Targeting ryanodine receptors for relief from increased myogenic tone following subarachnoid hemorrhage

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Abstract

Vasospasm following subarachnoid hemorrhage (SAH) is a major contributor to poor outcome following cerebral aneurysm rupture. Research has suggested that a contributing factor to vasospasm following SAH is a decrease in calcium spark frequency due to increased inhibition of ryanodine receptors (RyRs) by FK-506 binding protein (FKBP 12.6). Calcium spark stimulation of large-conductance calcium activated potassium (BK) channels represents a prominent vasodilation pathway. The objective of this study was to investigate the proposed involvement of FKBP 12.6 in vasospasm by examining whether pharmacological agents that prevent interactions between FKBP 12.6 and RyRs cause significant dilation in arteries from SAH model animals. Isolated superior cerebellar arteries from control and SAH model rats were mounted on a pressure myography chamber and diameter changes were analyzed using edge detection software. Assessment of myogenic tone at 60 mmHg showed that SAH arteries experienced significantly more tone than control arteries. Paxilline was shown to cause significant constriction in control arteries but elicited little response in SAH arteries, suggesting decreased BK channel activity. Rapamycin and FK-506, two chemicals that form complexes with FKBP 12.6, were applied in order to increase the activity of RyRs and alleviate the increase in myogenic tone. At various concentrations, neither rapamycin nor FK-506 was able to produce a significant change in arterial diameter. In summary, this study was able to show physiological differences between SAH and control arteries related to myogenic tone and BK channel activity. However, the vasoconstriction of arteries following SAH could not be alleviated with rapamycin.

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is the result of cerebral artery aneurysm rupture and the ensuing release of blood onto the brain surface.[1] SAH is associated with poor patient outcomes: 10-15% of patients die before receiving medical attention; overall SAH has a 50% mortality rate.[2] SAH accounts for a disproportionally high loss of person-years compared to other types of strokes and approximately 33% of survivors are faced with the need for lifelong care.[1] It is thought that a primary cause of the poor outcomes is due to vasospasm, which presents itself approximately four days following SAH leading to overly constricted brain arteries.[3] Vasospasm results in decreased cerebral blood flow and a critical decrease in the supply of oxygen and other vital nutrients required to maintain proper brain function.[4] The global objective of the current research is to determine what properties differ between arteries from healthy individuals and arteries exposed to subarachnoid blood.

Myogenic tone is the constriction that arteries exhibit in response to an increase in intravascular pressure and is a critical component of blood flow autoregulation.[5, 6] Brain surface (pial) arteries from SAH model rabbits exhibit a greater level of constriction than do similar arteries from control animals.[7] Studies have also shown that ion channels on the plasma membrane of cerebral artery myocytes play an important role in vasospasm.[8] Voltage-gated potassium channels (K_V) are important for hyperpolarization and were found to be down regulated following SAH.[9] It has been shown that the blood component oxyhemoglobin causes suppression of K_V currents independent of changes in expression of these channels.[10, 11] Several different studies have shown that R-type calcium channels play a role in vasospasm as well.[12-14] The present project will focus on the impact of SAH on a particular ion channel, the large-conductance calcium activated potassium (BK) channel.

Using a rabbit SAH model, Koide et al. (2011) showed that Ca^{2+} spark-induced BK channel activity is significantly reduced following SAH. Ca^{2+} sparks represent local, transient intracellular Ca^{2+} release events caused by the brief activation of a cluster of ryanodine-sensitive Ca^{2+} channels (ryanodine receptors or Ryrs) found on the sarcoplasmic reticulum.[15] This pathway is illustrated in figure 1. In healthy arteries, Ca^{2+} sparks occur in close proximity to the plasma membrane. This leads to the efflux of potassium ions through BK channels resulting in membrane potential hyperpolarization. A more negative membrane potential leads to decreased activity of the voltage dependent calcium channels (VDCC), resulting in vasodilation.[4] Following SAH, our laboratory has demonstrated a marked decrease in Ca^{2+} frequency and BK channel activity resulting in pathological vasoconstriction.

