Central Role of TRPM4 Channels in Cerebral Blood Flow Regulation

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Background and Purpose—The transient receptor potential channel TRPM4 is critically linked to the myogenic constrictor response of cerebral arteries that occurs when intravascular pressure increases. This myogenic behavior is thought to be fundamentally involved in the mechanisms of blood flow autoregulation. In this study, we tested the hypothesis that TRPM4 channels in cerebrovascular myocytes contribute to cerebral blood flow autoregulation in vivo.

Methods—In vivo suppression of cerebrovascular TRPM4 expression was achieved by infusing antisense oligodeoxynucleotides into the cerebral spinal fluid of 400- to 550-g Sprague-Dawley rats at 80 μg · day⁻¹ for 7 days using an osmotic pump that discharged into the lateral cerebral ventricle. Absolute cerebral blood flow measurements were obtained over a range of mean arterial pressures using fluorescent microsphere methods.

Results—Oligonucleotides infused into the cerebrospinal fluid were detected in the smooth muscle cells of pial arteries. Semi-quantitative RT-PCR indicated that the message for TRPM4 was decreased in the cerebral arteries of antisense-treated rats. Myogenic constriction was decreased by 70% to 85% in cerebral arteries isolated from TRPM4 antisense- versus sense-treated rats. Cerebral blood flow was significantly greater in TRPM4 antisense- versus sense-treated rats at resting and elevated mean arterial pressures, indicating that autoregulatory vasoconstrictor activity was compromised in TRPM4 antisense-treated animals.

Conclusions—In vivo suppression of TRPM4 decreases cerebral artery myogenic constrictions and impairs autoregulation, thus implicating TRPM4 channels and myogenic constriction as major contributors to cerebral blood flow regulation in the living animal. (Stroke. 2007;38:2322-2328.)

Key Words: autoregulation ■ cerebral blood flow ■ myogenic tone ■ TRPM4

Over the range of mean arterial pressures (MAP) from 50 to 150 mm Hg, a variety of intrinsic mechanisms adjust cerebrovascular resistance to prevent large changes in cerebral blood flow (CBF). This homeostatic process is referred to as autoregulation. The vascular myogenic response is thought to be a key contributor to the vascular resistance adjustments involved in CBF autoregulation.¹,² In isolated cerebral arteries, increasing the perfusion pressure depolarizes the smooth muscle cells.³ Depolarization activates voltage-sensitive Ca²⁺ channels, causing influx of extracellular Ca²⁺ and contraction. The degree of depolarization and contraction is proportional to the intravascular pressure. In this way, the vascular myogenic response may help to limit changes in blood flow that would otherwise occur with increased perfusion pressure.

We previously showed that the transient receptor potential channel TRPM4 is critically involved in the cerebrovascular myogenic response.⁴ TRPM4 channels are monovalent selective Ca²⁺-activated cation channels present in cerebral artery smooth muscle cells.⁴,⁵ These channels are potentially important regulators of membrane potential via their sodium permeability. When smooth muscle TRPM4 channel expression is suppressed in isolated cerebral artery segments, pressure-induced myogenic tone is inhibited.⁴

Because TRPM4 channels are critical to the in vitro myogenic response and the myogenic response is a likely component of CBF autoregulation, we hypothesized that TRPM4 channels are important regulators of CBF in vivo. This hypothesis was tested using antisense oligodeoxynucleotides (ODNs) to suppress TRPM4 function in vivo. We found that CBF was higher over a mean arterial pressure (MAP) range extending from 100 to 180 mm Hg in animals treated with TRPM4 antisense (AS) versus TRPM4 sense (S) ODNs. Thus in vivo, TRPM4 channels are important for autoregulation of CBF and may help prevent brain overperfusion under both resting and hypertensive conditions.

