Suppression Of Voltage-Gated Potassium Channels In Brain Parenchymal Arterioles: A Potential Role For Protein Kinase C Activation

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−Subarachnoid hemorrhage (SAH) caused by aneurysm rupture is often associated with decreased cerebral blood flow and the development of severe neuronal deficits in patients who survive. Persistent intracerebral (parenchymal) artery constriction after hemorrhage contributes to morbidity. This constriction may be due in part to suppression of voltage-gated potassium (K_V) channels causing membrane potential depolarization, enhanced Ca^{2+} influx and vasoconstriction. Previous studies have shown that protein kinase C (PKC) is activated following SAH and that K_V currents are suppressed in myocytes of larger diameter cerebral arteries from SAH model animals. Here, we examined PKC involvement in SAH-induced K_V current suppression in parenchymal arterioles using the conventional whole cell patch clamp technique. We found that K_V currents were suppressed in myocytes obtained from SAH model rats compared to cells obtained from control animals. We also observed that the PKC activator 1,2-dioctanoyl-glycerol suppressed K_V currents in control but not SAH myocytes, indicating PKC may be maximally active following SAH. PKC inhibitor chelerythrine did not have a significant effect on K_V channel activity in control or SAH myocytes. In summary, our data are consistent with increased PKC activity following SAH and contributes to suppression of K_V channels.

vascular smooth muscle; protein kinase c; voltage gated potassium channel; subarachnoid hemorrhage

ANEURYSMAL SUBARACHNOID HEMORRHAGE (SAH) is rupture of cerebral arteries leading to bleeding onto the brain surface. SAH accounts for 7% of all strokes, afflicting roughly 30,000 people in the United States annually (4, 8). Release of blood into the subarachnoid space from cerebral arteries leads to a sudden increase in intracranial pressure, which is responsible for significant mortality in patients prior to reaching a hospital. SAH is marked by very high mortality rates as 45% of individuals suffering from aneurysm rupture die within 30 days from the initial increase in intracranial pressure or from subsequent rebleeds at the rupture site (6). Survivors are typically treated surgically to decrease risk of rebleeding at the site of rupture (37). However, in these survivors, up to 50% suffer from a syndrome of delayed ischemic neurological defects beginning roughly 4 days after the initial bleed (37, 43). These deficits are the leading cause of death and disability in survivors of the initial insult (17). Cerebral artery constriction, called vasospasm, is thought to be responsible for the delayed morbidity. Despite aggressive intervention, vasospasm remains difficult to effectively treat (7).

Parenchymal arterioles play a critical role in supplying nutrients to the brain cortex. Following SAH, constriction of parenchymal arterioles reduces nutrient delivery, a dangerous situation due to the lack of collateral blood supply in the cortex. There is increasing evidence that vasospasm of parenchymal arterioles contributes to the morbidity and mortality after SAH (33, 44, 46). In the 20th century, the prevailing thought was the delayed deficits from SAH were a result of large cerebral conduit (pial) artery vasospasm. However, the recent CONSCIOUS 1 and 2 clinical trials showed that while clazosentan, an endothelin-1 receptor antagonist, significantly decreased large artery constriction, it did not relieve SAH symptoms suggesting large artery vasospasm is not the major contributor to delayed deficits (23, 24). These and other findings suggest that dysfunction of parenchymal arterioles may be playing a more significant role in

these delayed neurological deficits. Recent evidence in rabbit parenchymal arterioles suggests parenchymal arteriole myocytes may be more sensitive to oxyhemoglobin, a component of blood, than pial artery myocytes, suggesting they may be more responsive to SAH (21).

Voltage gated potassium (K_V) channels are a large family of distinct channels of several different subtypes. In smooth muscle, K_V1 and K_V2 have been observed (3, 14, 42). K_V channels regulate parenchymal arteriolar tone by controlling smooth muscle membrane potential (27). Normally, K_V channels open in response to membrane depolarization, allowing potassium (K^+) ions to flow out of the cell down their electro-chemical gradient. Decreased K_V channel activity promotes membrane potential depolarization, which results an increase in the open state probability of voltage dependent calcium channel (VDCC) and increased intracellular calcium ion (Ca^{2+}) concentration. Increased intracellular Ca^{2+} promotes contraction and vasoconstriction (19). Koide et al. in 2013 have showed K_V1 suppression in parenchymal arterioles following SAH is due to activation of the HB-EGF/EGFR pathway (21). However, activation of this pathway in control myocytes does not suppress the K_V current to the extent observed in SAH myocytes indicating other mechanisms may contribute to K_V current suppression after SAH.

The protein kinase C family is a group of serine/threonine kinases comprising at least 10 subtypes that regulate many different cellular effector responses from signals originating at the plasma membrane (5, 41). In smooth muscle, PKC has been shown to be involved in regulating contraction via phosphorylation of various effectors (22). Activation of PKC after SAH has been implicated in contributing to vasospasm in pial arteries (25, 29, 30). In addition, the concentration of diacylglycerol (DAG) an endogenous activator of PKC has been shown to be elevated in pial arteries 4-7 days following SAH (35). Koide et al. in 2007 demonstrated the HB-EGF/EGFR pathway responsible for suppression of K_V1 channel activity in rabbit cerebral

arteries following SAH is independent from PKC, and that addition of a PKC activator further suppressed K_V current after treatment EGF treatment (20). The role of PKC in parenchymal arterioles following SAH has not been determined.

