

Calcium Modulation of Adherens and Tight Junction Function

A Potential Mechanism for Blood-Brain Barrier Disruption After Stroke

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Background—This review deals with the role of calcium in endothelial cell junctions of the blood-brain barrier (BBB). Calcium is critical for adherens junction function, but it appears that calcium is also important in regulating tight junction function necessary for the barrier characteristics of cerebral microvessels.

Summary of Review—The BBB is critical for brain homeostasis and is located at the cerebral microvessel endothelial cells. These endothelial cells maintain their barrier characteristics via cell-cell contacts made up of adherens and tight junctions. Adherens junctions are calcium dependent; recent evidence suggests that calcium also affects tight junctions. After stroke, there is a disruption of the BBB. Interfering with calcium flux under hypoxic conditions can prevent BBB breakdown. Calcium may alter BBB junction integrity by a number of different signal transduction cascades, as well as via direct interaction of calcium ions with junction proteins. It remains to be determined whether clinical use of calcium channel antagonists is a viable means to reduce BBB disruption after stroke.

Conclusions—With the widespread use of calcium channel blockers as clinical treatments for hypertension, which is a risk factor for stroke, the exact role of calcium in modulating BBB integrity needs to be elucidated. (*Stroke*. 2002;33:1706-1711.)

Key Words: blood-brain barrier ■ calcium ■ endothelium ■ signal transduction ■ stroke

The blood-brain barrier (BBB) is present at the level of the brain capillaries¹ and is critical for ion homeostasis in the central nervous system. The physical BBB is due to a lack of fenestrations between brain capillary endothelial cells and the presence of adherens (AJ) and tight (TJ) junctions between adjacent endothelial cells, resulting in a lack of paracellular diffusion. TJ, in particular, are required for the prevention of ion flux across the BBB.

During normal function, ion transporters on the microvessel endothelial cells control ion flux across the BBB. The transport of other molecules occurs via specific transporters or endocytosis, while paracellular diffusion is tightly regulated. In pathological conditions, including stroke, diabetes, Alzheimer's disease, multiple sclerosis, and inflammatory pain, there is a disturbance of BBB integrity, with disruption of ion homeostasis and transporter function.²⁻⁷ In stroke, this loss of ion regulation is followed by passive diffusion of water, resulting in brain edema.⁸ This review will focus on the role of calcium in the integrity and function of the BBB in normal physiology and in stroke pathophysiology.

BBB Structure

The brain receives oxygen and nutrients from the peripheral circulation. However, the brain requires precise control of ion gradients for normal function. Ion control is maintained by

several barrier systems between the central nervous system and the periphery; key among these is the BBB. The BBB is formed by brain microvessel endothelial cells (BMEC)¹ and serves to prevent uncontrolled flux of ions, amino acids, and peptides into the brain; it acts as a physical blockade against immune cell migration as well. Paracellular diffusion is limited by TJ between microvascular endothelial cells.

Adherens Junctions

The AJ is an adhesive cell-cell contact found in many tissues. AJ consist of a cadherin dimer as the main mediator of cell-cell adhesion. Cadherins belong to a superfamily of cell adhesion molecules, all of which are single transmembrane domain proteins.⁹ E-cadherin is found in epithelial¹⁰ and endothelial cells¹¹ and mediates calcium-dependent cell adhesion by binding to actin via catenin accessory proteins.¹² This interaction is mediated by β -catenin, which links E-cadherin to α -catenin, which binds directly to actin (Figure 1). AJ are found in both epithelial and endothelial cells¹³; in the BBB the AJ is located near the basolateral side of endothelial cells (Figure 1).

