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Vascular KCNQ (Kv7) potassium channels as common signaling intermediates and therapeutic targets in cerebral vasospasm:

Kv7 channel openers to treat cerebral vasospasm

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Abstract

Cerebral vasospasm following subarachnoid hemorrhage (SAH) is characterized by prolonged severe constriction of the basilar artery, which often leads to ischemic brain damage. Locally elevated concentrations of spasmogenic substances induce persistent depolarization of myocytes in the basilar artery, leading to continuous influx of calcium (Ca^{2+}) through voltage-sensitive Ca^{2+} channels and myocyte contraction. Potassium (K^+) channel openers may have therapeutic utility to oppose membrane depolarization, dilate the arteries, and reduce ischemia. Here, we examined the involvement of vascular Kv7 K^+ channels in the pathogenesis of cerebral vasospasm and tested whether Kv7 channel openers are effective therapeutic agents in a rat model of SAH. Patch-clamp experiments revealed that three different spasmogens (serotonin, endothelin and vasopressin) suppressed Kv7 currents and depolarized freshly isolated rat basilar artery myocytes. These effects were significantly reduced in the presence of a Kv7 channel opener, retigabine. Retigabine (10 $\mu\text{mol/L}$) also significantly blocked L-type Ca^{2+} channels, reducing peak inward currents by >50%. In the presence of a selective Kv7 channel blocker, XE991, the spasmogens did not produce additive constriction responses measured using pressure myography. Kv7 channel openers (retigabine or celecoxib) significantly attenuated basilar artery spasm in rats with experimentally-induced SAH. In conclusion, we identify Kv7 channels as common targets of vasoconstrictor spasmogens and as candidates for therapeutic intervention for cerebral vasospasm.

Keywords

Potassium channel; membrane depolarization; cerebral vasospasm; basilar artery; retigabine; celecoxib

Introduction

Rupture of intracranial aneurysms results in extravasation of blood into the subarachnoid space. Cerebral vasospasm, particularly the intense constriction of the large conduit arteries

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Conflict of interest:

The authors have no conflicts of interest to disclose.

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(basilar artery and arteries of the Circle of Willis) near the site of a ruptured aneurysm, is a common occurrence following subarachnoid hemorrhage (SAH).^{1, 2} The consequent decrease in cerebral perfusion has been implicated as a contributing factor in stroke and death or prolonged disability in SAH patients. Though considerable progress has been made in understanding the pathogenesis of cerebral vasospasm, the cellular events that lead to the delayed vasospasm and ischemic events remain poorly understood. Elevated local concentrations of endothelin-1 (ET-1), serotonin (5HT, 5-hydroxytryptamine) and arginine⁸-vasopressin (AVP) along with blood components (oxyhemoglobin and bilirubin oxidation products) have been implicated in the initiation and maintenance of vasospasm.²⁻⁷

In previous studies of SAH using animal models, membrane voltages in vascular myocytes derived from basilar and cerebral arteries were found to be persistently depolarized.⁸⁻¹⁰ Membrane depolarization increases the open-probability of voltage-sensitive Ca^{2+} channels (VSCCs), resulting in Ca^{2+} influx and contraction of myocytes.¹¹ L-type calcium channel blockers (CCBs), particularly nimodipine, have therefore become the primary therapy to alleviate cerebral vasospasm.¹² CCBs are not, however, uniformly effective in overcoming vasospasm in patients and their utility is limited by their propensity to produce hypotension.^{13, 14} Understanding the mechanisms that lead to membrane depolarization and activation of VSCCs in basilar artery myocytes could potentially identify novel targets for more effective treatment of cerebral vasospasm.

Potassium channels are major determinants of resting membrane voltage and hence regulators of vascular tone.¹⁵ Dysfunction of 4-aminopyridine- (4-AP)-sensitive voltage-activated potassium (Kv) channels in vascular myocytes after SAH has been proposed as a mechanism contributing to cerebral vasospasm.⁹ However, presently there are no pharmacological modulators for 4-AP-sensitive Kv channels that are available in the clinic to modulate vascular or any other disorders.¹⁶

A different subtype of Kv channels, Kv7 (KCNQ) channels, were recently reported to be expressed in vascular myocytes and shown to regulate membrane voltage, thereby influencing the constriction of arteries in several vascular beds.¹⁷⁻¹⁹ We recently reported that Kv7 channels are expressed in rat basilar artery myocytes and provided evidence that K^{+} conductance through these channels determines their resting membrane voltage and contractile status.²⁰ K^{+} currents through Kv7 channels, unlike 4-AP-sensitive Kv currents, activate at very negative membrane voltages (~ -60 mV) and are not sensitive to 4-AP.^{19, 20} Perhaps more importantly from a therapeutic perspective, pharmacological Kv7 channel openers reversed spasmogen-induced rat basilar artery constriction²⁰ and attenuated myogenic tone of rat cerebral artery segments.²¹ These findings implicate activation of Kv7 currents as a possible mechanism for overcoming spasm of arteries in the base of the brain. Celecoxib, a clinically used cyclooxygenase-2 (COX-2) inhibitor, activated Kv7 currents and blocked voltage-sensitive Ca^{2+} currents in rat basilar artery myocytes and dilated basilar artery segments that were pre-constricted with spasmogens.^{20, 22} Here, using single cell path-clamp techniques and pressure myography, we provide evidence that suppression of Kv7 currents is a common intermediate step in the constrictor actions of multiple basilar artery spasmogens. We also demonstrate that drugs that enhance Kv7 currents in basilar artery myocytes significantly attenuate basilar artery spasm in rats with experimentally-induced SAH, providing the first direct evidence for a beneficial therapeutic effect of these drugs in the relief of cerebral vasospasm.

