Abstract

Two in vitro co-culture models of the blood-brain barrier (BBB) were developed using primary rat cortical astrocytes and ECV304 endothelial cells or primary rat brain capillary endothelial cells. We showed that intercellular calcium (Ca\(^{2+}\)) waves can mediate bidirectional astrocyte-endothelial Ca\(^{2+}\) signal communication in both co-culture models. It appears that two signaling pathways are involved. A first mechanism is related to intercellular diffusion of the Ca\(^{2+}\) mobilizing messenger inositoltrisphosphate (IP\(_3\)) through gap junctions as studied with flash photolysis of caged-IP\(_3\). A second pathway involves extracellular diffusion of a purinergic messenger as studied with the purinergic inhibitors apyrase and suramin. Using gap junction-deficient HeLa cells and connexin-GFP transfected HeLa cells, the existence of the two Ca\(^{2+}\) signaling pathways mentioned was confirmed and their subcellular characteristics and differences were further studied. We also suggest that astrocytic intercellular Ca\(^{2+}\) waves may be accompanied by and contribute to some of the ionic shifts observed in the brain upon traumatic brain injury. Further work will be needed to confirm our data in brain slices or even in the brain in vivo, and to establish the role and function of intercellular Ca\(^{2+}\) signals at the BBB.

Key words: Intercellular calcium waves ; astrocytes ; endothelial cells ; blood-brain barrier ; neurovascular coupling ; traumatic brain injury ; flash photolysis.

Introduction

Brain capillary endothelial cells (BCECs), the cells that form the actual blood-brain barrier (BBB), differ from endothelial cells in other capillary beds by their large number of interendothelial tight junctions and a low level of transcytosis (Bradbury, 1993). Extensions of astrocytes cover over 90% of the abluminal surface of brain capillary endothelium. They are believed to induce the barrier characteristics which render the brain capillary endothelium the least permeable of all vessels in the body. Ionic and molecular movements over the BBB thus rely on specific transport mechanisms. The regulation of the permeability of the BBB is currently a topic of large experimental and clinical interest as this barrier may be broken down in pathological conditions such as brain trauma, tumors or ischemia. The BBB is furthermore a major obstacle impeding treatment of brain diseases.

Astrocytes largely outnumber other cell types in brain tissue and constitute about half the volume of the brain. Astrocytes are no longer considered as passive supportive cells as studies have revealed a myriad of functions performed by these cells (Kimelberg and Norenberg, 1989). A striking feature is their very extensive gap junctional coupling, allowing the exchange of metabolites and second messengers (Dermietzel and Spray, 1993). Astrocytic intercellular calcium (Ca\(^{2+}\)) wave propagation has been observed both in vitro and in hippocampal slices, and consists of an increase in the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that is propagated from cell to cell (Charles, 1998). These waves can be induced by a variety of stimuli, including chemical, mechanical and electrical stimuli. They require the presence of gap junctions and are mediated by the intercellular diffusion of the Ca\(^{2+}\) mobilizing second messenger inositol 1,4,5-trisphosphate (IP\(_3\)) (Sanderson, 1995). As IP\(_3\) enters each cell, Ca\(^{2+}\) is released from the intracellular Ca\(^{2+}\) stores, mainly the endoplasmic reticulum (ER), through the action of IP\(_3\) on the IP\(_3\) receptor. Depending on the nature of the stimulus used to evoke an intercellular Ca\(^{2+}\) wave, also an extracellular factor, most likely adenosine triphosphate (ATP), is involved (Guthrie et al., 1999). The endothelial regulation of [Ca\(^{2+}\)]\(_i\), has also been extensively studied and the [Ca\(^{2+}\)]\(_i\) in these cells has furthermore since long been implicated as a messenger influencing BBB permeability.