Here we examined the effect of PKC activators and inhibitors on K_V current in parenchymal myocytes in control and SAH model rats. We found that the PKC activator 1,2-dioctanoyl-glycerol (DOG) significantly suppressed K_V currents in control but not SAH myocytes, indicating PKC may be maximally active following SAH. The PKC inhibitor chelerythrine had no significant effect on K_V current in control or SAH myocytes. The inability of PKC inhibition to increase K_V current may be a result of the downstream target of PKC already being phosphorylated. These data suggest PKC activity is increased following SAH and contributes to suppression of K_V channels.

MATERIALS AND METHODS

SAH Model. All experiments used 10-12 week old male Sprague-Dawley rats. SAH model rats used in this study are described by Nystoriak et al (2010) (32). Briefly, autologous unheparinized blood (0.5 ml) drawn from the tail artery was injected into the cisterna magna under isofluorane anesthesia. 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 36h, then as needed) as an analgesic. Animals were euthanized via pentobarbital (60mg/kg) and decapitated four days after the initial blood injection into the cisterna magna (32). 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 followed protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont.

Tissue Preparation. Parenchymal arterioles were dissected from rats euthanized the same day. Following decapitation, rat brains were kept in cold artificial cerebral spinal fluid containing (in mM) 125 NaCl, 3 KCl, 18 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, and 5 glucose, bubbled with 5% CO_2, 20% O_2 and 75% N_2 (pH 7.40) during dissection. A wedge was cut around the middle cerebral artery (MCA). The wedge tissue was agitated, and the MCA was carefully peeled from the cortex. Parenchymal arterioles branching from the MCA were extracted out of the cortex along with the MCA. Parenchymal arterioles were cut from the MCA and tied in bundles for digestion.

Isolation of Single Arteriolar Myocytes. Dissected parenchymal arterioles were enzymatically dissociated into single arteriolar myocytes. Arterioles were incubated in papain solution (0.3 mg/mL papain and 0.7 mg/mL 1,4-dithioerythriol (DTE)) in Ca^{2+} free glutamate isolation solution (GIS) that contains (in mM) 55 NaCl, 5.6 KCl, 80 L-glutamic acid, 2.0 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.3)) at 37° C for 17 minutes, followed by incubation in collagenase solution (0.7 mg/mL collagenase F and 0.3 mg/mL collagenase H in GIS containing 100 μM Ca^{2+}) at 37° C for 10 minutes. Arterioles were then washed in GIS containing 100 μM Ca^{2+} three times for 10 minutes each on ice. Arterioles were then gently triturated using a fire-polished Pasteur pipette to release individual myocytes (20).

Measurement of Whole Cell K^+ Currents. Outward whole cell K^+ currents were measured using the conventional whole cell configuration of the patch clamp technique (Figure 1A). The bath solution contained (in mM) 134 NaCl, 6 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 Glucose and 10 HEPES (pH 7.4). All electrophysiology experiments were conducted in the presence of the large conductance Ca^{2+} activated K^+ (BK) channel blocker paxilline (1μM). Micropipettes (5–10 MΩ) were filled with an internal pipette solution (in mM) 87 K^+ aspartate, 20 KCl, 1 $CaCl_2$, 1 $MgCl_2$,

10 HEPES, 10 EGTA, and 25 KOH (pH 7.2). Outward K^+ currents were elicited by a series of 10 mV depolarizing steps to +50 mV from a holding potential of -70 mV and were recorded using an Axon Instruments Axopatch 200B amplifier and Clampex software (20) (Figure 1B). Measurements were obtained before and after 10 minutes of exposure to either bath solution (control), 1μM 1,2-dioctanoyl-sn-g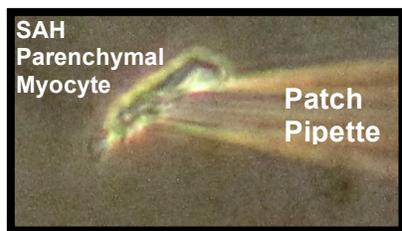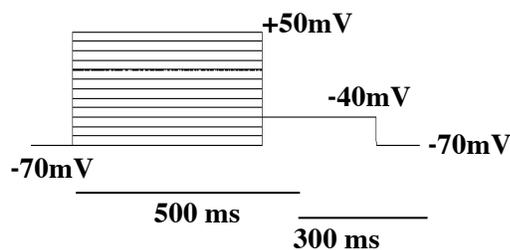lycerol (DOG) in bath solution, or 1μM chelerythrine in bath solution. To normalize for changes in cell size between myocytes, whole cell current density was calculated by dividing membrane current by cell capacitance for each cell.

Drugs. DOG was purchased from Caymen Chemical (Ann Arbor, MI). All other compounds were purchased from Sigma (St. Louis, MO).