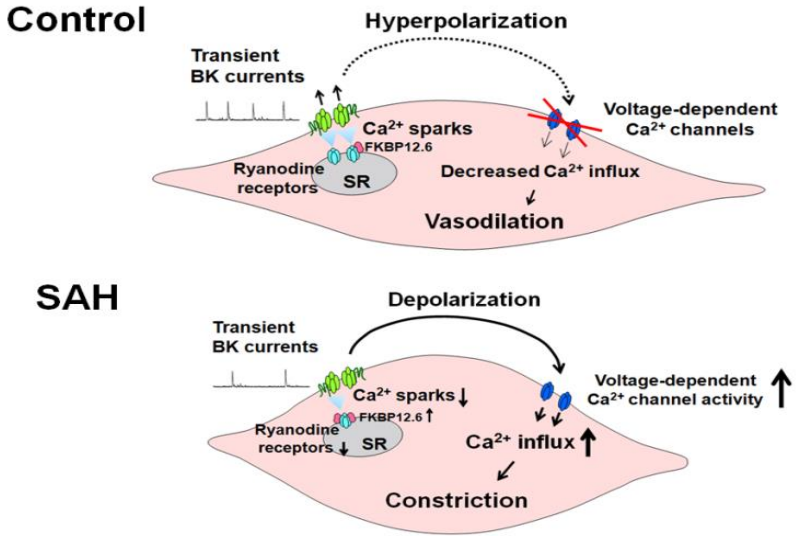

Figure 1. **Ca^{2+} spark/BK channel pathway in a healthy (control) artery and SAH artery**. Ca^{2+} is stored in the sarcoplasmic reticulum (SR) and is released by the ryanodine receptors as a calcium spark. The localized Ca^{2+} spark activates large conductance calcium activated potassium (BK) channels. The efflux of potassium ions causes an overall membrane hyperpolarization that decreases the open-state probability of voltage-dependent Ca^{2+} channels (VDCC). When VDCC are inactive the influx of Ca^{2+} is reduced leading to vasodilation of the artery. FK-506 binding protein (FKBP12.6) bound to the Ryanodine receptors regulates the Ca^{2+} spark activity. SAH model arteries have a greater concentration of FKBP12.6 and therefore a greater amount of FKBP12.6 bound to Ryrs. Increased binding of FKBP12.6 leads to reduced Ca^{2+} spark activity and reduced BK channel activity and a greater level of arterial constriction.

FK-506 binding protein (FKBP) 12.6 is a protein that regulates ryanodine receptors. Ryrs are held in a closed conformation when FKBP12.6 is bound, resulting in decreased calcium spark activity.[4] Thus, when more FKBP12.6 is present and bound to Ryrs there are fewer Ca^{2+} sparks.[16] Koide et al. (2011)[4] found that FKBP12.6 is upregulated following SAH. This was shown to decrease the frequency of calcium sparks and therefore K^+ efflux through the BK channels, leading to enhanced constriction. We believe that reducing FKBP12.6 binding to the ryanodine receptors is a possible target in the treatment of vasospasm following SAH. Briefly, removing FKBP12.6 from the Ryrs would enhance Ca^{2+} spark activity and promote vasodilation mediated via BK channels. Rapamycin and FK-506, both immunosuppressant drugs, were shown to specifically bind to FKBP12.6 in heart myocytes reducing FKBP12.6/Ryr interaction leading to an increase Ca^{2+} spark frequency.[16] Based on these observations, we hypothesized that the administration of rapamycin and FK-506 to SAH arteries would reduce the level of myogenic constriction seen (i.e. induce vasodilation).

Materials and Methods

Rat Subarachnoid Hemorrhage Model

Full details of the rat SAH model may be found in Nystoriak et al. (2010).[17] In short, autologous unheparinized blood (0.5 ml) drawn from the tail artery was injected into the cisterna magna, on day 0, with a 25-gauge butterfly needle. The animal was then positioned on an incline board at a 45° angle with the head down for 30 min. Twenty-four hours later, a second injection of blood was delivered by repeating the above procedure. Buprenorphine (0.01 mg/kg) was given every 12 h (for 36 h, then as needed) as an analgesic. On day 4, animals were euthanized under pentobarbital anesthesia followed by decapitation. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Pub. No.85-23,

revised 1996) and follow protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont (IACUC NO. 10-032).