Experimental work

To study Ca\(^{2+}\) signaling in and between both cell types composing the BBB, two in vitro co-culture models were developed. In both co-culture systems primary rat cortical astrocytes were used. As for the endothelial cells, most of the co-cultures were prepared with endothelial cells from the ECV304 cell line, that develop into a BBB phenotype upon coculture with astrocytes. The major observations
were confirmed in co-cultures of astrocytes and primary rat BCECs, an in vitro two dimensional reconstruction of the rat BBB. The cells were loaded with Ca\textsuperscript{2+}-sensitive probes (fura-2 and fluo-3) and changes of [Ca\textsuperscript{2+}] were monitored using a digital microfluorimetric Ca\textsuperscript{2+}-imaging setup. Intercellular Ca\textsuperscript{2+} waves were initiated in co-cultures of astrocytes and endothelial cells using flash photolysis of caged-IP\textsubscript{3} and mechanical stimulation of single cells. Mechanical stimulation was brought about by gently touching the plasmamembrane with a glass micropipette with a tip diameter of about 1 µm. Caged-IP\textsubscript{3} is chemically bound IP\textsubscript{3} that was brought into the cytoplasm through microinjection or electroporation. Biologically active IP\textsubscript{3} was subsequently liberated from its caged precursor by applying an UV flash to the cell and could diffuse towards neighbouring cells in the presence of gap junctions, initiating propagating Ca\textsuperscript{2+} increases. The presence of gap junctions was further investigated using several methods, including connexin immunostaining, dye coupling and electrical coupling studies. The extracellular purinergic Ca\textsuperscript{2+} signaling pathway was studied using mechanical stimulation of single cells in cultures with cell free gaps, using a fast superfusion flow, in the presence of the P\textsubscript{2} purinoceptor antagonist suramin and in the presence of the ATP-degrading enzyme apyrase. In a separate series of experiments ionic changes, possibly associated with intercellular Ca\textsuperscript{2+} waves, were studied. Intracellular sodium concentration ([Na\textsuperscript{+}]) and pH changes were measured using ion-sensitive fluorescent probes (SBFI and BCECF respectively) and the digital microfluorescence imaging setup. Extracellular potassium concentration ([K\textsuperscript{+}]) changes were measured using a K\textsuperscript{+}-selective micro-electrode.

Our results indicate that intercellular Ca\textsuperscript{2+} waves induced in co-cultures by flash photolysis of caged-IP\textsubscript{3} can propagate from astrocyte to endothelial cell and vice versa (Leybaert et al., 1998). The propagation of the Ca\textsuperscript{2+} signal from astrocytes towards endothelial cells in co-culture with this protocol is limited to a single layer of adjacent endothelial cells. These propagating Ca\textsuperscript{2+} signals did not cross cell free zones and were not affected by fast superfusion or by the purinergic inhibitors suramin and apyrase. Dye coupling, electrical coupling and connexin immunostaining further indicated the presence of a low degree of astrocyte-endothelial gap junctional coupling, sufficient to mediate intercellular IP\textsubscript{3}, diffusion and thus astrocyte-endothelial Ca\textsuperscript{2+} signaling. We studied whether in addition to the intracellular gap junction-mediated pathway also an extracellular pathway would contribute to astrocyte-endothelial Ca\textsuperscript{2+} signaling. We studied and compared the effects on BCECs of ATP and other extracellularly applied putative Ca\textsuperscript{2+} mobilizing agonists involved in astrocyte-endothelial Ca\textsuperscript{2+} signaling (Paemeleire et al., 1999). The largest fraction of BCECs responds to ATP (about 70 %), indicating that the purinergic hypothesis in astrocyte-ECV304 Ca\textsuperscript{2+} wave propagation is relevant to the native BBB endothelial cells. We demonstrated large (average radius 160 µm) intercellular Ca\textsuperscript{2+} waves induced by mechanical stimulation of a single cell in co-culture propagating from astrocytes to ECV304 cells and vice versa (Paemeleire and Leybaert, 2000a). An extracellular diffusible factor is involved in the propagation of such intercellular Ca\textsuperscript{2+} waves as these waves are biased by fast superfusion flow and can cross a cell-free lane between astrocytes and endothelial cells. This extracellular diffusible factor is purinergic in nature as the intercellular Ca\textsuperscript{2+} wave propagation induced by single cell mechanical stimulation is inhibited by the presence of the P\textsubscript{2} purinoceptor antagonist suramin and is inhibited by the presence of the enzyme apyrase. This inhibition is only partial which suggests an additional role for the intracellular IP\textsubscript{3}/gap junction-mediated pathway in such waves. Astrocyte-to-endothelial Ca\textsuperscript{2+} signaling was confirmed in the co-cultures of astrocytes and BCECs (Braet et al., 2001). BCECs in co-cultures organized into capillary-like structures loosely attached to the culture dish which made the adequate stimulation of a single BCEC impossible. Therefore endothelial-to-astrocyte Ca\textsuperscript{2+} signaling could not be tested in co-cultures of astrocytes and BCECs.

