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GROWTH AND ION-SPECIFICITY OF EXCITABILITY IN REGENERATING COCKROACH GIANT INTERNEURONES

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SUMMARY

The effects of lesions on cockroach giant interneurones (GIs) were studied to investigate the transient, Ca-dependent, action potentials which have been reported to appear near the tips of regenerating GIs (Meiri, Spira & Parnas, 1981). We were unable to repeat these observations.

Ligation of connectives, before cutting, delayed the degenerative changes in segments of GIs that were separated from their cell bodies. The short-term injury response (which was normally observed on cutting while recording close to the site of lesion) was not recorded when the axon was ligated.

The effect of cutting GIs at different distances from their cell bodies was also investigated. There was no apparent relation between the response of GIs to a lesion and the site of the lesion.

Sprouting of GIs was observed both at the tip of the axon and also at sites some distance from the lesion. No changes of the cell body or its dendritic tree in the terminal ganglion were seen but sprouting was observed in other ganglia.

INTRODUCTION

As in vertebrates and other higher invertebrates, the peripheral motor axons of insects are able to regenerate following lesions (Bodenstein, 1957) and can reform functional connections with their original target organs (Pearson & Bradley, 1972). Neurones in the central nervous system of insects also regenerate and can emit sprouts which may extend over relatively large distances (see Zeldes, Dorman, Hochner & Spira, 1982), unlike mammalian central neurones which show only limited growth (see Aguayo, David & Bray, 1981).

It has been suggested that the entry of calcium plays a regulatory role in neurite elongation (Llinas, 1979; Llinas & Sugimori, 1979). Meiri *et al.* (1981) concluded that their observations, on the electrophysiological responses of giant interneurones (GIs) of the cockroach nerve cord after injury and during subsequent regeneration, were consistent with this hypothesis. They reported the transient appearance of Ca-dependent action potentials (between 7 and 60 h after axotomy) near the regenerating tip of severed GIs.

The appearance of Ca-channels near the tips of severed axons in the early stages of

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regeneration raises a number of interesting questions. What are the characteristics of these channels? Is their appearance a purely local response, perhaps involving modification of existing ion-channels, or are these Ca-channels transported from the cell body? If it is not a local response, then what is the nature of the signal, or signals, that initiate the mobilization, transport and incorporation of the channels at the axonal tips?

To attempt to answer some of these questions we have repeated and extended the investigation of Meiri *et al.* (1981) and examined the ion specificity of excitability at different stages of regeneration near the tips of proximal and distal segments of ligatured and unligatured cut axons.

MATERIALS AND METHODS

Adult male cockroaches (*Periplaneta americana*) from the laboratory culture were used in all experiments. Animals were anaesthetized by immersion under water and then restrained, ventral surface uppermost, with plasticine. Dissection tools were initially dipped in alcohol and flamed. A flap of cuticle was lifted to allow lesions of one interganglionic connective at various levels of the abdominal nerve cord. In some experiments a ligature of surgical silk was tied around the connective before cutting. After the operation, the cuticle was sealed down using low melting point wax.

Operated animals were kept at 25–32 °C for various intervals and were then decapitated and the nerve cord removed and mounted in the experimental chamber for intracellular recording and injection of cobalt ions. Experiments were performed at room temperature.

Normal cockroach saline had the following composition: NaCl 157 mM; KOH 3 mM; $CaCl_2$ 2 mM; $MgCl_2$ 2 mM; trehalose 5 mM; HEPES buffer (pH 7·4) 8·6 mM. During attempts to record from the regenerating axon tip the following modified salines were used: NaCl 133 mM; $CaCl_2$ 20 mM; trehalose 5 mM; 4-aminopyridine (4-AP) 0·1 mM; Tris-HCl buffer (pH 7·4) 11·6 mM. Sodium-free salines were produced by replacing NaCl with Tris-HCl buffer. In some experiments, $CoCl_2$ (20 mM) was added by substitution of NaCl (Tris-HCl in Na-free salines) or tetrodotoxin (TTX, 3×10^{-7} M) was added hypertonically.

