3. (A) Variable retraction of stimulated Retzius cells sprouting on laminin-enriched substrate. Cells were stimulated with trains at $4\,s^{-1}$ for 6 s every 50 s. The assessment of retraction was carried out by examining photographs taken at various times and estimating total neurite length before and after stimulation. The percentage of retraction in each of the four classes was 'negligible' (0–5 %), 'minor' (5–10 %), 'obvious' (10–50 %) or 'extensive' (50–100 %). Retraction with (open bars) and without (filled bars) regrowth are compared. (B) Quantitative measurements of retraction of stimulated Retzius cells growing on leech laminin. The neurite length was measured from video pictures by computer and the percentage of retraction calculated. After retraction, all cells in this analysis showed regrowth.

some have retained their prestimulation length (star). Second, processes began to retract slowly and only after a delay. For the cells in which withdrawal occurred (N=101) the maximum effects occurred after 5±3 h (S.D.). The third conspicuous feature was a subsequent outgrowth that became apparent after approximately 3.5 h. This was accomplished both by regrowth of stunted processes and by initiation of new sprouts. 64 cells out of the total of 101 that showed retraction upon stimulation later showed such regrowth. The extent of neurite retraction was highly variable in different cells stimulated with similar parameters and growing on laminin-enriched extracts.

As a first step in quantification of the results, the total neurite length was assessed by inspection of photographs. Retraction was classified as negligible (0–5 %), minor (5–10 %), obvious (10–50 %) or extensive (50–100 %) as shown in Fig. 3A. Once this estimate of retraction had produced clear-cut results, a separate second series of experiments was made on 33 Retzius cells that had grown on laminin.

For these cells, precise quantitative measurements of retraction and outgrowth were made with a computer-imaging system. The results in Fig. 3B show that the measured retraction was very similar to that assessed by visual inspection. All of the 33 cells measured in Fig. 3B later regrew neurites. Fig. 4 shows the summed results for all Retzius cells grown on leech laminin substrate. Of the total of 112 cells only 11 failed to show retraction after stimulation.

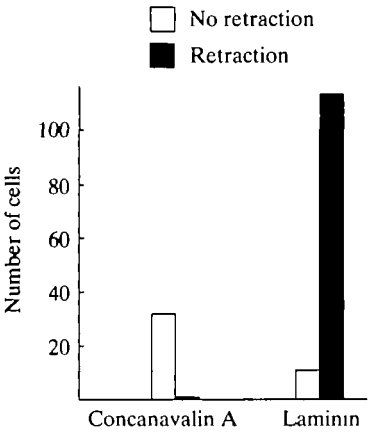

Fig. 4. Number of Retzius cells showing retraction of neurites after stimulation. Cells sprouting on Concanavalin A (3% showing retraction) are compared with cells sprouting on laminin (90% showing retraction).

The degree of retraction depended on the number of stimuli delivered to the cell. The minimum stimulation that was effective in promoting retraction consisted of 11 trains at $4\,s^{-1}$ lasting for 6 s and each delivered over about 10 min. No retraction was observed when only two trains of stimuli of the same frequency and duration were applied after a 50 s interval (six cells); in eight other cells, trains applied every 50 s for 5 min produced little or no retraction. The way in which impulses were grouped within the period of stimulation had little effect. Thus, in six cells, a continuous train of 48 impulses delivered in 100 s was not obviously different in its effect on retraction from the same number of impulses delivered in bursts according to our usual protocol at $4\,s^{-1}$ for 6 s once every 50 s. The relatively low frequency at which Retzius cells can fire – no higher than $4\,s^{-1}$ for prolonged periods – inevitably limited the range of stimulus parameters we tested.

Dependence of retraction on the phase of outgrowth

Careful inspection of 11 cells that failed to retract on laminin revealed that 10 of them were in a particular phase of growth: the cells that did not retract had all been stimulated soon after being plated, during the initial phase of profuse outgrowth. The sensitivity to electrical stimulation developed only later, when outgrowth was largely complete.

For example, a Retzius cell (Fig. 5) was first stimulated for 20 min (at a frequency of $4\,s^{-1}$ for 6 s every 50 s) 1 day after plating. At this time neurites were growing rapidly and impulse activity had no effect upon the rate of growth. Three days later (on day 5), 10 min of stimulation with the same parameters caused obvious retraction followed by regrowth. Similar results were obtained in four cells, when stimulation was applied first early and then at a later stage.

