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HOW WE TEACH | *Generalizable Education Research*

A simple laboratory exercise with rat isolated esophagus and stomach fundus to reveal functional differences between striated and smooth muscle cells

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Batista-Lima FJ, Gadelha KK, Oliveira DM, Vasconcelos TB, Brito TS, Magalhães PJ. A simple laboratory exercise with rat isolated esophagus and stomach fundus to reveal functional differences between striated and smooth muscle cells. *Adv Physiol Educ* 41: 291–297, 2017; doi:10.1152/advan.00150.2016.—This study describes an undergraduate student laboratory activity using isolated preparations from rat gastrointestinal tissues that possess contractile profiles typically exhibited by striated and smooth muscle cells. While students are introduced to an ex vivo methodology, they can compare differences in trace experiments, twitch aspects, phasic and tonic properties, force-frequency relationships, and pharmacological responsiveness of esophageal (striated) and fundic (smooth muscle) segments. Muscle strips were subjected to electrical field stimulation (EFS) applied by platinum electrodes immersed in the physiological solution. The contractile profile of EFS responses varied between these two types of gut preparations. Atropine and tubocurarine revealed differential inhibitory influences in esophagus or fundus tissues; caffeine and procaine produced similar effects, i.e., potentiation and blockade of the EFS-induced contractile response in these tissues, respectively. Experimental results obtained during the activity helped the improvement of student learning about basic concepts previously discussed in theoretical lectures. To measure student learning with this laboratory exercise, a questionnaire was applied before and after the activity, and the number of expected correct answers, concerning the mechanisms of contraction in striated and smooth muscle, could be clearly evidenced.

electrical field stimulation; gastrointestinal tract; acetylcholine receptor

Objectives and Overview

MUSCLE CONTRACTION IS ONE of the most important themes in physiology courses, and understanding the major differences between muscle cells constitutes a challenge for undergraduate students, especially if we consider the mechanisms involved in the excitation-contraction coupling for each muscle type. In general, skeletal muscle is the most common cell type that exemplifies the basic functions of the contractile apparatus of vertebrate voluntary striated muscles (10). In contrast, visceral organs are often referred to as the place where smooth muscle cells are found working under involuntary control (9). Here, we suggest a laboratory activity that reveals the conjunct presence of striated and smooth muscle cells in a visceral organ by means of functional experiments. For this purpose, small segments of esophagus and strips obtained from the fundic region of rat stomach served as experimental models to demonstrate several functional differences between striated and smooth muscle.

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Background

The striated muscle receives this denomination due to the alternate appearance of darker and clearer bands in its contractile apparatus observed under light microscopy. This aspect results mainly from the repetitive arrangement of major contractile filaments (actin and myosin) along the myofibrils. A single motoneuron can be responsible for the innervation of several muscle fibers (Fig. 1*A*). In its intimal contact with the muscle cell, the motoneuron synapses through a limited endplate region possessing the following general elements in its structure: a presynaptic membrane (neuron), a synaptic cleft, and a postsynaptic membrane (muscle). When action potentials reach the distal portion of the axon, voltage-operated calcium channels open, and calcium influx occurs to the cytoplasm, then signaling for the occurrence of acetylcholine (ACh) release from axonal end vesicles through exocytosis (4).

Once released, ACh diffuses into the synaptic cleft of striated myoneural junction and binds to nicotinic receptors of the postsynaptic membrane. This receptor is an anion channel by means of which monovalent cations, such as potassium (K^+) and sodium (Na^+), flow downhill of their electrochemical gradients. Such phenomenon creates an excitatory postsynaptic potential and membrane depolarization, and, whether the magnitude is enough to achieve a threshold voltage, a resulting action potential propagates throughout the sarcolemma to reach T-tubules (4). The terminal cisternae of the sarcoplasmic reticulum, where the calcium (Ca^{2+}) necessary for contraction is actively stored, are located near T-tubule membrane. In response to T-tubule depolarization, a complex receptor possessing a high affinity for dihydropyridines works as a "voltage sensor," and Ca^{2+} release occurs by the presence in sarcoplasmic reticulum membrane of channels identified by their high affinity to the plant alkaloid ryanodine. The opening of these channels (Fig. 1*C*), known as ryanodine receptors, leads to rapid leak of stored Ca^{2+} into the cytosol (7).

