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Autofluorescence as a tool to study mucus secretion in *Eisenia foetida* ☆

R.B. Heredia [a], S. Dueñas [d], L. Castillo [a], J.J. Ventura [b], M. Silva Briano [c], F. Posadas del Rio [a], M.G. Rodríguez [a,*]

[a] *Depto de Fisiología y Farmacología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Mexico*
[b] *Depto de Morfología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Mexico*
[c] *Depto de Biología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Mexico*
[d] *Depto de Fisiología, CUCS, Universidad de Guadalajara, Mexico*

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Abstract

Autofluorescence in living cells is due to the presence of endogenous substances that emit fluorescence upon excitation by incidental light. A type of fluorescence, bioluminescence, has been suggested to be linked to mucus secretion in earthworms; however, the origin and the physiological function of this fluorescence are not clear. The aims of this work were to describe autofluorescence in the earthworm *Eisenia foetida* by SEM, CLSM, and fluorescence microscopy and to examine the possible mechanism of mucus secretion by video microscopy. Earthworms were stimulated either chemically or electrically to induce the secretion of yellow mucus, which was subsequently studied by video microscopy. Mucus was released from the body wall and near the mouth. This phenomenon was associated with autofluorescence and involved at least four distinct stages: release of vesicles, formation of granules, muscular contraction, and organization of strands. The fluorescent molecules were stored in vesicles bound to the membranes. These vesicles were intact when shed from the body. The vesicles were stable but also changed to a granular material or formed strands. Video analyses demonstrated that secretion was dependent on the type of stimulus.

Keywords: Autofluorescence; Coelomic fluid; Earthworms; Mechanism; Mucus secretion; Videomicroscopy

1. Introduction

Fluorescence is the light emitted when a fluorescent molecule has been excited by the appropriate light. Native fluorescence is common in bacteria, algae, aquatic invertebrates, and some insects and moths (Abels and Ludescher, 2003). Fluorescence depends on the presence of endogenous fluorescent compounds such as nicotinamide adenine dinucleotide, flavins, and fluorophoric amino acids tryptophan, tyrosine, and phenylalanine (Albani, 2004). Other proteins such as green fluorescent protein and yellow fluorescent protein can also cause fluorescence (Daubner et al., 1987; Ya and Szalay, 2002).

The ability of earthworms to emit light has long interested researchers (Jamieson, 1981; Cardillo et al., 1997). Although much is known about the biochemistry of this earthworm luminescence, the cell biology of the phenomenon is less well understood. In *Diplocardia alonga*, bioluminescent light is emitted by a luciferin reaction; however, the anatomical origin of the bioluminescence has not been clarified (Otsuka et al., 1976). Early image-intensified microscopy studies suggested that fluorescence occurs in slime exudates, but the cellular or subcellular sources have not been elucidated (Rudie and Wampler, 1978; Wampler and Jamieson, 1986).

The mucus secreted on the surfaces and mucus membranes of earthworms plays a crucial role in locomotion, feeding, osmoregulation, defense, reproduction, and protection of

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* Corresponding author. Lab 6, Edif. 202 Centro de Ciencias Básicas, Avenida Universidad #940 col Bosques, Aguascalientes, Ags. c.p. 20100, Mexico. Tel.: +52 449 9108424; fax: +52 449 9 108401.
E-mail address: romag18@hotmail.com (M.G. Rodríguez).

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epithelial and other surfaces (Dayrup-Olsen et al., 1983; Denny, 1989). Appropriate to these diverse functions, mucus is composed of many components, including water, electrolytes, mucus glycoproteins, and mucopolysaccharides, as well as other large molecules such as lectins and hemocyanin (Jamieson, 1981). Secretion of yellow mucus is copious and varies considerably depending on the conditions and immediate physiological use (Dayrup-Olsen et al., 1983; Dayrup-Olsen and Luchtel, 1998).

Although bioluminescence and mucus secretion in earthworms have been linked in experimental studies, differences between the two are evident (Wampler and Jamieson, 1986; Rudie and Wampler, 1978). The cellular origin and physiological function are not clear. Thus, the aim of this work was to examine autofluorescence in the earthworm *Eisenia foetida* and to uncover a possible mechanism of mucus secretion. This model of secretion offers an exceptionally favorable system for the study of mucus secretion due to the copious levels of mucus and the precise control of this secretion. Furthermore, the mucus is released onto the exposed body surface rather than within body cavities or under the protection of shells as for other organisms.

