INNOVATIONS

Use of the Frog Heart Preparation to Teach Students about the Spontaneous Mechanical Activity of the Vena Cava

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Abstract: Most undergraduate physiology texts describe veins simply as reservoirs for blood and conduits for return of blood to the heart. This article describes a laboratory exercise that can be performed by students to demonstrate that veins are much more than reservoirs and conduits for blood flow: they possess a dynamic rhythmic contraction. In this exercise, we recorded the simultaneous beating of the frog postcaval vein (PCV; in mammals this is the inferior vena cava) and atrium by connecting them to separate force displacement transducers. *In vivo*, the PCV and atrium both contract ~ 35 beats/min; however, the contractions are not synchronous with each other. We developed a simple scoring method (comparative temporal analysis) to illustrate that the atrium contractions do not drive the contractions of the PCV. Instead the atrium and PCV contract independently of each other. To support our hypothesis that the atrium and PCV contractions were independent we removed the PCV from the frog and suspended it in an organ bath. The PCV rhythmically contracted as *in vivo*. The autonomic neurotransmitter, norepinephrine, did not affect the force of contraction and heart rate. In contrast, acetylcholine abolished the contractile activity. This investigation has encouraged discussions about the source and physiological significance of the rhythmic PCV contractions. This article provides some hypotheses about its significance, as well as possible evolutionary origins of the veins' mechanical activity. Overall, the implementation of this exercise into the classic frog heart preparation will deepen the students' understanding about the venous side of the cardiovascular system and give them insight into the integrative nature of physiology.

Key words: contraction, veins, physiology

INTRODUCTION

In many undergraduate physiology laboratories the classic frog heart preparation is used to teach Starling's Law of the heart, autonomic control of heart function, and the heart refractory period. In our senior-level animal physiology course students investigate Starling's Law by measuring the increase in magnitude of each heart beat with each incremental increase in tension applied to the apex of the heart. This is analogous to the increase in contractile strength of the heart with an increase in venous return. Autonomic regulation of the heart is explored by the application of norepinephrine and acetylcholine to activate the β-adrenergic and muscarinic receptors on the heart to increase and decrease heart rate, respectively. Likewise, students change the impulse frequency on an electrical stimulator to determine how much time needs to elapse after stimulation before another heart beat can be initiated (*i.e.* heart refractory period).

While performing these laboratory investigations, students recognized that the postcaval vein (PCV) next to the sinus venosus of the heart was 'beating.' In mammals the PCV is analogous to the inferior vena cava. In addition, the rhythmic PCV contraction appeared to be independent of the heartbeat. Traditionally, physiology courses depict veins as reservoirs for blood and as conduits for venous return of blood to the heart. However, there is a dynamic regulation of venous diameter by hormonal and autonomic influences. Veins contract in response to norepinephrine, endothelin-1, platelet-activating factor, thromboxane A_2, and leukotrienes (Gao and Raj, 2005; Xu *et al*., 2007). Relaxation is mediated by the inducers of nitric oxide formation, acetylcholine, and bradykinin (Gao and Raj, 2005).

Only a few studies have reported that the PCV contracts (*i.e.* beats) spontaneously (Victor and Nayak, 2003; Jones *et al*., 2003; Huizinga and Faussone-Pellegrini, 2005; Ghose *et al*., 2008). Victor and Nayak (2003) formulated a "cardiovascular hypothesis" to describe the beating of the major veins in vertebrates (including mammals) as a 'waltz and duet.' This hypothesis proposes that there is a systemic venous 'waltz' caused by the sequential contraction of the veins, venous sinus, and right atrium. The return of unoxygenated blood to the heart is followed by the pulmonary 'waltz;' this is the sequential contraction of the pulmonary veins, pulmonary venous sinus, and

left atrium. The 'duet' is created because the systemic veins contract before the pulmonary veins. The physiological significance associated with this rhythmic beating is unclear.