Smooth muscle cells do not have the same sarcomere arrangement seen in striated muscle cells. Neurotransmitter release occurs from axon varicosities in autonomic neurons, and there are no cleft and typical motor end plates, as observed in striated muscle (Fig. 1*B*). Released by parasympathetic or

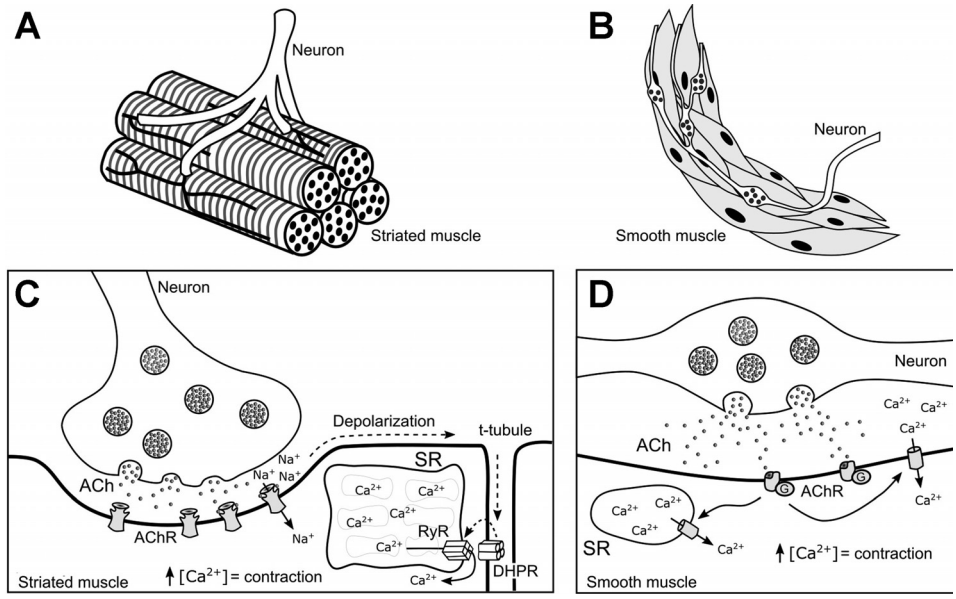

Fig. 1. Schematic structure of striated (*A*) and smooth muscle cells (*B*). *C*: the striated fibers receive innervations that release acetylcholine (ACh) from vesicles in synaptic clefts, and ACh receptors (AChR) are located on postsynaptic membrane. The AChR on striated muscle are ion channels, which initiate membrane depolarization when activated. This depolarization travels the sarcolemma and dives on T-tubules, reaching the dihydropyridine receptor (DHPR), which communicates with Ca^{2+} channels known as ryanodine receptors (RyR) to trigger Ca^{2+} efflux from sarcoplasmic reticulum (SR) to cytosol. *D*: neurons that stimulate smooth muscle cells possess varicosities where the vesicles are assembled. In this context, ACh diffuses to stimulate more than one single cell. The AChR on smooth muscle cells is coupled to G protein, which results in a production of second messengers. These second messengers are responsible for Ca^{2+} efflux from SR to cytosol, as well the Ca^{2+} entrance from extracellular medium to cytosol.

enteric neurons, ACh is one of the major excitatory neurotransmitters in the gastrointestinal tract (6). Once released, ACh might activate muscarinic receptors (mainly M2/M3 subtypes) associated with heterotrimeric G proteins located at postjunctional membranes. Such phenomenon is involved in the activation of phospholipases, formation of the second messengers inositol trisphosphate and diacylglycerol, Ca^{2+} release from the sarcoplasmic reticulum, and activation of L-type Ca^{2+} channels or nonselective cation channels to produce contraction (Fig. 1*D*) (15, 16).

Thus peculiarities in the architecture and signaling cascades between these two muscle types reveal notorious differences on the profile of their contractile responses, and the gastrointestinal tract establishes a good experimental model to compare them, especially in rats. In humans, the presence of striated muscle cells has been documented in the upper portion of the esophagus (5). In contrast, the rat contains striated muscle cells along the entire length of the esophagus (12, 14). On the other hand, smooth muscle is the major cell type present in the rat stomach. In this study, we employed electrical field stimulation (EFS) to induce contractions on isolated segments obtained from esophagus and stomach to construct a simple laboratory activity that reveals how these different muscle cell types behave.

Learning Objectives

After completing this activity, the student should be able to:

- Analyze experimental traces to obtain basic properties of isolated gastrointestinal tissues.
- Describe the major differences between the excitation-contraction coupling in striated and smooth muscle cells.
- List correctly the types of muscle present in the gastrointestinal tract.

Activity Level

This activity has been designed for undergraduate students as part of a basic physiology course.

Prerequisite Student Knowledge or Skills

Before doing this activity, students should have a basic understanding of cell physiology, synaptic transmission, and muscle contraction. To a better efficacy dealing with experimental data, students should be proficient in the use of computer data sheets. Additional theory about cell structure and human anatomy should be interesting for further discussion.