Materials and Methods

Animal procedures

All animal procedures described in this study were conducted in accordance to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23) and approved by the Institutional Animal Care and Use Committee, Loyola University Medical Center, Maywood, IL. All studies utilized male Sprague-Dawley rats (Harlan laboratories).

Basilar artery myocyte isolation

Sprague-Dawley rats (275-325g) were euthanized by cardiac extirpation while under 4% isoflurane anesthesia. Immediately following euthanasia, the brain was removed and placed in ice-cold dissection solution (in mM - 145 NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 2 CaCl₂·2H₂O, 2 pyruvic acid, 0.02 EDTA dihydrate, 3 MOPS, and 5 D-glucose with 1% bovine serum albumin (BSA), pH 7.4 at 0° C, 300 mOsm/L). The basilar artery was dissected from the brain and used for pressure myography or for isolation of myocytes for patch-clamp recordings. The myocytes were isolated from the basilar artery using the procedures described previously.²⁰

Electrophysiology

Individual basilar artery myocytes were allowed to attach to glass coverslips and whole cell currents or membrane voltage were measured using a perforated patch configuration (by including 120 µg/ml Amphotericin B in the internal solution). All experiments were performed at room temperature with continuous perfusion of bath solution as described previously.²⁰ Bath solution contained (in mM): 140 NaCl, 5.36 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-Glucose, pH 7.3, 298 mOsm/L; the internal (pipette) solution contained (in mM): 135 KCl, 5 NaCl, 10 HEPES, 0.05 K₂EGTA, 1 MgCl₂, 20 D-Glucose, pH 7.2, 298 mOsm/L. The effect of a treatment on membrane voltage was determined by comparing the mean of the values recorded over at least a 1 min period prior to the treatment with those recorded over at least a 1 min period at the end of the treatment.

Kv7 currents were recorded in isolation by application of 5 s voltage steps from a -4 mV holding voltage to test voltages ranging from -84 mV to +16 mV. GdCl₃ (100 µmol/L) was included in the bath solution to block L- and T-type Ca²⁺ channels, non-selective cation channels, and to shift activation of 4-AP-sensitive K_V currents to voltages positive to -20 mV.^{20, 23} Whole-cell K⁺ currents were digitized at 2 kHz and filtered at 200 Hz. Liquid junction potentials were calculated using Junction Potential Calculator provided by PCLAMP8 software and subtracted off-line. The steady-state K⁺ currents recorded during the last 1000 ms (2000 points) of each voltage step were averaged and normalized to cell capacitance after leak subtraction. Current-voltage (I-V) relationships were derived by normalizing the mean sustained currents measured at each voltage to the mean current measured at -20 mV before treatment. Time-course of enhancement or inhibition of isolated Kv7 currents was recorded by continuous measurement of outward currents at -20 mV holding potential.

Voltage-dependence of channel activation of Kv7 currents was derived by calculating the conductance from the steady-state currents at each voltage (according to the equation $G = I / (V - E_K)$, where I, steady-state current; V, step voltage; E_K reversal potential for potassium) and normalized to maximum conductance for each experiment. Normalized conductances were fitted by a Boltzmann function: $G/G_{max} = 1/[1 + e^{(V_{0.5} - V)/s}]$, where G/G_{max} is the fraction of maximal conductance, V_{0.5} is the voltage of half-maximal activation and s is the slope factor. Vasoconstrictor spasmogens (AVP, 5HT and ET-1) and drugs (retigabine,

XE991) were all prepared as stock solutions using deionized water; phorbol 12-myristate 13-acetate (PMA) was prepared as stock solution using dimethyl sulfoxide (DMSO). All stock solutions except retigabine were diluted by a factor of 1:10,000 in the bath solution to achieve the final concentration. Retigabine was diluted by a ratio of 1:1,000.

Currents through voltage-sensitive Ca^{2+} channels were recorded as previously described²⁰, using 10 mM Ba^{2+} as a charge carrier.

Pressure Myography

The effects of various treatments on the constriction of basilar artery were studied using a pressure myograph system (DMT-USA, Atlanta, GA) as described previously.²⁰ Basilar artery segments were pressurized to 80 mmHg and all artery contraction/dilation studies were performed at 37°C without intraluminal flow. Branches were tied off, if necessary, to maintain pressure and prevent flow within the artery segment.