Giant axons were stained by intracellular cobalt injection. Glass-capillary micropipettes filled with 3·3 % (w/v) cobaltic hexamine chloride in distilled water were used to inject Co ions by passing 0·5 nA current pulses of 0·5 s duration at a frequency of 1 Hz. The injected Co ions were allowed to diffuse and then precipitated by the addition of ammonium sulphide (Pitman, Tweedle & Cohen, 1972). The nerve cord was fixed in alcoholic Bouins for 1–2 h then washed and stored in 70 % alcohol, usually overnight, before being silver intensified (Bacon & Altman, 1977). Nerve cords were also washed for 30 s in destaining solution (Pitman, 1979) before intensification. Silver intensified preparations were dehydrated, cleared in methyl benzoate and whole mounted in Canada balsam.

RESULTS

The reaction of a nerve to a lesion depends, among other factors, on the type of trauma (Lieberman, 1974). In the cockroach nerve cord, Aviv, Hochner & Spi

[redacted]82) showed that the rate of degeneration induced by the Ca ionophore A-23187 was much faster than that induced by mechanical damage. Frizell (1982) showed that ligation combined with section caused a less marked increase in axonal transport of proteins than crushing the rabbit hypoglossal nerve. We have looked at the resting potential and action potential in GIs where the nerve cord was ligated, using fine surgical silk, before cutting.

To examine short-term changes, the isolated cord was cut on the distal side of the ligature from the cell body while recording intracellularly from a GI. The GIs which had been ligated before cutting maintained their normal resting and action potential for at least 5 h, *in vitro*, following lesioning, recorded at distances from 0·15 to 1·0 mm from the ligature (Fig. 1).