Time Required

The activity may be planned to occur in a single 2-h session, but requires a previous period of at least 1 h to prepare all physiological solutions, perform animal euthanasia, and obtain isolated tissues that should be mounted in the organ bath system before students arrive at the laboratory. It should be considered that adopters of this activity are responsible for obtaining permission for using animal tissues from their home institution.

METHODS

Equipment and Supplies

The following supplies and equipment are required to carry out this activity:

1. At least two isometric force transducers (one for each bath chamber; there are several suppliers worldwide, and, in this study, we used the ML870B60/C-V from ADInstruments–Australia).
2. Data acquisition and signal amplifier system (one device containing at least two channels, e.g., PowerLab 8/30, ADInstruments–Australia).
3. Microcomputer with software compatible with the data acquisition system.
4. Compressed gas cylinder containing a gaseous mixture (5% CO_2 in O_2) connected to the bath chamber (simple aquarium air pump also works to aerate bath chamber in this kind of experiment).
5. Thermal circulator pump to maintain the bath chamber temperature at 37°C (water bath coupled with an aquarium water pump also works to control temperature in the bath chamber).
6. Electrical field stimulator and a pair of platinum electrodes to each tissue (e.g., 132 LE12406, PanLab, Spain).

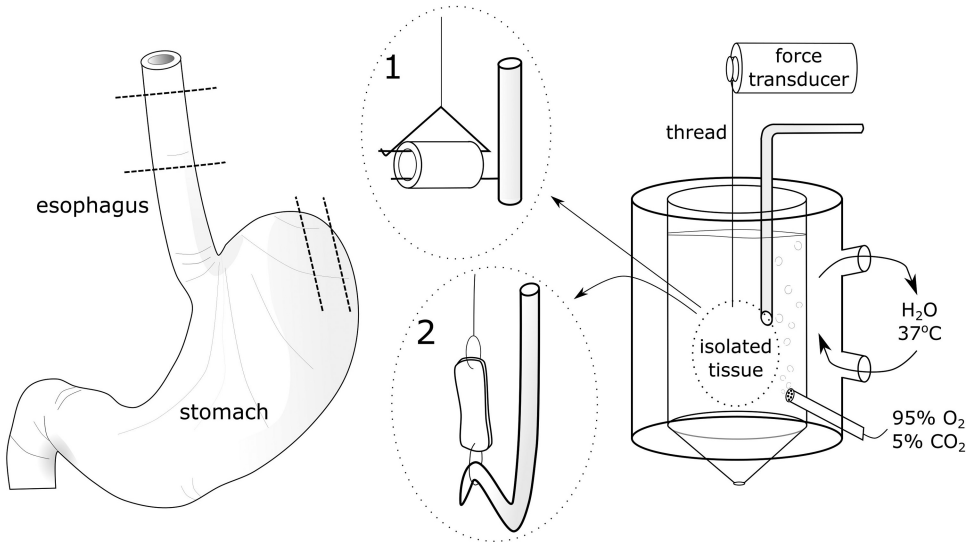

Fig. 2. Representation of gastrointestinal areas of rat esophagus and stomach used to obtain tissue pieces. Figure also depicts tissue disposal for mounting esophagus as rings (*1*) or stomach fundus as strips (*2*) in the isolated organ bath system.

7. At least two isolated tissue organ bath chambers with 5- to 10-ml capacity (one for each tissue preparation). Such devices are offered by several suppliers worldwide (e.g., ADInstruments, Radnotti, and Ugo-Basile).
8. Small hand-made triangular pieces (7-mm side length) made of stainless steel wire (0.3-mm diameter) as commonly used in dentistry (to produce tension in orthodontic brackets). Such pieces should be passed through the lumen of esophagus for tissue support to allow tension measurements.
9. Cotton thread to connect the triangular pieces containing the ring-like esophageal segments to the force transducer. Hand sewing needle can give support to attach strips from stomach fundus with the force transducer.
10. A petri dish for tissue handling before transfer to the bath chamber.
11. Salts and reagents of analytic grade (NaCl, KCl, $MgCl_2$, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, glucose, atropine, tubocurarine, procaine, and caffeine).
12. Distilled water.
13. Assay tubes (2–10 ml) for solution preparation and dilution.
14. Volume-adjustable, single-channel pipettes (volume range: 5–1,000 μl).

The activity requires the following solutions:

A. Tyrode solution (physiological saline solution) with the following composition (in mM): 136.00 NaCl, 5.00 KCl, 0.98 $MgCl_2$, 0.36 NaH_2PO_4, 2.00 $CaCl_2$, 11.90 $NaHCO_3$, and 5.50 glucose (pH should be adjusted to 7.4).