Rat model of subarachnoid hemorrhage

To determine the *in vivo* efficacy of Kv7 channel activators for relief of basilar artery spasm we used a well-established rat model of SAH. Adult male Sprague-Dawley rats (300-350g) were used according to procedures similar to those described previously.²⁴ The femoral artery was first cannulated under 3% isoflurane anesthesia. The rats were then anesthetized by intramuscular administration of ketamine (80 mg/kg) and xylazine (8 mg/kg) and secured in a stereotaxic apparatus under aseptic conditions. A small sub-occipital incision was made to expose the arch of the atlas, the occipital bone, and the atlanto-occipital membrane. The cisterna magna was tapped to remove 0.1 ml of CSF, followed by slow injection (over 2 min) of 0.3 ml of artificial cerebrospinal fluid (aCSF; solution contained, in mMol/L - 125 NaCl, 2.5 KCl, 1 MgCl₂·6H₂O, 1.25 NaH₂PO₄, 2 CaCl₂·2H₂O, 25 NaHCO₃ and 25 D-glucose pH 7.3 at 37° C, 300 mOsm/L) or autologous blood drawn from the femoral artery. Immediately after injection, the hole was sealed with glue to prevent fistula. The animal was tilted at a 30° angle for 30 min in a head lowered position to permit distribution of blood (or aCSF) into the subarachnoid space. Pain was relieved by subcutaneous injection of buprenorphine (0.03 mg/kg) after surgery. The rats were returned to the cage after suturing to allow recovery from anesthesia. Of the 51 rats that underwent the surgery, 13 rats, all of which were injected with arterial blood into the cisterna magna, died during the surgery. The surviving rats were grouped randomly and treated with intraperitoneal administration of retigabine (7.5 mg/kg), celecoxib (20 mg/kg) or the solubilizing vehicle (DMSO; 1 ml/kg) starting an hour after SAH and repeated twice a day until 48 h (4 doses in total). Doses of retigabine and celecoxib were adopted from previous studies in which the drugs were utilized for their anti-seizure and anti-inflammatory properties, respectively.^{25, 26} Two SAH rats, one treated with vehicle and the other treated with retigabine, died during the course of treatment. Body temperature was measured rectally using a digital thermometer (Warner Instruments, CT). Body temperature measurements were made every 24 h after the surgical induction of SAH before the impending drug treatment. All assessments were conducted by personnel who were blinded to the treatments given to the rats. The rats were humanely euthanized with 100% CO₂ inhalation 48 h after surgery (or 12 h after last drug treatment). The brain was removed immediately and the basilar artery was photographed using a dinocapture digital system (AnMo Electronics Corporation, Taipei, Taiwan). Measurements of the outer diameter of the basilar artery were made at 10 places along the length of the basilar artery. The measurements were averaged to obtain the basilar artery diameter of each rat and expressed in micrometers.

Arterial pressure measurements

The effects of Kv7 channel activators on mean arterial pressure (MAP) were measured in a separate cohort of rats. Thirteen male Sprague-Dawley rats (275–325g) were aseptically implanted with PhysioTel® PA-C40 or C50-PXT radio-telemetry transmitters (Data Sciences International (DSI), St. Paul, MN) to allow continuous recording of arterial pressure in freely moving rats. Under 3% isoflurane anesthesia, a ventral midline laparotomy was performed and the abdominal contents were gently exteriorized into a moist and warm sterile gauze. Blood flow through the abdominal aorta was briefly interrupted using a bulldog clamp. The catheter of the radio-transmitter was inserted through a puncture made posterior to the blockade of blood flow. The puncture hole was immediately sealed with medical glue and the blood flow was restored. The abdominal contents were then placed back into the peritoneal cavity. The transmitter body was placed in the cavity and secured with abdominal sutures. Rats were administered buprenorphine (0.03 mg/kg) and ampicillin (50 mg/kg) every 8–12 h for 2 days or as needed after surgery. The rats were allowed to recover from surgery for 10 days and then distributed into 2 groups such that MAP measured before treatment was approximately equal in each group. MAP (mm Hg) was sampled from each rat for 10 s every 10 min for 72 h (Dataquest A.R.T 3.1 Gold Telemetry System, DSI, with RPC1 receivers). Each group underwent two treatments separated by a 72 h recovery period. The first treatment was control (no treatment) or vehicle treatment (DMSO, 1 ml/kg i.p., every 12 h) and the second treatment was retigabine (7.5 mg/kg i.p., every 12 h) or celecoxib (50 mg/kg i.p., every 12 h). Prior to each treatment, baseline MAP was recorded for 24 h. This was followed by a 48-h recording during treatment. Data were exported offline using Dataquest ART analysis software (DSI) to a spreadsheet for further analysis.

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of the following. Amphotericin B and nimodipine were from Calbiochem (San Diego, CA), BSA fraction V was from Boehringer Mannheim (Germany), celecoxib was from LC Laboratories, Inc. (Woburn, MA), retigabine was from LGM Pharma, Inc. (Boca Raton, FL), XE991 was from Ascent Scientific (Princeton, NJ).

Data presentation and Statistics

Data are presented as mean $\pm$ standard error of mean. Data were analyzed using Clampfit (Axon Instruments) and SigmaStat (Systat Software, Inc.) software programs. Student's '*t*' test was used for comparison between 2 groups, Student's paired '*t*' test was used for comparisons of parameters measured before and after treatments. Data derived by multiple treatments on the same system were statistically compared by using repeated measures analysis of variance (RM-ANOVA) followed by a Holm-Sidak post hoc test. Comparisons among multiple independent treatment groups were evaluated by analysis of variance (ANOVA) followed by a Holm-Sidak post hoc test. Differences with *p*-values ≤ 0.05 were considered statistically significant.

Results

Retigabine activates Kv7 currents in basilar artery myocytes

We tested the effects of retigabine, a Kv7 (Kv7.2–Kv7.5) channel opener, on Kv7 currents in basilar artery myocytes. Retigabine (10 μ M) robustly enhanced Kv7 currents in freshly isolated rat basilar artery myocytes and this effect was completely reversed on wash out of retigabine (Figure 1A). Enhancement of currents with retigabine was significant at all voltages tested from -59.2 mV to $+0.8$ mV (Figure 1B). At -20 mV, the voltage at which

near maximal activation of currents was observed (Figure 1C), current amplitudes increased from 0.2 ± 0.09 pA/pF before treatment to 0.46 ± 0.17 pA/pF during treatment with retigabine, $n=5$. Retigabine also induced a significant leftward shift in the steady-state voltage dependence of activation; $V_{0.5}$ (control) was -30.2 ± 1.4 mV, $V_{0.5}$ (retigabine) was -41.4 ± 0.5 mV, $n=5$ (Figure 1C). The currents at negative voltages were fully suppressed by 10 μ mol/L XE991 (Figure 1A,B), but were not sensitive to 2 mM 4-aminopyridine (4-AP) (Supplemental Figure S1).

