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Electrical Coupling and Dye Transfer between Axon Segments in the Medium Giant Axon of the Earthworm*

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Abstract. Electrical coupling between axon segments has been studied in the medium giant axon, which had been partially isolated from the ventral nerve cord of the earthworm. Evidence has been obtained that in addition to the coupling structures in the septum there are diffusion- and current-pathways through the pseudo-myelin which seem to be more permeable to fluorescein than the channels in the septum. These findings offer an explanation for the discrepancy of the experimentally determined specific resistance of the septum and the expected values based on nexus density and channel size in the septum.

Key words: Intercellular communication – Dye-coupling – Electrical resistance of nexus membranes – Giant axons

Septa in giant axons of the earthworm are structures which are especially suitable for studies of the electrical properties and of the permeability of nexus (gap junctional) membranes. The specific electrical resistance of nexus membranes has been determined in the medium giant axon by applying a combined extra- and intracellular recording technique (Weidmann 1970) to an excised ventral nerve cord in which the giant axons are embedded (Brink and Barr 1977). However, from the values of those experiments a specific resistance has been calculated which is approximately three orders of magnitude larger than expected from a model based on nexus density and channel size in the septal membrane.

The present study supports the assumption that axon segments are not only coupled by nexuses in the septum but also by current- and diffusion pathways through the pseudomyelin of the axon. Attempts have been made to isolate electrically the septum from current pathways through the pseudo-myelin.

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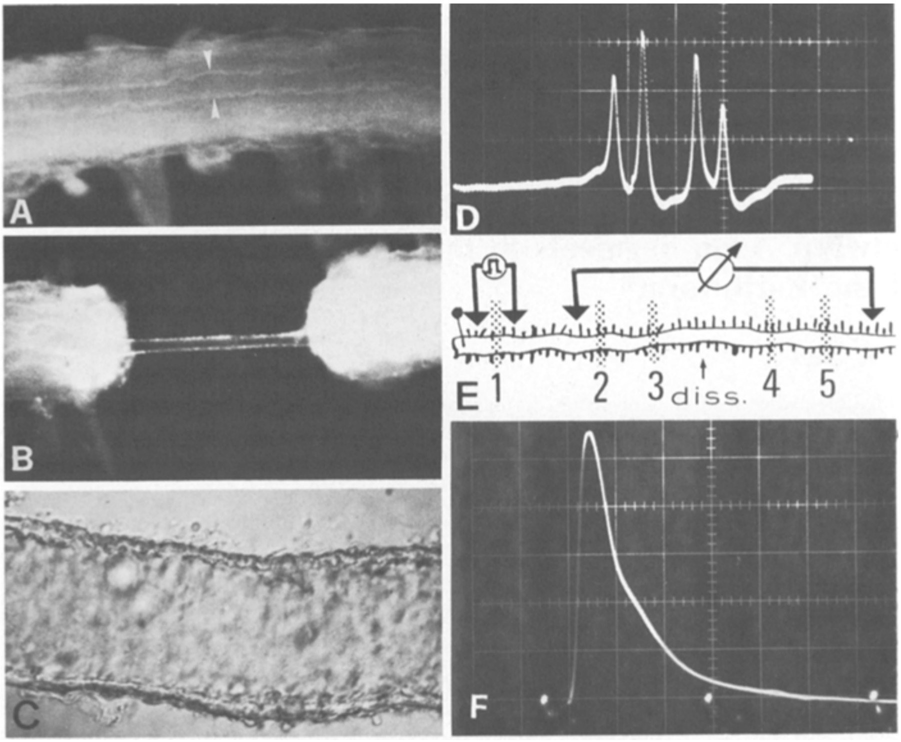

Fig. 1. **A** A medium giant axon (arrow heads) in an excised nerve cord of the earthworm × 40. **B** Isolated axon stretched by contractions of the muscular tissue in the sectioned nerve cord × 30. **C** Unstretched medium giant axon. The pseudo-myelin layers of 5 μm thickness appear wrinkled × 280. **D, E** Sequence of four action potentials recorded with external electrodes across four grease seals (2–5) in an experimental arrangement as described in E. Average conduction velocity = $33 \mathrm{m} \cdot \mathrm{s}^{-1}$. If during dissection the axon is stretched or injured the time delay between action potential 2 and 3 increases or conduction block develops which is indicated by the loss of action potential 3 and 4. 20° C and 1 mV resp. 1 ms per Div. **F** Action potential recorded via a fluorescein-filled microelectrode inserted into the isolated portion of the axon. 20° C and 20 mV resp. 1 ms per Div

