Blood–Brain Barrier KCa3.1 Channels
Evidence for a Role in Brain Na Uptake and Edema in Ischemic Stroke

Yi-Je Chen, DVM, PhD; Breanna K. Wallace, PhD; Natalie Yuen, PhD; David P. Jenkins, PhD; Heike Wulff, PhD; Martha E. O’Donnell, PhD

Background and Purpose—KCa3.1, a calcium-activated potassium channel, regulates ion and fluid secretion in the lung and gastrointestinal tract. It is also expressed on vascular endothelium where it participates in blood pressure regulation. However, the expression and physiological role of KCa3.1 in blood–brain barrier (BBB) endothelium has not been investigated. BBB endothelial cells transport Na\(^+\) and Cl\(^-\) from the blood into the brain transcellularly through the cooperation of multiple cotransporters, exchangers, pumps, and channels. In the early stages of cerebral ischemia, when the BBB is intact, edema formation occurs by processes involving increased BBB transcellular Na\(^+\) transport. This study evaluated whether KCa3.1 is expressed on and participates in BBB ion transport.

Methods—The expression of KCa3.1 on cultured cerebral microvascular endothelial cells, isolated microvessels, and brain sections was evaluated by Western blot and immunohistochemistry. Activity of KCa3.1 on cerebral microvascular endothelial cells was examined by K\(^+\) flux assays and patch-clamp. Magnetic resonance spectroscopy and MRI were used to measure brain Na\(^+\) uptake and edema formation in rats with focal ischemic stroke after TRAM-34 treatment.

Results—KCa3.1 current and channel protein were identified on bovine cerebral microvascular endothelial cells and freshly isolated rat microvessels. In situ KCa3.1 expression on BBB endothelium was confirmed in rat and human brain sections. TRAM-34 treatment significantly reduced Na\(^+\) uptake, and cytotoxic edema in the ischemic brain.

Conclusions—BBB endothelial cells exhibit KCa3.1 protein and activity and pharmacological blockade of KCa3.1 seems to provide an effective therapeutic approach for reducing cerebral edema formation in the first 3 hours of ischemic stroke. (Stroke. 2015;46:237-244. DOI: 10.1161/STROKEAHA.114.007445.)

Key Words: blood–brain barrier ■ brain edema ■ cerebral ischemia ■ KCa3.1 protein, mouse ■ stroke

KCa3.1, a Ca\(^2+\)-activated potassium channel, regulates ion and fluid secretion in the kidney, intestine, airway, pancreatic duct, and in colonic epithelial cells.\(^1\)–\(^4\) In these secretory epithelia, KCa3.1 recycles K\(^+\) out of the cell after accumulation of Cl\(^-\) through the Na–K–Cl transporter and thus facilitates Cl\(^-\) secretion by maintaining a favorable electrochemical gradient for Cl\(^-\) efflux.\(^5\)\(^,\)\(^6\) Accordingly, inhibition of KCa3.1 has been proposed as a therapeutic strategy for diarrhea,\(^1\) whereas KCa3.1 activators have been considered for increasing fluid secretion in cystic fibrosis.\(^7\) KCa3.1 is also known to be expressed on peripheral vascular endothelial cells where it participates in endothelium-derived hyperpolarization (EDH) for blood pressure regulation.\(^8\)\(^,\)\(^9\) Even though there is a large body of literature on K\(^+\) channel expression in peripheral blood vessel endothelial cells, little is known about the expression and physiological role of KCa3.1 in endothelial cells of the blood–brain barrier (BBB). BBB endothelial cells tightly regulate transcellular movement of ions and other solutes between blood and brain via transporters and channels that are asymmetrically distributed between luminal and abluminal membranes, much like secretory epithelial cells. In the healthy normoxic brain, BBB endothelial cells secrete Na\(^+\), Cl\(^-\), and water into the brain, producing ≤30% of brain interstitial fluid and also remove K\(^+\) from the brain as needed to maintain an appropriately low brain extracellular K\(^+\) concentration.\(^10\)–\(^12\) Much is still unknown about the ion transporters and channels that accomplish this although there is an evidence that Na\(^+\), Cl\(^-\), and water secretion into the brain involves luminal Na–K–Cl cotransport (NKCC) and Na/H exchange, together with the abluminal Na/K ATPase and abluminal Cl\(^-\) channels.\(^13\)–\(^18\) During the early hours of ischemic stroke, cerebral edema forms in the presence of an intact BBB because transendothelial secretion of Na\(^+\), Cl\(^-\), and water into the brain is greatly increased.\(^10\)–\(^12\),\(^17\),\(^18\) Previous studies have provided evidence that this involves ischemia-stimulation of luminal NKCC and Na/H exchange activity and that inhibition of these transporters by intravenously administered bumetanide or HOE642 effectively reduces edema and brain Na\(^+\) uptake.\(^17\) The primary anion accompanying Na\(^+\) in ischemia-induced BBB secretion is Cl\(^-\).\(^19\) In Na\(^+\) and