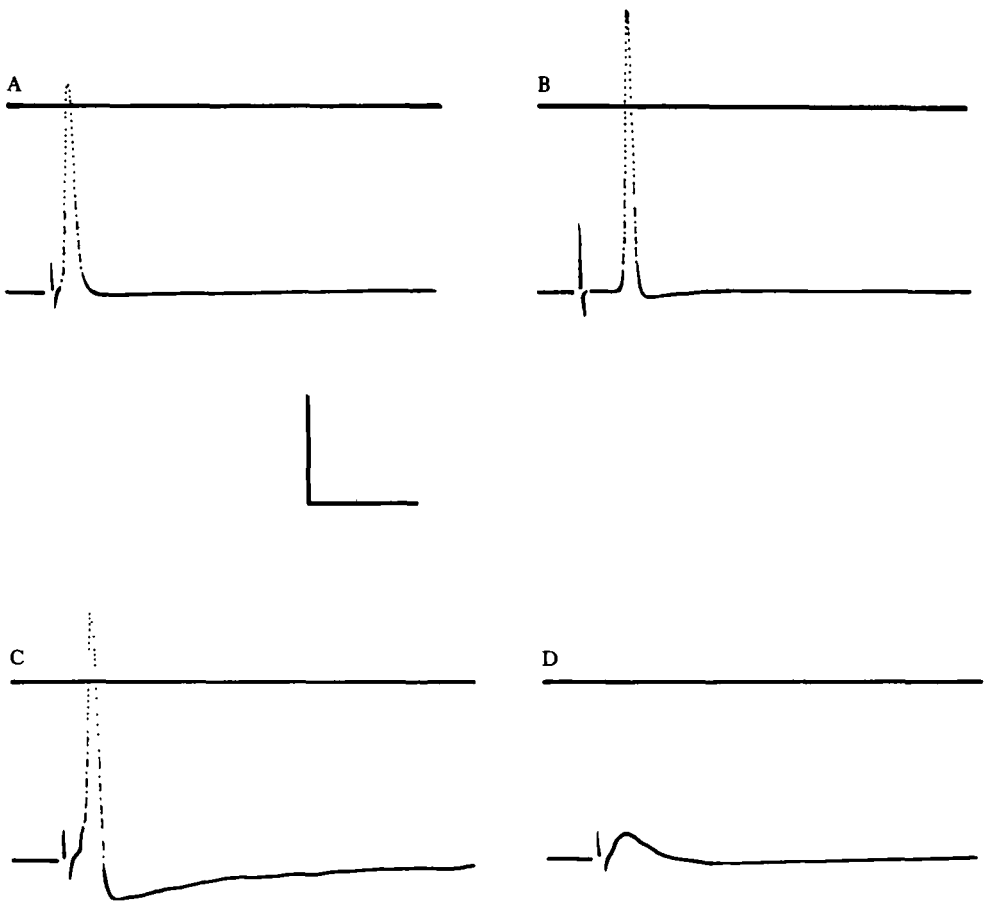


Fig. 1. Effect of cutting ligated nerve cords. (A) is a traced record of an action potential taken on initial impalement and immediately before cutting a ligated axon. The electrode was impaled 0·51 mm from the ligature, proximal to the cell body. (B) shows a record from the same axon 5 h after the cord was cut on the distal side of the ligature from the cell body. The increase in spike amplitude probably resulted from better sealing of the electrode tip. C and D are records from an axon in a connective which was ligatured and cut, *in vivo*, 24 h earlier. (C) was taken with the cord bathed in a saline containing 133 mM-Na; 20 mM-Ca. (D) was taken 5 min after the introduction of TTX (3×10^{-7} M) to this saline. The electrode was impaled 0·3 mm from the ligature, proximal to the cell body. Scale bars: 40 mV (vertical), 4 ms (horizontal).