Spasmogens suppress Kv7 currents and induce membrane depolarization in basilar artery myocytes

The cellular mechanism(s) by which the spasmogens induce membrane depolarization is not clear. Since K^+ conductance through Kv7 channels is a critical determinant of resting membrane potential in rat basilar artery myocytes and pharmacological modulation of these channels influences basilar artery tone,²⁰ we tested the hypothesis that Kv7 currents are suppressed by spasmogens to induce membrane depolarization. We tested three spasmogens (ET-1, 5HT and AVP) that have been implicated in cerebral vasospasm. All three spasmogens significantly suppressed Kv7 currents measured in the physiological voltage range (Figure 2, Supplemental figure S2). Currents measured at -20 mV were suppressed by 56%, 49% and 37% with the addition of serotonin (100 nmol/L), ET-1 (250 pmol/L) and AVP (250 pmol/L) respectively. Spasmogen concentrations were chosen to reflect the concentrations achieved in the cerebrospinal fluid (CSF) after SAH, as described previously.²⁰ The current amplitudes at -20 mV were 0.18 ± 0.04 pA/pF before and 0.07 ± 0.02 pA/pF during application of 5HT ($n=6$), 0.12 ± 0.02 pA/pF before and 0.06 ± 0.01 pA/pF during application of ET-1 ($n=4$), 0.37 ± 0.13 pA/pF before and 0.27 ± 0.12 pA/pF during application of AVP ($n=6$). These results suggest that suppression of Kv7 currents is a common effect of basilar artery spasmogens.

Inhibition of protein kinase C (PKC) was previously found to attenuate rat basilar artery constriction produced by each of the three spasmogens used in the present study,^{27, 28} indicating that activation of PKC is a common signal transduction intermediate in spasmogen-induced basilar artery constriction. Therefore, we tested whether activation of PKC is sufficient for suppression of Kv7 currents in basilar artery myocytes and constriction of the basilar artery using a direct activator of PKC: phorbol 12-myristate 13-acetate (PMA, 10 nmol/L). PMA suppressed Kv7 currents at voltages from -44.2 mV to +15.2 mV (Supplemental figure S3) and PMA (100 nmol/L) also constricted rat basilar artery segments (Supplemental figure S4). An inactive phorbol ester (4 α -PMA) was not effective in either suppression of Kv7 currents (not shown) or constriction of basilar artery (Supplemental figure S4).

Addition of the Kv7 channel activator retigabine (10 μ mol/L) more than fully restored Kv7 current amplitude in the presence of spasmogens (Figure 2, Supplemental figure S2). In the presence of 5HT, ET-1 and AVP at the concentrations indicated above, 10 μ mol/L retigabine increased current amplitude (measured at -20 mV) to 72%, 65% and 78% above the control levels, respectively.

Since Kv7 currents are active at resting membrane voltages (Figure 1) and Kv7 current suppression produces membrane depolarization,²⁰ we tested whether the spasmogens also produce membrane depolarization of myocytes. As expected, all three spasmogens produced significant depolarization: 5HT, from -57.1 ± 6.3 mV to -35.4 ± 2.4 mV, $n=4$; ET-1, from -62.1 ± 2.3 mV to -42.8 ± 1.3 mV, $n=4$; AVP from -53.2 ± 4.2 mV to -37.9 ± 1.4 mV, $n=4$ (Figure 3, Supplemental figure S5). More importantly, simultaneous addition of 10 μ mol/L retigabine to enhance activation of Kv7 currents prevented membrane depolarization by 5HT (Figure 3). The voltage measured was -65.2 ± 4.6 mV before and -64.7 ± 4.3 mV after

combined treatment with 10 $\mu\text{mol/L}$ retigabine and 100 nmol/L 5HT, $n=3$. These results indicate that activation of Kv7 currents can oppose the membrane depolarization induced by spasmogens involved in cerebral vasospasm.

Kv7 current suppression is sufficient to induce basilar artery constriction and may account for the majority of spasmogen-induced basilar artery constriction

The VSCC blocker nimodipine (1 $\mu\text{mol/L}$) dilated basilar artery segments pre-constricted by spasmogens (Supplemental figure S6). This implicates membrane depolarization and Ca^{2+} influx through VSCCs as the predominant mechanism for constriction produced by spasmogens at the concentrations measured following SAH. Since basilar artery spasmogens suppressed Kv7 currents and induced membrane depolarization (Figure 2, 3), we sought to determine whether spasmogen-induced basilar artery constriction involves suppression of Kv7 currents. To address this question, we tested whether addition of spasmogens in the presence of XE991 (10 $\mu\text{mol/L}$, a concentration that fully suppresses Kv7 currents) induces additive basilar artery constrictor responses. Of the three spasmogens tested, AVP (100 pmol/L) and ET-1 (100 pmol/L) produced a slight but non-significant additional constriction in the presence of XE991; 5HT (75 nmol/L) in the presence of XE991 produced a significant additional constriction compared to either XE991 alone or the spasmogen alone (Figure 4). In each case, the percentage constriction induced by the combined presence of spasmogen and XE991 was less than the sum of constrictor response produced by the spasmogen alone and XE991 alone.

5HT-induced basilar artery constriction is attenuated by retigabine

Retigabine, which opposed 5HT-induced Kv7 current suppression and membrane depolarization in isolated basilar artery myocytes (Figure 2 and 3), was also able to reverse the constriction of basilar artery segments induced by 5HT. Retigabine (100 $\mu\text{mol/L}$) produced 92.8% reversal of the constriction produced by 5HT. The EC_{50} for retigabine-induced basilar artery relaxation was 23.7 $\mu\text{mol/L}$, $n=5$ (Figure 5).