After observing the rhythmic PCV contraction in lab, a student (one of the coauthors) in our animal physiology class developed hypotheses about the physiological significance of the PCV's mechanical activity and the autonomous nature of its beat. This paper describes experiments that this student completed. These experiments can easily be done by undergraduate students to show that the PCV contracts independently of the atrium. Furthermore, we provide a discussion about possible function(s) of the spontaneously contracting PCV that can be used by instructors to promote student discussion and planning of future experiments. These experiments will deepen student knowledge of the cardiovascular system and improve their scientific skills by forcing them to formulate hypotheses that they will test.

METHODS

Animal Preparation

Bullfrogs ~ 5 inches in length were obtained from Carolina Biological Supply (Burlington, NC). Frogs were stored at room temperature (22° C) in a fiberglass container designed specifically for frogs. The container consisted of a "swimming pool" with a drain outlet and a shelf on which the frogs sat and were sprayed with charcoal-filtered tap water from an inlet nozzle. Before use in laboratory experiments, frogs were cooled in crushed ice for ~ 30 minutes and then double-pithed with a stainless steel needle. Next, the heart was exposed by cutting open the chest cavity with stainless steel scissors and kept moist by periodically dripping frog Ringers directly on the heart. The frog Ringers contained (in mM) 100 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, and 10 Tris buffer, pH 7.5. All salts and Tris buffer were obtained from Sigma (Saint Louis, Missouri). The use of the bullfrogs was approved by the University's IACUC.

In vivo experiments

The atrium and PCV for each frog were connected to separate UFI model 1030 force displacement transducers (MacLab) using thread tied to a size 6 fishhook. The transducers were connected to MacLab Bridge Amplifiers that were, in turn, connected to an ADinstruments Powerlab/4st. Data were acquired with ADinstruments Chart 5.4 software and the digitized data displayed on the screen of an Apple eMac computer.

To further explore the source of the PCV beat, the PCV (immediately adjacent to the sinus venosus) was pinched with stainless steel forceps. The ends of the forceps were covered with heat shrink tubing to minimize damage to the PCV; this maneuver stopped blood flow from the PCV to the sinus venosus and to the atrium.

Organ bath experiments

The PCV was removed from each frog with stainless steel scissors and mounted in a 25 mL organ bath (GlobalTown Microtechnology, Bradenton, Florida) that contained frog Ringers. The isolated PCV was connected to a tissue holder (TSC-100S, GlobalTown Microtechnology) that was connected to a force-displacement transducer (GlobalTown). The transducer was connected to an ADinstruments Powerlab/4st, and data were acquired as described above.

Neurotransmitters

Acetylcholine (ACH) and norepinephrine (NOREPI) (Sigma) were used to inhibit or stimulate the activity of the heart and the PCV, respectively. Both neurotransmitters were made as 1 mM stock solutions in frog Ringers, and dripped onto the heart with a plastic pipette or added to the organ bath to modulate the activity of the PCV. Because NOREPI oxidizes easily, it was freshly prepared the day of the experiment.

Statistical Analysis

We developed a simple scoring method (comparative temporal analysis, CTA) that undergraduates could do in lab to determine if the PCV contraction was independent of the atrium beat in their *in vivo* experiments. We made the

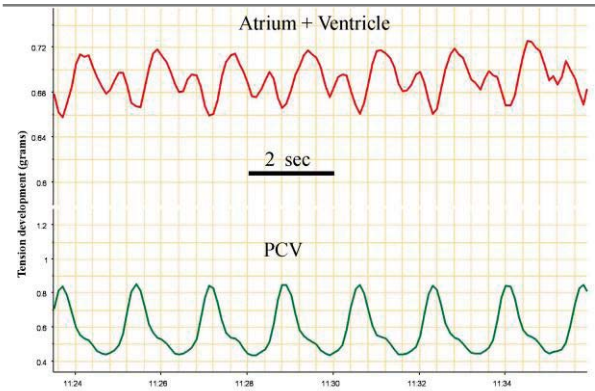

Fig. 1. Simultaneous recordings of the asynchronous contractions of the atrium and PCV in a bullfrog. Both the atrium and PCV contract at a frequency of ~ 35 beats/min. The smaller beat in the atrium trace is ventricular mechanical activity.