2. Materials and methods

2.1. Animals

E. foetida were purchased from a local dealer. Worms were maintained in a plastic case containing a mixture of humid soil, banana peel, and finely milled oat husk at a constant temperature of 20±2 °C. This medium favored their growth and allowed the emergence of many cocoons. Experiments were performed at room temperature with sexually mature earthworms that weighed more than 300 mg.

2.2. Electric stimulation

Worms were transferred to Petri dishes containing filter paper moistened with distilled water and were maintained for 24 h to depurate the intestine. Immediately prior to the electrical stimulation, earthworms were placed individually in Petri dishes containing 2 mL of saline solution (125 mM NaCl, 2.5 mM KCl, 2.0 mM Tris buffer, pH 7.4). Field stimulation was applied to the worm using platinum electrodes connected to a Grass S-2 stimulator (Grass Instruments Co., USA). A single electrical current was applied at the lowest frequency for 2 ms. The current intensity was increased from zero until a secretion was observed. The amount of current delivered during a single stimulation was 2–30 mA as monitored with a constant current unit.

Secretion phenomena were recorded by video microscopy using a CCD color camera (Panasonic GP-KR222) coupled to a 2.5×–10× zoom inspection microscope (Edmund Scientific. Inc., USA). The incident illumination was facilitated via a fiber optic ring light.

2.3. Quantification of mucus secretion

Individual worms were weighed in previously tared Petri dishes, and the electrical stimuli were applied (17±4 mA). Following mucus secretion, the worms were removed from the dishes, and the Petri dishes were again weighed to determine the quantity of the mucus secretion.

2.4. Microscopy techniques

At the end of the stimulus, worms were fixed for further examination by scanning electron microscopy. The worms were

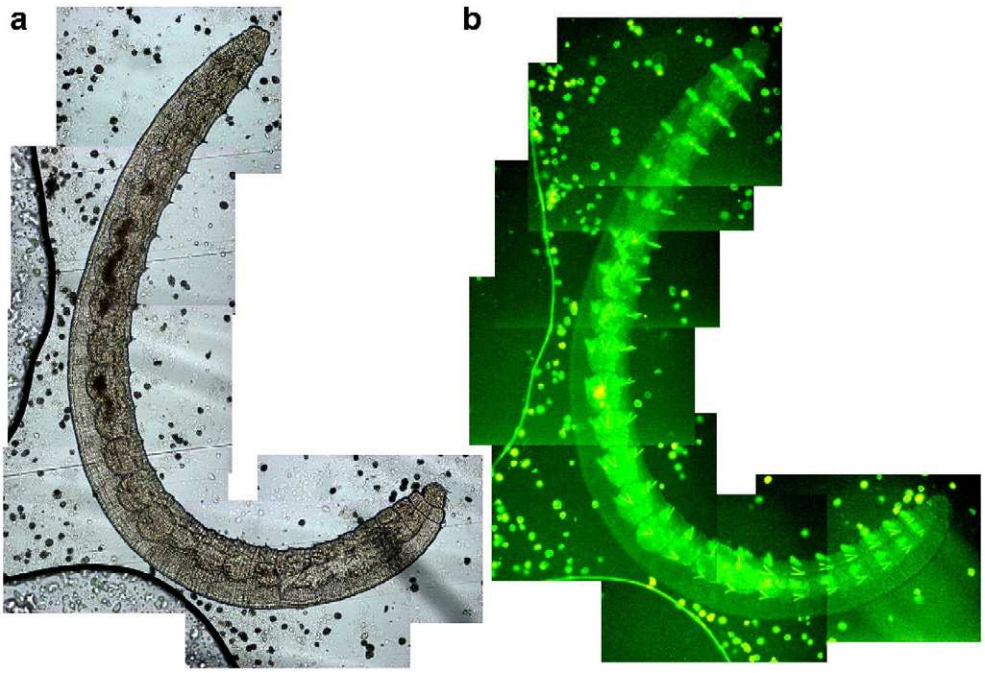

Fig. 1. Earthworm montage using bright field and fluorescence microscopy. (a) The anatomical structures shown are the digestive tube, body, and setae. (b) The entire body shows green fluorescence especially the digestive tube, coelomic cavity, and secretion vesicles.