The vasodilatory actions of retigabine may be due to in part to direct suppression of L-type VSCC activity. We found that retigabine (10 $\mu\text{mol/L}$) significantly inhibited L-type currents, reducing peak amplitude in basilar artery myocytes by more than 50% (control: -2.1 ± 0.6 pA; retigabine: -0.9 ± 0.3 pA, $p<0.05$, paired Student's t-test, Supplemental figure S7).

Kv7 channel openers attenuate basilar artery vasospasm in a rat model of SAH

Single-cell electrophysiology and pressure myography studies demonstrated the ability of Kv7 channel openers to attenuate spasmogen-induced membrane depolarization and basilar artery constriction (Figures 3 and 5 and ²⁰). We therefore tested the efficacy of Kv7 channel openers to relieve basilar artery vasospasm *in vivo* using a single-hemorrhage rat model of SAH. Rats were treated with either retigabine (7.5 mg/kg, i.p) or celecoxib (20 mg/kg, i.p; a dual Kv7 channel opener and VSCC blocker ^{20, 22}) twice daily starting an hour after hemorrhage and continuing for 48 h (4 doses in total). Basilar artery outer diameter measured at 48 h (12 h after the last injection) was used as the endpoint criterion to assess the severity of vasospasm and efficacy of treatments. SAH induced by an intracisternal injection of autologous blood produced significant basilar artery vasospasm compared to control rats that had no surgery or that were injected with artificial cerebrospinal fluid (aCSF) instead of blood (Figure 6). Both retigabine and celecoxib significantly attenuated the basilar artery spasm when compared with vehicle control in the rat model of SAH (Figure 6).

A striking observation was a pronounced hypothermia with administration of retigabine in SAH rats. Rats administered retigabine had significant hypothermia even 12 h after drug administration. The rectal temperature of rats in the retigabine group (33.1 ± 1.4 °C) was approximately 5°C lower than the mean temperature in all untreated control groups (Supplemental figure S8). Celecoxib also tended to induce mild hypothermia, though this effect was not statistically significant.

Kv7 channel openers induce transient hypotension, but do not alter long-term arterial pressure

We tested the effect of the Kv7 channel opener retigabine (7.5 mg/kg i.p.) and the dual Kv7 channel opener and CCB celecoxib (50 mg/kg i.p.) on systemic arterial pressure in conscious rats (MAP was monitored by radiotelemetry). The drugs were administered intraperitoneally every 12 h for 48 h (4 doses in total) to mimic the schedule of test drug administration in SAH rats. Both the drugs produced a transient drop in MAP usually lasting for 30-60 min after each administration. By comparison, vehicle administration tended to increase MAP transiently (Figure 7A). Despite the differences in MAP immediately after drug administration, the mean 24 h MAP (Figure 7B) and heart rate (not shown) were not significantly different between vehicle- and drug-treated groups.

Discussion

Cerebral vasospasm is a devastating clinical condition involving sustained contraction of vascular myocytes through unknown mechanisms. The condition has a poor prognosis owing to the paucity of effective therapeutic agents. The findings in the present study demonstrate that known basilar artery spasmogens, including the $G_{q/11}$ -coupled receptor agonists vasopressin, endothelin, and serotonin, potently suppress the activity of Kv7 channels in myocytes, and induce membrane depolarization similar to that observed in myocytes following SAH.⁸⁻¹⁰ Pharmacological Kv7 channel activators, retigabine and celecoxib, opposed the effects of spasmogens on Kv7 currents and membrane depolarization and also relaxed pressurized basilar arteries that were pre-constricted with these spasmogens (Figs. 3 and 5 and ²⁰). We also demonstrated that these same clinically used drugs were effective in relieving basilar artery spasm *in vivo* in rats with experimentally-induced SAH. These results provide the first evidence supporting the use of Kv7 channel activators to alleviate cerebral vasospasm.

Suppression of Kv7 currents as a mechanism of vasoconstriction by spasmogens

Though it is well established that basilar artery spasmogens depolarize the vascular myocytes and trigger Ca^{2+} influx through VSCCs to produce constriction, the signal transduction events that lead to membrane depolarization remain poorly understood. Our results indicate that suppression of Kv7 currents is a common signal transduction intermediate in the actions of multiple spasmogens that elicit cerebral vasospasm (Figure 2, Supplemental figure S2), and that this suppression of Kv7 currents results in significant membrane depolarization in basilar artery myocytes (Figure 3, Supplemental figure S5 and ²⁰).

Most previous evidence points to activation of non-selective cation channels as a mechanism for membrane depolarization.²⁹ Suppression of the K^+ channel activity at resting voltages would be expected to increase membrane resistance and thereby amplify the voltage change produced by inward leak currents, and to enhance membrane depolarization due to activation of inward (non-selective cation and/or chloride) currents. The additional constriction produced by 5HT when Kv7 currents were already fully inhibited by XE991 (Figure 4B,C) may therefore be due to 5HT-induced activation of inward currents²⁹ which

produces additional membrane depolarization under these conditions. Neither ET-1 nor AVP produced significant additional constriction in the presence of XE991, suggesting that Kv7 current suppression in the presence of resting inward leak conductances may be sufficient to account for their constrictor actions (Figure 4C).

The signal transduction intermediates involved in the spasmogen-induced suppression of Kv7 currents are not currently known. Activation of PKC, a common signal transduction intermediate following activation of $G_{q/11}$ -coupled receptors, is consistently observed in vasospastic myocytes after SAH.³⁰ In our experiments, the PKC activator PMA suppressed Kv7 currents (Supplemental figure S3) and constricted basilar artery (Supplemental figure S4), mimicking the effects of spasmogens and of the direct Kv7 channel blocker XE991. Hence, PKC activation during vasospasm is likely mediating the vasoconstrictor responses at least in part via the suppression of Kv7 currents in basilar artery myocytes.