B. Solutions of the following compounds directly dissolved in distilled water: atropine (1 mM), tubocurarine (5 mM), caffeine (200 mM), and procaine (100 mM; warmed until complete dissolution). In this activity, all compounds were purchased from Sigma (St. Louis, MO).

Animal Subjects

This activity requires the use of esophagus segments (0.5 cm) and strips from stomach fundus (0.7 × 0.2 cm). For this purpose, we obtained male Wistar rats (190–230 g) from the vivarium maintained by the Department of Physiology and Pharmacology (Federal University of Ceará, Fortaleza, Ceará, Brazil). Animals were previously housed under standard conditions in polypropylene cages (410 × 340 × 160 mm) with 12:12-h light-dark cycles with free access to food and water. Animal welfare and experimental procedures were undertaken in accordance with the Ethical Principles for the Care and Use of Laboratory Animals of the Brazilian Society for Laboratory

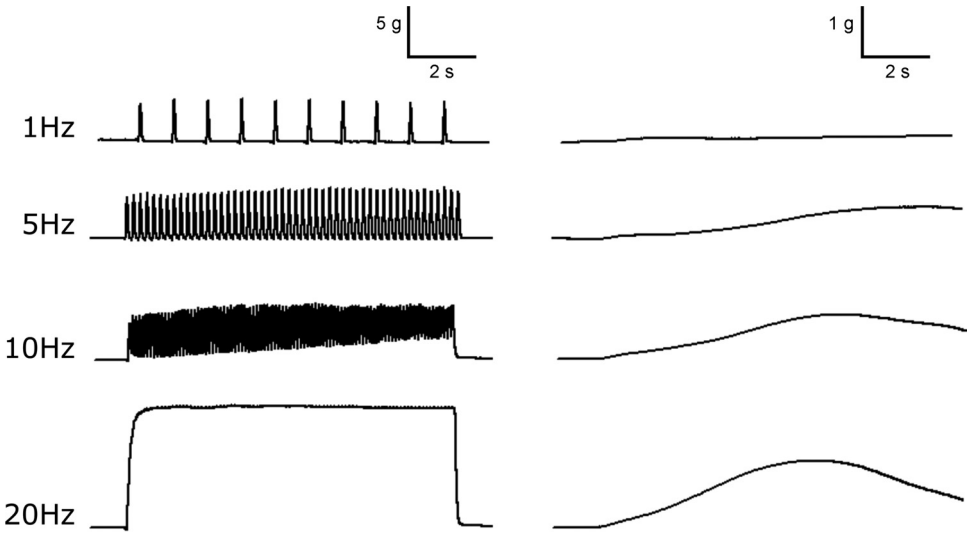

Fig. 3. Experimental traces typically recorded from an isolated esophagus segment (*left*) and stomach fundus strip (*right*) during electrical field stimulation (EFS) at increasing frequencies (1–20 Hz).

Animal Science (recorded in our institutional Animal Ethics Committee as protocol no. 46/2015).

Instructions

Setting up the preparations. Each tissue preparation was mounted in an organ bath filled with Tyrode solution (37°C, bubbled with 95% O_2 and 5% CO_2), as represented in Fig. 2. For this purpose, rats were anesthetized [tribromoethanol 250 mg/kg ip, Sigma (USA)] and then euthanized by exsanguination. Stomach and esophagus were removed and placed in a petri dish containing Tyrode solution and were cleaned of adhering fat and connective tissue. The distal part of the esophagus (0.5 cm near to stomach) was discarded. The stomach was opened through the lesser curvature and washed with Tyrode solution to remove any luminal food content. The upper portion of the stomach, easily identified by whiter and thinner aspect, was cut in strips. Small stainless steel triangles had one of the edges inserted through the esophageal lumen, while the opposite vertex of the first triangle was connected to the force transducer by cotton thread. The second triangle was connected to a fixed point at the bottom of a hooklike tissue holder. Strips of stomach fundus were attached by cotton thread in both endings: one connected to the force transducer, and the other fixed to a fixed point at the bottom of the tissue holder. Resting tension applied for each tissue was 1 *g*. For EFS, a pair of platinum electrodes was placed into the bath chamber, maintaining the experimental tissues between them.

Experimental protocols. The activity started with a brief explanation about the experimental system and about how to interpret the experimental trace recordings. The students were then encouraged to perform a frequency-response curve in both tissues. Electrical pulse parameters were trains of stimulation of 10 s and pulses of 1 ms and 20 V. Frequency was settled at 1, 5, 10, and 20 Hz. Comparison between tissue responses revealed the first valuable information about the differences of the two muscle types.