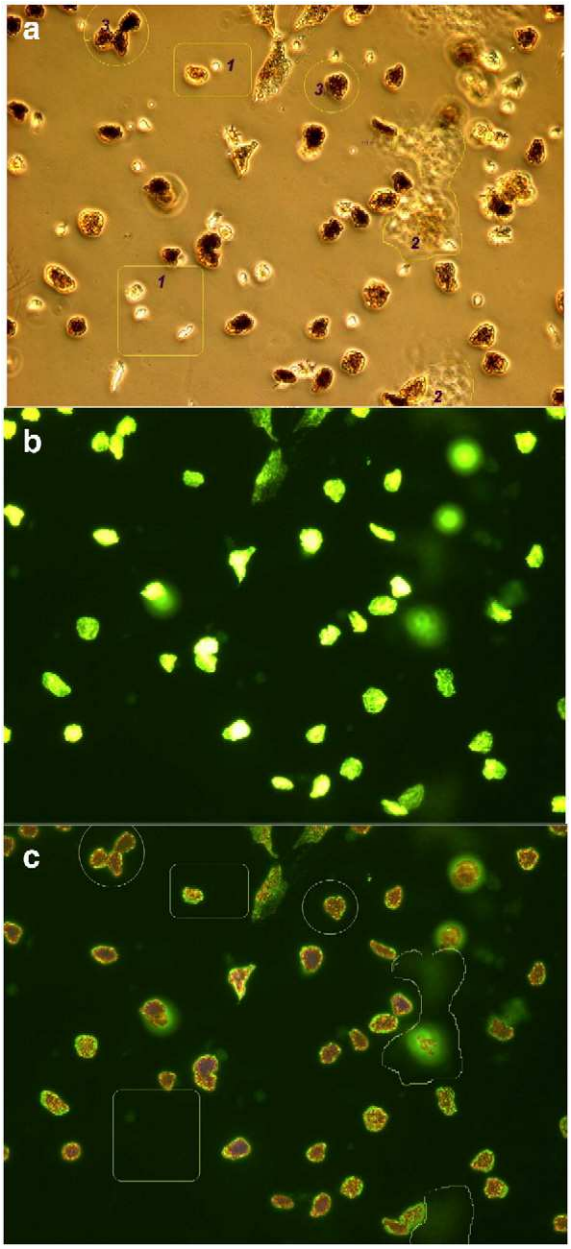

Fig. 2. Secreted mucus vesicles. (a) Bright-field microscopy of vesicles shows that the vesicles have small enclosed refractive granules. (b) The same field is shown using a fluorescence microscope. Fluorescent intact vesicles and vesicles disrupted by mechanical shearing (irregular areas, 2) are shown. (c) Overlay pictures of (a) and (b). The granules are more refractive. The granules look the same as in bright field microscopy but show green fluorescence. When the vesicles are disrupted, the fluorescence is lost (rectangular areas,1).

fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate in phosphate buffer overnight at 4 °C, rinsed several times with phosphate buffer, and dehydrated in an acetone series (50, 80, 90 and 100%). The worms were then subjected to critical point drying, coated with metal, and examined in a Jeol model JSM 25S scanning electron microscope.

Light microscopy studies were carried out using a low light video microscope. Transmitted light pictures were obtained using a Zeiss tungsten illuminator selected by colored glass filters and a Zeiss substage condenser system adjusted for bright field illumination. Fluorescence microscopy was performed with a Zeiss epi-illuminator microscopy (Axioscop 40/40FL, Zeiss, Germany) and set fluorescein filters. These filters were selected from one work previously reported in earthworms (Wampler, 1982).

The confocal microscopy system used to study the vesicles consisted of a FV300 laser scanning confocal unit interfaced with an Olympus upright microscope (Olympus Optical Co. Ltd., Japan). The earthworm was placed on a dish and stimulated with electrical current in order to induce mucus secretion. The observations of the worm on the stage were made with a water immersion lens (UPLFL 40×) that was submerged in the saline solution contained in the dish. An Argon laser operating with a set FITC filter was used to excite and detect the emission fluorescence. Repetitive scans were acquired and subsequently subjected to analysis. To collect serial z-sections, the software was run in Z-plane. Each optical section was obtained with Fluoview software and an image average was calculated at the end.

2.5. Elastic properties of the vesicles

To observe the elasticity of the vesicles, one worm was placed in a Petri dish and stimulated with electrical current. The secretion was allowed to settle and attach to the chamber floor for 10–15 min. After this time period, the remaining suspension was removed, and the dish was filled with worm saline solution.