Diameter Measurements in Isolated Arteries

These procedures were previously described by Koide et al. (2011).[4] Freshly isolated superior cerebellar arteries from control and SAH rats were cannulated in a 5 mL myograph chamber (Living Systems Instrumentation, Inc., Burlington, VT, USA) and perfused with PSS (pH 7.4) aerated with 20% O_2/5% CO_2/75% N_2 at 37°C. The arterial diameter was measured using video edge detection equipment and recorded using data acquisition software (Dataq Instruments, Inc., Akron, OH, USA). Arteries were discarded if an initial constriction representing less than 50% decrease in diameter was observed when arteries are exposed to elevated extracellular K^+ (60 mmol/L). The fully dilated (passive) diameter was determined at the end of each experiment by exposing the arteries to Ca^{2+} free aCSF (artificial cerebrospinal fluid) containing the vasodilators diltiazem (100 µmol/L) and forskolin (1 µmol/L). Myogenic tone is presented as a percent decrease from the passive diameter. Arterial changes to FK506, Rapamycin, and Paxilline are expressed as a percentage change in diameter from the basal level of pressure-induced myogenic tone at an intravascular pressure of 60 mmHg.

Statistical Analysis

Data are expressed as mean ± s.e.m. and analyzed by Student's unpaired **t**-test or one-way ANOVA (analysis of variance), followed by Tukey's multiple comparison test. Statistical significance will be considered at the level of $\mathbf{P} < 0.05$ (*) or $\mathbf{P} < 0.01$ (**).

Results

Increased Myogenic Tone Following Subarachnoid Hemorrhage

The initial comparison between isolated cerebellar arteries from control and SAH rats evaluated the level of myogenic tone at 60mmHg (figure 2). Increased myogenic tone has previously been shown in both pial arteries from rabbits and parenchymal arterioles from rat.[5, 17] Here, similar results were seen in rat pial arteries; arteries after SAH exhibited significantly more myogenic tone (26.03% ± 7.0, n=14) than control arteries (20.38% ± 5.4, n=15).