Kv7 channels are certainly not the only potassium channels that are involved in the regulation of vascular tone. There is abundant evidence that inhibition of other K^+ channel types, including 4-AP-sensitive Kv, inwardly rectifying (K_{ir}), ATP-sensitive (K_{ATP}) and Ca^{2+} -activated (K_{Ca}) potassium channels, induces or potentiates vasoconstriction in various arterial preparations^{31,32}. A number of previous studies have demonstrated that 4-AP treatment induces significant constriction of pressurized arteries or arterial rings³²⁻³⁶, suggesting that 4-AP-sensitive Kv channels may also be active in smooth muscle cells at physiological pressures. PKC-dependent suppression of 4-AP-sensitive K^+ currents by 5HT in pulmonary artery myocytes has also been reported³⁷, though a much higher concentration of 5HT (10 μ M) was used. In mesenteric artery myocytes, 4-AP-sensitive Kv currents were not inhibited by 100 pM AVP, whereas the same concentration of AVP suppressed Kv7 currents by 50% using the same preparation¹⁹, suggesting that Kv7 channels may be more favorable signal transduction targets at physiological agonist concentrations. In basilar arteries, which are relatively large conduit arteries, there is little if any evidence that 4-AP-sensitive Kv, K_{ATP} , K_{ir} , or K_{Ca} channels are active under resting conditions or are targeted by vasoconstrictor agonists to initiate vasoconstriction. However, these other channel types are likely to function as essential feedback regulators to limit influx of Ca^{2+} through VSCCs in constricted basilar arteries. Hence, pharmacological activators of these channels might be useful as therapeutic agents to oppose vasospasm. Unfortunately there are currently no pharmacological activators of 4-AP-sensitive Kv, K_{ATP} , K_{ir} , or K_{Ca} channels that have demonstrated clinical utility in the treatment of cerebral vasospasm.

Retigabine attenuates spasmogen-induced membrane depolarization and vasoconstriction

Retigabine opposed the spasmogen-induced suppression of Kv7 current amplitude (Figure 2) and attenuated membrane depolarization in basilar artery myocytes (Figure 3). These effects translated functionally into dilation of the pre-constricted basilar artery segments (Figure 5). In the presence of the spasmogens, when Kv7 currents were suppressed, retigabine still enhanced currents to about 65-80% above control levels (Figure 2, Supplemental figure S2). The increase in Kv7 current amplitude is accompanied by a hyperpolarizing shift in the voltage dependence of steady-state current activation (Figure 1C). Thus, in the presence of retigabine, Kv7 channels would have higher probability of opening and hence provide greater stabilization of negative membrane voltage. This would effectively oppose the spasmogen-induced membrane depolarization (Figure 3) and opening of L-type voltage-sensitive Ca^{2+} channels. The latter is the primary stimulus for the spasmogen-induced basilar artery constriction (see Supplemental figure S6). The vasodilatory actions of retigabine could result from enhanced voltage-dependent activation of the proportion of Kv7 channels that were not suppressed by the spasmogens. Alternatively (or perhaps in addition), retigabine may reverse the suppression of the Kv7

currents by spasmogens by virtue of its direct binding to the channel. Our findings do not distinguish between these two possibilities.

Direct suppression of L-type Ca^{2+} currents also likely contributes to retigabine-induced basilar artery dilation (Supplemental Fig. 7). This effect of retigabine as a calcium channel blocker has not previously been reported and is perhaps a bit surprising given that the same concentration of its close structural analog flupirtine, which is also a Kv7 channel opener, did not inhibit L-type currents in A7r5 rat aortic smooth muscle cells²². Notably, flupirtine was an effective vasodilator in both mesenteric¹⁹ and basilar arteries²⁰, despite its apparent inability to directly block L-type Ca^{2+} channels.

The agonist and drug concentrations used in the patch-clamp experiments and the vascular reactivity studies in pressure myography differ because of the varying sensitivities of the two systems used (Figure 2, 4, Supplemental figure S2). As noted previously¹⁷, a likely explanation for the differential sensitivity is that in intact arteries depolarization of a small proportion of smooth muscle cells leads to depolarization of the adjacent smooth muscle cells connected by gap junctions. Therefore the concentration of Kv7 channel activators required to oppose the concerted depolarization of vascular smooth muscle cells in a physiologically integrated arterial system is higher than in the single cell patch clamp studies. Conversely, lower concentrations of spasmogens are required in vascular reactivity studies compared to patch-clamp studies.^{17,18}

Kv7 channel openers as a potential therapy to relieve basilar artery vasospasm following SAH

Our *in vivo* SAH model study provided proof-of-concept that both retigabine and celecoxib effectively attenuate the sustained basilar artery vasospasm that persists 48 h after the initial injury (Figure 6).

Although cerebral vasospasm following SAH frequently results in neurological deficits in humans, a limitation in the present study is the inability to evaluate neurological benefits derived from Kv7 channel activator treatment. Despite the clear attenuation of basilar artery spasm by Kv7 channel activators in rats with SAH, obvious differences in behavior were not apparent. This is consistent with existing literature, which suggests that rat models of SAH do not produce neurobehavioral deficits comparable to those observed in SAH patients.³⁸ The time-course of development of vasospasm in this acute model is also different from delayed vasospasm observed in humans, another potential limitation of the animal model.