Statistics. All data were analyzed using Student's *t*-test (paired for drug treatments, unpaired for comparison between SAH and control). Analysis was performed using pClamp Clampfit electrophysiology software and Origin 6.0 statistical software. Statistical significance was considered at the level of $P < 0.05$.

A

B

Figure 1. The whole cell patch clamp technique was used to measure K_V current. A: Representative photograph of a parenchymal myocyte from SAH rat with patch clamp pipette B: Patch clamp protocol: Outward K^+ currents were elicited via 10 mV depolarizing steps from to +50 mV from a holding potential of -70 mV. Protocol was repeated 13 times with a 10 second lag period, with each successive cycle having a depolarization of 10 mV higher than the previous.

RESULTS

K_V current is significantly decreased in SAH parenchymal myocytes. Initial experiments were designed to compare K_V currents in control and SAH parenchymal myocytes. Using the

whole cell patch clamp technique, outward K^+ currents were elicited by a series of 10 mV depolarization steps from -70 mV to +50 mV. To minimize contamination from other outward potassium channels, all experiments were conducted with 1 μM paxilline in the bath solution, which blocks BK channels (21). Significantly lower current density was observed in SAH myocytes as compared to controls (Figure 2). These data are consistent with prior data suggesting K_V current in SAH myocytes is suppressed as compared to control (14).

K_V currents do not significantly decrease over a period of 10 minutes. Prior to drug treatment, we performed time control experiments. Since both DOG and chelerythrine require a 10-minute
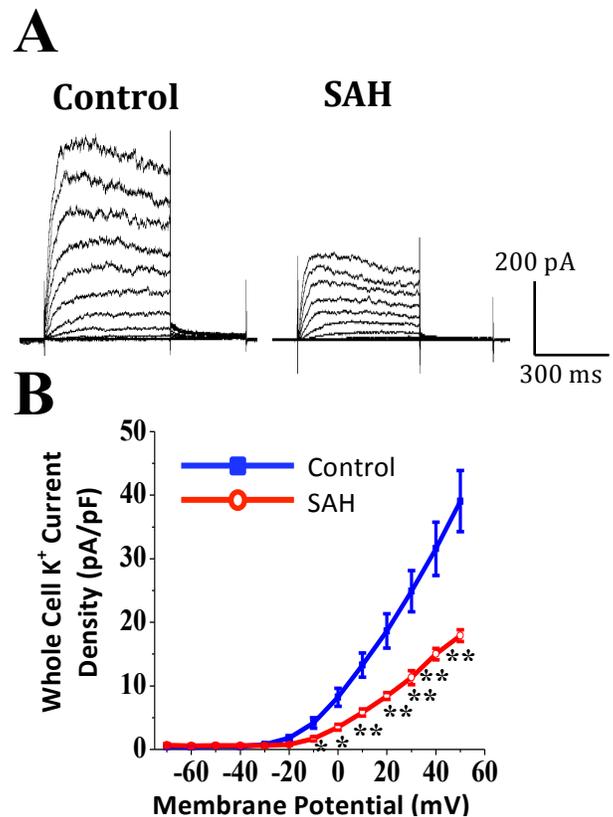
Figure 2. Whole cell K_V currents in parenchymal arteriolar myocytes from control and SAH animals. A: Representative traces of K^+ currents in control and SAH myocytes. B: Summary of current density in SAH and control myocytes, currents elicited by a series of 10 mV depolarizing steps to +50 mV from a holding potential of -70 mV. n=8 cells from 7 animals for control, 6 cells from 3 animals for SAH. * P < 0.05, ** P < 0.01 vs. control.

