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Recreating Intestinal Peristalsis in the Petri Dish

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Abstract

Here we describe a culture technique of cells dissociated from the external muscularis of the guinea pig small intestine, which allows us to maintain all the elements involved in the intestinal peristaltic reflex. After a few days in culture, these cells reorganize to form a small group of cells that permit the generation of pacemaker activity, spontaneous contractions, and the development of inhibitory and excitatory junction potentials in the petri dish, all elements involved in the peristaltic reflex. Therefore, these co-cultures are suitable to study the cellular and molecular aspects related to the development, maintenance, and modulation of motor intestinal functions.

Keywords

Smooth muscle
Neuromuscular junctions
Gastrointestinal tract
Small intestine
Enteric nervous system
Myenteric neurons
Slow waves
~~in~~Inhibitory junction potentials
Excitatory junction potentials

1. Introduction

The innate capability to generate spontaneous action potentials is evident in various tissues, including the sinoatrial node, the atrioventricular node, and the intestinal *external muscularis*. A common characteristic of these tissues is the presence of specialized transmembrane pores, the gap junctions, formed by the face-to-face association of two hemichannels (connexons), each one anchored in the membrane of cells found in juxtaposition to each other [1, 2, 3]. Gap junctions extend across the extracellular space, they are exchange routes for ions and other small molecules, and in excitable cells they provide a low resistance pathway for electrical coupling of tissues [1, 2, 3].

The peristaltic reflex was first described in guinea pig by Trendelenburg (1917; revised by [4]). This reflex consists in a stereotypic motor behavior, induced by gut distension activated by lumen filling, and it is supported by the coordinated contraction and relaxation of both layers of the external muscularis, longitudinal and circular.

Thus, this reflex consists in the relaxation of the circular muscle and contraction of the longitudinal layer of the distended portion and the contiguous anal segment. Whereas the circular muscle oral segment adjacent to the distended area contracts, the longitudinal muscle receives little inhibitory innervation, and therefore relaxation does not occur in the layer of this segment. Intrinsic intestinal neurons, located in the enteric nervous system, are essential for peristaltic reflex. Thus, distension activates intrinsic sensory neurons that modulate inhibitory (descending) and excitatory (ascending) motor neurons innervating the circular muscle. Conversely, the longitudinal muscle is modulated by excitatory (descending) and few inhibitory (ascending) motor neurons [5, 6].

In the gastrointestinal tract, two types of potentials can be associated with contraction of the *external muscularis*, slow wave-type and spike-like action potentials [7, 8]. In some tissues, the slow waves do not reach the threshold for contraction generation under basal conditions, but they do when excitatory nerves are activated. Slow wave-type action potentials are the pacemaker activity of the digestive tract and determine, together with intrinsic and extrinsic nerves, the involuntary gut contractions which force the intestinal content onward. Peristaltic reflex remains in resected intestinal segments, indicating that the reflex circuitry is not impaired [5, 6, 9].

In the present chapter, we describe a culture technique that allows the maintenance of all the elements involved in the peristaltic reflex in co-cultures of the external muscularis. This technique can be used to study the cellular and molecular basis that permits the reorganization of the tissue responsible for the generation of pacemaker activity, spontaneous contractions, and the development of inhibitory and excitatory junction potentials in the petri dish.

2. Materials

2.1. Reagents

1. Guinea pig serum obtained from our laboratory.

2. Sterile rat tail collagen (from our laboratory).

2.2. Culture Media and Other Solutions

1. Modified Krebs solution has the following components (in mM): 126 NaCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 5 KCl, 25 $NaHCO_3$, 11 glucose; the solution is continuously gassed with 95% O_2 and 5% CO_2.

2. Growth medium was 94.5% minimum essential medium, supplemented with guinea pig serum (2.5% v/v), L-glutamine (2 mM; from a 200 mM stock), penicillin (10 U/mL; from a 1000 U/mL stock), streptomycin (10 μg/mL; from a 1 mg/mL stock), and glucose (15 mM; from a 1.5 M stock).