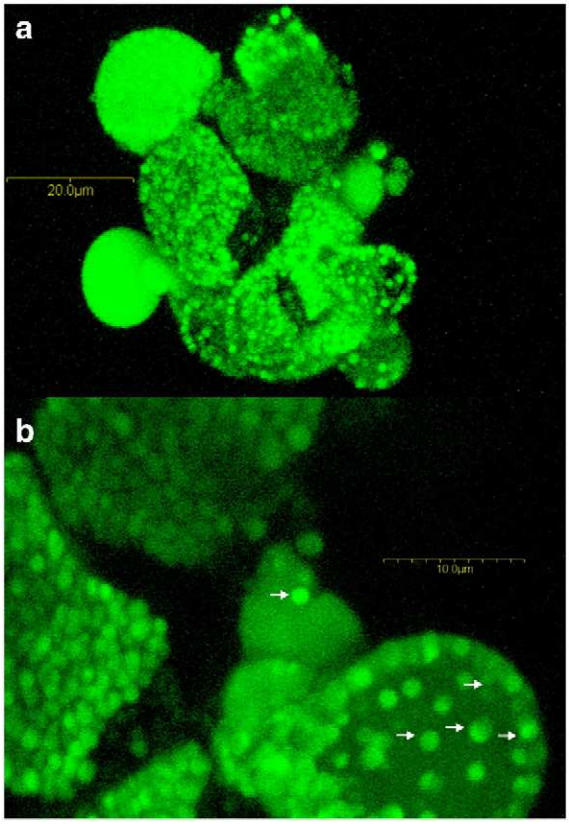

Fig. 3. Confocal microscopy of vesicles secreted by the earthworm *Eisenia foetida*. (a) Mucus vesicles containing granules. (b) Magnification of the vesicles with small fluorescent granules (white arrows).

The chamber was mounted on a stage of the inverted microscope (Zeiss) equipped with a video system. A vesicle was impaled with a micropipette and gentle pressure was applied. The software J-image (NIH, USA) was used for analysis.

2.6. Kinetics of mucus secretion

Viewing chambers made from glass slides and paraffin (1 mm W × 1 mm D × 5 cm L). The chambers were shallow with a narrow trough with dimensions slightly wider and longer than a single worm so that the worm fit snugly into the chamber. The chamber had hydrophobic walls and a hydrophilic floor. The hydrophobic wall ensured that a small volume of water used to bathe the worm, remained within the trough as an elongated column bound by its surface tension. Thus, the worm remained entrapped by the water surface tension. The worms also maintained contact with the solid walls and the electrodes under their body, thus minimizing crawling activity and optimizing mucus secretion viewing. Excess saline solution was removed from the chamber, and a cover slip was placed on the viewing chamber. Secretions were recorded by video microscopy using a CCD color camera (Panasonic GP-KR222) coupled to a 2.5×−10× zoom inspection microscope (Edmund Scientific, Inc. USA). Video clips of longer than 5 min in length were digitized with Pinnacle Studio version 7 software. To view the observation field, incidental illumination was facilitated with a fiber optic ring light. The worm mucus secretions were filmed at 30 frames/s. At least two minutes were digitized before the electrical stimulation. The fields were digitized every 33 ms. More than 50 individual animals were studied. For purpose of illustrate two sequences that yielded the greatest muscle contractions and mucus secretion were reported.

2.7. Statistics

Data obtained from experiments in this study reported as quantitative values are given as mean ± S.D. For each description of phenomena the same results were obtained in several preparations.

3. Results

3.1. Autofluorescence

Fig. 1a depicts a small *E. foetida* earthworm that was previously stimulated with an electrical current. The mucus secretion, which appeared as small vesicles, is clearly visible. The digestive tube and the body wall appeared to be separated by the coelomic fluid. Fig. 1b shows the same earthworm under fluorescence microscopy. The earthworm exhibited autofluorescence