After a brief interval to renew physiological solution in the bath chamber and discuss the results and details of the experimental procedures, the frequency-response curve was repeated in the presence of 1 μM atropine, 5 μM tubocurarine, 5 mM caffeine, or 1 mM procaine. Each drug was added at least 5 min before the construction of the second frequency response curve. The students compared the trace records obtained in the first round under EFS with the results seen in the presence of a given compound in the same tissue. In addition, the experimental results between esophagus and stomach were compared under similar conditions.

In general, data acquisition preceded discussion of theory between students and instructors concerning a given subject. Such procedure allowed that students connect their own results with theoretical concepts.

The data analysis was performed with raw data (e.g., tension, in g), although it could be compared in just a qualitative way, calling attention to the increase or decrease of response magnitude. However, aiming to present reliable results in the present work, our data were expressed as milligrams of tension per milligram of tissue. For this purpose, tissues were weighted immediately after the end of the experiments. Data were presented as means ± SE (standard error of mean). Significance of results was determined using ANOVA and, when significant, following a multiple-comparison test, as properly indicated. When $P < 0.05$, the result was considered significant.

Troubleshooting

Common difficulties related to this activity include nonappropriate conditions to maintain the isolated tissue viable (e.g., temperature, pH, and air bubbling). Attention should be also paid to set the volume pipette correctly. Platinum electrodes must be completely covered by physiological solution inside the organ bath during EFS. Procaine should be the last compound to be tested in this activity in virtue of the time necessary to full recovery of tissue response.

Safety Considerations

The activity was designed considering that the responsibility regarding animal handling, preparation of solutions, and adjustments of the entire data acquisition system belongs to the laboratory personnel to reduce ethical or safety concerns for students. However, students should be able to manipulate operational equipment by changing frequencies at the electrical stimulator and adding drug solutions into the bath chamber. They also should wear laboratory coat and gloves to avoid skin contact with substances employed in this activity.

RESULTS

Expected Results

Two patterns of contraction were observed when a given electrical stimulus was applied on experimental tissues (Fig. 3). In response to EFS, stomach fundus strips produced single and slow deflection on trace recordings whose magnitude increased as the frequency of stimulation was higher. At 1-Hz stimulus, peak deflections were almost undetectable. In contrast, esophagus produced individual twitch contractions of reproducible

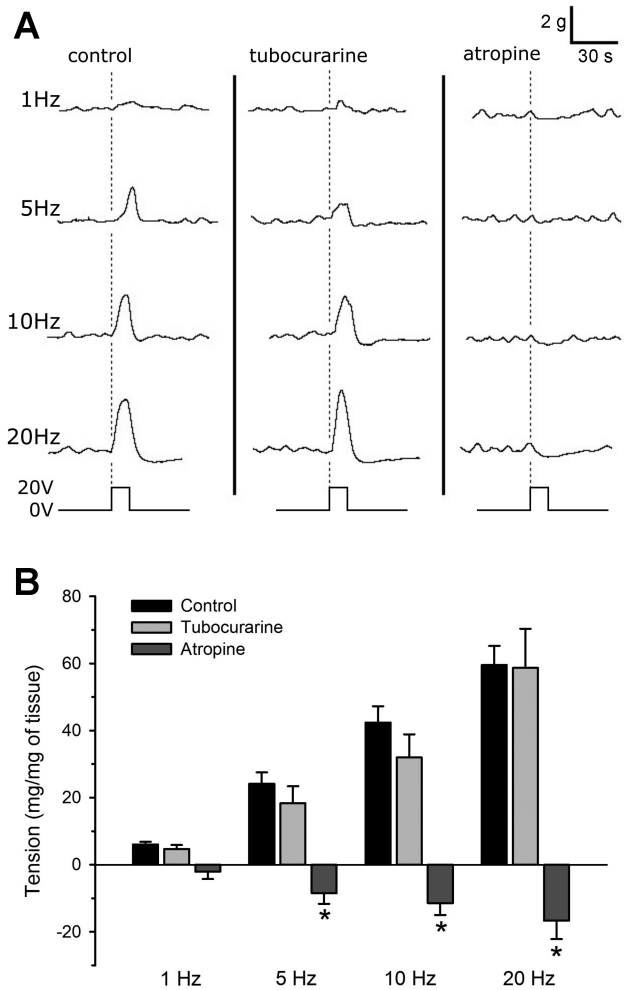

Fig. 4. *A*: experimental traces of fundic strips during electrical field stimulation (EFS) at increasing frequencies (1–20 Hz). These responses were obtained in the absence (control) or in the presence of either tubocurarine (5 μM) or atropine (1 μM). *B*: means ± SE of EFS frequency-response curves in fundic strips: control ($n = 36$) and in the presence of tubocurarine (5 μM; $n = 6$) or atropine (1 μM; $n = 6$). Inverted bars indicate reduced tonus in the presence of atropine. *$P < 0.05$, ANOVA, post hoc test Holm-Sidak.