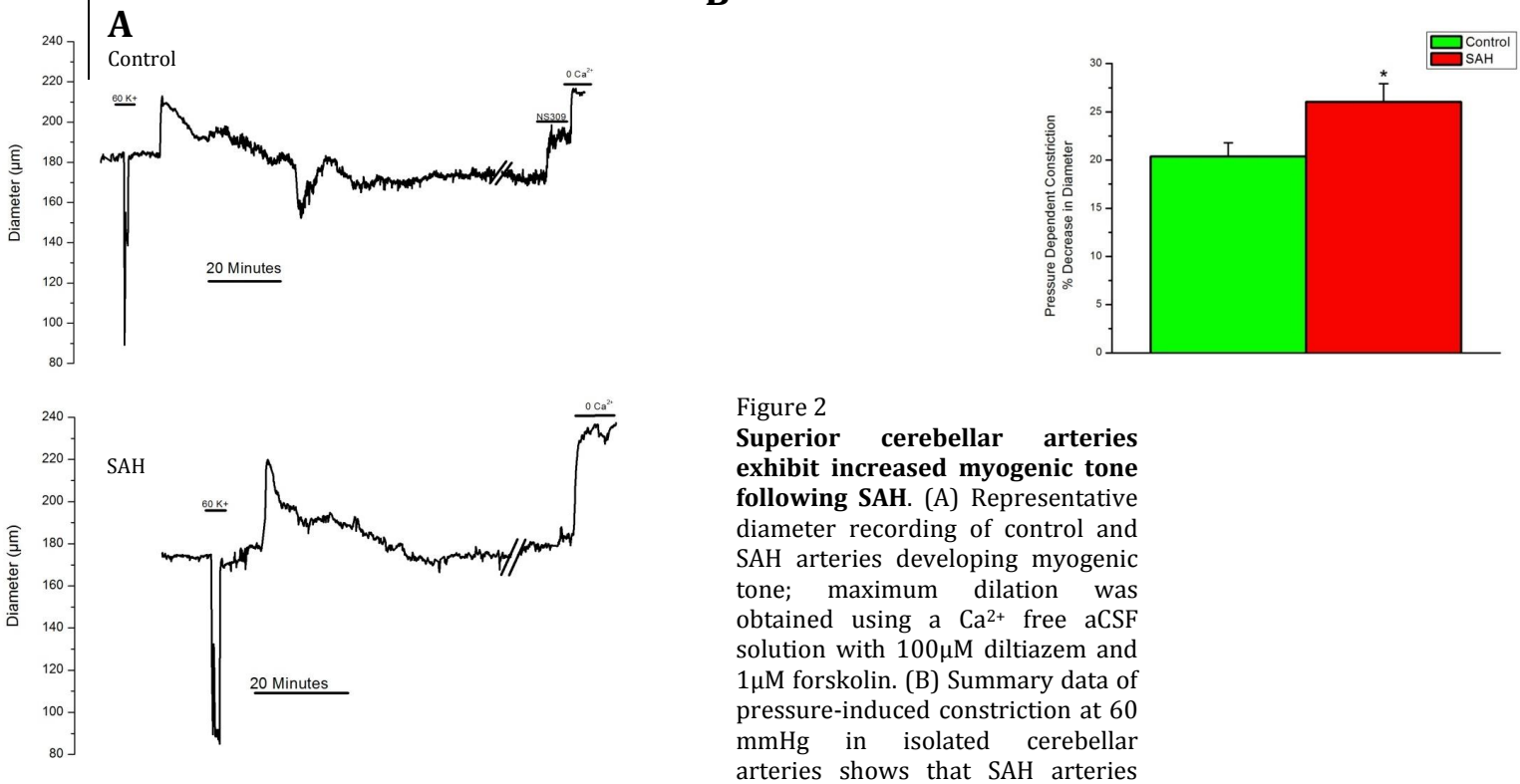

Figure 2
Superior cerebellar arteries exhibit increased myogenic tone following SAH. (A) Representative diameter recording of control and SAH arteries developing myogenic tone; maximum dilation was obtained using a Ca^{2+} free aCSF solution with 100µM diltiazem and 1µM forskolin. (B) Summary data of pressure-induced constriction at 60 mmHg in isolated cerebellar arteries shows that SAH arteries experience more myogenic tone than control arteries ($p < 0.05$).

Paxilline Induces Constriction in Control Arteries but not SAH Arteries

The proposed pathway leading to the enhanced myogenic tone in SAH arteries implicates a down regulation of BK channels. To assess BK channel function, paxilline, a BK channel blocker, was applied to the isolated arteries at 60 mmHg. Paxilline (1 μM) had differing effects on SAH arteries compared to control arteries (figure 3). In the arteries from control animals, paxilline caused an average of 9.46% ± 5.6 constriction (n=10). This represents a change in myogenic tone from 20.38% ± 5.4 to 27.73% ± 5.4 following the application of paxilline. However, in similar arteries from SAH animals, paxilline did not significantly change arterial diameter; SAH arteries experienced an average change in diameter of 0.04% ± 5.7 (n=6).

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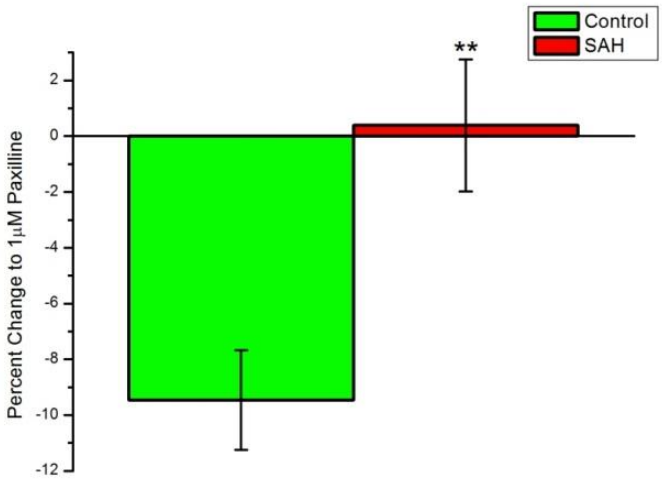