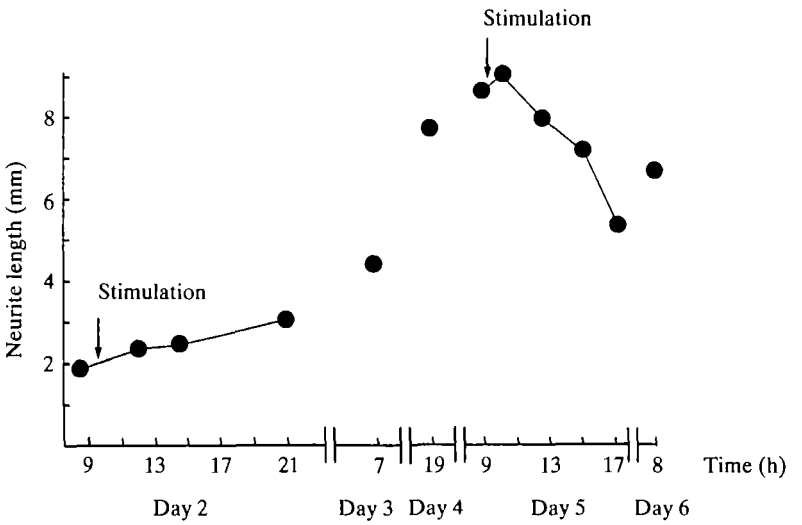

Fig. 5. Influence of early and late stimulation on outgrowth patterns. (A) A Retzius cell was plated on laminin 1 day before the first measurement. The first stimulation on day 2 lasted 20 min. The frequency was 4 pulses s^{-1} for 6 s every 50 s. This stimulation had no effect and the neurites continued to grow. Later, on day 5, the same regime of stimulation was followed by retraction with subsequent regrowth on day 6. Abscissa shows clock time of day.

Controls for damage

Several lines of evidence indicated that the retraction following extracellular stimulation was not caused by injury: (1) subthreshold stimuli that failed to evoke impulses did not lead to withdrawal of processes; (2) when a second Retzius cell was situated adjacent to and touching the one that was being stimulated it did not show retraction nor did other cells growing in the same microwell; (3) damage inflicted either deliberately or accidentally with the tungsten electrode gave a quite different retraction response. When the tungsten electrode accidentally pushed the cell, we observed a rapid and irreversible retraction that was not followed by regrowth. Such retraction affected all the processes of the cell, not just a few and, as the processes of the damaged cell drew back, they became characteristically beaded. Strong additional circumstantial evidence is provided by experiments described below in which Retzius cells were stimulated extracellularly on a different substrate, Concanavalin A.

Effects of stimulation on Retzius cells growing on Concanavalin A

The plant lectin Concanavalin A provides a suitable substrate for neurite outgrowth by Retzius cells. When cells growing on Concanavalin A were stimulated extracellularly by tungsten electrodes no retraction occurred. The action potentials in these cells have been shown to invade all the processes and are indistinguishable from those of cells growing on laminin. The striking differences

between the effects seen in cells growing on Concanavalin A and on leech laminin are shown in Fig. 4. Whereas 101 of 112 cells retracted on laminin, only 1 of 33 retracted on Concanavalin A and that cell did not regrow. Whether short (3 s) or long (10 s) trains of stimuli at $4\,s^{-1}$ were applied, the neurites of Retzius cells on Concanavalin A failed to retract and continued to grow. Retraction did not occur even if stimulation in bursts was maintained for as long as 1 h (Fig. 6). Continuous prolonged stimulation at $4\,s^{-1}$ also failed to produce retraction. Inspection of the cells at high magnification during stimulation showed minor retraction of filopodia but no decrease in the overall extent of outgrowth. These experiments further ruled out damage as a factor causing withdrawal in our experiments. However, they did suggest a possible role for calcium entry (see below).

Stimulation in high-Mg^{2+} fluid to block calcium entry

Experiments made with optical recordings of Ca^{2+} levels inside Retzius cells (Ross *et al.* 1988) have shown that there is a pronounced influx of Ca^{2+} following stimulation of Retzius cells plated on leech laminin. In contrast, no detectable Ca^{2+} entry followed stimulation of similar Retzius neurites on Concanavalin A. It is possible, therefore, that Ca^{2+} entry into processes growing on leech laminin might trigger retraction. Unfortunately, specific, potent calcium channel blockers are not effective on Retzius cells; Cd^{2+} and Mn^{2+}, which do block calcium entry, produce damage when applied for periods of more than a few minutes. Mg^{2+}, however, depresses Ca^{2+} entry reversibly and without damaging cells. Isolated leech neurones in culture will continue to grow normally on both substrates when placed in modified media containing $15\,mmol\,l^{-1}$ Mg^{2+} (Ross *et al.* 1988). The outgrowths from such cells show no obvious differences from the outgrowths seen in normal L-15 medium. When extracellular electrical stimuli were applied in high-Mg^{2+} solution the retraction was reduced but not abolished in cells growing on leech laminin. Thus, in normal L-15 medium the standard train of stimuli produced an overall rectraction of 31 % (to 69 ± 6.9 %, S.E.M., 9 cells). In high-Mg^{2+} medium the overall retraction was only 11 % (to 89 ± 3.3 %, 18 cells) ($P<0.01$).