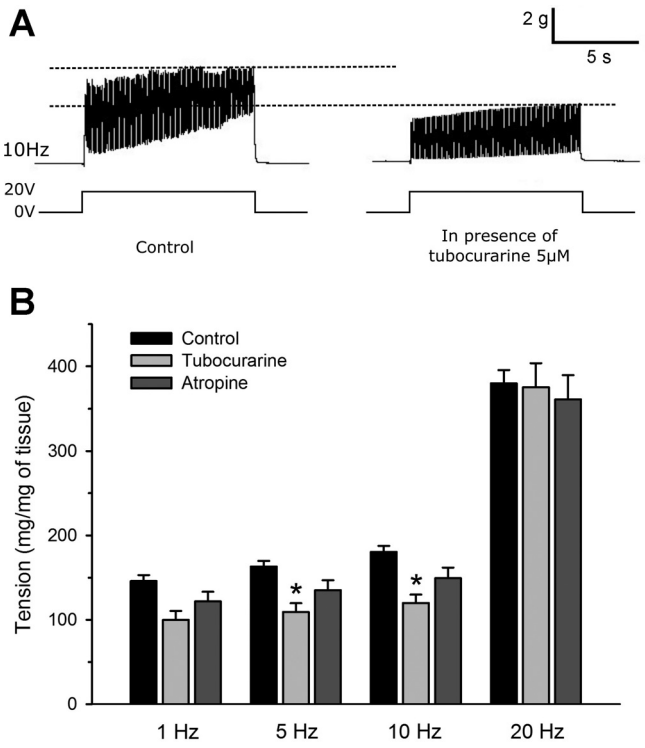

Fig. 5. *A*: experimental traces of isolated esophagus under electrical field stimulation (EFS) in a frequency of 10 Hz in the absence (control) or presence of tubocurarine (5 μM). *B*: means ± SE of contractions induced by EFS (1–20 Hz) in the absence (control, $n = 36$) or presence of tubocurarine (5 μM; $n = 6$) or atropine (1 μM; $n = 6$). *$P < 0.05$, ANOVA, post hoc test Holm-Sidak.

magnitude simultaneously with electrical pulses (not shown in Fig. 3), as easily observed at the lowest frequency of stimulation (1 Hz; Fig. 3). These findings reveal that contractile responses in esophagus differed from those in fundic strips.

At higher frequencies of electrical stimulus (5 and 10 Hz), frequency of contractions in esophageal preparations increased to a level that was enough to produce sustained force as the major consequence of a phenomenon typically known as mechanical temporal summation (10 Hz; Fig. 3) (8). At the highest frequency of stimulus (20 Hz), the muscle response reached maximal peak force that resulted in a plateau observed during almost the complete period of the stimulatory pulse train, a phenomenon named tetanic contraction (8).

From these initial observations, it would be interesting to connect the phenomena observed in esophageal and fundic tissues with theoretical classes dealing with the physiology of muscle contraction. For this purpose, students were encouraged to test the effects of adding compounds that interfere with ACh and its receptors. Atropine and tubocurarine were added to the medium, and the contractile responses induced by EFS were observed again. Students should observe that, whereas tubocurarine (5 μM) was inert on fundic contractions (Fig. 4), it significantly reduced the resulting contraction on esophageal tissue (Fig. 5). In contrast, atropine (1 μM) abolished the development of contraction in fundic strips (indeed, it also reduced the basal tonus, as observed by the significant reduction in baseline; Fig. 4, *A* and *B*, right traces and columns respectively), whereas the interference on EFS-induced contractions of esophageal preparations was minimal (Fig. 5*B*).