Materials and Methods

Animals
All protocols and experimental procedures used in this research project were approved by the University of Vermont Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (400 to 550 g; Charles River Laboratories, St Constant, Quebec, Canada) were used for all experiments.
In Vivo Suppression of TRPM4 Channels

As ODNs were used to downregulate TRPM4 expression by the cerebrovascular smooth muscle. ODN sequences (Qiagen Inc) were: TRPM4 AS-1, 5'-GTTGCCATCGCCTGCTCCACA-3'; TRPM4 AS-2, 5'-CTCGGATAGCCTGCGCACA-3'. Control animals were treated with S ODNs: TRPM4 S-1, 5'-TGTTGGACCGATGGCACCAC-3'; and TRPM4 S-2, 5'-TTTGCGAGTGCTATCGCAG-3'. The last 3 bases on both the 5' and 3' ends were phosphorothioated to limit ODN degradation. In some experiments, Texas Red fluorescent dye was conjugated to the 5' end of TRPM4 ODNs for assessment of cellular ODN uptake. ODNs were dissolved in bicarbonate-free artificial cerebral spinal fluid solution of the following composition (in mmol/L): 290 NaCl, 6 KCl, 2.8 CaCl2, 2H2O, 1.6 MgCl2, 6H2O, 3 NaH2PO4, 7H2O, and 0.45 NaH2PO4·H2O. Alzet Model 2001 mini osmotic pumps (1.0 µL/h pump rate; Durect Corporation) were filled with either TRPM4 S-1 plus TRPM4 S-2 (1 mmol/L each) or TRPM4 AS-1 plus TRPM4 AS-2 (1 mmol/L each) ODN solutions. Each pump was connected to a 28-gauge stainless steel brain infusion cannula using polyvinyl tubing (Alzet Brain Infusion Kit I; Durect Corporation). This configuration allowed the ODNs to be delivered at a rate of ~50 mmol·day⁻¹ for 7 days.

Cannula and Osmotic Pump Implantation Surgery

Under isoflurane anesthesia, the animals were fixed in a stereotaxic frame and a midline incision was made to expose the skull. The cannula was implanted into the lateral cerebral ventricle based on the following stereotaxic coordinates: bregma ~0.8 mm, lateral 1.4 mm, and depth 4 mm. The mini osmotic pump was placed in a subcutaneous scapular pocket. CBF measurements were made 7 days after surgery. The 7-day treatment period was chosen to allow ODN distribution throughout the CSF, uptake of ODNs, and time for message suppression and channel protein turnover.

RT-PCR for TRPM4 mRNA

Pial arterial RNA was prepared and semiquantitative RT-PCR was performed as previously described, using the following forward and reverse primers for TRPM4 and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH): TRPM4(F), 5'-GTCAATCGTGAGCAAGATGATGGAA-3'; GAPDH(F), 5'-TGAAGGCATACCTGTGGACATCGAGCA-3'; TRPM4(R), 5'-GTCACACCTTCTGGGAGCTGC-3'; GAPDH(R), 5'-CTGAGGCCTGAGCTGACACAC-3'. TRPM4 primers yielded a product of 707 base pairs, and the GAPDH primer products were 1000 base pairs.

Confocal Microscopy

Isolated pial artery segments were fixed in phosphate buffered saline containing 4% paraformaldehyde and then mounted on glass slides using a product of 707 base pairs, and the GAPDH primer products were isolated smooth muscle cells were viewed using a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging Inc).

In Vitro Diameter Recordings

Posterior cerebral or superior cerebellar artery segments were mounted in a myograph chamber (Living Systems) and superfused with warmed (37°C) gassed (20% O2/5% CO2) physiological saline solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 24 NaHCO3, 0.2 KH2PO4, 1.1 EDTA, 1.2 MgSO4, 1.6 CaCl2, and 10.6 glucose, pH 7.4. Endothelial cells were removed by passing an air bubble through the arterial lumen. Arteries were denuded to allow direct comparisons of myogenic tone between the present study and our previous study, which used an in vitro ODN strategy. Diameter was measured using a video dimension analyzer (IonOptix Corporation). Contractile responses to 60 mmol/L external KCl and 30 µmol/L uridine triphosphate (UTP) were assessed in arteries at 20 mm Hg intravascular pressure. Pressure-diameter response curves were created by raising intraarterial pressure in 20 mm Hg increments (20 to 100 mm Hg, 5 minute intervals). The passive diameter for each artery was measured by repeating the pressure steps using Ca²⁺-free PSS (0 CaCl2, 3 mmol/L EGTA, 1 µmol/L nisoldipine).