3. Modified L-15 medium. To prepare 100 mL of this medium, we add 87 mL Leibovitz's L-15 medium, 10 mL fetal calf serum, and 1 mL of each stock solution described before (*see* **item 2**). Thus, the final concentrations were 2 mM L-glutamine, 15 mM glucose, 10 U/mL penicillin, and 10 μg/mL streptomycin.

4. Collagenase-Hanks' balanced salt solution (calcium/magnesium-free), which is supplemented with collagenase (1 mg/mL) and dispase (4 mg/mL).

5. Papain-Hanks' balanced salt solution (calcium/magnesium-free), which is supplemented with papain (9 U/mL) activated with L-cysteine (0.4 mg/mL).

3. Methods

3.1. Dissecting the *Muscularis Externa* Preparation

Young guinea pigs (150–300 g; *see* **Note 1**), either male or female, are stunned and immediately exsanguinated by severing major neck blood vessels. Remove a segment of proximal jejunum (about 6 cm in length), wash out its contents, and then place in modified Krebs solution (see below) in a Pyrex petri dish with a bottom side covered with Sylgard® 184 (Dow Corning). Pin down the tissue to open it longitudinally using iris scissors. Next carefully pin down all the borders to keep the preparation flat and slightly distended, which is done with the help of pins to avoid tearing the tissue. Remove the mucosa and dissect the submucosal layer from the underlying layers of smooth muscle (*muscularis externa* preparation; MEP) using straight tweezers and dissecting microscope. Krebs solution in the petri dish is continuously gassed with CO_2 (5%) and O_2 (95%) and exchanged every 3 min.

3.2. Dissociation of the *Muscularis Externa* Cells

1. Incubate the MEP for 10 min at 37 °C in solution #5. Remove this solution and wash the MEP with solution #3.

2. Incubate the preparation for another 10 min in solution #4.

3. Gently triturate the MEP (*see* **Note 2**) with the long Pasteur pipette until the preparation is completely dispersed (usually 15–20 times) and single cells are observed under the microscope.

4. Centrifuge this solution (100 × *g*), remove and discard the supernatant, and resuspend the cell pellet in 5 mL of modified L-15 and centrifuge again. Discard the supernatant and disperse the cell pellet in growth medium. Plate the cells into culture wells of a 24-well plate containing 12 mm Ø round coverslips at the bottom. The coverslips were previously coated with collagen (*see* **Note 3**).

3.3. Validating Functional Properties and Interactions of *Muscularis Externa* Cells

1. Place the coverslips containing the cultured cells in a recording chamber (capacity about 1 mL) which is continuously superfused (2–3 mL/min) with modified Krebs solution maintained at 35–36 °C and gassed with 95% O_2 and 5% CO_2. An inverted microscope (×400) is used to visualize the cultures.

2. Intracellular recordings are performed using glass microelectrodes filled with 2 M KCl (resistance 40–80 MΩ; *see* **Note 4**). Membrane potential is measured with an Axoclamp-2A preamplifier (Axon Instruments Inc., Foster City, CA). The output of this preamplifier is displayed on an oscilloscope (Beckman 9020, Tucker Electronics Co., Garland, TX) and recorded on a Macintosh computer with Chart v 3.3.6 software (MacLab) and a sampling frequency of 1 kHz.

3. Apply drugs by changing the superfusion solution to one that differs only in its content of the drug.

4. Microscopic observation of dissociated cells indicates that most cells attach to the coverslips 2–4 h post plating. During the first several days, cultured cells are mainly singly distributed. However, by 5 days post plating, they reorganize and most cells are to be found as part of cellular clumps, with diameters between 50 and 150 μm. Single cells remain present between the cellular clumps (Fig. 1).

5. Most cellular clumps appear connected to each other by nerve-like strings. Clumps contain smooth muscle cells, interstitial cells of Cajal (ICC)-like cells (Fig. 2), and neuronal cells (Fig. 1). We confirmed

the presence of ICC by C-kit immunostaining. Clumps show spontaneous contractions (Fig. 2b) that are associated to slow waves.