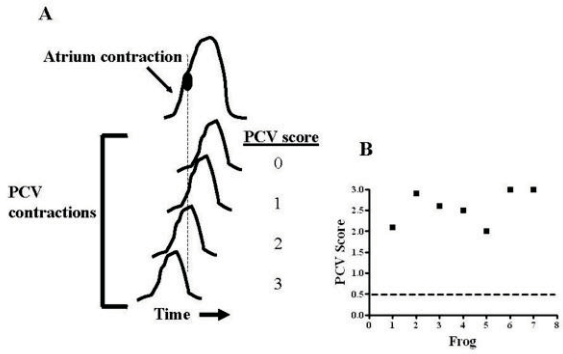

Fig. 2. Comparative temporal analysis of PCV and atrium contractions. A. Scoring method used to compare the time course of an atrium contraction to the PCV contraction. B. Individual PCV scores (n=7) illustrate that the PCV contraction occurs before the atrium contraction (P<0.05). A PCV score of ~ 0.5 would have suggested that the atrium contraction drives the PCV beat.

assumption that if the atrium beat creates a PCV contraction, then it must precede the PCV contraction. The CTA was achieved by comparing the time-course of atrium and PCV contractions to generate CTA scores (Fig. 2A). A CTA score of zero indicates that the middle of the atrium upstroke corresponds to a relaxed PCV, thus suggesting that the PCV beats after the atrium. A score of 2 indicates that the PCV is fully contracted and occurs before the atrium contracts. Data are represented as the mean ± standard deviation. Results were considered significant when P<0.05 using a paired t-test or the Wilcoxon sign ranks test (comparative temporal analysis).

RESULTS

In vivo contractile activity of the atrium and PCV

The atrium and PCV both beat at an average rate of 35 beats/min at room temperature (22°C; Fig. 1). Also seen in Figure 1 is the ventricular beat (smaller beat) that is superimposed on the atrium trace. Contraction of the PCV occurs after the ventricular contraction, and thus, precedes the atria contraction. In frogs there is a component of the electrocardiogram (ECG) that is attributed to the electrical activity of the PCV (Victor and Nayak, 2002, 2003). Although we did not record the ECG, students can record both the ECG and contraction of the PCV and correlate the two to demonstrate that the electrical event (ECG) precedes the mechanical event (contraction of PCV).

Comparative Temporal Analysis

Conceivably the contraction of the atrium could create a PCV 'pulse' that appears as an independent beat (Victor and Nayak, 2003; Jones *et al*., 2003). Therefore, we used a comparative temporal analysis (CTA) of the atrium and PCV contractions to test the hypothesis that the PCV beat is driven by the contractile activity of the atrium. According to this analysis, if the atrium beat induces a PCV contraction it will precede the PCV beat with a score of ~ 0.5. As shown in figure 2B, the CTA scores (n=7 frogs) ranged from 2.0 to 3.0. These high scores indicate that the PCV contraction occurs before the atrial contraction. Therefore, we reject our hypothesis that the atrial beat drives contraction of the PCV. The frequency of the atrium and PCV contractions for the seven frogs were 36.0±7.7 and 35.6±7.4 beats/min, respectively.