Measurement of CBF

**Surgical Preparation**

Rats were anesthetized with sodium pentobarbital (i.p. 60 mg/kg; Nembutal Oxvation Pharmaceuticals). A 24-gauge catheter was inserted into the left femoral vein for i.v. infusion of sodium pentobarbital (20 mg·kg⁻¹·hr⁻¹) to maintain anesthesia. A 22-gauge catheter was inserted into the right femoral artery to monitor MAP and obtain arterial blood samples. For microsphere infusion, a 20-gauge catheter was advanced into the left ventricular chamber via the right common carotid artery. Observation of the characteristic left ventricular pressure waveform confirmed proper placement of the catheter. All catheters were filled with 0.9% saline containing 200±1 mL⁻¹ heparin. A tracheotomy was performed to allow mechanical ventilation (A.D.S. 1000 Engler Engineering Corporation) with a 40%O2 60% N2 gas mixture. Animals were allowed to stabilize after surgery for 30 to 40 minutes.

**Experimental Protocol**

In each animal CBF was measured at 3 of 4 target MAPs: 40, 100, 150, or 180 mm Hg. Norepinephrine (6.5 µg·min⁻¹ to 85 µg·min⁻¹) was infused into the left femoral vein to raise MAP above the resting value of 100 mm Hg and lowered below 100 mm Hg by withdrawing blood volume. Once the animal was stable at the targeted MAP, 400 µL of 0.9% saline containing approximately 200 000 15-µm diameter fluorescent microspheres (Molecular Probes Inc) was introduced into the circulation at a rate of 1.0 mL·min⁻¹. A reference sample of arterial blood containing injected microspheres was simultaneously withdrawn at a rate of 1.3 mL·min⁻¹. Microspheres containing different fluorescent dyes were used to measure flow at different MAPs within an individual animal. The brain, kidneys, and spleen were harvested postmortem. Each tissue or reference blood sample was digested in ethanolic potassium hydroxide to recover trapped microspheres. The microspheres recovered from each sample were dissolved in 2-ethoxethyl acetate (3 mL) and the released fluorescent dyes were then assayed using a fluorometer (Model 814, Photon Technology International). Absolute tissue blood flow was calculated using the following equation:

\[
\text{Flow}_{\text{tissue}} = \frac{[\text{Rate}_{\text{tissue}} - \text{Rate}_{\text{ref}}]}{\text{Rate}_{\text{tissue}}} \times \text{tissue mass}_{(g)}
\]

Where, \(\text{Flow}_{\text{tissue}}\) is tissue sample fluorescence, \(\text{Flow}_{\text{ref}}\) is reference blood sample fluorescence, \(\text{Rate}_{\text{tissue}}\) is the reference blood sample withdrawal rate, and the tissue mass is the blotted wet weight. Autoregulatory function was assessed over 2 MAP intervals (100 to 150 mm Hg and 150 to 180 mm Hg) by determining autoregulatory index values using the following equation: Autoregulatory index = ΔCBF/ΔMAP.

**Chemicals, Drugs, and Enzymes**

Buffer reagents, collagenase type F, hyaluronidase, dithioerythritol, and 3 tissue blood flow was calculated using the following equation:

\[
\text{Flow}_{\text{tissue}} = \frac{[\text{Rate}_{\text{tissue}} - \text{Rate}_{\text{ref}}]}{\text{Rate}_{\text{tissue}}} \times \text{tissue mass}_{(g)}
\]

Where, \(\text{Flow}_{\text{tissue}}\) is tissue sample fluorescence, \(\text{Flow}_{\text{ref}}\) is reference blood sample fluorescence, \(\text{Rate}_{\text{tissue}}\) is the reference blood sample withdrawal rate, and the tissue mass is the blotted wet weight. Autoregulatory function was assessed over 2 MAP intervals (100 to 150 mm Hg and 150 to 180 mm Hg) by determining autoregulatory index values using the following equation: Autoregulatory index = ΔCBF/ΔMAP.

**Chemicals, Drugs, and Enzymes**

Buffer reagents, collagenase type F, hyaluronidase, dithioerythritol, norepinephrine, and UTP were purchased from Sigma. Papain was obtained from Worthington Biochemical. Nisoldipine was a gift from Miles Pharmaceuticals.

