Cyclic electrical activity in the intestine of marine nematode *Enoplus brevis*

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Summary. In the nematode *Caenorhabditis elegans* the defecation motor program (DMP) is initiated by calcium concentration oscillations between intestinal cells without participation of the nervous system. In the related nematode *Heterorhabditis megidis* it was shown that DMP is accompanied by electrical membrane potential oscillations in the intestinal cells. Calcium oscillations and membrane potential in these species are cell-autonomous, and they are synchronised between cells through gap junctions. In the midgut cells of distant from Rhabditida nematode *Enoplus brevis* (Enoplida) we discovered membrane potential cycling which resemble oscillations in *H. megidis*. We also found that the gut cells in *E. brevis* are electrically connected through gap junctions. This finding indicates that distant nematode species possess the same non-nervous rhythmical generation DMP mechanism. It appears that this mechanism was present in the nematode last common ancestor.

Key words: gap junctions, intercellular communication, membrane potential, central pattern generator, nematode defecation.

Nematode studies, especially on the model species *Caenorhabditis elegans*, have seeded numerous fundamental discoveries of general biological significance (www.wormbook.org). In particular, in *C. elegans* it was shown that intestinal motor function is controlled by a unique central pattern generator (CPG) (Dal Santo *et al.*, 1999). CPG are usually considered to be neural networks that generate periodic motor commands for rhythmic muscular contractions in the absence of external rhythmic inputs (Arshavsky *et al.*, 1996; Kuo, 2002). However, in the rhythmic defaecation of *C. elegans* the midgut itself plays the CPG role. It does not contain any neurons and is represented only by endodermal cells (Sulston *et al.*, 1983). Continuously eating, a *C. elegans* hermaphrodite defaecates about once per minute. Its defaecation motor program (DMP) is controlled by calcium concentration oscillations in the cytoplasm of intestinal cells (Espelt *et al.*, 2005; Teramoto & Iwasaki, 2006). These oscillations are cell-autonomous, and they are synchronised between neighbouring intestinal cells through gap junctions (Peters *et al.*, 2007; Liu *et al.*, 2013; Kuznetsov *et al.*, 2016). As a result, a regular calcium wave spreads periodically through the intestine and induces proton ejections with other signals from gut cells into pseudocoelome. pH decrease evokes posterior body wall muscle contractions – the first step of DMP. The molecular mechanism of this process in *C. elegans* has been studied in detail (Thomas, 1990; Branicky & Hekimi, 2006).

Intracellular ion (H^+ and Ca^{2+}) concentration changes are involved in DMP operation and could influence electrical properties such as voltage and conductance of the intestinal cell membrane. However, any electrophysiological experiments in *C. elegans* are complex due to small intestinal cell size. Therefore, gut cell electrophysiology of a related nematode species *Heterorhabditis megidis* that belongs to the same order Rhabditida was reported (Kuznetsov *et al.*, 2017). DMP cycles in both species have similar general features while *H. megidis* has bigger gut cells accessible by microelectrode techniques. As a result, electrophysiological experiments on *H. megidis* supplemented molecular biology studies in *C. elegans*. In intestine cells of *H. megidis* CPG activity

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Fig. 1. *Enoplus brevis* general view and gut autofluorescence. A: The fourth-stage juvenile of *E. brevis*. Bright field, scale bar = 1 mm. B: A fragment of the middle body of *E. brevis* under a fluorescence microscope with the filter set optimised for fluorescein: nuclei of intestinal cells are contrasted by the cytoplasm's bright autofluorescence. Scale bar = 100 µm.

was found to manifest itself as an unusual all-or-none hyperpolarisation 'action potential' with inverted to conventional spikes in muscle cells and neurons polarity, with an amplitude of about 60 mV, and the period of about 4 min . In the intact animal defaecation acts coincide with these hyperpolarisation 'action potentials'.

The unique 'non-nervous' CPG governing rhythmic defaecation was so far found only within the order Rhabditida (*C. elegans* and *H. megidis*).

It is not clear if this mechanism is a specific feature of Rhabditids characterised by their rapid evolution (Blaxter *et al.*, 1998), or it is a common feature of nematodes in general. Enoplida are probably the most evolutionary distant nematodes from Rhabditida. Therefore, we studied the electrophysiological properties of the intestine cells in *E. brevis* with a special focus on the membrane potential oscillations and electrical connections between the intestinal cells.

MATERIAL AND METHODS

The experiments were held at the White Sea biological station of the Russian Academy of Sciences "Kartesh" in the summer of 2017. *Enoplus brevis* Bastian, 1865 is a free-living littoral and sub-littoral marine nematode with the body length of an adult animal about 6-8 mm and the maximum diameter 0.13-0.17 mm (Fig. 1A). *E. brevis* were extracted from sandy littoral and kept in seawater at 4°C. The experiments were carried out at 15-17°C. Only larger worms (adult males, females, or forth-stage juveniles) were taken for experiments.