6. Intracellular recordings show the presence of slow-wave activity (Fig. 2) and spike-like potentials [10]. We can also determine the membrane potential, membrane resistance, and the amplitude, frequency, and duration of slow-wave and spiking activity.

7. In control experiments, inhibitory junction potentials (IJPs) or excitatory junction potentials (EJPs) ~~are~~were not affected by atropine (1 μM), hexamethonium (100 μM), or nifedipine (1 μM; Data not shown). Therefore, experiments can be performed in the presence of these three substances to prevent cholinergic effects and to decrease muscle contractions (*see* **Note ~~4~~5**).
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8. Electrical field stimulation of the nerve-like structures, using a bipolar electrode, induces IJPs in 60% of the recorded cells. The amplitude and IJP duration can also be measured. Single electrical pulses with a typical duration of 0.1 ms (always ≤0.2 ms) ensure that we are stimulating only neurons. In 30% of the cells, two to five pulses (at a frequency of 20–30 Hertz) are required to obtain a robust and reproducible IJP (Fig. 3). In about 30% of the recorded cells, EJPs could be observed (Fig. 4) following the IJP.

9. To further prove that IJPs are nerve mediated, one can use 1 μM tetrodotoxin (Fig. 5, a sodium channel blocker [11]) or 100 nM ω-conotoxin GVIA (Fig. 6, a N-type calcium channel blocker [12]). Both toxins prevented IJPs (Figs. 5 and 6) and EJPs.

Fig. 1

Microphotograph of intestinal co-cultures after 10 days of plating showing two cell clumps (as indicated by arrows). Some myenteric neurons are seen in both clumps, stained for the presence of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), as previously described [17]