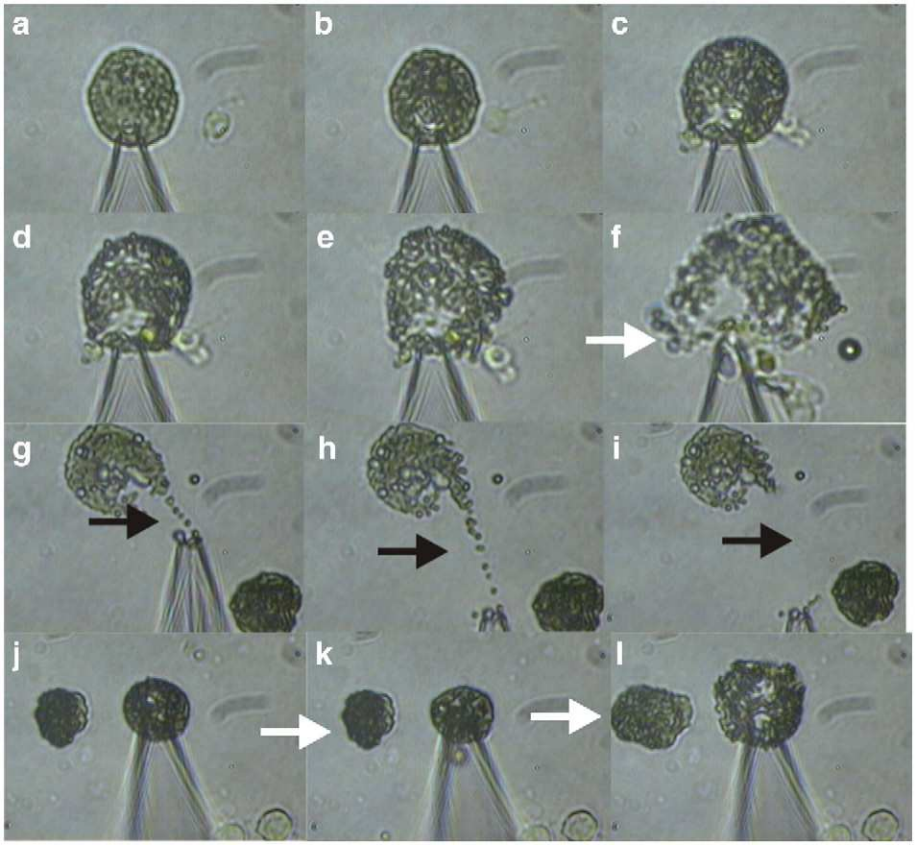

Fig. 4. Effects of shear stress on the mucus vesicles. A single vesicle was touched with a micropipette (a). When gentle positive pressure was applied, the volume of the vesicle changed (b–d). Application of excessive pressure resulted in breaking the vesicle and subsequent release of its granules (f). The micropipette retained a highly elastic fragment (g). Elongation resulted in a linear arrangement of the granules (h), and this arrangement was lost upon the application of more stress (i). The mechanical stress also deformed the adjacent vesicles (j–l).

in all parts of the body with principal fluorescence in the digestive tube, the setae, and the mucus secretions. When the mucus secretion was examined using fluorescence microscopy with fluorescein filters, green fluorescence was observed (Fig. 2b, c). As seen in Fig. 2a, some vesicles appeared to be refractive particles while some vesicles were broken and amorphous. The fluorescence was diffuse or absent in some vesicles. Other vesicles were elongated and lost their form over time. Small refractive granules, which adhered to vesicles, showed even greater green fluorescence than the typical granules (Fig. 2c).

In addition, the secretions in the liquid media were examined by confocal microscopy. The secreted mucus was composed of small vesicles arranged as clusters (Fig. 3a). The intact vesicles had an oval shape with diameters of major axis 35.7±3.9 μm and 29.5±2.9 μm minor axis (n=50). Other vesicles lost their shape upon exposure to mechanical stress. Confocal analysis in the Z-plane revealed spherical particles (diameter 1.44±0.06 μm, n=100) with strong fluorescence. These particles were associated with the basal membrane although occasionally these particles were free (Fig. 3b).

3.2. Elastic properties of the vesicles

The vesicles were quite elastic, and the volume was increased with gentle pressure by a micropipette tip (Fig. 4a, b, and c). Excessive pressure caused the vesicles to break and release granules (Fig. 4d, e, and f). Small pockets of refractive granules were seen while, in other cases, the granules were dispersed in Brownian motion. The elastic response of the vesicle to pressure resulted in retention of a vesicle fragment (Fig. 4g), elongation and a linear release of granules (Fig. 4h), and loss of this structure under higher stress (Fig. 4i). The stress also deformed adjacent vesicles (Fig. 4j, k, and l).

3.3. Electrical and chemical stimulation

Before stimulation, all worms had minimal mucus secretion (Fig. 5a), but following electrical or chemical stimuli, they secreted viscous, cloudy, yellow mucus-like fluid (Figs. 5b and 6). This effect was observed visually and also recorded by video microscopy. Scanning electron microscopy revealed that the space between the segments was the precise site of secretion (Fig. 5d). The threshold for secretion was 17.3±2.5 mA (n=30). The addition of NaCl or KCl to external medium induced mucus secretion only at high concentrations of 653±35 mM and 385±21 mM, respectively. When added to the worm medium, neurotransmitters such as acetylcholine (100 μg/mL), adrenalin (50 μg/mL), L-glutamate (100 μg/mL), and serotonin (150 μg/mL) also provoked mucus secretion in a dose-independent manner. As the quantity of mucus secretion was determined by the difference in body mass before and after secretion, the secretion of individual worms was 0.067±0.017 g (n=30), which corresponds to 12.2% of body mass. Examination by microscopy showed that the mucus was transformed into