The hypothermic effect observed with retigabine (Supplemental figure S8) may represent an additional beneficial effect for patients with SAH, as mild hypothermia (33-34°C) has been reported to improve the functional outcomes in patients with delayed cerebral vasospasm³⁹ and in a rat model of SAH.⁴⁰

Celecoxib was previously found to exhibit dual actions on vascular ion channels (Kv7 current enhancement and L-type VSCC inhibition).²⁰ Here we found that retigabine has the same dual activity, though it only partially suppressed L-type currents (Supplemental Fig. 7) compared with full suppression of L-type currents by celecoxib at comparable doses.²⁰ This may account for the tendency of retigabine to induce less dilation of the basilar artery in the SAH model compared with celecoxib (Figure 6).

Another potential benefit of Kv7 channel opener therapy is that these drugs will also likely reduce neuronal deficits after SAH through their direct neuroprotective effects⁴¹⁻⁴⁴. Retigabine has been found to exhibit neuroprotective effects, presumably by reducing

pathological axonal depolarization.^{41, 44} Celecoxib is likely to provide neuroprotective and anti-inflammatory effects via its clinically utilized COX-2 inhibitory activity.⁴⁵⁻⁴⁷

Kv7 channel openers do not produce sustained hypotension

As a general therapeutic strategy, treatment with potassium channel activators or calcium channel blockers (CCBs) would seem like good choices to relieve vasospasm. There are a number of potential subtypes of potassium channels that could potentially be targeted. Blood components have been reported to reduce the activity of 4-AP-sensitive Kv channels and large conductance Ca^{2+} -activated K^+ channels and these effects have been implicated in the development of vasospasm^{11, 48, 49}. However, despite their promise as therapeutic targets to modulate vascular tone, there are currently no drugs that are available for clinical use that directly activate 4-AP-sensitive Kv channels or large conductance Ca^{2+} -activated K^+ channels.¹⁶ Activation of another class of K^+ channels, the ATP-sensitive K^+ channels (K_{ATP}), with cromakalim relaxed pre-constricted basilar arteries and attenuated basilar artery vasospasm in animal models of vasospasm.^{50,51} But administration of K_{ATP} channel activators was also found to be associated with hypotension and tachycardia.⁵²

Sustained hypotension due to dilation of the resistance vasculature may limit the utility of other classes of K^+ channel openers (e.g. K_{ATP} channel openers) or CCBs in the effective treatment of cerebral vasospasm.^{13, 52} In contrast, neither of the Kv7 channel activators tested in the present study (retigabine and celecoxib) produced sustained hypotension (Figure 7) or altered heart rates in conscious rats. We speculate that vasodilatory responses to Kv7 channel activators would primarily result from relaxation of myocytes with depolarized membrane voltages (e.g. basilar artery myocytes following SAH), with little effect on resistance vessels where arterial myocytes generally have more hyperpolarized membrane voltages at which L-type VSCCs are not appreciably active. In this regard, the function of Kv7 channels in vascular myocytes is analogous to their function in excitability of neurons, where dysfunction, either by suppression of M-currents or inactivating mutations of Kv7 channels, results in hyperexcitability, precipitating seizures.⁵³ Retigabine, used as an anti-epileptic agent, dampens neuronal hyperexcitability with little effect on the normal function of the nervous system.⁵⁴ Similarly, in arteries, activation of Kv7 currents relaxes the constricted arterial myocytes (Figure 6) without producing marked systemic hypotension (Figure 7).

Conclusions

Our results suggest that Kv7 channels are critical mediators of cerebral vasospasm and that clinically used drugs that enhance Kv7 channel activity may be effective in reducing vasospasm and preventing or attenuating stroke following SAH. Our findings also provide the first evidence that suppression of Kv7 currents is a common mechanism that is shared by multiple vasoconstrictors that activate $\text{G}_{\text{q/11}}$ -coupled receptors. The significance of these findings may therefore extend beyond the role of Kv7 channels in the cerebral circulation, providing mechanistic insights into the signal transduction processes that contribute to physiological and pathological regulation of blood flow and blood pressure in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Cerebral vasospasm following subarachnoid hemorrhage (SAH) is characterized by prolonged severe constriction of the basilar artery, which often leads to ischemic brain damage. Locally elevated concentrations of spasmogenic substances induce persistent depolarization of myocytes in the basilar artery, leading to continuous influx of calcium (Ca^{2+}) through voltage-sensitive Ca^{2+} channels and myocyte contraction. Potassium (K^+) channel openers may have therapeutic utility to oppose membrane depolarization, dilate the arteries, and reduce ischemia. Here, we examined the involvement of vascular Kv7 K^+ channels in the pathogenesis of cerebral vasospasm and tested whether Kv7 channel openers are effective therapeutic agents in a rat model of SAH. Patch-clamp experiments revealed that three different spasmogens (serotonin, endothelin and vasopressin) suppressed Kv7 currents and depolarized freshly isolated rat basilar artery myocytes. These effects were significantly reduced in the presence of a Kv7 channel opener, retigabine. Retigabine ($10\text{ }\mu\text{mol/L}$) also significantly blocked L-type Ca^{2+} channels, reducing peak inward currents by $>50\%$. In the presence of a selective Kv7 channel blocker, XE991, the spasmogens did not produce additive constriction responses measured using pressure myography. Kv7 channel openers (retigabine or celecoxib) significantly attenuated basilar artery spasm in rats with experimentally-induced SAH. In conclusion, we identify Kv7 channels as common targets of vasoconstrictor spasmogens and as candidates for therapeutic intervention for cerebral vasospasm.