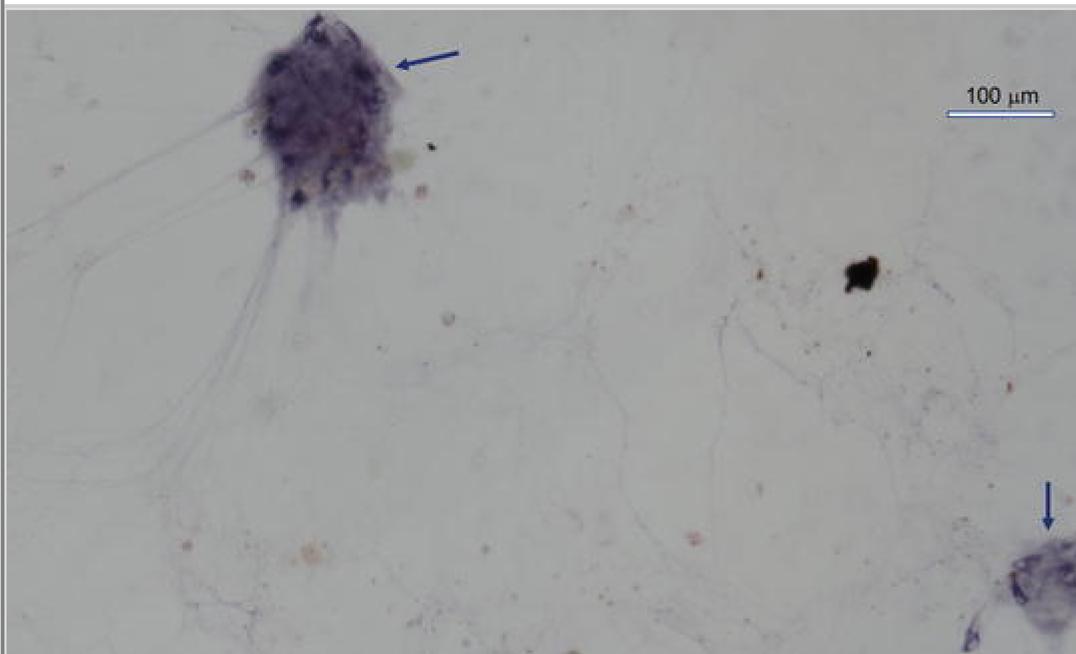


Fig. 2

Cells with a typical morphology of interstitial cells of Cajal (ICC) (indicated by the arrow head) are seen during the reorganization of cultured cells (5 days post plating). These cells have a triangular body and relatively thick processes. Cells with such characteristics are C-kit positive as expected for ICC [9]. A neuron-like cell is indicated by the arrows. These small clusters of cells contract spontaneously. Microphotographs were taken before (**a**), during (**b**), and after (**c**) a spontaneous contraction. Notice that during the contraction, almost all cells appear shortened and out of focus, including the ICC, except for the neuron-like cell. The lower panel shows the spontaneous slow-wave activity recorded in a cell clump after 21 days in culture. Each of these slow waves was associated with a contraction of the cell clump. In a previous study, we showed that application of octanol (a gap junction channel blocker; 1 mM) abolished these slow waves [10]