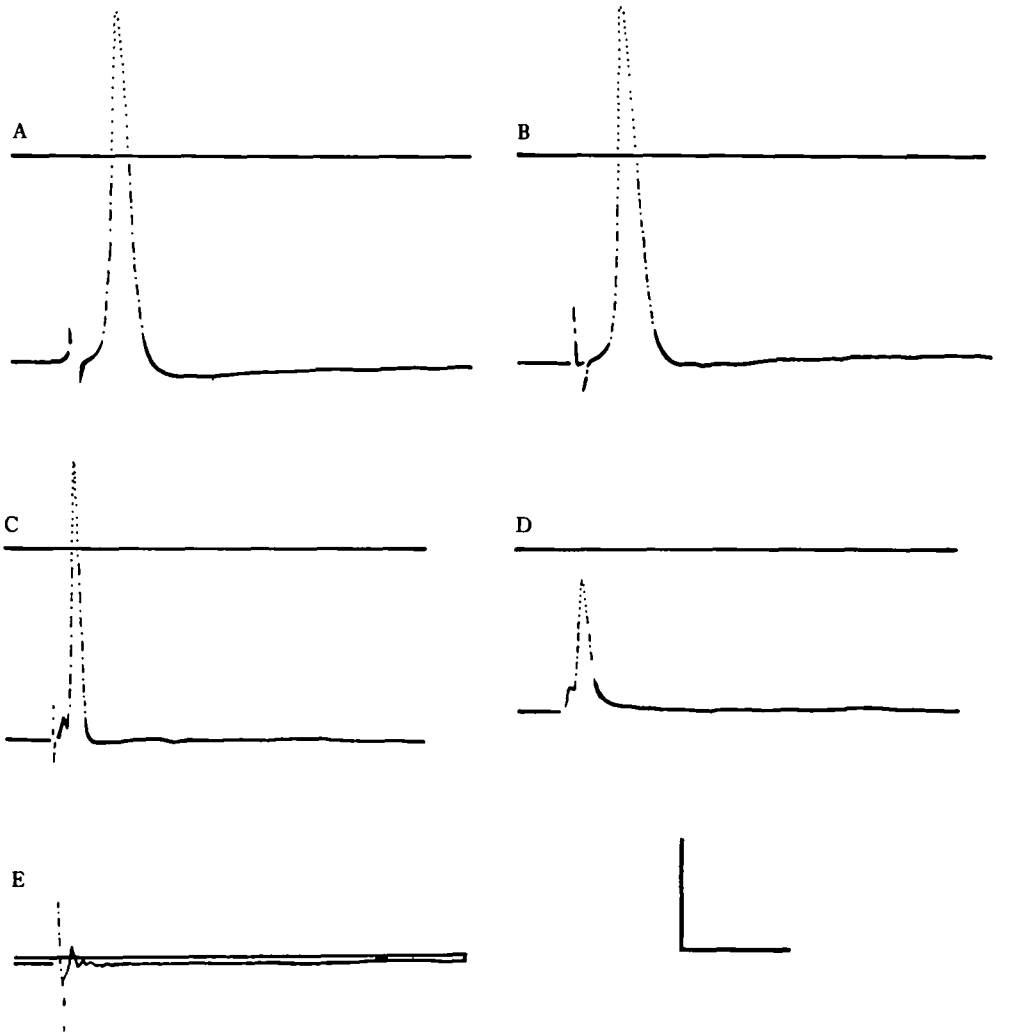


Fig. 2. Effect of cutting connectives while recording intracellularly. (A) shows a traced record of an action potential recorded immediately before cutting the connective. (B) was taken from the same axon 5 h after the connective was cut. In this case the axon appears to have sealed during the cut. (C), (D) and (E) show records from an axon before cutting (C), and 5 min (D) and 2 h (E) after cutting. This axon shows the normal injury response to cutting. Scale bars: 40 mV (vertical), 2 ms (A, B) or 4 ms (C, D, E: horizontal).

Long-term experiments were also performed. In these, a single connective was ligatured and cut *in vivo*. After 24 h, a Na-dependent, overshooting action potential could be recorded which was blocked by TTX but not by cobalt (Fig. 1). Similar results were obtained after 48 and 72 h.

Giant axons separated from their cell body degenerate, losing their electrical excitability within 8 days (Farley & Milburn, 1969). Connectives cut on the side of a ligature proximal to the cell body could maintain action potentials in the distal segment for up to 20 days.

In other experiments, unligatured connectives were cut close to the site of intracellular recording. In some cases, the resting and action potentials were maintained for at least 5 h (Fig. 2) suggesting that the cut end of the axon had sealed. More frequently, the resting potential fell rapidly accompanied by loss of the action potential (Fig. 2). These results are similar to those of Meiri *et al.* (1981) who showed also that during the first 3 h after axotomy it was possible to restore the action potential by hyperpolarizing the membrane. They found it impossible to restore invasion of the