Received September 12, 2014; final revision received October 30, 2014; accepted November 5, 2014. From the Department of Pharmacology (Y.-J.C., D.P.J., H.W.) and Department of Physiology and Membrane Biology (B.K.W., N.Y., M.E.O.), University of California, Davis. Presenting in part at the Experimental Biology Meeting, San Diego, CA, April 26–30, 2014. Correspondence to Yi-Je Chen, DVM, PhD, Department of Pharmacology, UC Davis, School of Medicine, University of California, 451 Health Sciences Dr, GBSF 3409, Davis, CA 95616. E-mail ljchen@ucdavis.ucdavis.edu © 2014 American Heart Association, Inc. Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.114.007445

237
Cl\(^-\) secreting epithelia, K\(^+\) channels serve the important role of maintaining an outwardly directed electrochemical gradient for Cl\(^-\) to support Cl\(^-\) efflux. Furthermore, NKCC is highly sensitive to inhibition by elevation of intracellular Cl\(^-\) concentration via a Cl\(^-\) sensitive kinase.\(^20\) In this regard, BBB K\(^+\) channels activity is predicted also to support transendothelial secretion of Na\(^+\) and Cl\(^-\) by supporting luminal NKCC activity. This study was conducted to investigate whether KCa3.1 is expressed on BBB endothelial cells and participates in ischaemia-induced cerebral edema formation.

**Materials and Methods**

**Cell Culture**

Bovine cerebral microvascular endothelial cells (CMEC) were maintained in DMEM containing 5 mmol/L \(\nu\)-glucose, 1 mmol/L Na-pyruvate, 2 mmol/L \(\nu\)-glutamine, 50 \mu\text{g}\text{mL}^{-1}\) gentamicin, 1 ng\text{mL}^{-1}\) bovine basic fibroblast growth factor, 5% calf serum, and 5% horse serum (DMEM growth medium) in an atmosphere of 95% humidified air with 5% \(\text{CO}_2\) at 37°C, as described previously.\(^20\) Cells were grown to confluence on collagen-coated and attachment factor-coated (Cell Systems, Kirkland, WA) 6-well plates or coverslips. Cells were refed fresh DMEM growth medium every 48 hours until 2 days before the experiments, when medium was replaced with a 50:50 mixture of DMEM growth medium and astrocyte-conditioned medium (ACM) to all CMEC culture. ACM was prepared by exposing primary cultured rat neonatal astrocytes to DMEM containing 10% fetal bovine serum for 48 hours and added to the CMEC cultures before functional assays because multiple previous studies had shown that ACM promoted a BBB phenotype in the cultured cells.\(^21\)

**Electrophysiology**

Bovine CMEC grown for 48 hours in ACM containing medium were studied in the whole-cell configuration of the patch-clamp technique as described.\(^22\) Briefly, the holding potential in all experiments was −80 mV. For measurement of KCa3.1 currents, the internal pipette solution contained (in mmol/L): 145 K\(^+\) aspartate, 2 MglCl\(^-\), 10 HEPES, 10 K, EGTA, and 8.5 CaCl\(^2-\) (3 \mu\text{mol free Ca}^{2+}). pH 7.2, 290 to 310 mOsm. External solution (in mmol/L): 160 Na\(^+\) aspartate, 4.5 KCl, 2 CaCl\(^2-\), 1 MgCl\(^2-\), 5 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.4, 290 to 310 mOsm. KCa3.1 currents were elicited by 200-ms voltage ramps from −120 to 40 mV applied every 10 s and the reduction of slope conductance at −80 mV by drug taken as a measure of channel block. TRAM-34, charybdotoxin, apamin, andiberiotoxin were used to identify KCa3.1 channels. TRAM-34 was synthesized in the Wulff laboratory as previously described.\(^22\) Charybdotoxin, apamin, and iberiotoxin were from Bachem Americas Inc (Torrence, CA).