incubation, time control experiments were designed to determine if K_V currents decreased over a period of 10 minutes. Control and SAH myocytes were treated with bath solution alone. Recordings were obtained before and after a 10-minute incubation in bath solution. K_V currents did not significantly decrease over the 10 minutes in either control or SAH myocytes (Figure 3). These data establish that in the absence of drug treatment, K_V currents do not significantly change over the period of time in which myocytes were incubated with drugs.
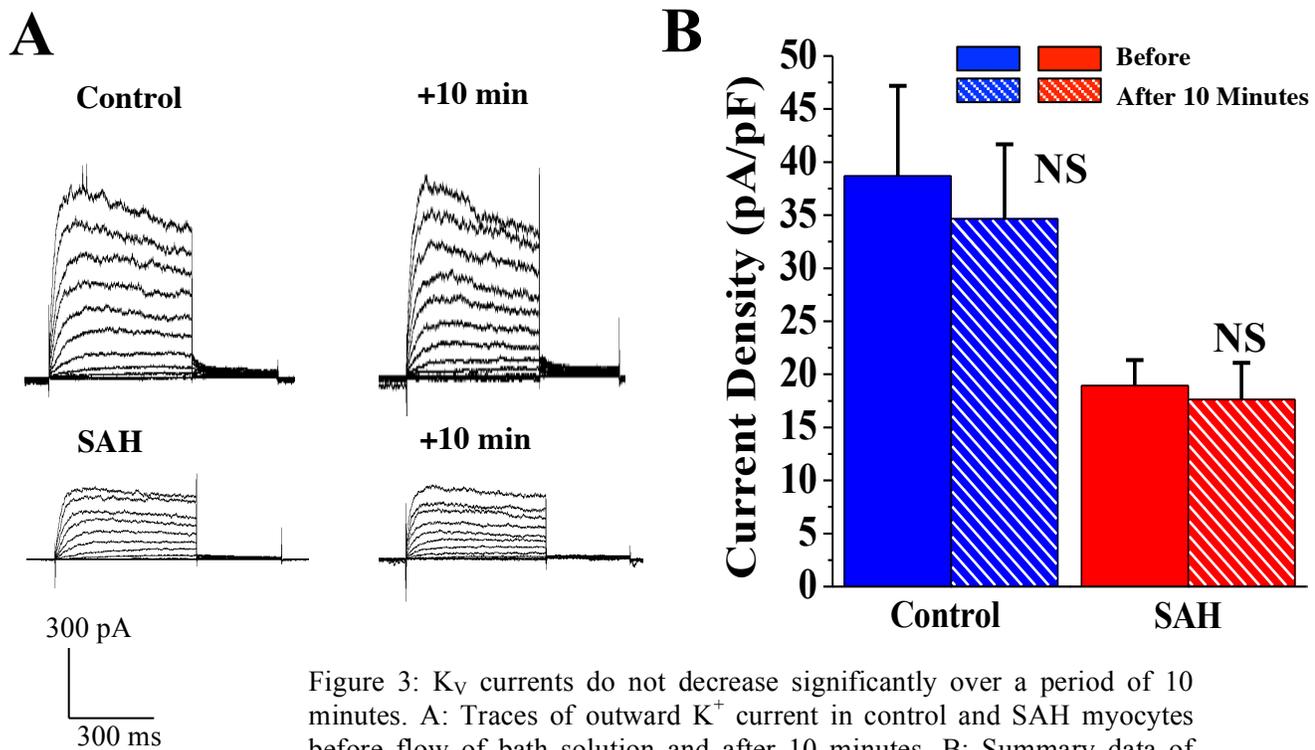
A

B

Figure 3: K_V currents do not decrease significantly over a period of 10 minutes. A: Traces of outward K^+ current in control and SAH myocytes before flow of bath solution and after 10 minutes. B: Summary data of current densities (at +50 mV) for time control experiments. K_V currents do not significantly diminish after 10 minutes. Control: n=5 cells from 5 animals. SAH: n=4 cells from 4 animals.

PKC activator 1,2-dioctanoyl-sn-glycerol (DOG) suppresses K_V currents in control but not SAH myocytes. To examine the impact of PKC activation on K_V currents, we examined the effect of DOG on K_V currents in SAH and control parenchymal myocytes. DOG is an analog of an endogenous PKC activator diacylgylcerol and a competitive agonist of conventional and novel PKC isoforms (9, 15). Previous work by others has indicated that active PKC suppresses K_V currents in vascular smooth muscle (1, 11). In SAH parenchymal myocytes, we hypothesized DOG would not cause further K_V current suppression if PKC was already active. In control myocytes, addition of DOG could activate PKC leading to a decrease in K_V currents. DOG caused a significant decrease in K_V currents in control parenchymal myocytes, from 35.4 ± 2.4 to 25.0 ± 0.98 pA/pF at +50 mV (Figure 4A, 4B). This suppression of K_V currents by DOG is

consistent with data reported by Koide et al in rabbit cerebral artery myocytes (20). In SAH myocytes, DOG did not cause a significant decrease in K_V current density (18.6 ± 1.4 to 16.0 ± 0.5 pA/pF at +50 mV). These data are consistent with our hypothesis and indicate that PKC may already be maximally active in myocytes from SAH parenchymal arterioles such that DOG is unable to further activate PKC. In control myocytes, PKC is not maximally active, so addition of DOG is able to elicit a decrease in K_V current.

PKC inhibitor chelerythrine does not significantly alter K_V current in control or SAH myocytes. To determine the effect of inhibiting PKC, we then applied chelerythrine to control and SAH myocytes. Chelerythrine is a selective, competitive inhibitor of PKC (12). Multipe studies have demonstrated that the suppression of K_V currents by DOG was abolished when vessels were treated in concert with chelerythrine (1). We hypothesized that chelerythrine would have no effect on K_V current in control myocytes, whereas in SAH cells it might rescue K_V current such that it increased after 10 minutes. An alternative hypothesis suggested chelerythrine would not increase K_V current in SAH animals, because as the channel or downstream effector from PKC is already phosphorylated, thus blocking PKC at this point would have no effect. We applied chelerythrine to control and SAH cells. Chelerythrine did not significantly alter K_V currents in either control or SAH myocytes (Figure 4C, 4D). These data suggest inhibiting PKC in isolated smooth muscle cells does not alter K_V channels, either because PKC was not active or because the pathway to inhibiting K_V channels had already been initiated.