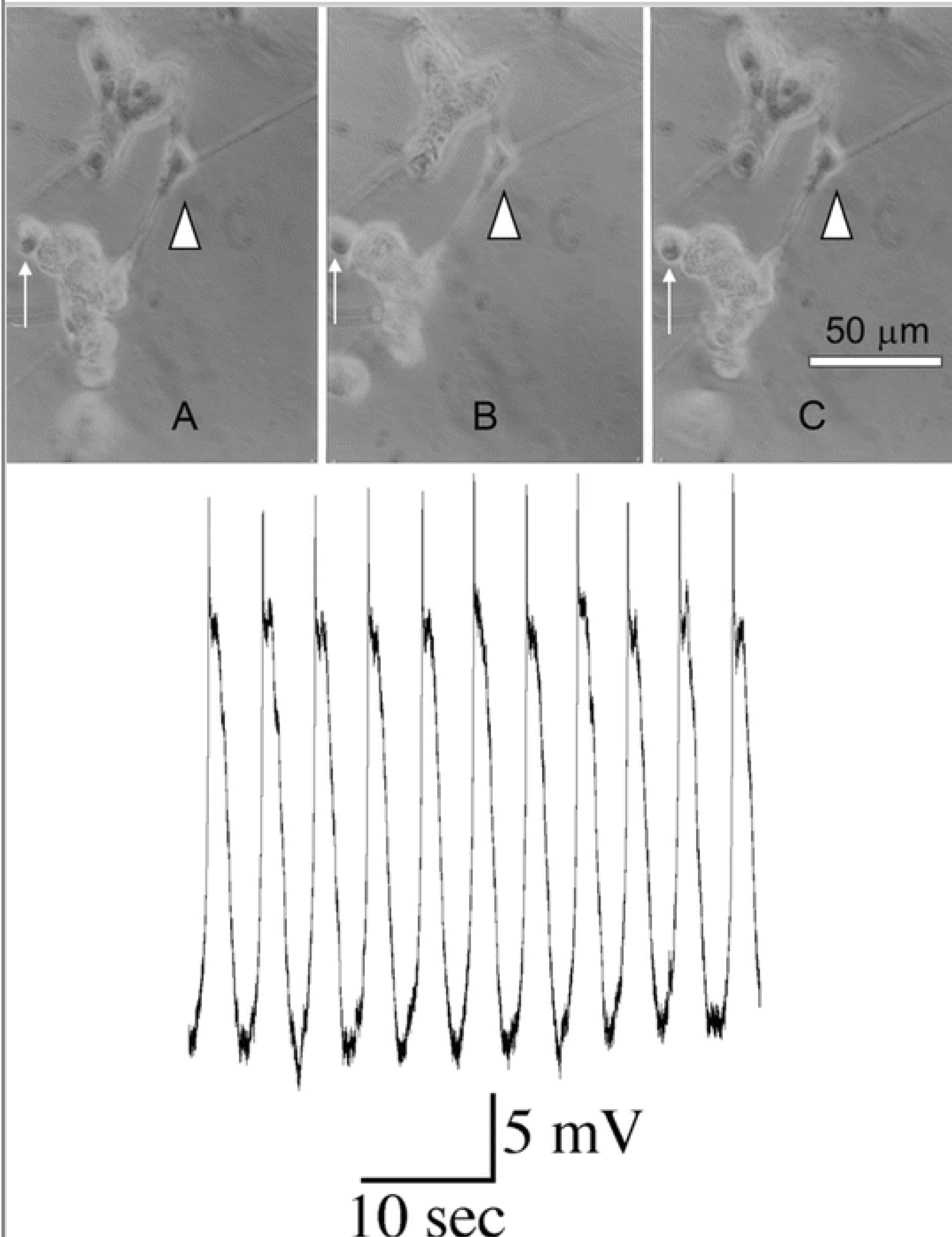


Fig. 3

Most clumps display spontaneous transient hyperpolarizations with an amplitude of a few mV up to 25 mV, which have a duration of less than a second, as shown in the upper trace. Application of a single voltage square pulse of sufficient intensity induces a larger transient hyperpolarization, whereas five pulses with the same intensity increase mainly the duration of the hyperpolarization. These hyperpolarizations resemble those recorded in intestinal smooth muscle strips, in vitro, known as inhibitory junction potentials [6, 18]

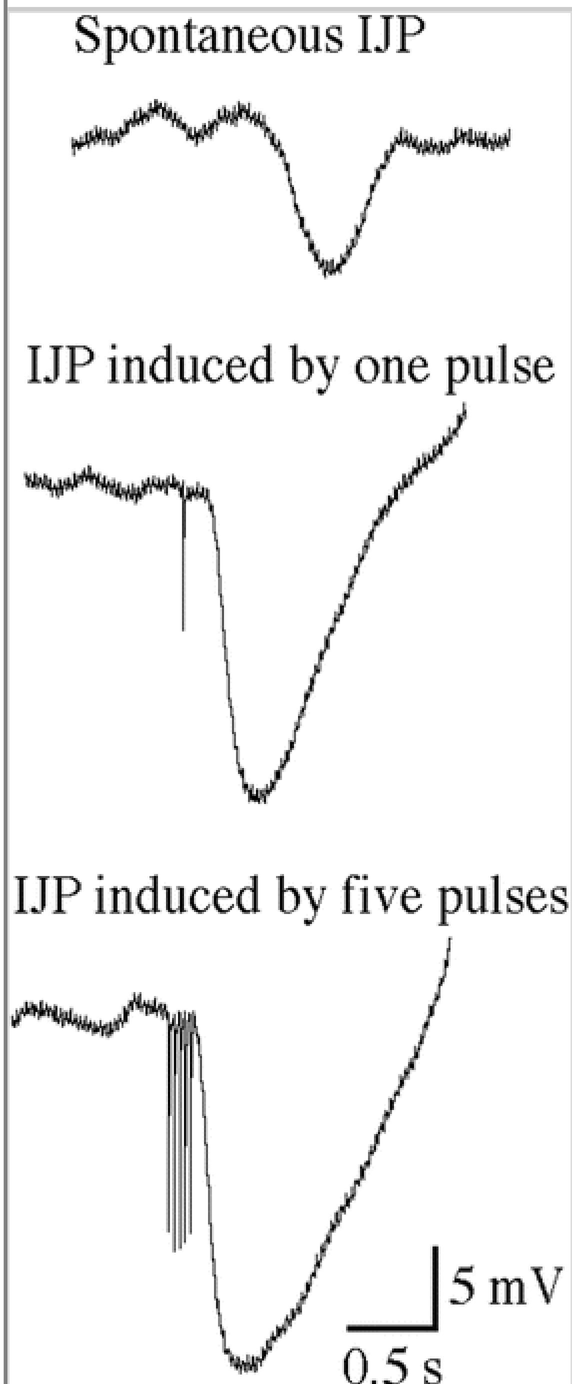


Fig. 4

Inhibitory junction potentials (IJPs) and excitatory junction potentials (EJPs) can be evoked by nerve electrical stimulation in various clamps. Here a single electrical voltage pulse evoked an IJP followed by an EJP as indicated by the dashed line. Spontaneous slow waves were seen in this cell