**Immunocytohistological and Immunofluorescence Staining**

CMEC monolayers grown on collagen-coated glass slides in the presence of ACM were subjected to immunocytohistological and immunofluorescence assays because multiple previous studies had shown that ACM promotes a BBB phenotype in the cultured cells.\(^21\)

**Western Blot**

Bovine CMEC whole cell lysates from cells grown for 48 hours in ACM containing medium and freshly isolated rat brain microvessel lysates were subjected to Western blot analysis. Rat brain microves-
sels were isolated as described.\(^23\) Five milligram protein from each sample was denatured with 50 mmol/L dithiothreitol in SDS sample reducing buffer (NP0004; Life Technologies) and heated at 70°C for 10 minutes. Protein samples were then separated on 12% Tris-HCl gel (No.5952, PAGE\(_{\text{G}}\) Gold Precast Gel; Lonza) using electrophoresis and electrotransferred to polyvinylidene fluoride membranes (BioRad XCell SureLock, Hercules). Membranes were blocked with 7.5% milk in PBS with 0.1% Tween-20 (PBST) for 1 hour, incubated with anti-KCa3.1 antibody (AV35098, rabbit polyclonal, 1:3000; Sigma-Aldrich) in milk/PBST overnight at 4°C, washed with PBST, and incubated with horseradish peroxidase-conjugated secondary antibody (A16096, goat antirabbit, 1:2000; Life Technologies) in milk/PBST for 1 hour at room temperature. After washing with PBST, bound antibody was detected using enhanced chemiluminescence Kit (RPN2133, ECL plus; GE Healthcare) and visualized on a Fuji Film LAS-4000 Imaging Machine (Medford, United Kingdom).

**KCa3.1 Activity Assay**

KCa3.1 activity was assessed by Ca\(^2+\)-sensitive and KCa3.1 channel blocker–sensitive K\(^+\) efflux using \(^{86}\text{Rb}\) as a tracer for K\(^+\) as described previously.\(^7,16\) Bovine CMEC monolayers grown for 48 hours in ACM containing medium on 24-well plates were placed in a hypoxia chamber (COY Laboratory Products; Grass Lake, MI) preset to 37°C with an atmosphere of 5% \(\text{CO}_2\) (normoxia). Cells were incubated in DMEM with \(^{86}\text{Rb}\) (1 \mu\text{Ci}\text{mL}^{-1}) for 2 hours. Media was removed and cells were washed 3 times with Low-Ca\(^+\) Ringers (138 mmol/L NaCl, 10 mmol/L 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, 4.5 mmol/L KCl, 1 mmol/L MgCl\(^2-\), and 250 \mu\text{mol CaCl}\(^2-\)). Treatment media was added to the cells for 10 minutes: high-Ca\(^+\) Ringers (20 mmol/L CaCl\(^2-\), 120 mmol/L NaCl, and 20 mmol/L 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) or high-Ca\(^+\)-

**Middle Cerebral Artery Occlusion in Rats**

This study was approved by the University of California, Davis, Animal Use and Care Committee and conducted in accordance with the guidelines for survival surgery in rodents. Adult male Sprague Dawley rats weighing 110 to 130 g (Charles River, Wilmington, MA) were acclimatized to the new vivarium for 3 to 7 days and used for the surgery when they weighed 190 to 200 g. Rats were anesthetized using box induction with 5% isoflurane in medical grade oxygen and mounted in Fluormount-G (SouthernBiotech) with 4,6-diamidino-2-phenylindole and imaged with a Zeiss LSM-510 confocal microscope. In situ expression of KCa3.1 was evaluated in rat and human brain sections by double-labeling immunofluorescence with the rat BBB endothelial cell–specific marker (Clone: SMI-71, mouse monoclonal, 1:1000; Covance), von Willebrand factor for human endothelial cells (Clone: RFF-VIII B/1, mouse monoclonal, 1:100; Serotec), and KCa3.1. Staining was visualized by Alexa Fluor 405 (A-51556, Goat antiamoine, 1:500; Life Technologies) and Alexa Fluor 647 conjugated secondary antibodies (A-20991, Goat antiabbit, 1:500; Life Technologies). The results were analyzed by confocal microscopy. Human brain samples were provided by the Alzheimer Disease Center at the University of California, Davis, funded by National Institutes of Health/National Institute of Aging (P30 AG10129). The tissue procurement was approved by the Institutional Review Board. Informed consent to share research tissue after death was obtained from all patients or a representative before their death.
then maintained on 0.5% to 1.5% isoflurane via a facemask. To assure consistent reduction of cerebral blood flow throughout the procedure, a Laser Doppler (Moor Instruments, Wilmington, DE) was used to monitor cerebral blood flow throughout the middle cerebral artery occlusion (MCAO) surgery. Permanent focal cerebral ischemia was then induced by occlusion of the left middle cerebral artery as previously described. Briefly, the left common carotid artery was surgically exposed, the external carotid artery was ligated distally from the common carotid artery, and a silicone rubber-coated nylon monofilament with a tip diameter of 0.43±0.02 mm (Doccol Corp, Redlands, CA) was inserted into the external carotid artery and advanced into the internal carotid artery to block the origin of the middle cerebral artery. Vehicle (Miglyol 812 neutral oil, 1 μL/g; Spectrum Chemicals, Gardena, CA) or TRAM-34 (40 mg/kg, intraperitoneal injection) was given 20 minutes before MCAO.