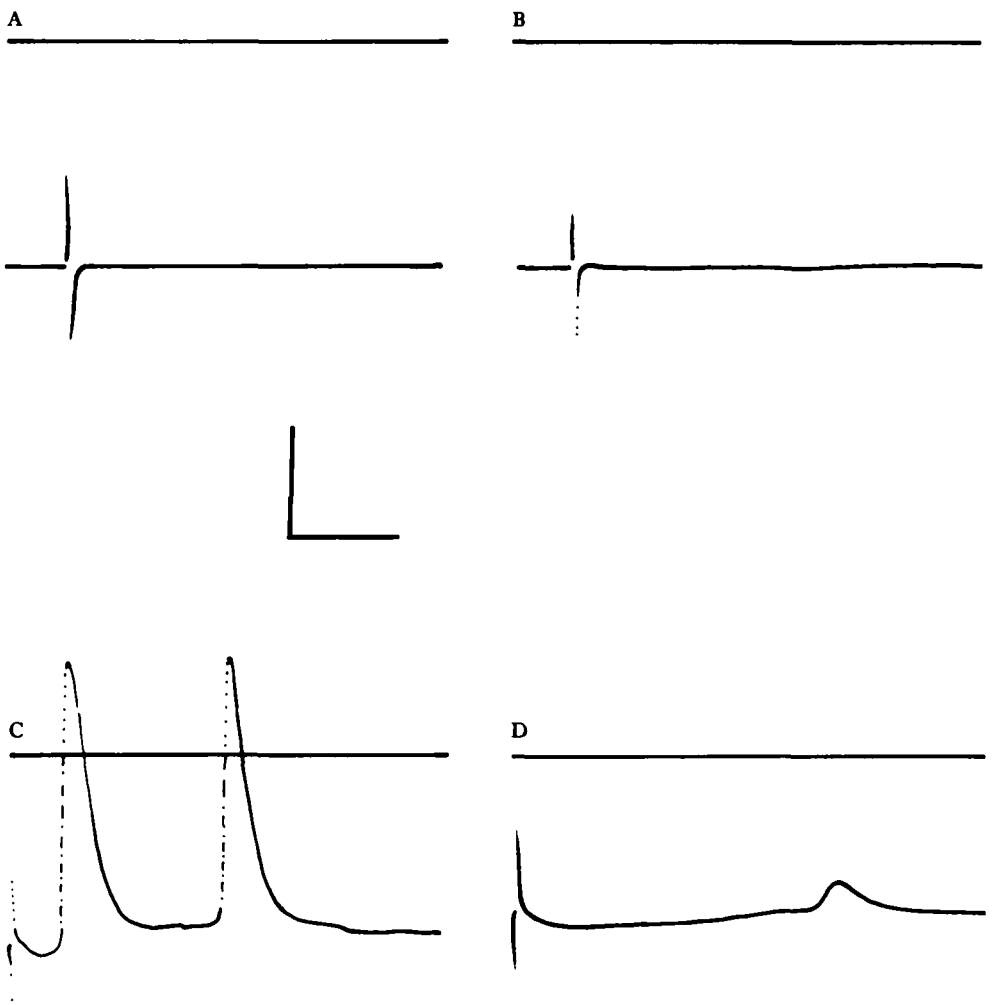


Fig. 3. (A) and (B) show records from a nerve cord cut *in vivo* 4 h previously. (A) was taken with the nerve cord bathed in normal cockroach saline. The axon was hyperpolarized, using a suction electrode, from −9 mV to −80 mV. Stimulation, near the cell body, failed to produce an action potential which propagated into the region of the cut end. The electrode was impaled 0·4 mm from the cut end. (B) is from a different axon in the same preparation after washing for 20 min with 133 mM-Na; 20 mM-Ca saline. This axon was hyperpolarized from −4 mV to −80 mV and stimulation again failed to produce an action potential, recording at 0·3 mm from the cut end. (C) and (D) are records taken 0·2 mm from the cut end before (C) and after (D) the addition of TTX to the 133 mM-Na, 20 mM-Ca saline. These records are from a single axon cut, *in vivo*, 51 h earlier. The upper trace in each record marks the zero potential level. Scale bars: 40 mV (vertical), 2 ms (horizontal).

action potential to the cut end between about 3 and 48 h following nerve section ev[illegible] if the membrane was hyperpolarized. Our results confirm this observation (Fig. 3).

Recording anterior to ganglion A3 near the cut end of a connective [bathed in a Na-free (Tris-substituted) saline containing 4-AP] and by hyperpolarizing the membrane to −80 mV, Meiri *et al.* were able to record action potentials which were not blocked by TTX (10^{-7} M), but were reversibly blocked by cobalt and showed a dependence on extracellular Ca concentration close to that predicted from the Nernst equation.

We attempted to repeat these experiments by recording anterior to A3 at 0·2–0·7 mm from the cut end of the connective. Our observations have failed to confirm the earlier work of Meiri *et al.* (1981). We were unable to record overshooting action potentials (Fig. 3) at less than about 48 h after axotomy. Small spikes (approx. 10–40 mV) were observed rarely but these could not be conclusively identified as being Ca-dependent. At later times, overshooting spikes could be recorded which were always blocked by TTX (3×10^{-7} M; Fig. 3).

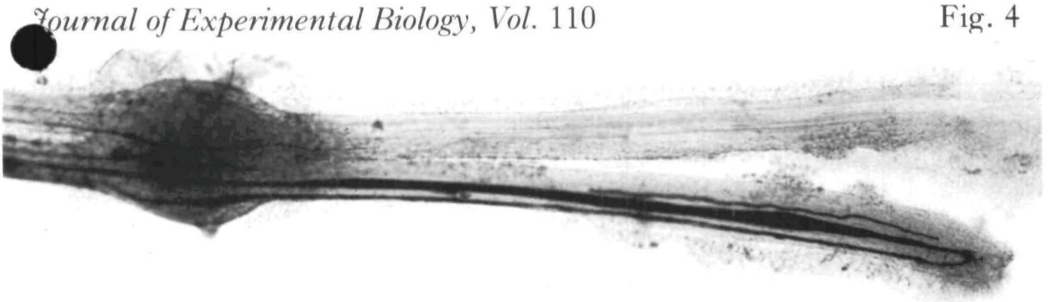
When we ligated GIs before cutting, normal resting and action potentials could be maintained for 3–48 h after axotomy. In contrast, the resting potentials were generally low and action potentials absent in cut, unligatured GIs. This result supports the idea that the response of GIs depends on the type of lesion.

