LABORATORY DEMONSTRATION OF VASCULAR SMOOTH MUSCLE FUNCTION USING RAT AORTIC RING SEGMENTS

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This laboratory exercise uses a simple preparation and a straightforward protocol to illustrate many of the basic principles of vascular biology covered in an introductory physiology course. The design of this laboratory allows students to actively participate in an exercise demonstrating the regulation of arterial tone by endothelial and extrinsic factors. In addition, this hands-on laboratory allows students to gather data using well-known basic biomedical research techniques. Specifically, students are introduced to an isolated organ-chamber technique that is widely used to study cellular mechanisms of many tissues including vascular smooth muscle contraction and dilation. On the basis of student evaluations, participation in the experiments and interpreting data reinforce lecture materials on smooth muscle and endothelial cell function and illustrate mechanisms regulating vascular tone. Students come away with a greater understanding of vascular biology, a deeper appreciation of integrative physiology, and an understanding of the process of conducting tissue-chamber experiments.

Key words: endothelial cell; teaching; physiology

Smooth muscle cell physiology is a topic represented in major medical physiology textbooks as a small section of muscle physiology. However, this is a rapidly changing, complex area of research that has seen recent significant advances impacting many diverse scientific fields. Therefore, it is advantageous for graduate students majoring in the field of biomedical sciences to understand the function and regulation of vascular smooth muscle (VSM). In our graduate program, we address this by preparing a bibliography of key publications, presenting lecture material covering cellular mechanisms of VSM contraction and dilation, and by requiring students to actively participate in a VSM laboratory that demonstrates the regulation of vascular tone by various extrinsic and intrinsic factors.

This laboratory exercise is part of a medical systems physiology course offered for students enrolled in our Biomedical Sciences Graduate Program. On the basis of student reviews and exam scores, this exercise has greatly enhanced students' understanding of basic physiological concepts in vascular biology. Before participating in this laboratory exercise, students are encouraged to review and discuss Poiseuille’s law of fluid dynamics. This law states that resistance to flow through a cylindrical tube is equal to \((8 \eta L)/(\pi r^4)\), where \(\eta\) is the viscosity, \(L\) is the length of the tube,
and \( r \) is the radius of the tube. Therefore, small changes in vessel diameter have very large opposite effects on resistance.

In the vasculature, the radius of a blood vessel is altered by contraction of VSM cells (11). Thus it is possible to predict the effect of stimuli on vascular resistance and blood pressure by observing contraction of VSM in isolated preparations. One way to demonstrate VSM contraction is to attach strips or ring segments of arteries to force transducers and record changes in tension development after exposure to different agents. These responses can subsequently be used to predict the effect of these agents on blood pressure. Additionally, this approach can be used to illustrate the role of intracellular calcium concentration in regulating vascular tone (3).

In this laboratory exercise, students perform two protocols. Protocol 1 demonstrates the principles of calcium regulation of vascular contraction. Agents that elevate intracellular calcium contract VSM, and those that lower intracellular calcium relax VSM. The source of calcium can be determined by manipulating extracellular calcium concentrations and by depleting intracellular stores. The purpose of this protocol is to determine the sources of activator calcium for three different contractile agents: norepinephrine (NE), a receptor-mediated vasoconstrictor that activates phospholipase C, high potassium chloride solution (KCl), a solution that depolarizes VSM cell membrane potential to activate voltage-operated calcium channels, and caffeine, a methyl xanthine that penetrates the plasma membrane and acts directly on the sarcoplasmic reticulum to release intracellular calcium stores (1). Protocol 2 examines the ability of endothelial cell products to modulate vascular tone. In this protocol, students examine endothelium-dependent relaxation to ACh and endothelium-independent relaxation to a nitric oxide donor in the presence or absence of nitric oxide synthase (NOS) inhibition, muscarinic receptor blockade, and guanylyl cyclase inhibition.

Together, these protocols illustrate the basic mechanisms of VSM contraction and relaxation. A discussion after the laboratory exercise puts this into the context of blood pressure regulation providing a way to discuss vascular physiology integrating molecular, cellular, tissue-level, and whole animal hemodynamic responses.