Magnetic Resonance Chemical Shift Imaging of Brain Na⁺

Magnetic resonance chemical shift imaging (CSI) was used to determine extravascular brain Na⁺ content of rat brains with the nuclear magnetic resonance chemical shift/excitation reagent dysprosium triethylene tetramine hexaacetic acid as described previously. After ligation of both sides of the renal artery and vein, 250 mmol/L of dysprosium triethylene tetramine hexaacetic acid (Sigma-Aldrich) was infused intravenously at 0.3 mL/min to achieve a final dose of 1.5 mmol/L per kilogram and allowed to equilibrate across the various body compartments for 20 minutes before MCAO. A 7-T Bruker Biospec magnetic resonance spectroscopy/MRI system (Bruker) with a double-tuned 1H/23Na probe (Doty Scientific, Columbia, SC) was used for Na⁺ CSI. Two-dimensional Na⁺ CSI images were acquired via the standard Bruker CSI protocol with Paravision 2.1 software (Bruker Biospin GmbH, Rheinstetten, Germany) and each 23Na CSI data set was acquired in 21 minutes. Na⁺ CSI spectra were further analyzed using MATLAB 2011b (MathWorks, Natick, MA) to integrate over the unshifted extravascular Na⁺ peak and that of an external standard to calculate the extravascular Na⁺ concentration in the core of the infarct ≤170 minutes after MCAO.

Magnetic Resonance Diffusion-Weighted Imaging of Edema Formation

Resonance diffusion-weighted imaging analysis of apparent diffusion coefficient (ADC) values was performed to evaluate edema formation using a 7-T Bruker Biospec magnetic resonance spectroscopy/MRI system (Bruker) with a double-tuned 1H/23Na probe (Doty Scientific, Columbia, SC) was used for Na⁺ CSI. Two-dimensional Na⁺ CSI images were acquired via the standard Bruker CSI protocol with Paravision 2.1 software (Bruker Biospin GmbH, Rheinstetten, Germany) and each 23Na CSI data set was acquired in 21 minutes. Na⁺ CSI spectra were further analyzed using MATLAB 2011b (MathWorks, Natick, MA) to integrate over the unshifted extravascular Na⁺ peak and that of an external standard to calculate the extravascular Na⁺ concentration in the core of the infarct ≤170 minutes after MCAO.

Statistics Analyses

Data were analyzed for significance using analysis of variance or by Student t test. P<0.05 were considered to indicate significant difference.

Results

KCa3.1 Is Expressed in Isolated Rat Brain Microvessels and Cultured Bovine CMEC

To test the hypothesis that the KCa3.1 channel participates in edema formation at the BBB during ischemic stroke, we first evaluated KCa3.1 protein expression on the BBB endothelium using Western blot and immunocytohistological staining. A KCa3.1 protein-specific band at ≈47 kDa was detected in whole cell lysates of freshly isolated rat brain microvessels and cultured bovine CMEC (Figure 1A). Immunocytohistological and immunofluorescence further confirmed the expression of KCa3.1 channel protein on bovine CMEC (Figure 1B, left, and 1C, right). The specificity of the polyclonal KCa3.1 antibody was confirmed by staining HEK-293 cells stably expressing hKCa3.1 (Figure 1C, left). We next used the whole-cell patch-clamp technique to assess the magnitude of the KCa3.1 current in bovine CMEC and determine whether the cells expressed any other significant K⁺ conductances. Voltage ramps from −120 to +40 mV in the presence of 3 μmol free Ca²⁺, a concentration that fully activates KCa3.1 currents, in the patch pipette-elicited Ca²⁺-activated K⁺ currents that exhibited the biophysical and pharmacological properties of KCa3.1 in the majority of cells. The current was voltage-independent, reversed ≈−80 mV and was not visible in the absence of Ca²⁺ (not shown). The current was sensitive to the classical but not KCa3.1 selective scorpion venom peptide charybdotoxin (Figure 2A), insensitive to the KCa2 blocker apamin and the KCa1.1 blocker iberiotoxin (data not shown), and dose-dependently inhibited by the KCa3.1 selective small molecule KCa3.1 blocker TRAM-34 (Figure 2B). In addition to KCa3.1, CMEC also expressed small inward-rectifier currents in roughly 50% of cells (Figure 2A), but no
significant voltage-dependent conductance, demonstrating that KCa3.1 is the major K⁺ channel in BBB endothelial cells.