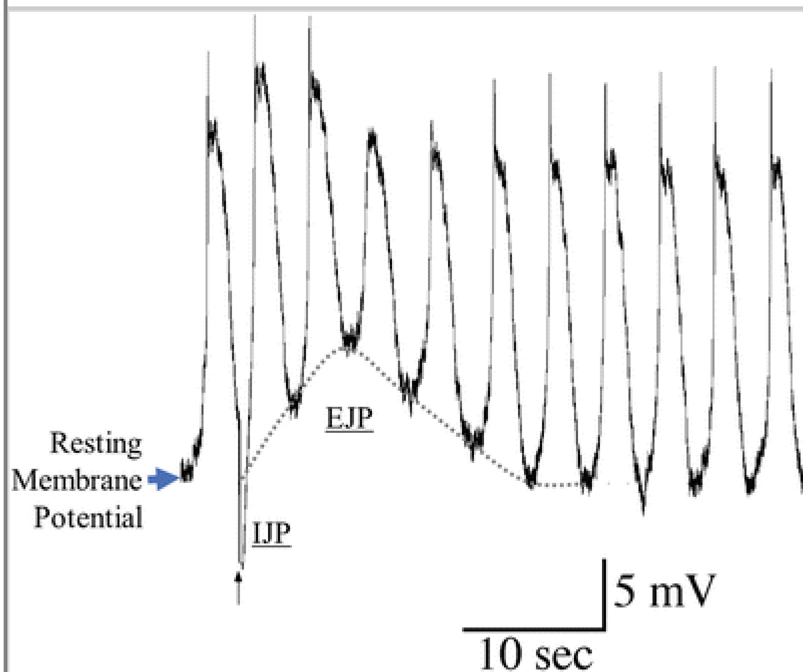


Fig. 5

To demonstrate that inhibitory junction potentials are mediated by nerve stimulation, we investigated the effects of tetrodotoxin (TTX), known to inhibit voltage-activated sodium channels and axon action potentials. In our preparation, TTX completely blocks the IJPs

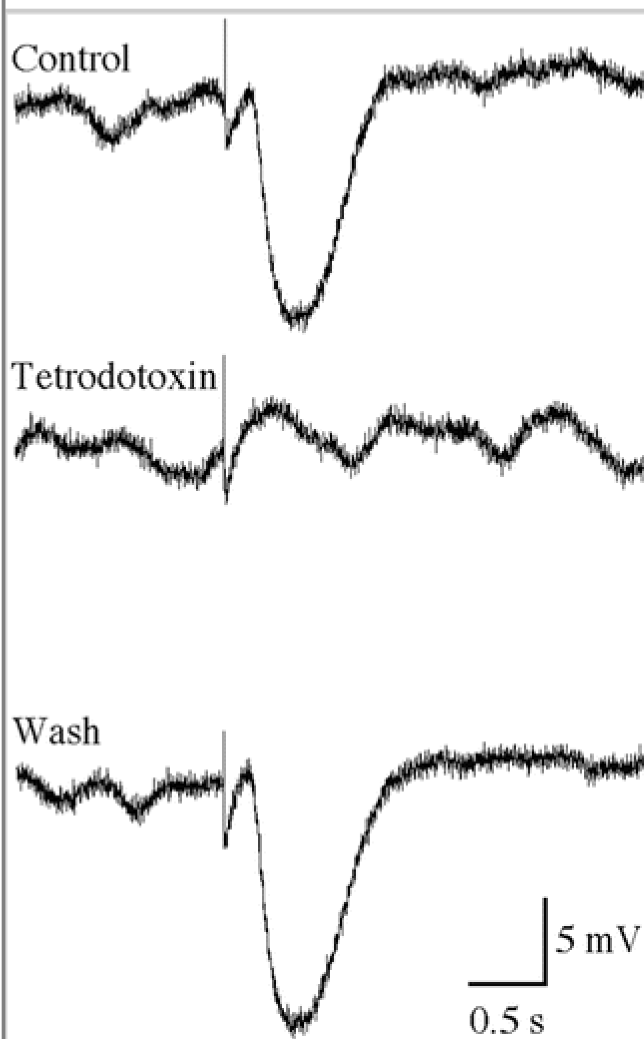
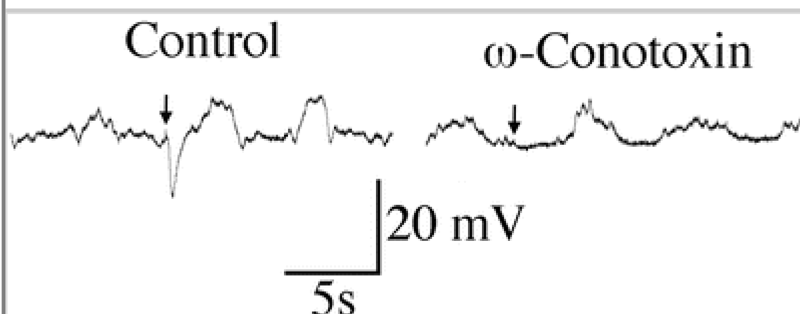