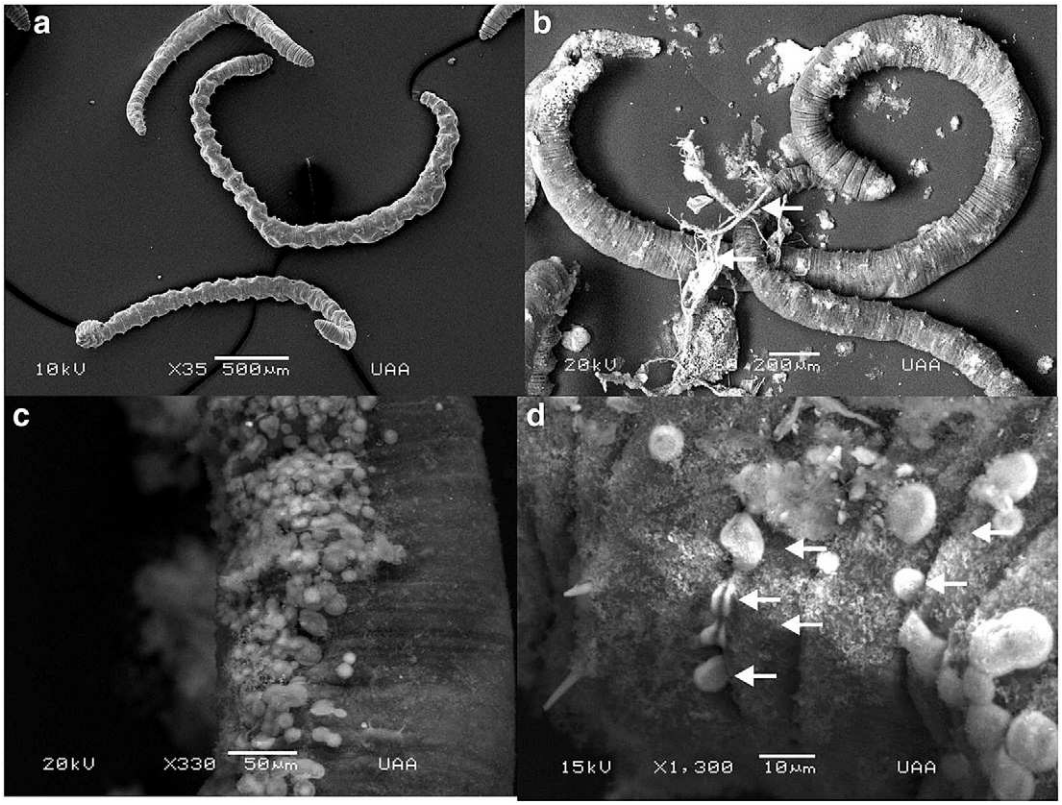

Fig. 5. Scanning electron micrographs of *Eisenia foetida*: (a) control conditions and (b) following electrical stimulation at 15 mA. Mucus strands formed and adhered to the body wall. SEM of earthworm segments: (c) secreted vesicles adhered to body wall and (d) magnification showing the emergence of vesicles from the inter-segment space.