Tight Junctions

The restrictive nature of the BBB is due to TJ between adjacent endothelial cells at the apical membrane.¹⁴ TJ restrict

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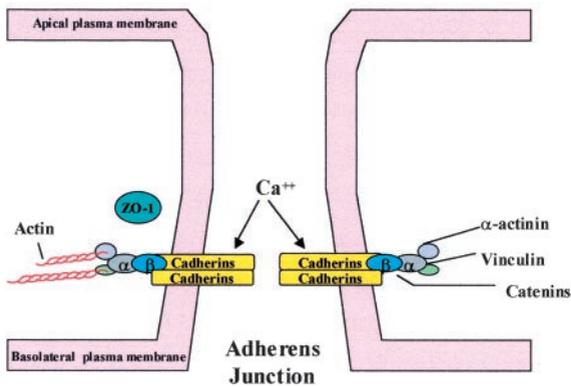


Figure 1. AJ of the BBB. AJ of the BBB are located at the basolateral plasma membrane. Calcium is critical for the normal functioning of cadherins, which have calcium-binding sites in their extracellular domains. Cadherins are linked to actin by α - and β -catenin. Reduction of extracellular calcium leads to dissociation of cadherin-cadherin linkage, disruption of cytoskeletal integrity, and loss of cell-cell adhesion.

ion flux and paracellular diffusion. A number of TJ proteins have been identified; these include claudins,¹⁵ occludin,¹⁶ and the zonula occludens proteins ZO-1, ZO-2, and ZO-3. Claudin-1 and occludin have been demonstrated at the BBB (Figure 2).^{7,14} Claudins and occludin have multiple transmembrane domains and form heterodimeric bridges with adjacent cells, blocking paracellular diffusion.¹⁷ Stabilization of TJ involves a complex network of occludin, claudins, and ZO proteins linking the transmembrane proteins to the actin cytoskeleton. ZO proteins mediate this linkage in a manner similar to that of the catenins of the AJ, by binding to the cytoplasmic tails of occludin and claudin and to actin (Figure 2).

ZO proteins are members of the membrane-associated guanylate kinase (MAGUK) family and interact with the

cytoplasmic domains of claudins¹⁸ and occludin.¹⁹ The best-studied ZO protein in the TJ is ZO-1, which has binding sites for occludin and claudin, as well as a conserved guanylate kinase domain, and SH3 and PDZ domains, which mediate protein-protein interactions with signal transduction molecules.²⁰ All MAGUK proteins are membrane associated, and for ZO-1, this association is key for membrane targeting of occludin¹⁹ and claudin.²¹ ZO-1 is also associated with the AJ during cell polarization²² and can bind α -catenin and actin filaments in nonepithelial cells,²³ implicating ZO-1 in both TJ and AJ function. The precise actions of the ZO proteins in TJ formation and maintenance remain to be determined; the roles of ZO-2 and ZO-3, as well as other accessory proteins localized to the TJ, have been reviewed in detail elsewhere^{24,25} and will not be discussed here.

Role of Calcium in AJ and TJ

Calcium is critical for the maintenance of cell-cell junctions in various cell types. In Madin Darby canine kidney (MDCK) epithelial cells, reduced extracellular calcium decreased transepithelial resistance readings, which were restored by replacing calcium.²⁶ In primary pulmonary endothelial cultures, chelation of extracellular calcium increased albumin transfer by 125%, decreased transepithelial resistance, and caused retraction of adjacent cells. Restoration of extracellular calcium restored normal barrier function.²⁷

In systems in which AJ are the primary mechanism of cell-cell contact and adhesion, calcium is necessary for normal function.²⁸ Transfer of epithelial cells to low-calcium media resulted in AJ dissociation mediated by protein kinase A (PKA), causing redistribution of E-cadherin and ZO-1.²⁹ Low levels of calcium disrupt AJ in endothelial cells,³⁰ presumably by removing calcium from binding sites on E-cadherin extracellular domains (Figure 1), causing a conformational change,³¹ and interrupting cell-cell adhesion. TJ are also sensitive to extracellular calcium. Incubation of Caco-2 epithelial cells in calcium-free solution rapidly decreased transepithelial resistance and increased paracellular permeability, indicating a decreased tightness of TJ. These effects were reversed by replenishing extracellular calcium.³² Mouse mammary epithelial monolayers cultured in calcium-deficient media lost membrane association of ZO-1, ZO-2, and occludin, decreased transepithelial resistance, and increased permeability. Association of ZO-1, ZO-2, and occludin with actin was not disrupted.³³ When calcium was removed in the presence of PKA inhibitors, barrier characteristics were preserved,³³ indicating a role for PKA in modulating calcium-dependent TJ and AJ function. The effect of low calcium on TJ can be overcome by activation of protein kinase C (PKC),³⁴ implicating PKC signaling in calcium-modulated function of TJ. Rapid changes in extracellular calcium may lead to changes in BBB permeability by disrupting the AJ between microvessel endothelial cells. This, in turn, may lead to disruption of the TJ both by the removal of extracellular calcium, as discussed below, and also by the potential removal of the supporting structure of the AJ, causing tension on the TJ and leading to loosening. However, these rapid changes in extracellular calcium levels may also affect BBB permeability by altering the activity of signaling