**METHODS AND EXPERIMENTAL PROTOCOLS**

All protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine. On the day of the laboratory exercise, two male Sprague-Dawley rats (300 g; Harlan Industries) are anesthetized with pentobarbital sodium (50 mg/kg) and exsanguinated. Thoracic aortic segments are isolated, cleaned of adventitia, and cut into rings (6). We use aortic segments for our demonstration because they are relatively hardy and easily prepared. Other laboratories could easily adapt these protocols to different VSM preparations, e.g., mesenteric or tail blood vessels. The aortic segments are kept in cold physiological saline solution (PSS) containing (in mM): 130 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄·7H₂O, 14.9 NaHCO₃, 5.5 dextrose, 0.026 CaNa₂EDTA, 1.6 CaCl₂, pH 7.3. For some protocols, calcium-free PSS is prepared by omitting the CaCl₂ and adding the calcium chelator EGTA (1 mM). Once isolated, aortas are cut into six rings (3 mm). Three segments from each rat are denuded of endothelium by gently rubbing the lumen with the closed tips of microforceps. Two aortic segments from the same rat, one endothelium intact and one endothelium denuded, are mounted on stainless steel hooks and suspended in a water-jacketed 25-ml tissue bath filled with PSS maintained at 37°C and aerated with 95% O₂ and 5% CO₂. Six tissue baths are required for the two protocols. Isometric force generation is recorded with FT03 Grass transducers (Quincy, MA) connected to chart recorders (Gould Instruments, Cambridge, MA). Aortic rings are stretched to the equivalent of 2,000 mg of passive tension to allow the maximal detection of active tension.

After the stretch, aortic rings are equilibrated 1 h, during which time they are washed every 15 min and treated for the final 30 min with indomethacin (10 µmol/l, cyclooxygenase inhibitor) to eliminate the role of cyclooxygenase products in this preparation. Next, arterial segments are contracted with NE (1 µM), and the presence or absence of endothelium is confirmed by adding ACh (0.1 µM). A contraction of 1,000 mg or greater to NE is considered viable,
relaxation of 60% or more to ACh is considered endothelium-intact (Fig. 1, top trace), whereas relaxation of <5% is considered successfully denuded (Fig. 1, bottom trace). Only the segment with endothelium-intact (+) relaxed in response to ACh (top trace).

Preparation of tissues and the endothelium check are completed before student arrival to shorten the time of the experiment. When the students arrive, they are divided into groups of four to six to allow active participation. The tracings from the curves generated by the addition of NE and ACh are left on the chart recorder, and students are instructed to observe and record the magnitude of the responses to these agents on a worksheet (Table 1). Students are now ready to participate in protocols 1 and 2.

Protocol 1

Calcium sources for VSM contraction in response to NE, KCl, and caffeine. Three sets of two-ring segments, one endothelium-intact and one denuded, are mounted in separate tissue baths as described above. The purpose of this protocol is to evaluate the contribution of intracellular and extracellular calcium on VSM contraction for three different agents, as described previously (4). Briefly, after the equilibration period, PSS in tissue baths is replaced with calcium-free PSS containing EGTA. Aortic rings are then stimulated with one of three agonists: NE (1 μM), KCl (65 mM), or caffeine (30 mM). Sample tracings of contractile responses are shown in Figs. 2–4. Next, rings are washed for 30 min with calcium-containing PSS to remove the contractile agents. After the wash period, artery rings are stimulated again with the same agents. The first response demonstrates contraction mediated by release of intracellular calcium stores because there is no calcium available for influx whereas the second response is dependent on calcium release and calcium influx.

Description of Responses

VSM response to NE. In the absence of extracellular calcium, NE stimulates a transient contraction that returns nearly to baseline. This contraction is caused by production of the second messenger inositol trisphosphate, which binds to receptors on the sarcoplasmic reticulum to release calcium from this intracellular store (8). Washing with calcium-containing buffer stimulates contraction dependent on calcium entry through open calcium channels such as the voltage-gated calcium channel (VGCC) (2, 13). This contraction diminishes as the ligand is washed off the receptor. In the presence of extracellular calcium, NE causes a rapid, sustained contraction that is significantly larger than that obtained in the absence of extracellular calcium. The differences in time course, duration, and magnitude between the two contractions illustrate the contribution both of intracellular and extracellular calcium stores in mediating receptor-activated contraction.

The endothelium-denuded artery segments develop a larger contraction compared with the endothelium-intact segment. The diminished response in the intact segment is caused by NE binding to α2-adrenergic receptors on endothelial cells to stimulate the release of nitric oxide and other vasodilators (5). The release of these vasodilators opposes and attenuates the VSM contraction.