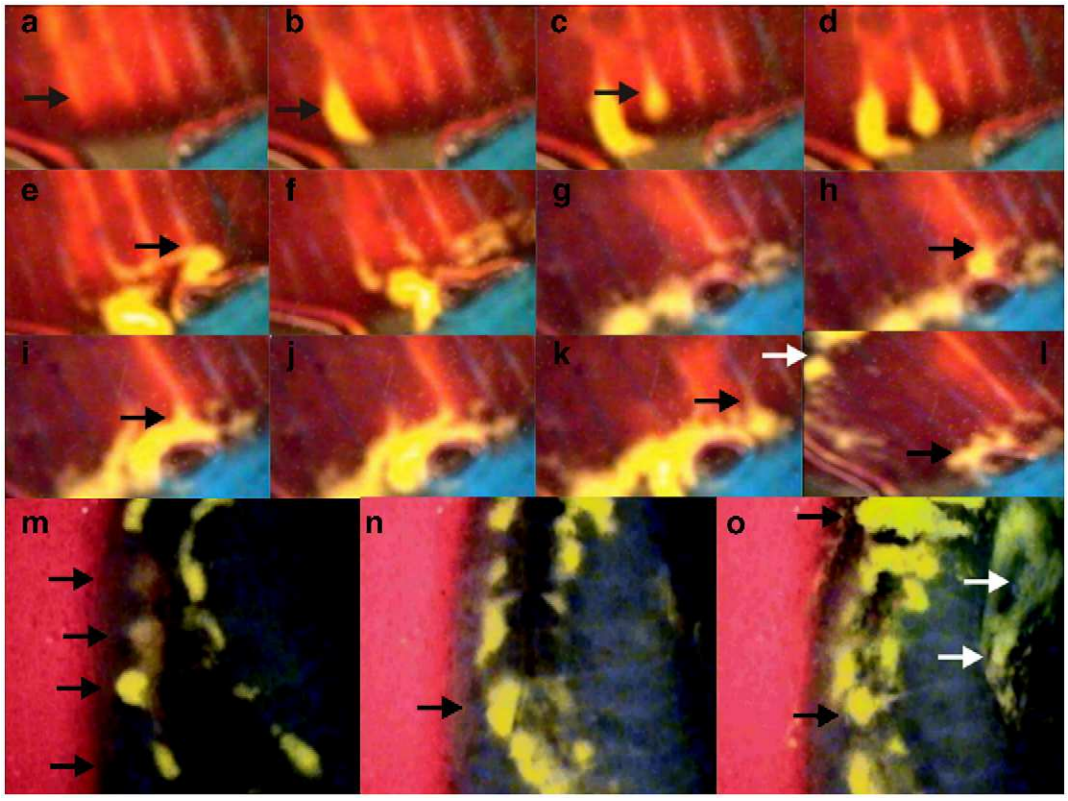

Fig. 6. Kinetics of mucus secretion in the segments in earthworms. The earthworm was stimulated with an electric current. A video sequence of the resultant mucus secretion was analyzed. (a) Earthworm before stimuli (0 ms). (b–l) Sequential time frames obtained each 33 ms. (m, n, o) Frames from a different earthworm under the same conditions. The frames (m) and (n) show lateral side views of the earthworm before (m) and after (n) stimulation Arrows indicate the points of secretion.

strands by shearing stress (Fig. 5b). In addition, the mucus was very adhesive and remained bound to the cuticles even after being processed for scanning microscopy (Fig. 5c).

The electrical stimulation of worms in saline solution resulted in secretion of large quantities of fluid containing ovoid vesicles of varying sizes (35.7±3.9 μm major axis, 29.5± 2.9 μm). These vesicles were sedimented by centrifugation (5 min at 750 ×*g*). The vesicles were unstable and sometimes swelled and ruptured. The vesicles were converted to a densely sticky or granular mass upon treatment with Triton X-100 or lipid solvents (ethanol, acetone, and ether mixed 1:5 with vesicle suspensions). Intact vesicles exposed to water or glutaraldehyde (2% in cacodylate buffer) suffered swelling and loss of the ovoid shape. These vesicles were observed via transmission microscopy to become an amorphous mass containing small granules. The supernatant fluid that had been separated from the secreted mucus after centrifugation was a faint yellow color with none of the other obvious physical properties of mucus.

3.4. Kinetics of mucus secretion

Composite video images (Fig. 6) have been constructed to illustrate the general sequence of events that occur during mucus secretion. Generally, the worm exhibited yellow pigment in the coelomic fluid. Upon electrical stimulation, the worm moved backwards, and all of the segments contracted. Subsequently, the worm resumed forward movement, and the mucus was secreted at the first segment (Fig. 6b) as a yellow fluid. The mucus then appeared at the lateral side, and when the worm continued moving, the secretion appeared in the next segments (Fig. 6c, d, e, f, g and h). Concomitantly, the mucus secretion increased in the first segment. As the posterior segments contracted, the mucus secretion was increased in the proximal segments (Fig. 6i, j, k) Thus, these video images indicated that the earthworms secreted mucus along the body wall by the lateral sides(Fig. 6l, m, n, and o white arrows) and most abundantly from the proximal side near the prostomious.