Discussion

Fine extracellular tungsten electrodes offer advantages for stimulating individual leech neurones. Unlike patch electrodes (Cohan and Kater, 1986) or intracellular electrodes (Anglister *et al.* 1982), they can be applied to a cell repeatedly for prolonged periods without damaging it. For stimulating individual Retzius cells, tungsten electrodes were simpler to construct and use than the multielectrode arrays built into dishes and described by Regher *et al.* (1988). A chamber for stimulating a large number of sensory neurones for a prolonged period has been developed by Fields *et al.* (1990). Using this chamber they have studied the effects of different patterns and frequencies of stimulation and have

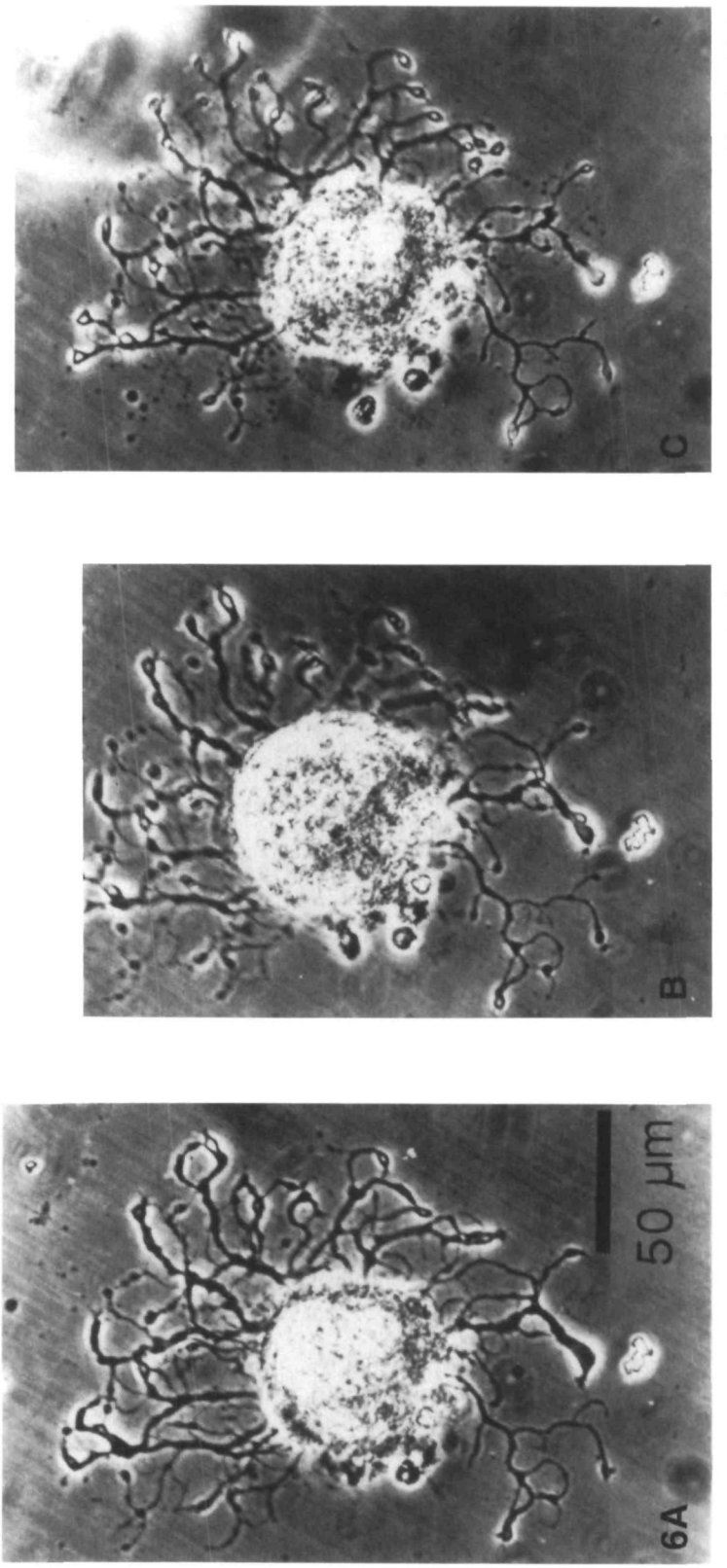
Concanavalin A
Before
Stim 10:30 h
→ 11:15 h
17:20 h
Next day 8:00 h
6A
50 µm
B
C

Fig. 6. Retzius cell plated on Concanavalin A 4 days before stimulation. (A) Before stimulation. The cell was then stimulated for 45 min with bursts at $4\,s^{-1}$ for 6 s every 50 s. (B) After 7 h, no change in the pattern of outgrowth was seen. (C) No changes were observed the next day.

observed retraction of filopodia. The effects they saw were far smaller than the major retraction of large neurites produced by stimulation of Retzius cells.