The intestine of *E. brevis* consists of approximately 6000 cells, each with a diameter about 20 µm (Fig. 1B). The intestine was cut out under the dissection microscope with micro scissors and tungsten needles then placed in a 50 mm Petri dish with the bottom covered by 1% agarose gel prepared on seawater and covered with filtered seawater. Membrane potential recordings in the intestinal cells were performed by means of glass microelectrodes filled with 2.5-3M KCl (tip resistances 20-60 MOm) and were amplified *via* a conventional electrophysiological apparatus, digitized in a PC computer using the LCard E-154 digitizer by LGraph2 (www.lcard.ru) program. The plots were constructed by Microsoft Excel and SimInTech (simintech.ru) programs.

RESULTS AND DISCUSSION

Accurate electrical coupling coefficient measurements require simultaneous impaling of each cell by two intracellular microelectrodes. Due to the small intestinal cell size of *E. brevis* only one electrode per cell could be inserted which allowed us to obtain only qualitative but not quantitative data about cell-to-cell coupling. Two cells, either adjacent or lying relatively close to each other, were simultaneously penetrated by microelectrodes (Fig. 2A). One microelectrode was used to inject positive or negative electrical current into the cell, and the second microelectrode was used to identify the presence of electrical coupling by corresponding membrane potential changes. The recording demonstrating the presence of electrical coupling between the intestinal cells as shown in Fig. 2B: negative 20 nA current pulse I_2 was injected into the cell, at the same time the second electrode inserted in a nearby cell recorded the deviation of its membrane potential of around –5 mV. In control experiments, where one of the electrodes was inserted into the intestinal cell and the second remained in the extracellular milieu, no electrical coupling was observed. Thus, intestinal cells are electrically connected *via* gap junctions.

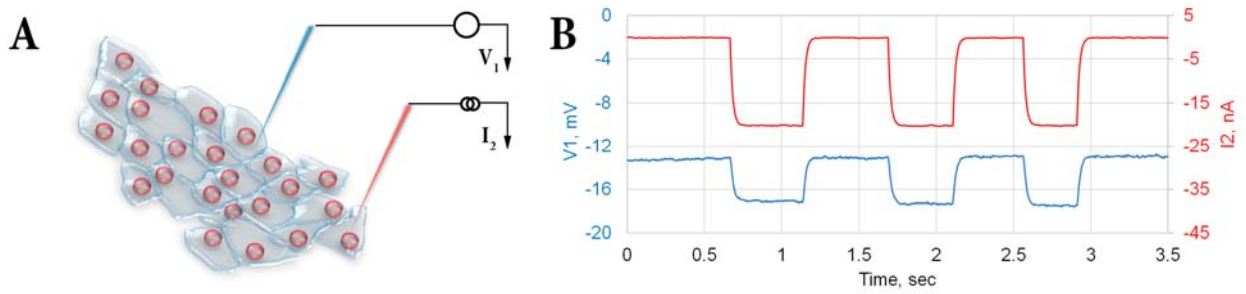

Fig. 2. Electrical coupling between intestinal cells. A: Scheme of the experiment: an area of the intestine with two electrodes. Membrane potential measurement electrode V_1 (blue) is impaled into one cell, and the electrode for intracellular injection of the electrical current I_2 (red) is inserted into another cell. B: –20 nA current pulse injected into one intestinal cell spreads through a series of intestinal cells *via* gap junctions and induces a –5 mV membrane potential shift in a remote cell. Horizontal axis – time, sec; vertical axes – voltage, mV (blue) and current, nA (red).