Rat and Human BBB Endothelium Express KCa3.1 In Situ

In these studies, we also evaluated KCa3.1 expression in BBB in situ using rat and human brain sections and confocal immunofluorescence microscopy. BBB endothelial cells were identified in rat brain sections by colabeling with anti-IST-57 antibody, which stains rat BBB endothelial cells (Figure 3, upper panel). Anti-Von Willbrand Factor antibody was used to identify BBB endothelial cells in human brain sections (Figure 3, lower panel). In both cases, KCa3.1 was clearly expressed on the same cells labeled with the endothelial markers.

KCa3.1 Blockers Inhibit K⁺ Flux on Bovine CMEC

To determine whether KCa3.1 is involved in K⁺ movement across the BBB, we tested the effect of KCa3.1 inhibitors in a well-established ⁸⁶Rb flux assay, which was previously used to evaluate activity of the NKCC and Na/H exchange activity in CMEC. All experiments were performed in the presence of 20 mmol/L Ca²⁺ and results were ratioed to ⁸⁶Rb flux in DMSO-treated control cells. In cells exposed to 0.25 mmol/L external Ca²⁺, the ratio dropped to 0.84 demonstrating that the K⁺ efflux is sensitive to changes in external Ca²⁺ concentration and thus to the resulting changes in Ca²⁺ driving force for Ca²⁺ entry. In CMEC exposed to 20 mmol/L Ca²⁺, application of the KCa3.1 blockers NS1680 and TRAM-34 reduced K⁺ efflux in a dose-dependent manner. As expected, the KCa1.1 blocker paxilline (1 μmol) and the KCa2.2 inhibitor NS8593 (10 μmol) did not significantly reduce K⁺ efflux (Figure 4).

We exclusively used small molecule inhibitors instead of peptides for this assay because they are more membrane permeable and less sticky and, therefore, much easier to handle in the flux experiments.

TRAM-34 Reduces Na⁺ Accumulation and Edema Formation in a Rat MCAO Model

To test our hypothesis that KCa3.1 inhibition can attenuate edema formation, we evaluated the effects of TRAM-34 on brain Na⁺ and water uptake in the rat permanent MCAO model of focal ischemic stroke. After induction of MCAO extravascular Na⁺ accumulation in the brain was visualized and quantitated by Na magnetic resonance spectroscopy CSI over a time course of 60 to 170 minutes from the onset of occlusion. The nuclear magnetic resonance software used converts the integrated signals into pseudocolor (orange in this case) correlating the Na⁺ concentration to color intensity (higher

Figure 2. KCa3.1 current in cultured bovine cerebral microvascular endothelial cells (CMEC). A, Current recorded from a CMEC with a ramp-pulse from −120 to +40 mV and 3 μmol/L free Ca²⁺ in the patch-pipette. After blocking the KCa current with 100 nmol/L charybdotoxin (ChTX), a small Kir current (red) remains. B, The KCa current is blocked by the KCa3.1-specific inhibitor TRAM-34 (IC₅₀, 20 nmol/L) but is insensitive to the KCa2 blocker apamin or the KCa1.1 blocker iberiotoxin (not shown).

Figure 3. KCa3.1 is expressed on blood–brain barrier (BBB) endothelial cells in situ. Sections of rat brain (upper panels) and human brain (lower panels) were stained with rat BBB endothelial cell-specific marker (SMI-71), von Willebrand factor (vWF) for human endothelial cells, and KCa3.1 antibody. Representative images are shown.