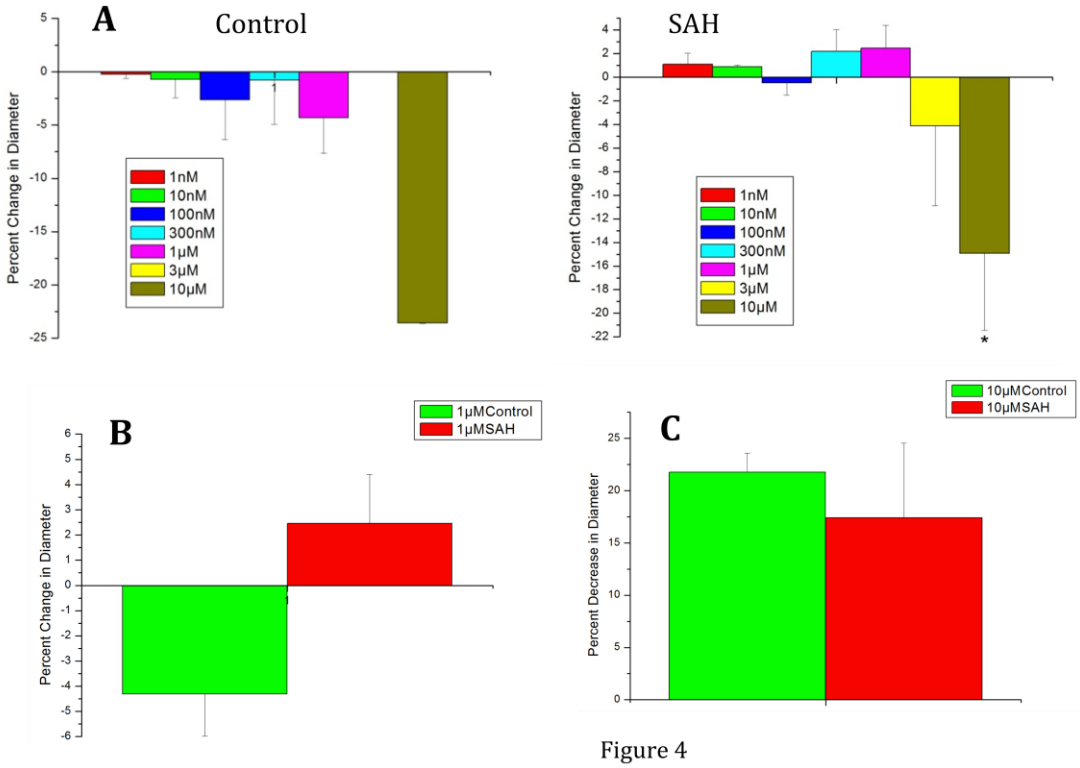
Figure 3.
Paxilline, a BK channel blocker, constricts arteries from control, but not SAH model animals. Summary of data on the effect of paxilline on isolated arteries from control and SAH animals. Data show that control arteries experience significantly more constriction to 1 μM paxilline compared to SAH arteries, which exhibit little change.

Targeting Ryanodine Receptors with Rapamycin

Previous research has shown that BK channels maintain their expression and properties following SAH suggesting that differences in BK channel activity are from other sources.[4] Therefore the ryanodine receptors were targeted in an attempt to relieve the

increased myogenic tone that was seen. Various concentrations of rapamycin and FK-506 were administered to isolated control and SAH arteries. Initially, trials using both rapamycin and FK-506 were pursued to assess whether the two similar drugs would have similar or varying effects. After progress was made using both drugs, it was determined that rapamycin had a generally more consistent effect than FK-506 and was therefore the sole focus of this investigation. All data shown is from experiments using rapamycin (figure 4).