The pinch experiment

The PCV was pinched with modified stainless steel forceps to stop blood flow from the PCV to the sinus venosus and atrium. In three out of five bullfrogs, pinching the PCV on the atrial side of the fishhook completely eliminated the beating of the atrium after several larger beats but had little effect on PCV contractile activity (Fig. 3); this effect was

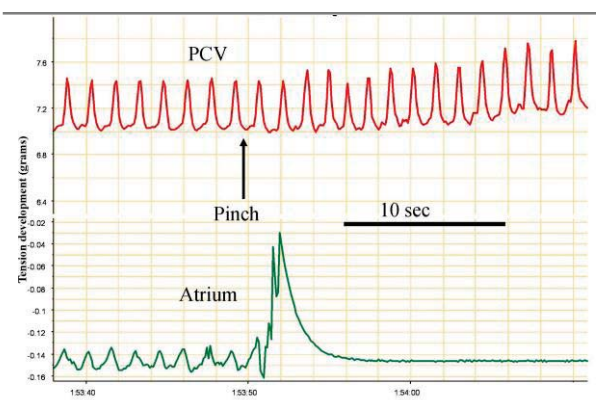

Fig. 3. A representative trace of a pinch experiment shows that the mechanical activity of the atrium is independent from the PCV contraction.

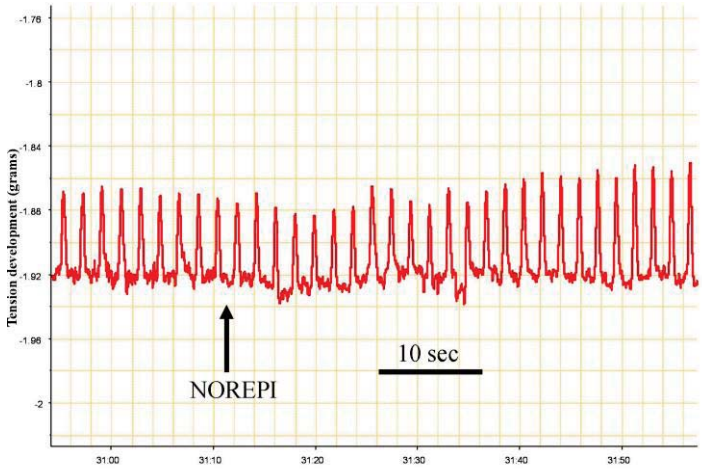

Fig. 4. The adrenergic agonist, NOREPI, has little effect on the spontaneous contractile force and rate of the PCV contraction.

reversible.

In the other two frogs, pinching the PCV reduced but did not completely abolish atrial mechanical activity. These experiments support the CTA results. We attribute the loss or reduction of contractile activity of the atrium to the marked decrease in venous return of blood to the heart via the PCV because this major vein returns more blood to the heart than does the anterior caval vein.

Organ bath experiments

The *in vivo* experiments detailed above imply that the mechanical activity of the atrium is not responsible for the PCV's rhythmic contractions. To isolate contractions of the PCV, the PCV was suspended in an organ bath. In three out of six PCVs that were mounted in organ baths, beating was spontaneous (Figs. 4 and 5). We suspect the lack of beating in the other three PCVs was due to "pacemaker" damage caused by the dissection. Application of 4 µM NOREPI caused a slight increase ($P>0.05$) in PCV contractile activity (Fig. 4). NOREPI increased the force of contraction from 0.01±0.01 g to 0.02±0.01 g and heart rate remained unchanged from 35±17.6 to 37±14.2 beats/min. In contrast, 4 µM ACh gradually abolished the mechanical activity of the PCV (Fig. 5). ACh significantly decreased the contractile force from 0.02±0.01 g to 0.00±0.01 g and attenuated the heart rate from 36.00±10.40 to 4.00±6.93 beats/min.

DISCUSSION

In our animal physiology course students are required to write in scientific journal format about some of the experiments reported here as part of a formal laboratory report on frog cardiovascular physiology. Because undergraduate physiology texts do not mention the beating of the vena cava, students were particularly excited about the possibility that they were researching a physiological process that was not described in their textbook. These experiments provide evidence that the PCV rhythmically contracts with the same frequency as the atrium contraction. However, the PCV contraction is independent of the atrium contraction and occurs after the ventricular contraction, and thus, precedes the atrium contraction. The muscarinic agonist, acetylcholine, effectively inhibited the contractile activity of the PCV. In contrast, the β-adrenergic agonist, norepinephrine, had little effect on the PCV contraction. The student excitement and desire to learn more about the spontaneity of the PCV contraction is a tremendous confirmation about the success of these experiments to inspire thinking about the cardiovascular system.