The proximity of a lesion to the cell body is known in vertebrates to affect the response of the neurone (see, Lieberman, 1974). We have cut cockroach nerve cords at different levels to discover whether this affected the regenerative response of GIs.

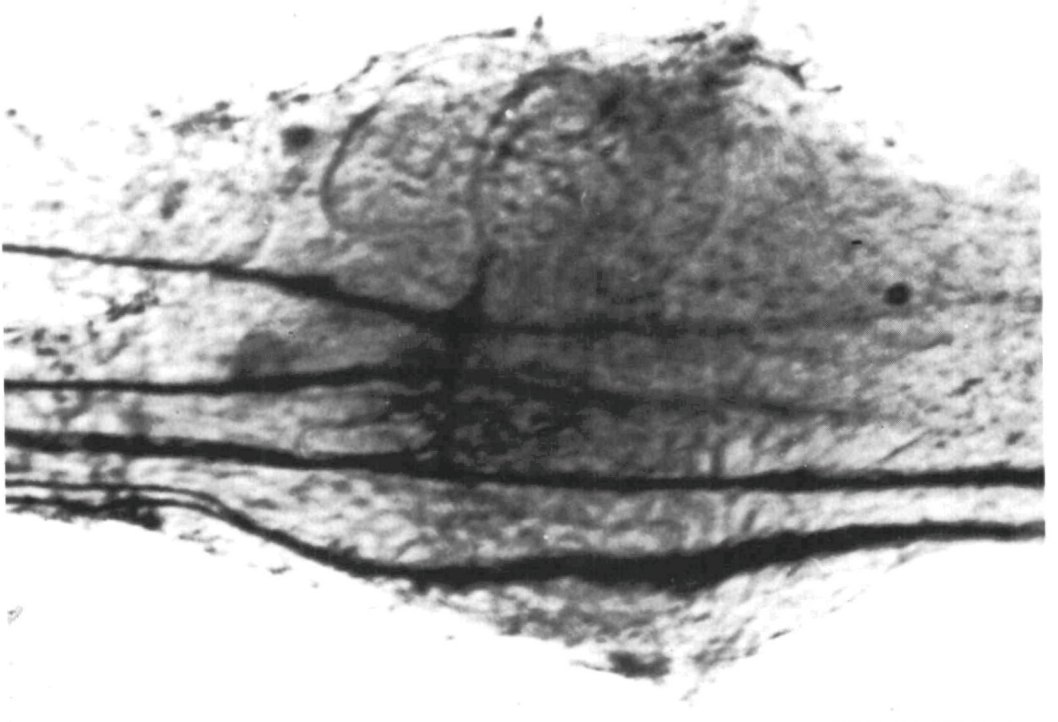
We cut single connectives between ganglia A2 and A3 (A2–3), A3 and A4 (A3–4) or A4 and A5 (A4–5). The approximate distances between the lesion site and the terminal ganglion (A6) were 10·3 mm (mean value; $N = 9$) for A2–3, 8·4 mm ($N = 6$) for A3–4 and 4·6 mm ($N = 8$) for A4–5. Cut connectives were observed to reconnect on several occasions making it difficult to establish the exact location of the lesion site. Such reconnections were never observed by Meiri, Dormann & Spira (1983). The connective caudal to that of the lesion site was impaled at various times after axotomy and a GI injected with cobalt (Pitman *et al.* 1972). Meiri *et al.* (1981) showed that GIs severed at A2–3 began to sprout after approximately 7 days. We found that after 7 days, four out of nine GIs cut at A2–3, four out of six cut at A3–4 and five out of eight cut at A4–5 showed sprouting. In other preparations, axons injected with cobalt at 14, 21 and 28 days after cutting showed a considerable variation in their pattern of growth but the response did not appear to be affected by the site of the lesion.