Figure 4. Bovine cerebral microvascular endothelial cells (CMEC) exhibit a TRAM-34 sensitive K⁺ flux. TRAM-34 and NS6180 reduced K⁺ efflux from bovine CMEC in a dose-dependent manner, whereas neither Pax nor NS8593 significantly altered K⁺ efflux from the cells. K⁺ efflux in the presence of inhibitors relative to 20 mmol/L Ca²⁺ control efflux was 0.86±0.05 (P<0.01) for 1 μmol/L NS1680, 0.9±0.057 (P<0.01), 0.76±0.086 (P<0.01), 0.63±0.198 (P<0.01), and 0.58±0.269 (P<0.01) for TRAM-34 at 100 nmol/L, 250 nmol/L, 500 nmol/L, and 1 μmol/L, respectively. Lower doses of NS1680 250 nmol/L (0.93±0.14; P=0.052) reduced ⁸⁶Rb⁺ efflux but the effect was not statistically significant. **Significantly different from Ringer with 20 mmol/L Ca²⁺; P<0.01.
intensity represents higher concentration). Rats administered TRAM-34 immediately before induction of MCAO showed significantly lower brain Na+ uptake compared with vehicle-treated rats. Figure 5A shows representative brain Na+ images obtained at 104 and 170 minutes after onset of MCAO in vehicle-treated and TRAM-34–treated rats. Figure 5B depicts the quantitated changes in brain extravascular Na content. In vehicle-treated rats subjected to MCAO, the extravascular Na+ content rose linearly, reaching 1.92±0.06 by 170 minutes. For rats given TRAM-34 (40 mg/kg IP) and then subjected to permanent middle cerebral artery occlusion, the increase in brain Na+ content ratio was significantly attenuated at all time points ≤170 minutes in permanent middle cerebral artery occlusion (Figure 4A and 4B; ratio=1.45±0.4 at 170 minutes). The ratio of ipsilateral to contralateral extravascular Na+ content in rats with sham surgery was 1.0 (data not shown).

Edema formation was evaluated in a separate group of animals by diffusion-weighted imaging analysis of ADC values over a time course of 38 to 236 minutes from the onset of permanent MCAO. Figure 5C shows representative diffusion-weighted imaging images from vehicle-treated and TRAM-34–treated rats at 104 and 236 minutes after induction of MCAO. Lower ipsilateral to contralateral ADC ratios indicate greater edema formation. We found that TRAM-34 treatment attenuated the MCAO-induced fall in ADC ratios. In vehicle-treated rats, ADC ratios fell from 1.0 (Sham rats) to 0.69±0.08 at 38 minutes, and 0.54±0.03 at 236 minutes; whereas ADC ratios fell to only 0.84±0.09 at 38 minutes and 0.65±0.15 at 236 minutes in TRAM-treated rats. This attenuation of edema formation by TRAM-34 was sustained throughout the nearly 4-hour experiment. Statistically significant differences in ADC ratios of vehicle-treated versus TRAM-treated rats were observed from 38 to 148 minutes from the start of MCAO (Figure 5D).

Discussion

In this study, we demonstrate that the Ca2+-activated K+ channel KCa3.1 is expressed on bovine, rat, and human BBB endothelial cells and is a significant participant in K+ fluxes at the BBB. Moreover, we show that the KCa3.1 blocker TRAM-34, a compound that is highly selective for KCa3.1,22,26 significantly attenuates edema formation and Na+ accumulation in the brain of rats in the early stage of ischemic stroke, suggesting that KCa3.1 constitutes a new therapeutic target for reducing brain edema. K+ channels have been proposed to participate in K+ homeostasis of the brain for decades; however, few studies have investigated K+ channels on BBB endothelial cells. Most of those studies were performed before KCa3.1 was cloned in 199727,28 and were conducted with limited tools, such as antibodies or specific channel blockers. In 1991, Hoyer et al29 reported Kv and inward rectifier (Kir) currents in freshly isolated and primary-cultured porcine brain microvascular cells. In 1995, Van Renterghem et al30 described a K+ current with biophysical and pharmacological properties resembling a KCa3.1 channel in cultured rat brain capillary endothelial cells. More recently, Millar et al31 described Kv1 and Kir family channels in cultured rat brain endothelial cells by electrophysiology and demonstrated the presence of mRNA for Kv1.3, Kir2.1, and Kir2.2 channels by reverse transcriptase polymerase chain reaction. In this study, we have confirmed the observations of Van Renterghem et al,30 who described an intermediate-conductance KCa current (but could not assign it a molecular identity because KCa3.1 had not yet been cloned), and now show that KCa3.1 is one of the major K+ channels in BBB endothelial cells and is involved in ischemia-induced K+ fluxes and in edema formation in vivo. In the electrophysiology experiments conducted in this study, we also observed Kir currents with properties resembling Kir2.1 and Kir2.2 but did
not study them further because we do not consider Kir2.1/2.2 realistic targets for stroke treatment.