**Statistical Analysis**

Data are expressed as means±SEM, and n indicates the number of animals. Vascular tone in the presence of contractile agonists or as a function of intravascular pressure (myogenic tone) was calculated as follows:

\[
\% \text{Tone} = \left( \frac{\text{Diameter in Ca}^{2+}-\text{free PSS} - \text{Diameter in PSS}}{\text{Diameter in Ca}^{2+}-\text{free PSS}} \right) \times 100
\]

Data were compared using Student t test or a 2-way repeated measures ANOVA where appropriate. Means were considered significantly different at P<0.05.
Results

**In Vivo Suppression of TRPM4 Channels**

Infusion of TRPM4 ODNs into the lateral cerebral ventricle for 7 days was an effective method for delivering the ODNs to cerebrovascular smooth muscle cells. Fluorescent tagged ODNs were clearly visible in whole cerebral arteries (Figure 1A, upper left panel). The pattern of fluorescence indicated that the fluorophore was present in the arterial smooth muscle cells. A similar pattern of fluorescence in smooth muscle was observed in 8 different pial artery segments. Fluorophore was not apparent in endothelial cells or perivascular nerves, suggesting a rather specific uptake of the oligonucleotides by the smooth muscle cells. The presence of oligonucleotide in smooth muscle was confirmed by examining individual vascular smooth muscle cells enzymatically isolated from the cerebral arteries of animals treated with ODNs (Figure 1A, upper middle and right panels). Fluorescence was observed in individual smooth muscle cells obtained from arteries of animals treated with ODNs containing fluorophore. If the rats were exposed to unlabeled ODNs, fluorescence was not observed (Figure 1A, lower middle and right panels).

Semi-quantitative RT-PCR performed on pial arteries of animals treated with either TRPM4 sense or TRPM4 antisense ODNs revealed that the TRPM4 AS ODNs effectively reduced TRPM4 mRNA levels (Figure 1B). The signal for GAPDH mRNA was not different between samples prepared from arteries of TRPM4 S- or AS-treated animals (Figure 1B).

**In Vitro Assessment of Artery Function**

Cerebral arteries isolated from TRPM4 S- or AS-treated animals contracted similarly to external 60 mmol/L KCl (S 65±5%; AS 53±6%; Figure 2A) and 30 μM UTP (S 55±4%; AS 48±9%). In contrast, pressure-induced myogenic responses of cerebral arteries from animals treated with TRPM4 AS ODNs were 70% to 85% lower than the responses of arteries from TRPM4 S-treated animals at all pressures above 20 mm Hg (Figure 2B and 2C).

**Blood Flow Measurements**

Implantation of the infusion cannula into the lateral cerebral ventricle produced no obvious side affects in the animals. Ventilation with 40% O₂ resulted in hyperoxia, but there were no differences in P<sub>O<sub>2</sub></sub>, P<sub>CO<sub>2</sub></sub>, or pH between the TRPM4 S- and AS-treated rats (Table 1).

Renal and splenic blood flows were measured to validate the fluorescent microsphere approach under the conditions used in the present study (Table 2). Resting renal blood flows were comparable to reported values for the rat. Infusion of norepi-
nephrine reduced renal blood flow as expected, through \( \alpha_1 \) adrenergic receptor–mediated constriction of the renal vascular smooth muscle. This response was similar in TRPM4 S- and AS-treated rats. There were no differences in blood flows between the right and left kidneys at resting or elevated MAPs, indicating that the injected microspheres were uniformly mixed in the left ventricular chamber.

Splenic blood flow was also reduced by infusion of norepinephrine; no difference in splenic blood flow was observed between the treatment groups.