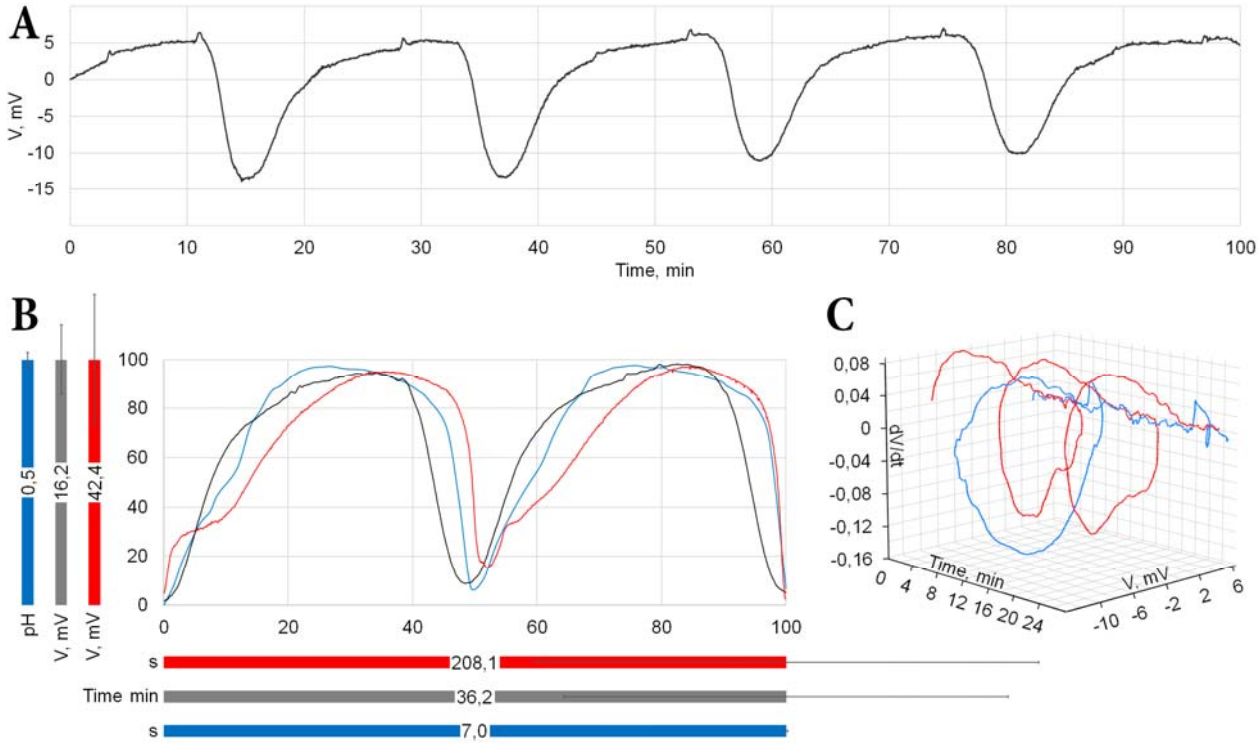

Fig. 3. Electrophysiology of nematode gut oscillations. A: An example of membrane potential oscillations in the intestine cell of *Enoplus brevis*. Horizontal axis – time, sec; vertical axis – membrane potential, mV. B: Membrane potential of the intestinal cells in the *E. brevis* (black) and *Heterorhabditis megidis* (red). Rhythmical changes of the membrane potential and period normalisation to 100%: horizontal axis – time percentage; vertical axis – voltage percentage. The average mean trajectory for *H. megidis* eight cycles were taken from four different experiments and for *E. brevis* eight cycles were taken from two different preparations. In *C. elegans*, (blue) the membrane potential of the intestinal cells has not been studied, therefore for comparison the cyclic changes of pH in the intestinal intracellular space were used. Data for this species was received from Pfeiffer *et al.* (2008) and also normalised to 100%: horizontal axis – time percentage; vertical axis – pH percentage. For *C. elegans* all possible cycles acquired by GetData Graph Digitizer (getdata-graph-digitizer.com) from Pfeiffer *et al.* (2008) Fig. 1B were used to average the mean trajectory. The pattern of cyclic changes for all species is similar. Actual time, voltage and pH mean ± SD values are presented via bars with trajectory color matching. C: 3D plot obtained by SimInTech program in coordinates t (time), V (voltage), dV/dt (membrane potential rate of change). The red and blue lines depict fragments of recordings from two different *E. brevis* specimens.

We performed a total of 15 experiments in the isolated gut of *E. brevis* and succeeded to receive 30 h of the membrane potential recordings in two different preparations with the regular membrane potential oscillations in intestinal cells (Fig. 3). The oscillation amplitude could vary in a range of 15-25 mV, while the mean period was about 20 min. The variations in the pattern of *E. brevis* intestinal oscillations are depicted in Fig. 3C. Membrane potential (V) and dV/dt changes are quite similar in two preparations, while the period differs about two-fold. The potential starts at the low positive level and transits to a rapid all-or-none hyper-polarisation 'action potential', which later slowly tends back to previous values (Fig. 3A). As a result, midgut (endoderm) cells of *E. brevis* are able to generate stable oscillations of the membrane potential without any other cell type involvement, including nervous cells. Such oscillations are very similar to those observed in the intestine cells of *H. megidis* (Kuznetsov *et al.*, 2017), but differ from them by a longer period and smaller amplitude (Fig. 3B). Normalised membrane potential oscillations in intestinal cells (two cycles) for both species *E. brevis* (black) and *H. megidis* (red) have the same pattern. In both species, the oscillation cycle can be decomposed into a relatively long positive 'plateau' and a shorter hyperpolarisation 'pit'. In both species, the intestinal cells are electrically connected and could be synchronised through gap junctions. The similarity of cyclic membrane potential changes in intestinal cells of *E. brevis* and *H. megidis* makes it plausible that the defaecation motor program in both species is based on the same mechanism.