DISCUSSION

This study used the whole cell configuration of the patch clamp technique to investigate the effect of activating and inhibiting PKC on K_V currents in control and SAH myocytes. Our
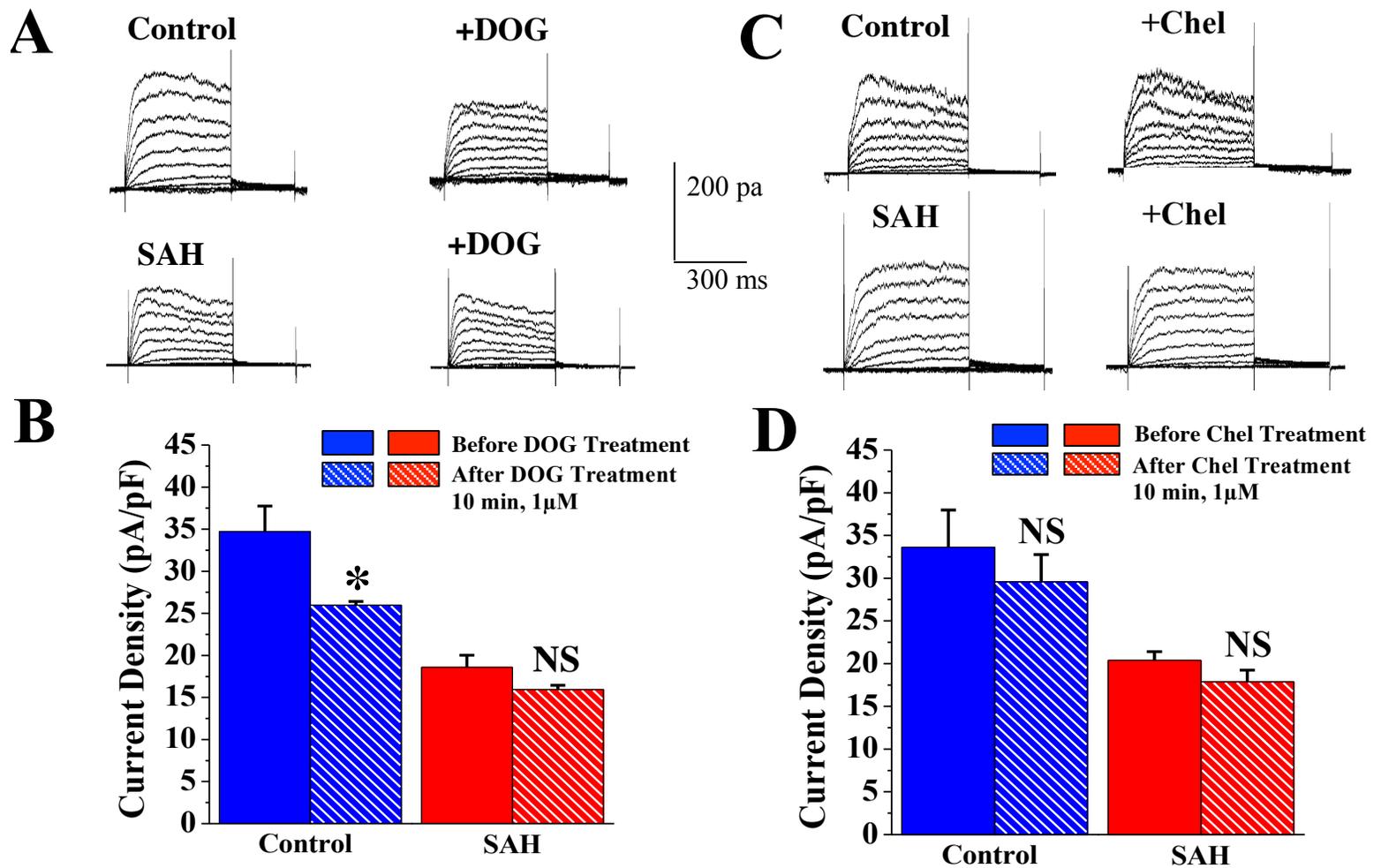
Figure 4: PKC activator DOG induced K$_V$ current suppression in parenchymal arteriolar myocytes from control but not SAH animals and PKC inhibitor chelerythrine did not significantly change current in control or SAH myocytes. A: Traces of outward K$^+$ currents in control and SAH myocytes before and after addition of PKC activator, DOG (1 μM, 10 min). B: Summary data of current densities (at + 50 mV) before and after DOG treatment. DOG decreased currents in control, but not SAH, myocytes. Control: n=5 cells from 5 animals; SAH: n=4 cells from 3 animals. C. Traces of outward K$^+$ currents in control and SAH myocytes before and after adittion of PKC inhibitor chelerythrine (1μM, 10 min). D. Summary data of current densities (at +50mV) before and after chelerythrine treatment. Chelerythrine did not significantly chance outward K$^+$ currents in either control or SAH myocytes. Control: n=4 cells from 4 animals; SAH: n=5 cells from 3 animals. * P<0.05 vs. before DOG, NS: not significant, Chel: chelerythrine.

results indicate basal K$_V$ currents are suppressed in SAH parenchymal myocytes as compared to

control. Time control studies demonstrate K$_V$ current density does not significantly decrease in

control of SAH myocytes, which allows for meaningful data to be obtained from drug treatment

studies. We report PKC activation significantly suppresses K$_V$ currents in control but not SAH

parenchymal myocytes. The PKC activator DOG suppressed K$_V$ current in control myocytes but

not in SAH myocytes. These data indicate that PKC may be maximally active following SAH.