Figure 1. Retigabine enhances Kv7 currents in freshly isolated rat basilar artery myocytes

A. Time-course of XE991-sensitive Kv7 currents with the addition of 10 μ mol/L retigabine, recorded from a single basilar artery myocyte by continuous holding at -20 mV. B. Current-voltage curves (I-V) of mean steady-state Kv7 currents before (control; ●) and during treatment with 10 μ mol/L retigabine (○) and 10 μ mol/L XE991 (▼), normalized to control current measured at -20 mV before treatment. *Significantly different from control at indicated voltages using paired Student's "t" test, $p < 0.05$, $n = 5$. C. Normalized conductance plotted against membrane voltage was fitted by a single Boltzmann function to show the effect of retigabine on voltage dependence of channel activation. Retigabine induced a significant hyperpolarizing shift in the steady-state voltage dependence of activation; $V_{0.5}$ (control) was -30.2 ± 1.4 mV, $V_{0.5}$ (retigabine) was -41.4 ± 0.5 mV, $p < 0.05$ based on paired Student's "t" test, $n = 5$.

Figure 2. Serotonin (5HT) suppresses Kv7 currents in freshly isolated rat basilar artery myocytes

A. Kv7 current amplitude measured in a single basilar artery myocyte by continuous holding at -20 mV during application of 100 nmol/L 5HT and subsequent addition of 10 μ mol/L retigabine as indicated. B. I-V curves of steady-state Kv7 currents measured before (Control; ●), during treatment with 5HT (○) and following addition of 10 μ mol/L retigabine in the continued presence of 5HT (▼) (currents at each voltage step are normalized to control currents measured at -20 mV before treatment). 5HT significantly suppressed currents at voltages from -34.2 mV to -14.2 mV (using repeated-measures (RM) one-way ANOVA followed by post hoc Holm-Sidak test, $p < 0.05$, $n = 6$).

Figure 3. Serotonin depolarizes membrane voltage in basilar artery myocytes; Kv7 channel opener retigabine inhibits serotonin-induced membrane depolarization
A. Time-course of change in the membrane voltage with the addition of 100 nmol/L 5HT as indicated, in the absence (top) or presence of 10 μ mol/L retigabine (bottom). B. Summarized result of membrane voltage measured before (control; white bars) or approximately 25 min after (grey bars) addition of 5HT in the absence (n=4) or presence (n=3) of 10 μ mol/L retigabine as indicated. *Significantly different from control using paired Student's "t" test, $p < 0.05$; N.S— Not significant.

Figure 4. Kv7 current suppression is sufficient to induce basilar artery constriction; lack of additivity with basilar artery spasmogens

A. Representative time-course of change in basilar artery outer diameter shows constriction produced by application of Kv7 channel blocker, XE991 (10 $\mu\text{mol/L}$) and subsequent addition of 100 pmol/L AVP (left) or 75 nmol/L 5HT (right) during the continued presence of XE991. B. Summarized bar graph shows the percentage basilar artery constriction produced by 10 $\mu\text{mol/L}$ XE991, spasmogens (100 pmol/L AVP, 75 nmol/L 5HT, 100 pmol/L ET-1) alone and addition of spasmogens in the continued presence of 10 $\mu\text{mol/L}$ XE991. *Significant additional constriction compared to constriction produced by XE991 alone and 5HT alone using one-way ANOVA followed by post hoc Holm-Sidak test, $p < 0.05$. Addition of 100 pmol/L AVP or 100 pmol/L ET-1 in the presence 10 $\mu\text{mol/L}$ XE991 did not produce significant additional constriction, $n = 4-8$ in each group.

Figure 5. Retigabine reverses serotonin-induced basilar artery constriction

A. Representative time-course shows the effect of increasing concentrations of retigabine on outer diameter of a basilar artery segment pre-constricted with 5HT (75 nmol/L). B. Bar graph shows concentration-dependent retigabine-induced basilar artery dilation *Significant dilation compared to absence of retigabine, using RM one-way ANOVA followed by post hoc Holm-Sidak test, $p < 0.05$, $n = 5$.

Figure 6. Kv7 channel openers attenuate basilar artery spasm in a rat model of SAH

A. Illustrative photographs show basilar artery in the base of brain from rats in different treatment groups. Brains were photographed 48 h after injection of blood or aCSF and compared with brains of control rats (no surgery). B. Bar graph shows the mean basilar artery outer diameter measured in each of the treatment groups—Control (no surgery), aCSF control (sham surgery), SAH rats (black bars) treated with vehicle (DMSO, 1ml/kg, i.p., b.i.d), SAH rats treated with retigabine (7.5 mg/kg, i.p., b.i.d) and SAH rats treated with celecoxib (20 mg/kg, i.p., b.i.d). ***Significant difference between the treatment groups as indicated using one-way ANOVA followed by post hoc Holm-Sidak test, $p < 0.001$, $n = 9$.

Figure 7. Kv7 channel openers induce a transient drop in mean arterial pressure

A. Change in mean arterial pressure (MAP) during treatment, compared to the 24 h baseline MAP measured before treatment. Areas in grey shade are dark hours and areas in white are light hours during the 12 h dark/light cycle. Arrows indicate treatment times. Each data point represents the average change in MAP during a 30 min interval. Standard error is not represented for clarity. *Significantly different from vehicle group, using one-way ANOVA followed by post hoc Holm-Sidak test, $p < 0.05$, $n = 6-7$. B. Line graph shows the 24 h mean MAP recorded in untreated rats (control) or rats treated with vehicle (DMSO, 1ml/kg, i.p., b.i.d), retigabine (7.5 mg/kg, i.p., b.i.d) or celecoxib (50 mg/kg, i.p., b.i.d), N.S— no significant difference from control, $n = 6-7$.