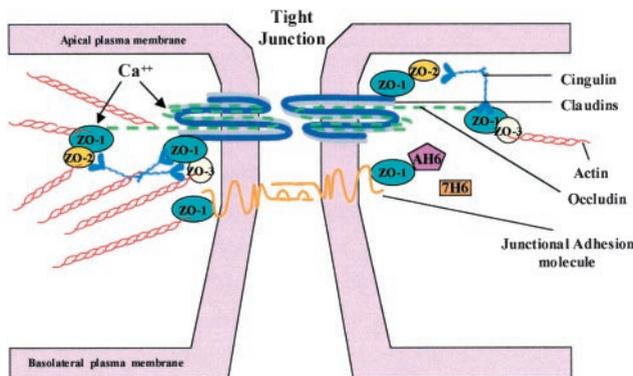


Figure 2. TJ of the BBB. TJ of the brain capillaries are composed of a number of different proteins. Three proteins serve as cell-cell adhesion molecules: claudin, occludin, and junctional adhesion molecule. Claudin is necessary for the formation and maintenance of the TJ. Occludin correlates with “tightness” of the TJ; higher occludin expression decreases paracellular permeability. Junctional adhesion molecule is important in mediating leukocyte adhesion and migration. These transmembrane adhesion molecules are anchored to the actin cytoskeleton in a manner similar to that of the AJ. The ZO proteins link claudin, occludin, and junctional adhesion molecule to the actin cytoskeleton. The exact role of calcium in the modulation of the TJ is not clear, but it is important for the normal functioning of ZO-1 and occludin.

cascades, such as the PKC cascade, potentially leading to changes in TJ or AJ protein expression.

Intracellular calcium is important for TJ integrity. In contrast to extracellular calcium chelation, which does not alter ZO-1/actin interactions, lowering intracellular calcium changes ZO-1/actin binding and alters the subcellular localization of occludin.³⁵ Increasing intracellular calcium by disrupting intracellular stores also interferes with TJ formation.³⁶ This indicates that regulation of intracellular calcium is critical for normal function of TJ, although different compartments of intracellular calcium may be more important than others in regulating TJ function.

Stroke, Calcium, and BBB Function

Stroke is the third leading cause of death in the United States and is a leading cause of disability.³⁷ Neuronal damage after stroke is due to deprivation of oxygen and nutrients during ischemia and to the production of reactive oxygen species (ROS) after reperfusion.³⁸ Ischemic damage to the brain endothelial cells results in dysregulation of ion flux, leading to a net influx of solutes and water, which results in edema.^{11,39}

Hypoxia causes transient increases in intracellular calcium levels in many different cells. In human umbilical vein endothelial cells, hypoxia causes an increase in intracellular calcium not blocked by dihydropyridine calcium channel blockers.⁴⁰ In rabbit carotid cells, hypoxia augments calcium current, indicating calcium flux into the cell. This augmentation was sensitive to PKC inhibitors.⁴¹ Hypoxia induces permeability in aortic endothelial cells that can be dose-dependently blocked by nifedipine, an L-type calcium channel blocker.⁴² Nifedipine did not block intracellular calcium increase but blocked PKC α and PKC ζ activation. This suggests that calcium flux through L-type channels is important for PKC activation under these conditions. This PKC activity, in turn, is important for modulating permeability because phorbol ester treatment increased permeability. However, the intracellular increase in calcium may be derived from other pools within the cells themselves and may not be due to L-type channels.⁴² Increases in intracellular calcium can be very limited spatially and specifically localized, triggering different signaling pathways.⁴³