Fig. 6

To demonstrate that inhibitory junction potentials are mediated by vesicular neurotransmitter release, we investigated the effects of ω-conotoxin GVIA, known to inhibit voltage-activated calcium channels at the presynaptic membrane and, subsequently, synaptic transmission. Similar than TTX, ω-conotoxin treatment also completely blocks the IJPs



3.4. Concluding Remarks

Enteric neurons have been co-cultured with visceral smooth muscle cells [13] and with striatal neurons [14]. These latter studies were concerned with the effect of cholinergic neurons on smooth muscle growth and the study of soluble factors from myenteric neurons on neurite outgrowth from postnatal striatal neurons. The group of Mayer reported [15], using co-cultures of sensory neurons (from the dorsal root ganglion) and colonic smooth muscle cells, the presence of gap junctions between nerves and smooth muscle cells. Here we describe co-cultures of *external muscularis* cells, which reorganize into cell clumps after a week in culture. These clumps display spontaneous contractions associated with slow-wave activity which, as in vivo [16], require the presence of gap junctions [10]. Inhibitory and excitatory myenteric neurons in culture reinnervate smooth muscle cells, and their stimulation can induce typical IJPs or EJPs, respectively. Pacemaker activity, smooth muscle contractions, and IJPs and EJPs are crucial for peristalsis in vivo [5, 6], and all these activities are present in our co-cultures. Therefore, this preparation is suitable to study the cellular and molecular aspects related to the development, maintenance, and modulation of pacemaker activity, smooth muscle contraction, IJPs, and EJPs.

4. Notes

1. The major problem encountered with intestinal cultures is infection. In our experience, there is one infection (likely viral) that one is unable to identify; however, these contaminat~~ing~~ed cells do not attach to the glass. Changing the collagen or the culture appears ineffective. We have found that requesting the animal supplier to provide animals from a different room may work. The next day, our cultures were again working.

2. Tissue trituration with the Pasteur pipette should be done avoiding the formation of gas bubbles.

3. The coverslips are coated with rat tail collagen using a Pasteur pipette and exposed to UV light for 10 min before placing them in the wells of the cell culture plates.

4. Glass microelectrodes are made in a Brown and Flaming puller (Sutter Ins. Co., Model P-80/PC).

5. Stock solutions (0.1–10 mM) are made in ethanol (nifedipine) or in water (all other substances). Final experimental concentrations are obtained by dilution of stock solutions with the superfusion solution. Stock solutions were always kept at −4 °C.

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