Global CBF values in TRPM4 S- and AS-treated rats at 4 targeted MAPs are shown in Figure 3. Blood flow was significantly greater in TRPM4 AS-treated compared with TRPM4 S-treated animals at resting MAP and the 2 elevated MAPs.
blood pressures. Resting CBF values in S-treated rats were comparable to published values.9

Regional CBF was also measured (Table 3). The general pattern of increased global CBF at resting and elevated MAPs in TRPM4 AS-treated rats was mirrored in the regional blood flow measurements, including in those regions supplied by the arteries studied in vitro, ie, the cerebral cortex and the cerebellum. Between 106 and 145 mm Hg MAP, global CBF increased by 192 mL/min/100g brain and 44.8 mL/min/100g brain in TRPM4 AS- and TRPM4 S-treated animals, respectively. Calculated autoregulatory indices for this pressure range were 2.2 ± 0.5 (AS) and 1.4 ± 0.4 (S; P = 0.13). Between 145 and 181 mm Hg, blood flow increased by 192 ± 27 mL/min/100g brain and 71 ± 19 mL/min/100g brain in TRPM4 AS- and TRPM4 S-treated animals, respectively, resulting in calculated autoregulatory indices of 7.3 ± 2.8 (AS) and 2.2 ± 0.4 (S; P < 0.05). CBF at elevated MAPs in AS-treated rats approached the maximal CBF that could be obtained in these animals. Maximal flow rates were induced by hypoxic hypercapnic challenge, conditions under which the cerebral vasculature is expected to be maximally dilated and autoregulation abolished.10 Hypoxic hypercapnic CBF values were 476 ± 42 mL/min/100g brain at 158 ± 4 mm Hg and 461 ± 80 mL/min/100g brain at 161 ± 5 mm Hg in TRPM4 S- and AS-treated rats, respectively. Taken together, these CBF measurements indicate that the autoregulatory vasconstrictor activity in TRPM4 AS-treated rats is substantially reduced versus control.

Discussion

Major New Findings
The major new findings of this study are: (1) an antisense oligonucleotide approach can be used to suppress ion channel function in cerebrovascular smooth muscle cells in vivo, and (2) the transient receptor potential channel TRPM4 plays a significant role in the autoregulatory vasoconstrictor response of cerebral arteries in vivo.

In Vivo Suppression of TRP Channels
Methods for gene silencing represent selective and powerful approaches for elucidating the contributions of various proteins, including ion channels, to regulation of cellular function both in vitro and in vivo. In the present study, we validated the efficacy of a technique of in vivo application of antisense ODNs directed against the TRPM4 gene. ODNs were taken up by cerebrovascular smooth muscle cells and TRPM4 message was reduced in cerebral arteries from animals treated with TRPM4 AS ODNs in vivo. Suppression of cerebrovascular TRPM4 message using this approach inhibited myogenic tone by 70% to 85% in cerebral arteries isolated from these animals.

The suppressing effect of ODNs administered via the cerebral spinal fluid in this study was as effective as our previous in vitro, cell permeabilization, and organ culture approach, where TRPM4 AS suppressed myogenic tone by ~60% to 70%.4 ODN uptake by the cerebrovascular smooth muscle cells may be attributable to the presence of an endocytosis mechanism that results in effective intracellular delivery of the oligonucleotides.11 Indeed, we have seen evidence for some, albeit modest, uptake of fluorescent tagged oligonucleotides without permeabilization in our previous in vitro studies.4,12 Efficient uptake of oligonucleotides by vascular myocytes in vivo may have resulted from the long exposure time during which the osmotic pumps continuously infused the ODNs in the CSF over many days. In addition, intrinsic oligonucleotide degradation mechanisms, ie, DNAase activity, may be lower in the in vivo versus

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<td><strong>TRPM4 antisense</strong></td>
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Values are mean ± SEM. MAP indicates the mean arterial blood pressure; n, No. of animals studied.
the in vitro situation. Other investigators have used a similar approach to determine the functional roles of various proteins in the vascular wall and have also observed remarkable suppression of protein function. For instance, intracisternal injection of AS ODNs targeting angiotensin II receptor mRNA decreases angiotensin II receptor expression and protects rats against ischemic insult. Similarly, Onoda et al found that cerebroventricular injection of preproendothelin-1 AS ODNs inhibited arterial constrictions in a rat model of cerebral vasospasm. Intracisternal injection of AS ODNs targeted to the EGF receptor or to adenylyl cyclase reduced expression of these proteins, with an associated decrease in EGF-mediated BK channel modulation by EGF in basilar artery myocytes. Thus, this approach represents a highly feasible strategy for evaluation of the functional roles of different types of proteins in regulation of cerebral artery vasomotor activity.