Models of the BBB have been used to investigate the effects of hypoxia. The *in vivo* middle cerebral artery occlusion model showed increased leakage of Evans blue dye after transient focal ischemia in rats.⁴⁴ The increase in permeability is correlated with tissue damage.⁴⁵ The effects of hypoxia/aglycemia have been investigated with the use of *in vitro* models of the BBB. Hypoxia/aglycemia decreased E-cadherin expression in BMEC while increasing permeability.² Nifedipine and SKF 96365, an inhibitor of receptor-mediated/voltage-gated calcium entry, blocked this change.² Hypoxia/reoxygenation increased intracellular calcium levels but inhibited calcium oscillations and capacitive calcium entry in BMEC.⁴⁶ These impairments were blocked by superoxide dismutase and inhibitors of mitochondrial electron transport, indicating that ROS play a role in calcium signaling after hypoxia/reoxygenation. High intracellular calcium levels after reoxygenation are likely to be due to release

from mitochondrial stores, and this mitochondrial dysfunction is important in toxic ROS production. These studies indicate that hypoxia/reoxygenation generates ROS and impairs calcium mobilizing mechanisms,⁴⁶ suggesting a role for calcium ions and ROS generation in modulation of BBB function.

What signaling pathways mediate hypoxia-induced disruption of BBB function? In BMEC, hypoxia caused a rapid increase in intracellular calcium,^{46,47} and blockade of calcium flux^{2,47} or calcium-regulated signaling cascades^{42,47} prevent hypoxia-induced BBB disruption. However, the mechanisms by which calcium flux alters signaling cascades or modulates BBB function are as yet undefined, as are the precise protein targets of calcium signaling pathways. Furthermore, a distinction should be made between rapid events mediated directly by the direct interactions of calcium with proteins, ie, E-cadherin, and slower events that involve changes in gene expression, regulated by calcium-sensitive signal transduction mechanisms or by other factors released by injured cells.

Potential Mechanisms by Which Calcium Affects BBB Function After Stroke

Calcium is a potent second messenger. Intracellular levels of calcium are strictly regulated by membrane calcium channels and calcium pumps that remove calcium from the cytoplasm and return it to the extracellular space or intracellular storage, ie, the endoplasmic reticulum.⁴⁸ Activation of calcium signaling pathways occurs after a rise in intracellular calcium. This increase can be extremely localized, triggering specific signaling systems that vary with cell type and with the mode of calcium entry.^{43,49}

Increased intracellular calcium after stroke can trigger several signaling cascades (Figure 3). One major pathway involves calcium/calmodulin-dependent protein kinases. Calmodulin is a ubiquitous calcium receptor that binds 4 calcium ions and activates calmodulin-dependent kinase (CaMK).⁴⁹ Many CaMKs maintain their activity long after intracellular calcium has returned to normal, allowing for extensive signal transduction. CaMK can activate transcription factors such as cAMP response element binding protein (CREB) and c-fos.⁴⁹ Calcium regulates other cascades, such as those modulated by mitogen-activated protein kinases such as extracellular signal-regulated kinase (ERK)1 and ERK2⁴⁹ and PKC.

Hypoxia can trigger calcium-dependent and independent changes in gene expression. But how does this directly affect AJ and TJ integrity? The E-cadherin promoter has sites that could be potentially regulated by calcium,⁵⁰ but this has not yet been clearly demonstrated. The occludin promoter contains a number of regulatory sites,⁵¹ but there is as yet no evidence of direct regulation by calcium. The promoters for other junctional proteins have been identified, but little is known about how their expression is regulated. Therefore, it seems more likely that the modulatory effects of calcium on TJ and AJ are due to modulation of existing proteins, although the possibility still exists for calcium or calcium signaling to directly regulate TJ and AJ protein expression.