Figure 5 depicts a proposed signaling pathway from PKC activation to myocyte contraction.

The PKC inhibitor chelerythrine did not significantly change K$_V$ current in either control or

SAH myocytes. This may be the case because PKC had already phosphorylated the channel or a

regulatory unit, so inhibiting PKC would not prevent futher phosphorylation but would not

remove the phosphate already added. To clarify this question, electrophysiological experiments

will need to be conducted in the presence of a phosphatase to cleave phosphate from the channel

or regulatory unit. Cleavage of the phosphate from the effector PKC targeted may relieve the

suppression of K$_V$ currents. As phosphates are peptides and are not easily transported across the

cell membrane, application would need to proceed through the pipette. Phosphatase would be

added to the pipette solution, and when the cell membrane is ruptured upon obtaining a pipette

seal and applying suction, the phosphatase in the pipette solution would be able to diffuse into

the cell and act on its targets. PKC inhibitors could also be applied *in vivo* following injections
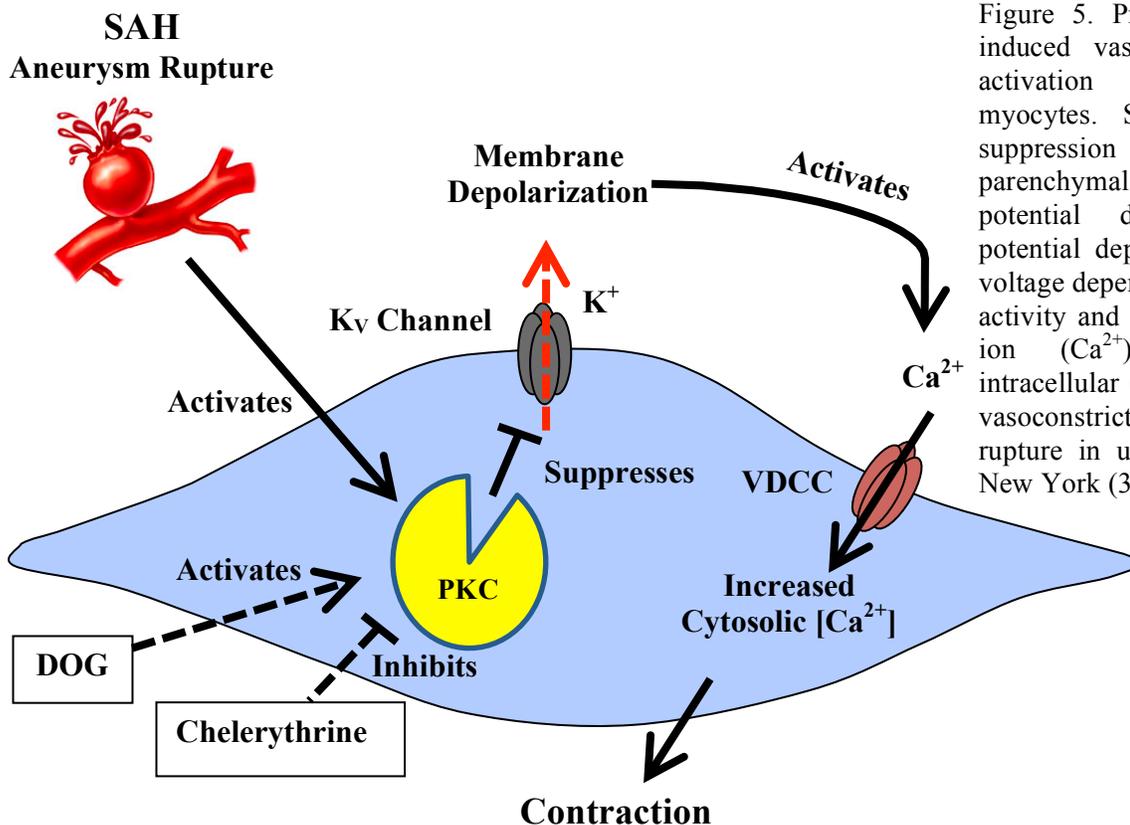

Figure 5. Proposed mechanism of SAH-induced vasoconstriction involving PKC activation in parenchymal arteriolar myocytes. SAH activates PKC leading to suppression of K$_V$ channels promoting parenchymal arteriolar myocyte membrane potential depolarization. Membrane potential depolarization leads to increased voltage dependent calcium channel (VDCC) activity and increased intracellular calcium ion (Ca^{2+}) concentration. Increased intracellular Ca^{2+} promotes contraction and vasoconstriction. Image of aneurysm rupture in upper left from Vascular Care New York (31)

of blood into the cisterna magna. This application could potentially prevent suppression of K$_V$ channels by PKC, thus myocytes from chelerythrine treated animals would be expected to be greater than animals without treatment.