Initial concentrations used ranged from 1nM to 10μM, with a 10 fold increase in concentration at each step. This wide range of concentrations was used to identify the approximate concentration at which the drugs were most effective. Data showed that the lowest concentrations of 1nM and 10nM did not have significant effects so half log steps were added at 300nM and 3μM to help determine a maximum effective concentration. Figure 4a shows summary data in control and SAH arteries along the spectrum of concentrations used. In general, SAH arteries showed dilation in response to rapamycin exposure while control arteries showed constriction. This is shown further in figure 4b where responses to 1μM rapamycin are compared. In SAH arteries, 1μM was the concentration that showed the greatest average level of dilation, however this response did not reach statistical significance. In both control and SAH arteries, the only concentration that had a significant impact on the atrial diameter was 10 μM, where significant constriction was seen (4c).



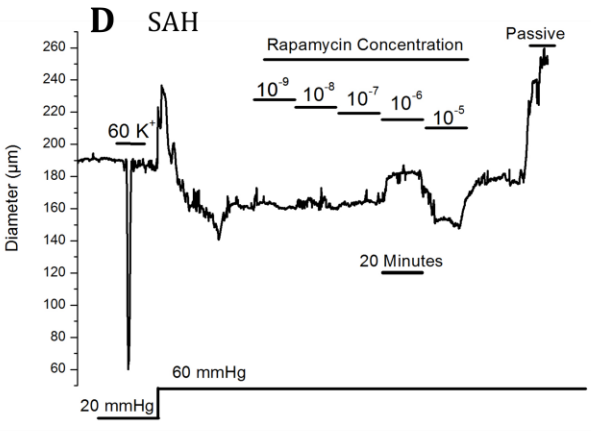


Figure 4

Summary data of the effects of rapamycin on isolated control and SAH arteries at 60 mmHg. (a) Concentrations of rapamycin ranged from 1nM to 10μM. No statistically significant variations in the diameter were seen except at 10 μM. At other concentrations control arteries tended towards slight constriction with rapamycin whereas SAH arteries tended towards slight dilation; (b) comparison of effects of 1μM rapamycin in control and SAH arteries (n=9) shows that SAH arteries tended to dilate, control arteries (n=4) tended to constrict; (c) comparison of effects of 10μM rapamycin on SAH (n=7) and control arteries (n=4) shows that both sets of arteries experience significant constriction; (d) representative trace of the development of myogenic tone, application of rapamycin, and determination of passive diameter.

Discussion

This study was able to confirm fundamental changes in the physiology of rat pial arteries following SAH, therefore providing further support for prior research.[4, 5, 17] Following SAH it was shown that myogenic tone is significantly increased. Myogenic tone is a fundamental property of pial arteries that allow for the autoregulation of cerebral blood flow. Enhanced myogenic tone, or vasospasm, is a hallmark of SAH and is one of the leading causes of poor outcome following a subarachnoid hemorrhage. The onset of vasospasm is a complex phenomenon that has been linked to numerous intracellular targets and membrane channels including oxyhemoglobin, protein kinase C, Ryrs, K_V channels, R-type calcium channels, and BK channels.[4, 5, 10-14] This study specifically focused on changes to the activity of ryanodine receptors and BK channels and how these relate to the onset of vasospasm.

The BK channel blocker, paxilline, was used to assess the function of BK channels which are directly associated with Ca^{2+} sparks from the ryanodine receptors. The results from the application of 1 μM paxilline show that BK channel function is altered in the SAH arteries compared to the control arteries. In arteries from healthy rats, paxilline caused significant constriction by limiting the outward flow of potassium ions as is shown in figure 1; this result is to be expected with control arteries. However, in arteries from SAH animals, paxilline had minimal affect. The discrepancy in the effect of paxilline suggests that there is a decrease in potassium efflux occurring in the diseased arteries. There could be several reasons for this decrease but prior research has suggested that an increased in FKBP12.6 on the ryanodine receptors is responsible.[4] Koide et. al (2011) discredited other possible mechanisms for decreased BK channel function such as altered BK channel properties and

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a decrease in Ca^{2+} spark amplitude following SAH in a rabbit model. The increase in FKBP12.6 on the ryanodine receptor would cause a decrease in calcium spark frequency and therefore lead to vasoconstriction.