4. Discussion

The present study reports several findings that, although relatively simple in nature, are of profound importance for understanding the integrative properties of the mucus secretion. The experiments were conducted on the earthworm *E. foetida* as these organisms secrete yellow mucus under basal conditions (Stepheson, 1930; Needham, 1966; Jiang et al., 1989) and exhibit autofluorescence in the entire body especially in the body wall, setae, and digestive tube (Fig. 1). In general, the pigment of the body wall of earthworms is usually red, brown, or purple and occasionally olive or green. The setae and intestinal tube have been described as a faint yellow color (Stepheson, 1930). Wampler and Jamieson (1980), on the other hand, described bioluminescence in twelve species of

earthworms. A broad spectrum of emissions was detected with a maxima ranging from to 500 nm to 570 mn. This bioluminescence was associated with chloragocytes. Recently, Albani et al. (2003) reported autofluorescence in the coelomic fluid in *E. foetida* and suggested that the fluorescent compound 4-methylumbelliferyl β-d-glucuronide may be responsible for the fluorescence. The pigmentation of earthworms has been conferred to compounds such as flavins (Needham, 1966), carotenoids, flavones, coumarins (Roots and Johnston, 1966), and erythrocruorin (Cardillo et al., 1997). These compounds are conjugated to lipids and proteins and show fluorescence upon excitation with ultraviolet light. A study to isolate the fluorescent compound has not yet been conducted, and only a partial characterization of the one fluorescent compound has been published (Jiang et al., 1989). The confocal microscopy studies revealed that the fluorescence was localized to small fluorescent granules in contact with the vesicles. This result is consistent with a previously described specific packing mechanism in which fluorescent pigment joins to other macromolecules such as proteins or lipids (Roots and Johnston, 1966). Following centrifugation, the secreted mucus supernatant was free of visible particles suggesting that the fluorescent pigment is polar. Needham (1966) and Jiang et al. (1989) have also isolated water-soluble yellow pigment from earthworms.

Our results demonstrate that the yellow secretion of earthworms was autofluorescent when illuminated with the appropriate light. The color of the mucus is related to the metabolic process (Stepheson, 1930; Albani, 2003). Richards (1977) identified yellow cells in the epidermis. Furthermore, he described the possible interaction of these yellow cells with mucus cells (Richards, 1975). Other researchers have found a close relationship between the intestinal tissue (chloragogenous tissue), the coelomic fluid, and the yellow pigment in earthworms (Needham, 1966; Roots and Johnston, 1966; Erno and Molnár, 1992; Valembois et al., 1992).

The phenomenon of this secretion in earthworms is quite common in invertebrates and occurs in slugs, sea stars, anemone, and squid. In fact, most of the 90% of animals without backbones are bursting with mucus secretions which serve a far broader spectrum of functions than in vertebrates. Examples include navigation, defense, desiccation resistance, structural support, feeding, and locomotion (Denny, 1989). In earthworms, mucus originates from the tissues in response to noxious stimuli. The mucus secretion is accompanied by extrusion of intact vesicles that contain mucopolysaccharides and glycoproteins (Jiang et al., 1990). The 1-μm granules are adherent and can be transformed to strands. Shearing stress participates in the characteristic organization of the mucus strands. Such forces may be developed by contact with surfaces.

Chemical or electrical stimulation provoked the expulsion of mucus from the coelomic fluid (Jiang et al., 1990). Following stimulation, the earthworms responded with a fast impulse similar to the escape response described by Roberts (1962). The earthworm secreted mucus from the prostomious to elude the stimulus (IV segment, Fig. 6). This response has been described many times previously (Wampler et al., 1980; Jiang et al., 1990). The mucus was then secreted from the adjacent segments. In our system, the earthworm was unable to move freely, and this confinement caused the mucus to remain crowded together. The adhesive properties of this mucus facilitated the adhesion to the cuticle. On the contrary, worms freely swimming in saline solution secreted mucus and resulted in isolated vesicles. The muscular contraction of subsequent segments provoked the secretion of mucus from the lateral sides along the body. Therefore, this process involved the release of intact vesicles, deformation, adhesion, and conversion to strands. Similar production of vesicles and their disruption were observed in slugs of the genera *Arion* and *Limax* and in the snail *Helix aspersa* (Dayrup-Olsen et al., 1983; Prince et al., 1998). The mucus was secreted promptly upon exposure to high levels of heavy metals and resulted in mucus strand formation (Bouché et al., 2000). The expulsion of mucus near the prostomious could signal a defense mechanism similar to the expulsion of ink in octopus and squid (Thompson and Kier, 2001). As a defense mechanism, the mucus adhesive properties may serve to delay the predator and facilitate the earthworm locomotion (Denny, 1989).

In conclusion, the earthworm *E. foetida* exhibited autofluorescence in the intestine, setae, and coelomic fluid. The secreted mucus also yielded autofluorescence. This mucus was extruded from the earthworms in intact vesicles containing small granules. Exposure to shear stress converted the vesicles to strands. This mechanism may be used as a secretion model to offer an exceptionally favorable system for the study of mucus secretions due to the copious levels of mucus, the precise control of these secretions, and their release onto the exposed body surface and not within body cavities.

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