The mechanism by which K$_V$ suppression promotes smooth muscle contraction. Active PKC mediated suppression of K$_V$ channels would lead to membrane depolarization and subsequent influx of Ca^{2+} through VDCCs. Normally, K$_V$ channels are activated by membrane depolarization. Upon activation, K$_V$ channels would facilitate outward K$^+$ flow across the membrane, effectively repolarizing the cell (27). If K$_V$ channels are blocked, this repolarization would not occur leading to activation of VDCCs, which facilitate the flow of Ca^{2+} into the cell (26).

Arterial smooth muscle contraction is primarily regulated through VDCCs. Increased cytosolic Ca^{2+} concentrations stimulate muscle contraction. Smooth muscle cells tightly regulate their free Ca^{2+} concentrations. Cytosolic free Ca^{2+} concentration is in the sub-micromolar scale as opposed to total intracellular, which includes Ca^{2+}, contained in the sarcoplasmic reticulum, and extracellular concentrations, both of which are in the millimolar scale (13). Influx of Ca^{2+} through VDCCs increases the free Ca^{2+} concentration, permitting Ca^{2+} to bind calmodulin. The calmodulin-calcium complex can activate myosin light chain kinase, which in turns phosphorylates myosin light chain promoting contraction (16).

Mechanism of PKC mediated K$_V$ channel suppression. Activation of PKC following SAH would suppress K$_V$ channels by an as of yet undetermined mechanism. Aiello et al proposed three possible mechanisms of K$_V$ channel suppression via PKC. These mechanisms include PKC catalyzed phosphorylation of the K$_V$ channel, phosphorylation of a regulatory subunit that would then inactive the channel, and phosphorylation of another kinase, triggering a kinase cascade that

results in phosphorylation of the channel or regulatory subunit (1). One method to determine if PKC directly phosphorylates K$_V$ channels may involve isolation of the channel in both control and SAH myocytes followed by characterization of mass to charge ratio using mass spectrometry. If a phosphate is added to K$_V$ channels by PKC following SAH, mass spectrometry would detect the additional mass and negative charge covalently linked to the channel as compared to channels from control myocytes. We report inhibition of PKC does not change K$_V$ current in SAH myocytes, which may be due to the target of PKC already being phosphorylated.

PKC isotype involved in K$_V$ suppression following SAH. There are three large families of PKC isoforms: 1) conventional, Ca^{2+} dependent isoforms including PKCα and PKCß, 2) novel, Ca^{2+} independent isoforms including PKCδ and PKCε (among others), and 3) atypical PKC isoforms including PKCζ (41). Atypical PKC is not activated by DAG and is not Ca^{2+} dependent. Different isoforms are expresed in vascular smooth muscle, including PKCα, PKCß, PKCγ, PKCδ, and PKCε (18). There are some atypical isoforms expressed as well, but since our data demonstrates DOG treatment activated PKC leading to K$_V$ suppression, it is unlikely the PKC subtype involved is a member of the atypical PKC family. Evidence of angiotensin II and endothelin-1 (ET-1) mediated PKC suppression of K$_V$ current suggests different PKC isozymes may be responsible for K$_V$ suppression in arterial smooth muscle (39). Rainbow et al. reported angiotensin II inhibits K$_V$ channels through PKCε while ET-1 inhibits K$_V$ channels through PKCα. It has been reported that following SAH PKCα and PKCδ are active in arterial smooth muscle (28, 30, 38). Other evidence demonstrates PKCδ translocates to the membrane before PKCα, suggesting PKCδ is involved in establishing PKC mediated vasospasm while PKCα works in maintaining that vasospasm over a prolonged period of time (28). These data suggest

PKCα may be controlling prolonged K_V current suppression following SAH, but further research is required to test this hypothesis. PKCδ was not found to be involved in K_V suppression by Rainbow et al, but as it is active following SAH, further study should investigate the potential role of PKCδ in K_V channel suppression following SAH.

Mechanism of PKC activation following SAH. The mechanism by which PKC is activated following SAH is most likely two fold, as both PKCα and PKCδ are activated. Rho kinase has been reported to be active following SAH and contributing to vasospasm (45). In addition to phosphorylating myosin light chain kinase directly, there is evidence Rho contributes to the activation of PKCδ (34). However, the role of PKCδ in K_V suppression is not clear after SAH.

Following SAH, oxyhemoglobin triggers the release of ET-1 from astrocytes, neurons, and pituitary cells as well as from endothelial and smooth muscle cells into the cerebrospinal fluid (36). However, Pluta et al suggest that ET-1 is not responsible for initial vasospasm, rather it is a result of ischemia stemming from that vasospasm (36). PKCα is not active immediately after exposure to oxyhemoglobin, which suggests the activation of PKCα may also be a result of ischemia following SAH, rather than from oxyhemoglobin itself. If this is the case, PKCα functions in a feed-forward system in which SAH induces vasospasm through a mechanism independent of PKCα resulting in cerebral ischemia, production of ET-1, and activation of PKCα, which in turn functions to suppress K_V channels and maintain SAH. A time course experiment investigating the role of PKC in SAH induced K_V suppression could provide additional information of the onset of PKC mediated K_V suppression. Potential time points of interest may before the second injection of blood into the cisterna magna, immediately after this injection, day four (as was used in the experiments reported here), and day seven.