In this study, we have shown for the first time that inhibition of BBB KCa3.1 reduces edema formation during the first 4 hours of permanent MCAO. In the early stage of stroke when the BBB is still intact, the activity of a luminal membrane BBB NKCC is stimulated by ischemic factors and, together with the abluminal Na/K ATPase that mediates active extrusion of Na⁺ at the abluminal membrane, contributes to secretion of Na⁺, Cl⁻, and water from the blood into the brain and formation of brain edema.32 Our previous studies have shown that the K⁺ content of the BBB cells does not increase for several hours, despite the fact that both NKCC and the Na/K ATPase keep bringing K⁺ into the BBB endothelial cells during ischemia.32 This lack of K⁺ increase might be caused by K⁺ channel activity, most likely KCa3.1, allowing extrusion of K⁺ from the cells and thereby maintaining an appropriate intracellular K⁺ concentration and membrane potential (Figure 6).

The KCa3.1 channel regulates Cl⁻ secretion across secretary epithelia by maintaining the electrochemical driving force for Cl⁻ efflux through apical Cl⁻ channels. In airway, gastrointestinal, and pancreatic duct epithelia, inhibition of KCa3.1 channels slows down Cl⁻ efflux and increases intracellular Cl⁻ ([Cl⁻]i).2,33–36 Because NKCC activity is sensitive to [Cl⁻]i through a Cl⁻-sensitive kinase signaling mechanism, with elevated [Cl⁻]i inhibiting and reduced [Cl⁻]i stimulating NKCC, increased [Cl⁻]i inhibits not only Cl⁻ influx but also Na⁺ and K⁺ influx.29 In addition to the regulation of ion secretion, KCa3.1 plays an important role in regulating Ca²⁺ signaling and membrane potential in different cells, such as T-cells and microglia.37–39 K⁺ efflux through KCa3.1 channels helps to maintain a negative membrane potential for Ca²⁺ influx through inward-rectifier type Ca²⁺ channels.40 In vascular endothelial cells, Ca²⁺ similarly plays a crucial role in initiating signal transduction cascades with the transient receptor potential (TRP) channels TRPC3, TRPV3, and TRPV4 serving as the major type of Ca²⁺-permeable channels in cultured human, rat, and mouse microvessel endothelial cells.41 In iv in vitro studies, intracellular Ca²⁺ increases in 10 to 15 s after ischemic factor stimulations. Both intracellular-stored and extracellular Ca²⁺ are crucial for the Ca²⁺ elevation.42–44 Considering the evidence that blockade of KCa3.1 slows down intracellular Ca²⁺ elevation, increases [Cl⁻]i, and reduces NKCC activity, we hypothesize that blocking KCa3.1 will elevate BBB [Cl⁻], and reduce intracellular Ca²⁺, which both reduces NKCC activity in BBB endothelial cells, and should, therefore, reduce Na⁺ secretion in to the brain in ischemic stroke (Figure 6) as indeed observed in the MRI experiments after KCa3.1 blockade.

Although K⁺ channels may participate in edema formation and transport of K⁺ across the BBB, it should be recognized that K⁺ channels also participate in the regulation of cerebrovascular tone. In cerebral capillaries that form the BBB, the endothelium is surrounded by astrocyte endfeet. However, in larger vessels, where vascular smooth muscle cells underlie the endothelium, K⁺ channels are involved in EDH-induced vasodilations.45–47 KCa2.3 and KCa3.1 channels open in response to increases in intracellular Ca²⁺ and hyperpolarize the endothelium. This hyperpolarization is then transmitted to the smooth muscle cells by direct electric coupling through myoendothelial junctions or by the accumulation of K⁺ ions in the intercellular myoendothelial space.46,48,49 EDH-mediated vascular responses have been observed in human, rat, and guinea pig cerebral arteries,50–52 and several studies have reported that EDH-mediated responses remain intact during hypoxia,50,53 and even become more prominent during ischemia/reperfusion.54 In the middle cerebral artery, combined inhibition of KCa2.3 and KCa3.1 is required to abolish EDH responses; however, blocking KCa3.1 alone is sufficient to abolish EDH when nitric oxide synthase is inhibited.46,55 We are, therefore of course, aware of the fact that blocking KCa3.1 might affect EDH responses in the brain vasculature; however, the in vivo effects and physiological roles of EDH in ischemic stroke are currently not well understood and we, therefore, are unable to determine how partial or complete inhibition of EDH responses contributes to the effects on edema formation as we report here.