The rhythmic contractions of the PCV and atrium *in vivo* experiments allow students to visualize and hypothesize why the PCV and atrium contractions are out-of-phase with each other. The CTA provides evidence that the PCV contraction precedes the atrium contraction. This suggests that the atrium contraction does not drive the contractile activity of the PCV. Also, in the pinch experiment we were able to reduce/eliminate the atrial beat with little effect on the rhythmic activity of the PCV (Fig. 3). However, the contractile force of the PCV increased after the pinch was applied. This may be due to a myogenic reflex mechanism (Berne *et al.*, 2004) in which the increased force of contraction was due to blood building up in the pinched PCV, stretching the walls of the vessel. This stretch would then elicit a compensatory contraction by the venous smooth muscle. Taken together, these *in vivo* experiments support our conclusion that mechanical activity of the atrium does not drive the contraction of the PCV. The possibility exists that the electrical activity of the atrium is able to spread (via gap junctions and nerve pathways) to the PCV and drive its rhythmic contraction. However, once we isolated the PCV in an organ bath the rhythmic contractions

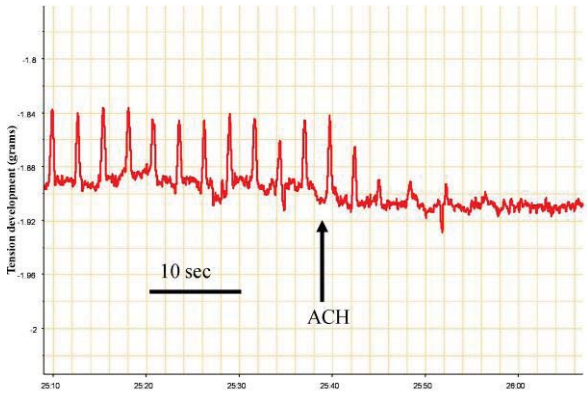

Fig. 5. The cholinergic agonist, ACh, abolished the rhythmic PCV contractile activity.

(~ 35 beats/min) were still identical to the frequency we recorded *in vivo*. Students have to be careful in removing the PCV from the frog because of damage to the 'pacemaker.' Ghose *et al*. (2008) similarly reported that the interstitial cells of Cajal in the frog, which are responsible for the rhythmic contractions of the gastrointestinal tract and closely associated blood vessels, can be easily damaged and lose their 'pacemaker' ability. Overall, our data support our hypothesis that the PCV possesses its own pacemaker ability.

When students perform experiments on the frog PCV, they will generate two important questions: (1) What is the source of the PCV beat? and (2) what is its physiological significance? The cardiovascular hypothesis formulated by Victor and Nayak (2003) proposes that there is a sequential contraction of the systemic and pulmonary veins ('waltz and duet'). Importantly, this suggests that there is a poorly understood rhythmic 'pacemaker' in the venous circulation. Similar to our study, Ghose *et al*. (2008) described that the frog PCV demonstrates spontaneous rhythmicity of 36-40 beats/min. They attributed this 'pacemaker' ability to the interstitial cells of Cajal. However, they used the distal end of the PCV adjacent to the hepatic veins, which demonstrated some differences from our results using the proximal end of the PCV. In contrast to our data, Ghose *et al*. (2008) found that norepinephrine significantly decreased the frequency of contraction until the beat was completely abolished. In addition, the rhythmicity of the distal end of some PCVs demonstrated interrupted periods of rest in which the rhythmic contraction resumed a few minutes later.