It must be acknowledged that, although reliable, the technique of using tungsten electrodes was not easy. A disadvantage was that stimulus artefacts made it difficult, though not impossible, to record impulse activity while actively stimulating through the same extracellular electrode. Nevertheless, the success rate was such that every spot test we made with an intracellular electrode showed that the stimuli were effective in initiating impulses. We did, however, observe that single shocks could trigger two or three impulses instead of one, particularly at the beginning of a train. Hence, precise counts of impulse numbers were not possible. Stimulating with tungsten wires had the additional advantage of allowing us to study activity of neurones growing on different substrates. This would be far more difficult on glass multielectrode dishes. One can envisage the use of tungsten electrodes for a variety of other purposes, including stimulation and recording of activity in intact cells within leech ganglia.

Several features of our results obtained in this way from Retzius cells were clearly different from those reported elsewhere. First, the effect we saw was not a deceleration of outgrowth or minor growth cone retraction. Instead, massive withdrawal of selected neurites occurred, to be followed by regrowth. We never observed enhancement of outgrowth. Second, the sensitivity to stimulation was virtually absent at early stages, while Retzius cells were sprouting extensively. Only at later stages, when growth was almost complete, did retraction become evident. Although the pattern and degree of withdrawal and regrowth varied considerably from cell to cell, the results were qualitatively similar. Third, the parameters of stimulation were relatively unimportant for Retzius cell retraction. By comparison, in snail neurones or dorsal root ganglion cells, patterned bursts of specific frequencies were required to cause cessation of growth cone activity (Cohan, 1990; Fields *et al.* 1990). Furthermore, leech neurones did not accommodate to prolonged periods of stimulation. Retzius cells, unlike some other leech neurones, such as T cells, do not fire at high frequencies and $4\,s^{-1}$ is about the maximal rate of stimulation that can be sustained for prolonged periods. What clearly was important was the duration of the activity. Two minutes of stimulation was insufficient to produce any effect, 5 min was barely effective and 10 min or longer produced maximal effects.

A further major difference from observations made on other types of nerve cells was that the susceptibility of Retzius cell neurites to impulse activity depended critically upon the substrate on which the cells were growing. D. Neely (unpublished observations) has confirmed this result by depolarizing Retzius cells plated on Concanavalin A and on leech laminin with high potassium concentrations. As

with extracellular electrical stimulation, massive retraction was evident only in cells growing on laminin.

The different responses of leech neurones to electrical activity compared to other types of cell may depend in part on the way in which the experiments were designed. We used only one type of cell – adult Retzius cells with well-defined growth properties on substrates that have been extensively investigated. At the same time, in view of the bewildering array of phenomena attributable to impulse activity in intact animals and the absence of a clear explanation for mechanisms of retraction, it is hardly surprising that different cells respond differently. It has been suggested that calcium entry may play a key role in neurite outgrowth (Mattson and Kater, 1987; Anglister *et al.* 1982). Thus, calcium can regulate the motility of growth cones and their elongation rate. The range and optimal levels of calcium concentrations are very different in different cells (Mattson and Kater, 1987). Silver *et al.* (1989), using calcium indicators, found distinct differences in internal calcium concentrations in quiescent, in mobile non-advancing, in mobile advancing, in flattening and in retracting growth cones. A clear example that argues against a simple unitary hypothesis is provided by the experiments of Usowicz *et al.* (1990). They showed that, while one strain of PC12 cells sends out neurites in culture and has abundant voltage-sensitive membrane calcium channels in the plasma membrane, another type of PC12 cell that also grows well is virtually devoid of them. No influx of Ca^{2+} is necessary for neurite outgrowth of these latter cells. Does calcium entry play a part in the massive retraction of Retzius cells? The results obtained with the stimulation of cells in the presence of higher concentrations of magnesium are inconclusive. What is clear is that growth is able to continue well in solutions containing magnesium at concentrations high enough to block calcium channels (15 mmol l^{-1} magnesium drastically reduces transmitter release and blocks chemical synapses between leech neurones). A further puzzling observation is the long delay of at least half an hour between the onset of stimulation and the beginning of retraction. If calcium entry is indeed the trigger, the slow development of subsequent events is likely to be complex and involve numerous steps.

A question that remains concerns the physiological significance of effects observed in tissue culture. The frequencies we used were within the range observed in intact leech Retzius cells. Cells in the adult leech, which do not of course retract, are part of a functional network with pre- and postsynaptic connections and well-defined target organs in the periphery. Leech neurones do, however, sprout extensively after injury, after destruction of their glial surround or even after neighbouring territories have been denervated. Activity as a modulatory factor could regulate such a propensity for sprouting.

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