Students should be aware about the role of ACh as the major excitatory neurotransmitter in neuromuscular junctions. Neurotransmitter release occurs from presynaptic membrane to activate receptors on postsynaptic cell. Tubocurarine is a selective blocker of cholinergic postsynaptic nicotinic receptors, and its historical importance is often considered in chapters dedicated to physiology of voluntary striated muscle contraction in most of the physiology textbooks (1, 13). By its turn, atropine is a well-recognized blocker of muscarinic receptors (3) and would be apparently obvious and expected by students regarding a putative inhibition of EFS-elicited contraction, not only in the fundic strips, but also in the esophageal tissue. However, the experimental procedures did not confirm this last

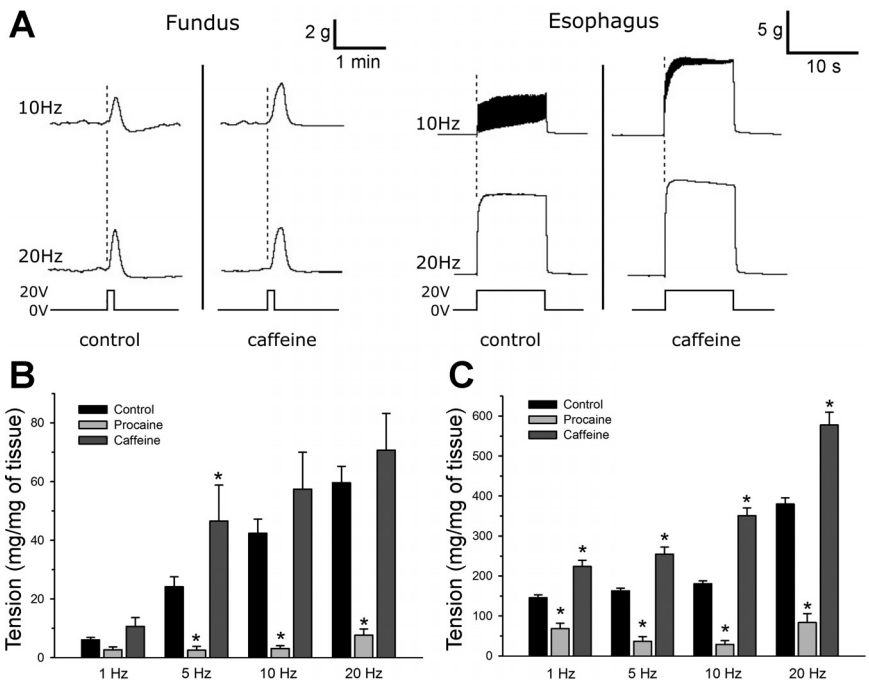

Fig. 6. *A*: experimental traces obtained from strips of stomach fundus (*left*) or esophagus (*right*) under electrical field stimulation (EFS) at 10 and 20 Hz in the absence (control) or presence of caffeine (5 mM). *B* and *C*: means ± SE of contractile responses to EFS in fundus strips (*B*) or esophagus (*C*): control ($n = 36$) and previous addition of procaine (1 mM; $n = 6$) or caffeine (5 mM; $n = 6$). *$P < 0.05$, ANOVA, post hoc Holm-Sidak.

Table 1. *Inquiry for the students*

1. What kind of muscle is present in the gastrointestinal tract?	*a*) Striated muscle *b*) Smooth muscle *c*) Striated and smooth muscle*
2. According to the options below, which best defines tetanic contraction?	*a*) Muscular contraction debilitated due to inefficient release of neurotransmitters in the synaptic cleft. *b*) Muscular contraction impaired due to excessive action of enzymes responsible for cleaving neurotransmitters. *c*) Intense and sustained muscle contraction due to successive stimulation not allowing relaxation.* *d*) Intense muscle contraction and sustained resulting from excessive production and release of neurotransmitters.
3. What is the major excitatory neurotransmitter released from the parasympathetic system in the gastrointestinal tract?	*a*) Epinephrine/adrenaline *b*) Dopamine *c*) Acetylcholine* *d*) Vasoactive intestinal polypeptide (VIP)
4. Select the correct option regarding the location of the ryanodine receptor in the muscle cells.	*a*) Presynaptic membrane *b*) Postsynaptic membrane *c*) Membrane of the sarcoplasmic reticulum* *d*) In the cytosol of muscle cells

*Expected correct item.

hypothesis because the inhibitory action induced by atropine was not evident in esophagus. So, certify that such compounds differentially interfere with neuronal signaling on a visceral organ, which constitutes a valuable gain to the student comprehension regarding the diversity of synapses on gastrointestinal tract.

The addition of caffeine (5 mM) into the organ bath before electrical stimuli enhanced the contractile response in both tissues, although in a more evident way in esophagus than in stomach fundus. Under our experimental conditions, responses increased significantly at 5 Hz in caffeine-treated stomach fundus (Fig. 6*B*), while esophageal preparations confirmed statistical augment to all frequencies tested in the presence of caffeine (Fig. 6*C*; $P < 0.05$, ANOVA). Such aspect was further confirmed by observing interesting details on experimental recordings. At frequency of 10 Hz on esophageal tissue maintained in the presence of caffeine (5 mM), the contractile response shifted from a profile typical of temporal summation to an aspect more compatible with the occurrence of tetany (Fig. 6*A*). Caffeine acts on ryanodine receptors, Ca^{2+} channels present on sarcoplasmic reticulum membrane, to increase the general cell sensitivity for Ca^{2+} release (11).