Methods

The excised ventral nerve cord from Lumbricus terrestris is placed in a dissection- and recording-chamber with a central glass-bottom and Sylgard-covered side troughs. Silicon-grease seals of 1 mm width are placed on each side of the central portion of the nerve cord (approximately 4 mm long) where dissection will be performed (Fig. 1E). For suppression of the spontaneous muscular activity the nerve cord is bathed in saline which contains 0.3 mg Carbachol/l (Brink and Barr 1977). The medium giant axon, located between two smaller lateral giant axons has the lowest threshold and can be stimulated by electrical pulses of 0.1 ms duration and 1–3 V amplitude across the first seal. Propagation of an action potential is indicated by a series of 3–5 mV action

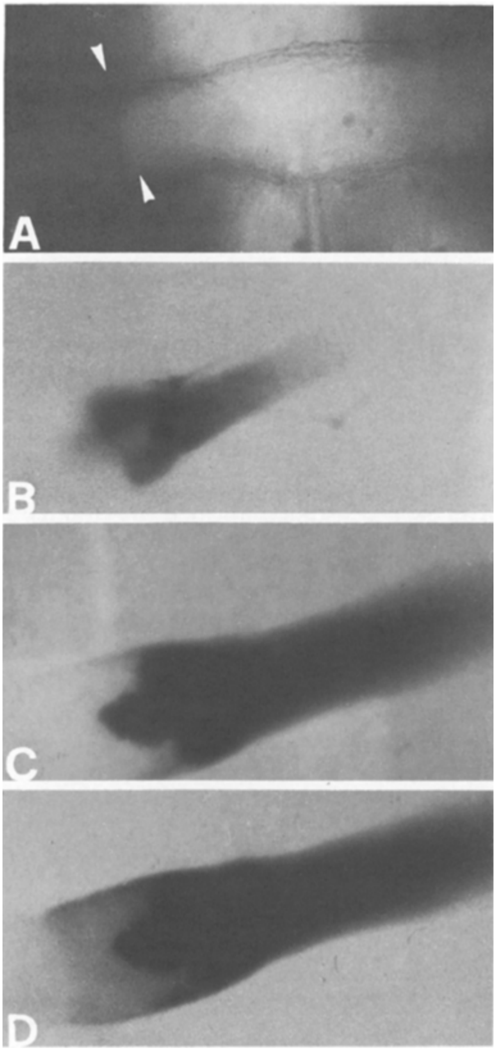

Fig. 2. A Isolated medium giant axon impaled with a fluorescein-filled microelectrode. Superimposed double-exposed pictures of bright-field and fluorescence images. **B** Filling-phase of the axon segment. The bright fluorescence of the injected fluorescein has been printed as negative in **B–D** and appears therefore black. Block of fluorescein diffusion at the septum in the left lower corner is already visible. **C** 5 min after the begin of fluorescein injection. A sharp funnel-shaped boundary of fluorescence marks the position of the septum. A faint marginal fluorescence extends already into the pseudo-myelin of the adjacent segment. **D** 10 min after the begin of fluorescein injection. The sharp boundary at the septum has not much changed in position while the adjacent segment is enveloped by fluorescence in the periphery of the axon

potentials which can be recorded with external bath electrodes because a voltage transient develops at each of the seals when the action potential passes along this region. From the peak-to-peak time delay and the distance between the seals conduction velocity can be calculated. A decrease in conduction velocity or conduction block indicates axon stretch or damage during the dissection phase. For visualization of the septum, which cannot be easily detected within the nerve cord, fluorescein is injected into a segment by diffusion or by electrical pulses from an inserted fluorescein-filled microelectrode. Septa block the diffusion of fluorescein through the axon temporarily (for 20–30 min) and sharp boundaries of the fluorescence mark the in vivo-position of the septa (Fig. 2A–D). Two microelectrodes (a voltage sensing electrode and a current electrode) are inserted into the fluorescein-injected segment and two other micro-electrodes are inserted behind the septum into the adjacent axon segment. During current pulses the voltage drop V_1 at the membrane of the impaled segment is compared to the voltage drop V_2 at the membrane of the adjacent segment (caused by current flow through the septum) and the coupling ratio V_2/V_1 is determined.