**Role of TRPM4 in Regulation of CBF**

Altered ion channel activity and regulation of membrane potential are key factors that regulate vascular tone. This is reflected in the central role of the L-type voltage-dependent calcium channel as a primary pathway for the regulation of calcium entry in vascular smooth muscle, particularly in the small diameter arteries that regulate vascular resistance and blood flow. Recent studies have indicated that several members of the TRP family of cation channels play important roles in control of vascular smooth muscle membrane potential and vasomotor tone. For instance, Inoue et al found, using AS ODNs, that downregulation of TRPC6 expression in primary cultured portal vein myocytes resulted in suppression of α-adrenoceptor-activated TRPC6-like cation currents. Using a similar AS ODN approach, we found that TRPC3 mediates UTP-induced depolarization and constriction of cerebral arteries in vitro. TRPV4 channels, members of the vanilloid receptor TRP channel subfamily, appear to be receptors for epoxyeicosatrienoic acid compounds (EETs) in vascular endothelial and smooth muscle cells, and mediate a signaling cascade leading to smooth muscle hyperpolarization and vasodilatation.

Our study of the contributions of TRPM4 in CBF regulation is the first to demonstrate an in vivo role for these channels. Normally, CBF changes only modestly over an arterial pressure range of approximately 50 to 150 mm Hg. This is the classic autoregulatory response observed in many vascular beds, which is well-characterized for the cerebral circulation. However, despite extensive study, the fundamental mechanisms underlying this response have been debated and the molecular mechanisms involved remain undefined. Brain blood flow at different perfusion pressures is determined by the integration of information from 3 major signaling inputs, ie, via, myogenic, metabolic, and neurogenic mechanisms. In the present study, we found that CBF was greater in TRPM4 AS- versus TRPM4 S-treated animals at resting and elevated MAPs. Further, autoregulation as reflected in the slope of the pressure/flow relationships was somewhat compromised in TRPM4 AS-treated rats at MAPs between 100 and 150 mm Hg and greatly compromised at pressures above 150 mm Hg. These data point out a crucial role for TRPM4 channels in regulation of CBF.

Our previous study found that TRPM4 channels are critical mediators of pressure-induced depolarization and myogenic tone in isolated pial arteries. It has been proposed the myogenic tone plays a major role in the autoregulation of blood flow in several...
organ systems, but perhaps most notably in the kidney and brain. The simplest explanation for the effects of TRPM4 channel suppression on CBF in the present study is that after channel suppression, vascular myogenic reactivity to changes in systemic arterial pressure elevations is reduced, and this compromises autoregulation. Previous studies have reported a major role for voltage-dependent calcium channels in the mechanism of CBF autoregulation in vivo. These findings are consistent with our proposal that TRPM4 channels are regulators of membrane potential, myogenic constriction, and autoregulation. Interestingly, in the present study, the inhibitory effect of TRPM4 AS treatment on autoregulation was largest at MAPs greater than 150 mm Hg. In contrast, TRPM4 AS treatment severely depressed myogenic tone in vitro at all pressures studied. This suggests that the contribution of myogenic tone to autoregulatory vasoconstriction may be proportionately greater at high blood pressures, where protection from breakdown of autoregulatory vasoconstriction is paramount. Of course such extrapolation from in vitro findings to the in vivo setting is speculative, pointing to the need for additional studies using in vivo strategies which might provide direct evidence for mechanistic links between myogenic tone and autoregulation.

Our study did not address the possibility that signals generated by metabolic or neurogenic autoregulatory mechanisms may also act through TRPM4 channels to alter vascular resistance. TRPM4 channel activity is modulated by a number of intracellular and metabolic factors such as phophatidylinositol bisphosphate, intracellular nucleotides and polyamines, and decavanadate. To date there are no reports implicating these factors as mediators of myogenic tone or autoregulation. However, based on the present report, investigations of the possible cellular factors involved in regulation of TRPM4 channels and myogenic tone in vitro and in vivo could lead to important new insights regarding control of CBF in health and disease states.

In conclusion, the present study demonstrates the efficacy of AS oligonucleotide treatment in vivo in suppressing vascular smooth muscle ion channel function in brain arteries. Further, this work implicates the transient receptor potential channel TRPM4 as a major contributor to regulation of CBF in the living animal.

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Disclosures
None.

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