This hypothesis was tested by applying rapamycin or FK-506, pharmaceuticals that bind to FKBP12.6, to the isolated arteries and observing the affects. It was anticipated that application of rapamycin or FK-506 would release the inhibition on the ryanodine receptors by forming a complex with FKBP 12.6. Removing FKBP 12.6 from the RyRs should increase the frequency of the calcium sparks, and lead to vasodilation in the SAH arteries. In the control arteries, slight dilation was expected due to the fact that there should only be minimal inhibition on the ryanodine receptors by FKBP 12.6. As previously described FK-506 was used initially but was eschewed in later experiments in favor of focusing on rapamycin.

Figure 4a shows data for control (left) and SAH (right) arteries following administration of rapamycin at 60 mmHg. Changes in arterial diameter are described based on the percent of change seen compared to initial myogenic tone at 60 mmHg. In the control arteries none of the concentrations caused significant changes to diameter compared to initial levels of myogenic tone except for the highest concentration of 10μM. The robust constriction at 10μM may have been caused by widespread Ryr activation resulting in a large efflux of Ca^{2+} from the sarcoplasmic reticulum. The proposed excessive discharge of calcium is different than the usual local calcium spark events that lead to the activation of the BK channels. Experimental substantiation of the large constriction being a global calcium effect is beyond the scope of this experiment, however evidence could be provided by: (a) blocking RyRs with ryanodine to prevent Ca^{2+} from exiting the SR or (b)

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depleting the SR of all Ca^{2+} before adding 10μM rapamycin. The SAH artery data from figure 4a shows 30nM and 1μM rapamycin caused the greatest average dilation but was not a statistically significant change from initial myogenic tone diameters. As with the control arteries, the only concentration of rapamycin to cause a significant change in diameter was 10 μM.

Although there were no significant dilations seen at any concentration, there were noticeable differences to how control and SAH arteries reacted to rapamycin. This is highlighted in figure 4b, which compares arterial responses to 1μM rapamycin. Data suggests that SAH arteries typically experience slight dilation to this concentration whereas control arteries show a tendency to constrict. Despite the fact that responses to 1μM rapamycin are not statistically significant compared to initial levels of myogenic tone, statistical power analysis suggests the difference between the response of SAH and control arteries is significant. Coupled with dissimilarity in BK channel activity, this suggests that there may be physiological changes to Ca^{2+} spark activity following SAH. However, this could not be explicitly shown through experiments attempting to reduce myogenic tone following SAH by restoring Ca^{2+} activity with rapamycin.

An explanation for why rapamycin was not able to cause significant dilation as hypothesized could be due to the fact that rapamycin has many intracellular targets that could affect the artery. An optimal concentration for vasodilation could be masked by various off-target effects. Additionally, a larger sample size may be necessary to highlight any changes seen or a longer exposure to rapamycin may be needed to allow the chemical to enter the cell and have a full effect. Typical administration of rapamycin lasted between 20 and 25 minutes before returning to an aCSF wash. Future work with isolated arteries

may benefit from administration between 45 and 60 minutes. In addition to continued arterial measurements with pressure myography, alternative techniques could be used to investigate the proposed pathway in the rat SAH model. These other techniques could include direct measurement of Ca^{2+} spark activity, measurement of BK channel activity by patch clamp electrophysiology, or PCR analysis of Ryr/FKBP12.6 levels.

In conclusion, the shown data do not provide direct evidence to support the hypothesis that rapamycin or FK-506 would be able to help lessen the increased myogenic tone following SAH in a rat model. However, it was found that myogenic tone was significantly increased and BK channel activity was significantly decreased following SAH. Rapamycin was not able to significantly lessen the myogenic tone seen in the arteries as was hypothesized. Regardless, this study and prior research provide evidence that the calcium spark and BK channel pathway could present a possible target in the treatment of vasospasm following to SAH.

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