Based on the present findings, the students can conclude that EFS is a technique able to induce neurotransmitter release in the experimental preparations and, more importantly, that neurotransmitter release may exert differential effects, depending on the gastrointestinal portion. To ensure the influence of neurons on these contractile events, the students added procaine (1 mM) that completely blocked the EFS-induced contractions in both tissues (Fig. 6). Procaine is a Na^{+} channel blocker that inhibits action potentials on neuronal cells (2).

Misconceptions

Initially, students did not realize the presence of striated muscle in the gastrointestinal tract before the first EFS responses. After observing basic differences on the profile of contraction between gastric and esophageal tissues, the presence of the two muscle types became more evident. It is important the students perceive that striated muscle cells are not restricted to skeletal voluntary or cardiac involuntary muscles. Similarly, the activity allowed the students to know that smooth muscle cells are not the unique type involved in the motor function of visceral organs.

Although possessing a simple experimental design, the activity is rich in evidence found in the experimental traces that illustrate several basic physiological principles. For instance, it would be important to discuss the existence of neuronal cells in the gastrointestinal wall, which can constitute an enteric nervous system. In addition, why are esophageal muscle fibers faster than fundic strips to produce force? In this aspect, the instructors should highlight the different architecture of striated and smooth muscle synapses, the existence of a synaptic cleft in striated but not in smooth muscle cells, and the more diffuse nature of the neurotransmitter release from varicosities typically found in smooth muscle tissues.

Table 2. *Evaluation of students' knowledge regarding contractility of gastrointestinal tract*

	Student Answer, %		
	Before	After	*P* Value
1. What kinds of muscle are present in the gastrointestinal tract?			
Striated muscle	2.27	0	<0.001
Smooth muscle	81.82	20.45	
Striated and smooth muscle	15.91	79.55	
2. What is tetanic contraction?			
Right	70.45	93.18	0.001
Wrong	29.55	6.82	
3. What is the main neurotransmitter released from the parasympathetic system in the gastrointestinal tract?			
Right	79.55	100	0.002
Wrong	20.45	0	
4. Select the correct option regarding the location of the ryanodine receptor in the muscle cells.			
Right	54.55	81.82	<0.001
Wrong	31.81	18.18	
No answer	13.64	0	

The questionnaire was anonymously given to 47 undergraduate students of biology and health sciences. For *P* value, Wilcoxon signed ranks test was used, compared with the response obtained before the activity.

Evaluation of Student Work

To assess the impact of this activity on the student's comprehension regarding the theme, a short questionnaire (Table 1) was applied before and after the class to a group of 47 students. Analysis of the answers revealed that the number of correct responses increased considerably after the activity. For instance, *question 1* (Table 2) revealed that 81% of students considered only smooth muscle present in the gastrointestinal tract before the activity, an opinion that clearly changed after the laboratory activity. This and other results show the success of the activity.

Inquiry Applications

The instructors prepared questions. Students carried out the experiments under the instructor's supervision. Students were free to propose changes in experimental protocols, such as the order of substance addition. Care was taken only in the case of procaine because of the long time necessary for full functional recovery of the preparation. During each experiment, the instructors ask students about what they observed, and students discuss it collectively and make conclusions before subsequent steps.

Wider Educational Applications

This activity can be useful to demonstrate the action of drugs that increase or decrease gut motility. Although the activity was designed to be presented during the courses dealing with muscle contraction, it should also be interesting for students who begin studies on gastrointestinal physiology.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

F.J.B.-L., K.K.L.G., D.M.N.O. and P.J.M. conception and design of research; F.J.B.-L., K.K.L.G., D.M.N.O, T.B.V. and T.S.B. performed experiments; F.J.B.-L., K.K.L.G., D.M.N.O. and P.J.M. interpreted results of experiments; F.J.B.-L., K.K.L.G., D.M.N.O. and P.J.M. drafted manuscript; F.J.B.-L., T.B.V., T.S.B. and P.J.M. edited and revised manuscript; F.J.B.-L., K.K.L.G., D.M.N.O., T.B.V., T.S.B. and P.J.M approved final version of manuscript; F.J.B.-L., K.K.L.G. and D.M.N.O. analyzed data; F.J.B.-L., K.K.L.G. and P.J.M. prepared figures.

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