VSM response to KCl. In the absence of extracellular calcium, KCl did not stimulate a contractile response. However, in the presence of extracellular calcium, KCl produced a sustained contraction. Increasing extracellular KCl from 7 to 72 mM reduces the driving force for K+ across the sarcolemma causing membrane depolarization. This more positive
membrane potential increases the open probability for VGCC. Increasing the extracellular concentration of KCl should not cause a contraction in the calcium-free PSS in either the endothelium-intact or -denuded aortic rings, because no calcium is available to enter through the open VGCC. In the presence of extracellular calcium, however, there is calcium influx. This influx increases intracellular free calcium and increases calcium-calmodulin binding to activate myosin light chain kinase and cause contraction. In addition, the increased calcium may stimulate calcium-sensitive calcium release from intracellular stores and activate calcium-sensitive Cl\(^{-}\) channels to further depolarize the cell (7).

**VSM response to caffeine.** Millimolar concentrations of caffeine penetrate the cell membrane to activate calcium release from the sarcoplasmic reticulum. Caffeine contraction of VSM is entirely dependent on calcium release from these intracellular stores (1). In the absence of extracellular calcium, there is a transient contraction that diminishes as calcium is removed from the cytosol by the cytosolic calcium transporters. In the presence of extracellular calcium,
Caffeine produces a more pronounced contraction that is augmented by recycling of calcium through the sarcoplasmic reticulum. The presence of endothelial cells may slightly diminish the contractile response due to basal release of endothelial relaxing factors.

**Protocol 2**

**Examination of endothelium-dependent dilation.** Six aortic segments are mounted in three tissue baths on stainless steel hooks with two segments (endothelium intact and denuded) in each tissue bath. These segments are equilibrated similarly to those in protocol 1, and the viability of segments and endothelium were determined as described above. After a viability and endothelium check, aortic rings in the three tissue baths are treated with inhibitors or vehicle for 30 min to demonstrate the mechanism of endothelium-derived nitric oxide-mediated relaxation of VSM.

One bath is treated with \(\text{N}^\text{G}\)-nitro-L-arginine (LNA; 100 \(\mu\)M), an NOS inhibitor, one with atropine (30 \(\mu\)M; muscarinic antagonist), and one with vehicle (ddH\(_2\)O). The tissue segments are all contracted with NE (0.1 \(\mu\)M), and contractile response is allowed to plateau. Then, without washing out the NE, increasing concentrations of the cholinergic agonist ACh are added to the bath (0.01 to 10 \(\mu\)M). After finishing the concentration response curve, the nitric oxide donor \(S\)-nitrosylacetylpenicillimine (SNAP, 1 \(\mu\)M) is added to the baths. Next, after the dilation reaches a plateau, the guanylyl cyclase inhibitor \(1H\)-[1,2,4]-oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ, 1 \(\mu\)M) is added to the
bath (10). Again, the final tension at the plateau is recorded.

Description of Responses

Relaxation in LNNA-treated aortic segments. In the bath treated with the NOS inhibitor, the response to NE is augmented compared with the response of segments treated with either atropine or vehicle. The augmented response is due to inhibition of basal nitric oxide production. The response should not be different between the endothelium-intact and -denuded segments in the presence of the NOS inhibitor. Furthermore, addition of ACh will not elicit relaxation in either segment in contrast to the initial check of viability when relaxation was observed in the endothelium-intact segment (Fig. 1). The addition of SNAP causes maximal relaxation in both segments that is reversed by the addition of the guanylyl cyclase inhibitor ODQ (Fig. 5). Because the contribution of nitric oxide should be eliminated by LNNA, the final contraction should be equivalent to the initial contraction to NE in both segments.

Relaxation in atropine-treated aortic segments. Atropine treatment will not affect NE contraction, and there should be a greater contraction in the endothelium-denuded segment compared with the endothelium-intact segment. Relaxation to ACh will be blocked by the muscarinic receptor antagonist. However, both denuded and intact segments will relax after addition of the nitric oxide donor SNAP. Addition of the guanylyl cyclase inhibitor ODQ restored contraction to pre-SNAP tension in the endothelium-denuded segment and above pre-SNAP tension in the endothelium-intact segment, demonstrating basal nitric oxide stimulation of guanylyl cyclase.