Further, some unidentified cells of the PCV may have migrated from the sinus venosus of the heart and/or become 'entrained' from the pacemaker (*e.g.* sinus venosus) of the frog heart. Overall, the *in vivo* autorhythmicity of these veins may be modulated by its inherent autonomic regulation (Victor and Najak, 2003, Jones *et al*., 2003). For example, vagal stimulation suppresses rat vena cava contractility independent of heart rate (Jones *et al*. 2003). Likewise, in our isolated bath experiments, ACh abolished the PCV contractile activity (Fig. 4B). Thus, the medulla oblongata may regulate the chronotropic and inotropic effects of the PCV (*i.e.* vena cava).

Victor and Nayak (2002, 2003) developed two hypotheses to explain the physiological significance of the beating veins. First, contraction of the veins prevents backflow of blood from the atrium into the sinus venosus and therefore also the vena cava. However, the sinoatrial valve (also present in frogs) may prevent backflow after the atrium contracts. If the sinoatrial valve closes during PCV relaxation and atrium contraction, the contraction of the PCV is functionally insignificant in preventing backflow. Second, contraction of the vena cava aids venous return of blood to the heart and ventricular filling (Victor and Nayak, 2002). Both hypotheses are not mutually exclusive and need to be tested experimentally.

Our experiments support Victor and Nayak's (2003) cardiovascular hypothesis, which describes how the veins rhythmically contract in a sequential manner whereby the systemic veins contract before the pulmonary veins (*i.e.* 'waltz and duet'). This 'waltz and duet' is poorly understood, and in humans may have clinical significance (Victor and Nayak, 2002). Nevertheless, the possibility exists that the rhythmic contraction of the veins has no physiological significance. However, Victor and Nayak (2002, 2003) have described the cardiovascular hypothesis in a wide range of animals that includes snakes, turtles, crocodiles, fish (sharks), frogs, birds, and mammals. Therefore, the environment (aquatic *vs.* terrestrial) has little effect on the presence of this phenomenon. Because the "waltz and duet' is present in a wide variety of animals we argue that it is physiologically relevant.

The evolutionary origin of the veins gives insight into the pumping action of these vessels. For example, in the Cephalochordata (*Amphioxus*) some

vessels contract and act as pumps to create blood flow (Solc, 2007). In these chordates, the primary pumping action is performed by the aorta ventralis, but the vena subintestinalis, vena portae, and vena hepaticae also contract autonomously (Solc, 2007). Further, these veins contract sequentially (reminiscent of the 'waltz and duet' of vertebrates) to pump blood in this primitive circulatory system. Hence, the beating veins in mammals and other vertebrates may have evolved from the beating vessels in the primitive chordates, supporting the notion that the 'waltz and duet' is physiologically relevant, as argued above. If true, the evolutionary origins of the beating veins suggests, as proposed by Victor and Nayak (2002), that the primary function of the contracting PCV and vena cava is to facilitate venous return of blood to the heart.

By studying the rhythmic beating of the PCV and the cardiovascular hypothesis, students will also gain insight into integrative physiology and the emergent properties of living organisms (Schultz, 1996). In this situation, the emergent properties are illustrated by the specialized 'pacemaker' cells found within the PCV that coordinate their function with the rest of the venous circulatory system to move blood throughout the vasculature to sustain life. Because the cardiovascular system distributes endocrine and neuroendocrine messenger molecules to target tissues, the cardiovascular system is integral to the regulation of the coordinated activities that maintain homeostasis.

In summary, students have much to learn by experimentally investigating the beating of the PCV in bullfrogs. They will learn about a cardiovascular phenomenon, 'waltz and duet', not covered in most, if not all, physiology texts. (We examined 14 widely used undergraduate physiology texts and found nothing on the spontaneously beating veins.) They will also learn that there is still much to discover about cardiovascular physiology, because the physiological significance and the mechanisms responsible for the regular mechanical activity of the veins are poorly defined (Jones *et al*., 2003). Furthermore, students will strengthen their scientific skills in hypothesis formulation, experimental design, and drawing conclusions from experimental results. Finally, by investigating the mechanical activity of the major veins, students will gain a deeper knowledge of the cardiovascular system.

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