For *H. megidis* whole animal preparations it was shown that the autonomous membrane potential cycles in intestinal cells are associated with the defaecation, and the act of defaecation corresponds to the negative potential peaks (Kuznetsov *et al.*, 2017). In another rhabditean species, namely *C. elegans*, DMP is also controlled by the autonomous central pattern generator (CPG) located in the intestine (Dal Santo *et al.*, 1999; Nehrke *et al.*, 2008; Pfeiffer *et al.*, 2008). For *C. elegans* it was shown that CPG activity is associated with Ca^{2+} and H^{+} concentration oscillations in the cytoplasm, and is probably also accompanied by cell membrane electrical potential cycling (Espelt *et al.*, 2005; Pfeiffer *et al.*, 2008). Fig. 3B demonstrates that intestine cell intracellular pH in *C. elegans* (blue) shows similar pattern of oscillations to membrane potential oscillations in *E. brevis* (black) and *H. megidis* (red).

The CPG in individual intestinal cells of *C. elegans* are synchronised through gap junctions junctions (Peters *et al.*, 2007; Kuznetsov *et al.*, 2016). Therefore, the intestine of all three nematode species under consideration contains a CPG, and what is more it consists of cells that could be synchronised through gap junctions between them.

It is important to note, that *E. brevis* intestine differs significantly in size (*E. brevis* is much bigger than *C. elegans* and *H. megidis*) and cell number (6000 cells in *E. brevis*, 20 cells in *C. elegans*, and about 24-28 cells in *H. megidis*) from two other considered species. At the same time, the general features of intestinal DMP oscillations are similar. In three studied species the generation frequency is the highest in *C. elegans* and lower in *H. megidis* and *E. brevis*. It is possible that the DMP frequency is adapted to the mechanical properties of the animal. Bigger-size animals may tend to move slower than smaller ones. In mollusk *Clione limacina* (Panchin, 1990; Arshavsky *et al.*, 1993) it was shown that CPG frequencies and electrical properties of the pacemaker neurons change (frequency getting lover and spikes become longer) with animal growth. It is quite possible that similar adaptation is also true to the nematode DMP and this assumption could be tested in future studies on other nematode species and in specimens of different stages in the same species.

The electrical activity discovered in a nematode intestine is an unusual example of excitability in tissue that is not usually considered to be excitable. For *H. megidis*, it was earlier shown that in the nematode intestine cell membrane potential cyclic changes are functionally analogous to the action potentials (spikes) observed during excitation of the nerve and muscle cells, but with reversed polarity (Kuznetsov *et al.*, 2017). If the action potential of nerve and muscle cells is associated with rapid growth of the membrane potential with subsequent return to a baseline level, the initiation of the DMP in the intestinal cells of *H. megidis* is associated with the peak of the membrane hyperpolarisation. The artificial membrane depolarisation of the nerve and muscle cells, exceeding a certain threshold, causes an action potential, and in the same way, but with the opposite sign, artificial membrane hyperpolarisation of the nematode intestinal cells causes the hyperpolarising peak generation in the defaecation program. Although the endoderm is considered to be non-excitable tissue, the self-supporting or regenerative electrical wave in nematode gut is similar to the action potential in excitable cells. The described phenomena calls for a reevaluation of the definition of excitable cells.

We suggest that distant nematode species possess the same non-nervous rhythmical DMP generation mechanism and that this mechanism was present in the nematode common ancestor.

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Г.А. Сливко-Кольчик, В.П. Кузнецов, Д.А. Воронов и Ю.В. Панчин. Циклическая электрическая активность в кишечнике морской нематоды *Enoplus brevis*.

Резюме. В нематоде *Caenorhabditis elegans* моторная программа дефекации (МПД) запускается изменением концентрации кальция в клетках кишки без участия нервной системы. Было показано, что в родственной нематоде *Heterorhabditis megidis* МПД сопровождается осцилляциями мембранного потенциала клеток кишки. Изменение концентрации кальция и мембранного потенциала в этих видах нематод автономны и синхронизированы между клетками кишки при помощи щелевых контактов. В клетках средней кишки нематоды *Enoplius brevis*, удаленной от Rhabtitida, мы обнаружили ритмы мембранного потенциала, похожие на колебания мембранного потенциала из *Heterorhabditis megidis*. Также мы показали, что клетки кишки *Enoplius brevis* электрически связаны при помощи щелевых контактов. Это исследование показывает, что далекие виды нематод обладают схожим механизмом генерации МПД без участия нервной системы. Мы предполагаем, что такой механизм присутствовал у последнего общего предка нематод.