Relaxation in vehicle-treated aortic segments. NE contraction should be greater in the endothelium-denuded segment. ACh will elicit relaxation only in the endothelium-intact segment, whereas SNAP will relax both segments to baseline (Fig. 7). The addition of SNAP causes maximal relaxation in both segments that is reversed by the addition of the guanylyl cyclase inhibitor ODQ. This ODQ-mediated contraction should be augmented in the endothelium-intact segment compared with the contraction elicited by NE in the absence of ODQ. The contraction is augmented due to the loss of guanylyl cyclase stimulation by endothelium-derived nitric oxide.
of ODQ will restore the NE contraction with an augmented response in the endothelium-intact segment.

DISCUSSION AND SUMMARY

The laboratory exercise is designed to encourage thought and discussion about the mechanisms involved in vascular contraction and relaxation. Furthermore, students are provided with the opportunity to actively participate in generating data and interpreting results. This laboratory exercise complements any smooth muscle physiology lecture and encourages students to relate experimental results to lecture topics. The interaction of students during this exercise also allows students to generate discussions and share ideas about the mechanisms of intracellular regulation and endothelium regulation of vascular tone. Most students have very favorable comments on this exercise. Several comments that we have received include:

“Explaining what was going to happen first and then letting us experience it ourselves was a very effective and straightforward way of learning.”

“Participation of the whole group was an important aspect of the lab. Not only could I relate to the material visually, but I could also use the dialogue between myself and the other students as a supplemental way of grasping the material. I felt it was easy to consult with my peers as well as with a mentoring professor to clarify the material.”

“Being able to see the instantaneous response of the vasculature via the chart recording to various pharmacological agents was important in understanding how quickly and dramatically the contraction happens!”

STUDY GUIDE QUESTIONS

These questions are designed to be used during the laboratory session to stimulate discussion among the students. Written responses are turned in after the laboratory session.

**Question 1: Why must segments be stretched to detect "active tension generation?" Describe "passive tension" and the elements of the cell that contribute to it.**

Answer: The intermediate filaments of the cell make up the cytoskeleton of the smooth muscle cell and maintain the cell in an elongated shape under resting conditions. These filaments also anchor the contractile units (sarcomeres) to the cytoskeleton at the dense bands and dense bodies. Initial contraction of the thin and thick filaments are thought to stretch these intermediate, elastic filaments without generating cell shortening. This contraction cannot be observed experimentally unless the cells are first loaded with a passive force to fully stretch the intermediate elastic filaments (9).

**Question 2: Why must the strips be contracted first to evaluate the presence of endothelial cells?**

Answer: Relaxation of the smooth muscle cells cannot occur in the absence of active tone. The initial stretch provides passive tone; however, only increasing intracellular calcium above resting levels will generate active tone. Active tone is generated in this experiment by stimulating α-adrenergic receptors with NE (12).
Question 3: What agents caused a contraction in the absence of extracellular calcium? If the agent caused a contraction, was it dependent or independent on the presence of endothelium?

Answer: Only caffeine and NE contracted arteries in the absence of extracellular calcium. The presence of endothelium attenuated the contraction to the NE due to the release of nitric oxide from the endothelium.

Question 4: What do the results in protocol 1 indicate regarding the source of calcium for each of the three agents examined?

Answer: The results suggest that both NE and caffeine can stimulate calcium release from intracellular stores. The augmented contraction in every case by the addition of extracellular calcium indicates that all three contractile stimuli stimulate calcium entry into the VSM cells.

Question 5: What would have happened in each case if an antagonist to voltage-gated Ca\(^{2+}\) channels had been present?

Answer: A voltage-gated calcium-channel blocker would have prevented contraction to KCl and would have diminished contraction to NE and caffeine. The resulting contraction should have looked much like the one in zero calcium solution.

Question 6: What effect on ACh relaxation do you observe with each of the inhibitors, and how is it working?

Answer: The muscarinic antagonist atropine blocks relaxation by ACh. This is through competitive antagonism at the muscarinic receptors on endothelial cells. In these cells, there will be neither receptor activation nor an increase in intracellular calcium and NOS activation. LNNA (NOS inhibitor) blocks ACh-induced relaxation by competing with the endogenous NOS substrate L-arginine. In this case, ACh will bind to its receptor, activate G\(_{\alpha}\), and increase intracellular calcium. This will increase endothelial NOS affinity for substrate, but no nitric oxide will be produced because NOS will be bound to the inhibitor LNNA, which is in excess of the endogenous sub-

SUGGESTED READINGS

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