There are other mechanisms by which stroke may alter gene expression. Hypoxia can trigger transcription factors directly regulated by oxygen, including hypoxia inducible

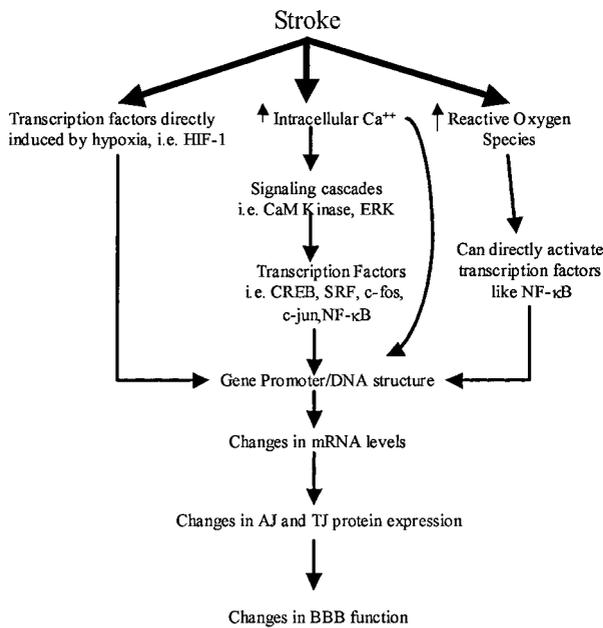


Figure 3. Calcium regulation of gene expression after stroke. Stroke can potentially change BBB AJ and TJ protein expression, and thus BBB function, via a number of mechanisms. Direct activation of oxygen-sensitive transcription factors such as HIF-1 can alter gene expression. ROS can directly activate transcription factors such as nuclear factor- κ B (NF- κ B). Changes in intracellular calcium levels can trigger multiple signal transduction cascades activating transcription factors that regulate short- and long-term changes in proteins. Calcium flux is the most easily modulated of these 3 potential stroke pathways and therefore presents the most attractive intervention strategy for prevention of stroke-induced BBB disruption. SRF indicates serum response factor.

factor-1 (HIF-1) and immediate early genes. This activation of oxygen-sensitive transcription factors may depend on intracellular calcium and can be blocked by inhibitors of PKC.⁵² Hypoxia and reoxygenation also increase ROS, which can directly activate transcription factors such as nuclear factor- κ B.^{53,54}

Use of Calcium Channel Blockers to Treat Hypertension and Stroke

Hypertension is a risk factor for the development of stroke, and calcium channel antagonists are widely used as a treatment. Numerous studies have examined how calcium channel antagonists decrease risk of stroke in hypertensive patients. Most of these studies found that calcium channel antagonists decrease the total rate of stroke from 25% to 59% depending on whether the antagonists are used alone or in combination with diuretics and β -blockers.^{55–59} However, there is no convincing evidence that calcium antagonists can improve clinical outcome after stroke,⁶⁰ although there are potential benefits, including improvement of cerebral blood flow and metabolic rates.^{61–64} Animal studies have indicated that treatment with calcium channel antagonists can decrease ischemic brain damage after focal ischemia,^{65–67} suggesting that calcium has an important role in stroke-induced brain (ie, neuronal) damage, as is well known. Furthermore, these drugs have demonstrated protective effects in peripheral

organs after ischemic events, including the heart⁶⁸ and kidney.⁶⁹ The results of these studies, however, can be linked to protective effects of the calcium channel antagonists against neuronal injury after stroke but do not address the issue of the effects of calcium on the BBB. Interestingly, in an animal model of spontaneous hypertension, nimodipine and nifedipine preserve microvascular integrity in the cerebral cortex as the animals age,⁷⁰ demonstrating a potential direct effect of calcium channel antagonists on the aging BBB.

Concluding Remarks

On the basis of the wealth of evidence available, it is clear that calcium is an important player in the normal function of the BBB. Regulation of calcium homeostasis seems to be critical: if calcium levels, either extracellular or intracellular, become too high or too low, disruption of the BBB will occur. Evidence from clinical studies also indicates that calcium channel antagonists may have beneficial effects in reducing the rate of stroke in hypertensive patients, although there are no data about poststroke clinical outcome in these studies. It remains to be seen which calcium compartments within the cell and which specific signaling cascades are triggered after an ischemic event. The exact role of calcium in the modulation of BBB function and integrity still must be clarified.

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