K$_V$ channel subtype involved in PKC mediated suppression after SAH. Previous work has suggested that at least two independent mechanisms contribute to suppression of K$_V$ current following SAH. The HB-EGF/EGFR pathway characterized by Koide et al appears to be specific to K$_V$1.2 and K$_V$1.5 channels and is independent of the PKC mediated pathway (20). Activation of this pathway in rabbit cerebral myocytes by exposure to oxyhemoglobin pathway suppressed K$_V$ current in control myocytes but this suppression did not match that observed in SAH myocytes. Treatment with DOG in addition to oxyhemoglobin further suppressed K$_V$ current. This suggests that DOG is involved in a pathway independent of the HB-EGF/EGFR pathway and indicate additional K$_V$ channel subtypes may be suppressed following SAH in addition to K$_V$1.2 and K$_V$1.5. If the PKC pathway solely targeted K$_V$1.2 and K$_V$1.5 channels, addition of DOG would not have suppressed K$_V$ currents in the presence of EGF. An additional experiment in this project would be to apply DOG to control parenchymal myocytes in the presence of correolide, a specific inhibitor K$_V$1 family channel inhibitor (10). We would predict that DOG would further suppress K$_V$ currents in the presence of correolide as it is working through a separate pathway.

A third subtype of K$_V$ channel, K$_V$2.1, is expressed in arterial smooth muscle and has been shown to be suppressed in a PKC dependent fashion following PKC activation by angiotensin II (2). Based on these data and on our own observations, we propose K$_V$2.1 as a possible end target for the PKC pathway following SAH. An experimental plan involving electrophysiological experiments with the use of K$_V$ subtype specific inhibitors could provide evidence as to the specific subtype. Correolide and stromatoxin, a specific inhibitor of K$_V$2 family channels (40) could be used in such an experimental series. If K$_V$2.1 is suppressed by PKC activation, then treatment with DOG after stromatoxin would result in no further decrease in total K$_V$ current. If

DOG was treated prior to stromatoxin, addition of stromatoxin should not then further decrease K_V currents as DOG has already initiated the inhibition of the stromatoxin sensitive, $K_V2.1$ channel. If PKC is suppressing $K_V2.1$, DOG treatment after correolide would suppress total K_V current further.

Implications of PKC mediated suppression of K_V channels in parenchymal arteriole myocytes following SAH. The PKC mediated pathway of K_V suppression following SAH is important in the severe, prolonged vasospasm responsible for high morbidity and mortality. In parenchymal arterioles, this vasospasm is especially devastating, as the cerebral parenchyma is not supplied by other blood vessels. Characterization of PKC activity following SAH in parenchymal arterioles may provide a therapeutic target. A specific PKC inhibitor for the isoform responsible for K_V channel suppression could potentially relieve K_V inhibition and subsequent vasospasm.

PKC Activity in Intact Arteriole. In follow up to the data reported here, we plan to perform an assay for PKC activity in intact parenchymal arterioles. We have been storing intact parenchymal arterioles by removing them as described for electrophysiology experiments and storing at -80˚C. For the PKC assay, we will homogenize these arterioles in a a lysis buffer containing (in mM) 10 MOPS, 50 ß-glyceraldehyde phosphate, 50 sodium fluoride, 1 sodium orthovanadate, 5 EGTA, 2 EDTA, 1 diothiothreitol, 1 benzamidine, 1 PMSF, 10μg/mL leupeptin, 10μg/mL aprotinin, and 1% IGEPAL CA 630. As active PKC translocates to the membrane, membrane fractions will be obtained by multiple rounds of centrifugation. PKC activity will be determined using an Enzo Life Sciences kit. Protein concentrations in samples used in the PKC assay will be measured using the Bradford assay, which is a spectroscopic assay of protein concentration in a solution, with bovine serum albumin as the standard.

PKC activity has not been characterized in parenchymal arterioles following SAH. Given the data reported here, and based on previous PKC assay experiments in pial arteries (30), we hypothesize PKC is active in parenchymal arterioles from SAH rats four days following the initial injection of blood into the cisterna magna. This study would provide further data PKC is active in the arteriole. Coupled with electrophysiology experiments showing PKC is active in smooth muscle, these data would provide a strong case for active PKC playing a role in vasospasm following SAH.

In summary, here we provide evidence of PKC activation in parenchymal arterioles following SAH leading to K_V current suppression. As K_V suppression contributes to vasospasm and subsequent neurological deficits following SAH, these data may contribute to future therapeutic approaches. Future research into the mechanism by which PKC is activated following SAH and how this activation suppresses specific K_V subtypes will elaborate on this contribution.

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