In sucrose-gap experiments the isolated portion of the axon is continuously superfused with isotonic sucrose-solution. The sucrose-gap is separated from adjacent saline-superfused compartments by thin partitions.

For experiments where electrical isolation of the septum membrane is required 50–80 μm capillaries, which were ground flat at the tip, are inserted horizontally into the axon on both sides of a septum. When the capillary in the fluorescein-injected segment is slowly moved, touching of the septum becomes visible by bulging of the fluorescence boundary. The second capillary inserted in the adjacent segment is then moved towards the septum until both capillaries meet, slightly compressing the septum between the edges of both electrodes. The seal-resistance formed by the compression of the septum is usually 10–15 times greater than the resistance of the septum.

Results

Electrical Coupling at the Septum

The coupling ratio V_2/V_1 is close to 1. Iontophoresis of ions into the axon like La^{3+}, Ca^{2+} and H^+ – known to uncouple vertebrate gap junctions – (for ref. Loewenstein 1976) decreases the coupling ratio to 0.6–0.2. However, time-course of decoupling and the final value of the coupling ratio vary very much, despite of all attempts to place the electrodes as close as possible to the septum (20–30 μm).

Conduction velocity as calculated from the time delay between externally recorded action potentials and the distance between seals ranges between 25–37 m/s at 20° C. Conduction velocity is usually not effected when microelectrodes are inserted into the isolated portion of the axon, however, after withdrawl of the electrodes occasionally decreased conduction velocity and even block of propagation has been observed. At high magnification leakage of axoplasm from the impalement sites is visible in such cases. In order to reduce the possibility of axon damage by microelectrode impalements an external current injection mode via a sucrose gap has been used.

Sucrose-Gap Experiments

When the isolated portion of the medium giant axon is superfused with isotonic sucrose solution, action potentials up to 100 mV amplitude have been recorded with external electrodes. However, after 10–15 min structural alterations of the superfused axon portion appear. The pseudo-myelin with a normal thickness of 5–7 μm swells and thickness increases up to 15 μm. The axon diameter also increases but due to the loose pseudo-myelin the exact diameter is difficult to measure (approximately 10–30% increase). These structural alteration can be suppressed when 1 mMol of $CaCl_2$ is added to the sucrose-solution, but conductivity of such sucrose-solution is too high to be useful for the sucrose-gap technique. When the perfused axon is bathed in normal saline after superfusion with sucrose-solution, action potentials will propagate across this region only when the superfusion has not exceeded 3–5 min. After this time there is no recovery of the ability to conduct action potentials. Because the conductivity of the axoplasm (75 Ω · cm) as measured with microelectrodes has not changed (i.e., there appears to be no measurable loss of ions from the axon) and because the only visible effect are the alterations in the structure of the pseudo-myelin, it has been concluded that the pseudo-myelin is involved in the mechanism of action potential propagation across the septum, eventually by electrical connections between the axon and the spaces in the myelin.

Coupling Between Myelin Spaces and the Axon

A fluorescein-filled microelectrode can be pushed tangentially into the pseudo-myelin of an axon loop without penetrating the axon. This is indicated by fluorescein leaking out of the electrode, which remains in the myelin region and does not stain the axoplasm. When under such conditions current pulses are delivered from a microelectrode inside the axon, the voltage drop at the myelin membranes equals the voltage drop at the axon membrane. However, microelectrodes pushed into the myelin may sometimes break and can pick up signals like pressure-electrodes (Bortoff 1961), even when normally no coupling between axon and the pseudo-myelin is present. Occasionally a potential of −70 mV is recorded by a microelectrode in the pseudo-myelin spaces, which is the same polarisation as present at the axon membrane.