Meiri *et al.* (1981) could not detect any changes in morphology of GIs except for sprouting in a region 0·2–0·4 mm from the cut end. We observed this type of sprouting but have also seen, on one occasion, what appears to be a neurite growing to cross a ganglion and extending both caudally and rostrally in the contralateral connective (Fig. 4A, B). Sprouts traversing ganglia have previously been observed to cross to the contralateral side and grow in a similar way following a nerve crush (Zeldes *et al.*

Fig. 4. Growth of neurites demonstrated in Co injected axons. (A) and (B) show a neurite in the 5th abdominal ganglion from a nerve cord cut, *in vivo*, between the 4th and 5th ganglia 21 days earlier. The axon was identified as GI2 from the position of its cell body. Scale bars: 500 μm (A); 100 μm (B). (C) is a camera lucida drawing of a neurite sprouting in ganglion A5 11 days after cutting the connective, *in vivo*. In this case the axon (GI3) appears to have died back to the ganglion. Scale bar: 200 μm.



4A



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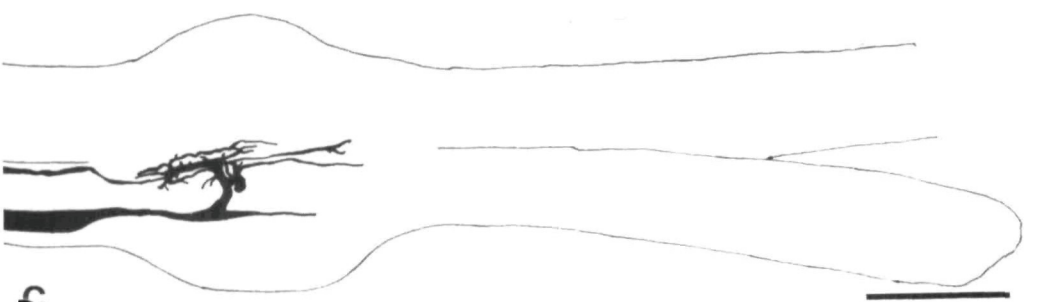

C

1982). Neurites were also seen to grow on other occasions, the lesion site in the example shown in Fig. 4C was 1·06 mm rostral to the ganglion (A5). No changes were apparent in the dendritic tree in the terminal abdominal ganglion.

DISCUSSION

Our failure to record Ca-dependent action potentials in the vicinity of regenerating axon tips could result from a number of factors. It could, for example, arise from some subtle difference in the cutting of connectives or, conceivably, from variations between the stocks of cockroaches used. However, similar results to our own have also been obtained in another laboratory (M. E. Spira, personal communication). Furthermore, it is important to note that, in our experiments, axonal sprouting occurred without a measurable, transitory, incorporation of voltage-sensitive Ca channels. This implies that neurite elongation can occur in the absence of calcium entry through specific voltage-sensitive channels.

The calcium action potential recorded by Meiri *et al.* (1981) could only be recorded by them at a time when (from their Fig. 1) the resting potential of GIs appears to be between about −10 mV and −30 mV. This raises questions as to the functional significance of these channels. Previous work under voltage-clamp conditions has shown that calcium channels in mouse neuroblastoma cells are inactivated completely at membrane holding potentials less negative than −30 mV (Moolenaar & Spector, 1979). In other systems, however, calcium channels are not inactivated by prolonged depolarization and inactivation seems to be dependent on intracellular calcium (see reviews by Hagiwara & Byerly, 1981; Tsien, 1983).

The calcium action potentials reported by Meiri *et al.* (1981) were present only between about 7 and 60 h after axotomy. The function of such transient calcium channels is not clear. It is certainly difficult to assign them a role in the initiation of sprouting (which begins after about 7 days) or subsequent elongation of the neurites. In both cases they would be expected to persist for a longer time. The possibility remains that calcium channels may be confined to the growing tip of the sprouts but it would be extremely difficult to record from these in the nerve cord.

The nature of the stimuli which inform the neurone of damage and initiate regeneration are not known. It has been shown, for example, that dendritic sprouting can occur in undamaged cockroach motoneurones as a result of neural lesions (Pitman & Rand, 1982). Excess intracellular calcium has been suggested as a possible trigger for degenerative processes (see, for example, Aviv *et al.* 1982), though the mechanism through which excess calcium may be involved is not clear.

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