Besides the potential benefit of reducing edema in the early stage of stroke, we have also recently reported that TRAM-34 reduces infarct area, microglia activation, and neurological deficits in rat MCAO with 7 days of reperfusion even if treatment was started at 12 hours after reperfusion.24 In this case, TRAM-34 inhibited microglia activation and decreased neuroinflammation through acting on KCa3.1 channels on microglia. Since our experiments were performed between 38 minutes and 3 hours after MCAO, we do not think microglia activation is involved at this early time point. Because these experiments showed that TRAM-34 effectively penetrates into the brain,24 we also cannot completely exclude that in addition to acting on the endothelium in early stroke, TRAM-34 could have affected astrocyte functions. Whether KCa3.1 is expressed or functionally important on astrocytes is currently not completely clear. Three groups cloning KCa3.1 did not detect mRNA for KCa3.1 (which was called hSK4, hIK1, or hKCa4 at that time) in the brain in 1997 when they evaluate the distribution of KCa3.1 in different type of tissue.57–59

When we performed IHC staining of KCa3.1 in brain sections from rats at 7 days after reperfusion ischemic stroke, we only observed staining on activated microglia and the vascular endothelium.28 However, other studies have suggested that KCa3.1 might be expressed on astrocytes in some pathological conditions, such as spinal cord injury, glioblastoma, and astrogliosis.46,55,57 Longden et al58 reported that KCa3.1 might

---

**Figure 6.** Hypothesized role of blood–brain barrier (BBB) KCa3.1 channels in edema formation during cerebral ischemia. During cerebral ischemia, a BBB luminal Na–K–Cl cotransport (NKCC) works with an abluminal Na/K pump and a Cl⁻ channel to cause transport of Na⁺ and Cl⁻ (with water following) from blood to brain. This secretion is greatly increased during ischemia by vasopressin, hypoxia, and aglycemia, 3 prominent factors present during cerebral ischemia. Blocking KCa3.1 might reduce NKCC activity by reducing intracellular Ca²⁺ and increasing intracellular Cl⁻ concentration.
express on astrocyte endfeet. More experiments with higher detecting resolution, like immunoelectron microscopy, will be needed to clarify this question. It will further need to be investigated whether pericytes express KCa3.1.

On average, a stroke occurs every 40 s in the United States and 87% of these cases are ischemic strokes.59 Cerebral edema is a major cause of neuronal death in stroke. Yet, acute therapies for stroke induced cerebral edema are currently limited. This is the first study evaluating a link between BBB endothelial K+ channels and edema formation in the brain and it might open up a new therapeutic strategy for the prevention or treatment of ischemia-induced cerebral edema. Other useful applications for our findings that KCa3.1 blockers reduce brain edema would be the treatment of traumatic brain injury or premedication of patients undergoing brain surgeries. In this context, we would like to point out that pharmacological KCa3.1 blockade has been shown to be safe and well tolerated in various mouse, rat, pig, and sheep models of autoimmunity60 and that senicapoc, a compound structurally similar to our TRAM-34, was safe and well tolerated in a Phase-1 clinical trial in healthy volunteers.61 Senicapoc was afterward found to significantly reduce hemolysis and increase hemoglobin levels in a 12-week, multicenter, randomized double-blind Phase-2 study in sickle cell disease patients.62 However, in a subsequent Phase-3 study, which was designed to compare the rate of acute vaso-occlusive pain crisis occurring in sickle cell disease patients, senicapoc failed to reduce this desired clinical end point, despite again reducing hemolysis and increasing hemoglobin levels and not inducing any significant adverse events.63 Senicapoc was subsequently deposited in the National Institutes of Health National Center for Advancing Translational Sciences (NCATS) library and would, therefore, theoretically be available for investigator initiated clinical trials.

Sources of Funding
This work was supported by National Institutes of Health (NIH) RO1 GM076063 to Dr Wulff, NIH RO1 NS039953 to Dr O’Donnell, the Alzheimer Disease Center at the University of California, Davis, funded by NIH/National Institute of Aging (P30 AG10129) and a postdoctoral fellowship from the American Heart Association Western States Affiliate (09POST2260973) to Dr Chen.

Disclosures
Dr Wulff is an inventor on a University of California patent claiming TRAM-34 for immunosuppression. However, because no pharmaceutical companies expressed any interest in the subsequently filed disclosure claiming TRAM-34 for ischemic stroke the University of California decided not to file an addendum to the TRAM-34 patent with this indication.

References
lial cell Na-K-Cl cotransporter activity is V1 receptor and 

Med Biol W

Vong CO, Yao X. TRP channels in vascular endothelial cells. 


Blood–Brain Barrier KCa3.1 Channels: Evidence for a Role in Brain Na Uptake and Edema in Ischemic Stroke
Yi-Je Chen, Breanna K. Wallace, Natalie Yuen, David P. Jenkins, Heike Wulff and Martha E. O'Donnell

Stroke. 2015;46:237-244; originally published online December 4, 2014;
doi: 10.1161/STROKEAHA.114.007445

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/1/237

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/