Dye-Coupling Between Axon Segments

The septum acts only as a temporary barrier to the diffusion of fluorescein. The early phase of transfer of fluorescein across the septum is difficult to observe, because stray-light from the injected axon segment makes the faint fluorescence in the adjacent segment almost invisible. An image intensifier has therefore been used for an analysis of the transport of fluorescein across the septum when the injected segment contains only a very low concentration of fluorescein. Fluorescence moves during the first 5–10 min only into the marginal regions of the next segment. At high magnification it appears that the fluorescein has penetrated the myelin spaces and forms a mantle-like coat around the adjacent axon segment. The fluorescence boundary at the septum changes position very little and only after 30 min the sharp boundary is no longer visible.

Electrical Isolation of the Septum Membrane

As the dye-coupling experiments indicate the presence of diffusion pathways across a septum through the pseudo-myelin, the coupling resistance between axon segments as measured with microelectrodes is determined by two different current pathways (through the septum and through the pseudo-myelin) in parallel and therefore cannot be related to the coupling structures in the septum alone. It is possible to isolate the septum membrane electrically from adjacent coupling structures by compressing the septum between the edges of the two capillaries, which have been ground flat. The seal resistance of two capillaries compressing a non-junctional cell membrane is in the range of 2–3 MΩ using capillaries of 50 μm internal and 70 μm external diameter. As the septum membrane presumably has a relatively low electrical resistance (100–200 KΩ) the seal resistance can be expected to exceed the membrane-patch-resistance at least 10 times.

In five preliminary experiments the following data have been obtained: The average electrical resistance of a septum segment compressed between glass capillaries of 70 μm internal diameter is 181 KΩ. From our freeze-fracture replicae of the septum one can assume that approximately 30% of the septal membrane are nexuses. The calculated specific resistance of the nexus membrane is 7 $\Omega \cdot cm^2$ and, if uniform distribution of nexus is present in the septa, the resistance of the total septum would be 70 KΩ. Thus, with electrical isolation the resistance of the septum is roughly 2× larger than without isolation.

Discussion

Diffusion of fluorescein into the pseudo-myelin indicates that the axon segment is functionally coupled to the myelin spaces. A similar dye-communication between sheath glia and the axon has been observed in crayfish axons where the dye, Lucifer Yellow CH, injected intraaxonally can enter the axon sheath glia within minutes (Viancour et al. 1981). However, several possible sources of error have to be considered. Staining of the pseudo-myelin could occur during the penetration of the fluorescein-filled microelectrode through the myelin spaces. This possibility can be excluded because microelectrodes were also inserted horizontally into the axon puncturing two to three septa before a segment was filled. Identical patterns of fluorescence migration develop under those conditions. As fluorescein also enters the myelin sheath in an undissected axon within the nerve cord increased permeability due to injuries of the isolated axon can also be excluded. Fluorescein could be transported by pinocytotic-endocytotic processes, though the rapid appearance of fluorescein in the myelin within 1 min makes this process less likely. However, in this case, it has to be assumed that the fluorescein-permeability of the septum is low and that therefore the channel size of the junctions is smaller than expected.

It appears, however, more likely to assume dye-permeable membrane junctions between the axon membrane and the pseudo-myelin. The rapid dye-transfer, the simultaneous swelling of pseudo-myelin and axon in sucrose-solutions, the electrical coupling between myelin-spaces and axon, the presence of morphological coupling structures between axon and innermost myelin layers as visible in our electron-micrographs and finally the increase in resistance when the septum is electrically isolated from adjacent axon membranes favour alltogether the hypothesis that pseudo-myelin and axon segments are functionally coupled by membrane junctions. Coupling structures between the axon membrane and the myelin spaces appear to be more permeable to fluorescein than coupling structures in the septum. The existence of nexuses with intramembraneous particles of different size and presumably different permeabilities has been reported (for ref. Gilula 1972). Recently the presence of A-type nexuses in the septum of the earthworm and of B-type nexuses between epithelial cells of the same animal has been observed (Brink et al. 1981). A coexistence of A- and B-type junctions in the axon could explain the difficulties in relating electrical data to the coupling structures of the septum alone. Also the variations in the time course of decoupling during iontophoresis of ions could be caused by the presence of different types of membrane junctions.

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