As evidence accumulated for both an intracellular IP\textsubscript{3}/gap junction-mediated pathway and an extracellular purinergic pathway in astrocyte-endothelial Ca\textsuperscript{2+} signaling, we developed a model system allowing experimental ‘dissection’ of the two propagation pathways (Paemeleire et al., 2000). We prepared cultures from gap junction-deficient HeLa cells to investigate the extracellular purinergic pathway. A separate culture was set up of HeLa cells that were transfected with plasmids encoding for GFP-labeled connexins. HeLa cells expressing GFP-labeled gap junctions were used to study the intracellular gap junction-mediated pathway, by eliminating the contribution of extracellular purinergic messengers using apyrase. These experiments demonstrated subcellular differences between the two propagation modes. In untransfected HeLa cells and in the absence of apyrase, cell-to-cell propagating [Ca\textsuperscript{2+}]\textsubscript{c} changes were characterized by initiating so-called Ca\textsuperscript{2+} puffs associated with the perinuclear ER. By contrast, in connexin-GFP transfected HeLa cells and in the presence of apyrase, [Ca\textsuperscript{2+}]\textsubscript{c} changes were propagated without initiating perinuclear Ca\textsuperscript{2+} puffs and were communicated between cells at the sites of the GFP-labeled gap junctions. These experiments furthermore illustrate that any of the pathways in separation is sufficient for intercellular Ca\textsuperscript{2+} wave propagation.
As brain trauma also involves a sizeable component of traumatic damage to the astrocytes, we used a stronger mechanical single cell stimulus to invoke single astrocyte damage in some experiments. We studied whether single astrocyte damage produces, in addition to intercellular Ca\textsuperscript{2+} waves, propagating changes in other intra- and extracellular ions (Paemeleire and Leybaert, 2000b). We observed a significant [Na\textsuperscript{+}]\textsubscript{i} increase in cells located up to 66 µm away from the stimulated cell. The kinetics of these changes suggest the [Na\textsuperscript{+}] changes are secondary to the Ca\textsuperscript{2+} wave event. [Ca\textsuperscript{2+}] changes appear to be preceded by [K\textsuperscript{+}] changes. The origin of the [K\textsuperscript{+}] changes is unclear but a likely explanation is that they derive from K\textsuperscript{+} released from the stimulated and damaged cell.

**Conclusion**

We have presented evidence for bidirectional astrocyte-ECV304 Ca\textsuperscript{2+} signaling in co-culture, involving both an extracellular purinergic pathway and an intracellular IP\textsubscript{3}/gap junction-mediated pathway (Fig. 1). The existence of both pathways was confirmed using HeLa cells including a subcellular analysis of their distinct features. Using co-cultures of astrocytes with primary endothelial cells isolated from brain capillaries, we showed astrocyte-to-BCEC Ca\textsuperscript{2+} signaling in an *in vitro* model of the rat BBB. The existence and role *in vivo* of the two Ca\textsuperscript{2+} signaling pathways mentioned, is unclear. The extracellular purinergic pathway seems most relevant to the native BBB, as ATP induces a Ca\textsuperscript{2+} increase in a large fraction of BCECs and as endothelial cells and astrocytes are separated by a continuous basement membrane. The purinergic messenger involved in astrocyte-endothelial Ca\textsuperscript{2+} signaling could exert its paracrine effect by diffusion across the basement membrane at the BBB. Purinergic Ca\textsuperscript{2+} signaling from astrocytes towards endothelial cells may be a mechanism influencing BBB permeability and transport mechanisms, such as fluid phase endocytosis and glucose transport. A role for an intracellular pathway involving IP\textsubscript{3} diffusion through gap junctions is uncertain at present as gap junctions between astrocytes and BCECs have not yet been detected at the BBB. However, a low level of gap junctional...
coupling is sufficient for significant IP₃-mediated Ca²⁺ signaling raising the possibility that a very small density of gap junctions at the BBB might play a role and have escaped morphologic detection so far. Alternatively, astrocyte-endothelial gap junctional coupling may be present during embryogenesis or may be induced in certain pathophysiological conditions affecting the BBB. Our results also suggest that astrocytic intercellular Ca²⁺ waves may be accompanied by and contribute to some of the ionic shifts observed in the brain in vivo upon traumatic brain injury (Doberstein et al., 1993). We noticed especially that mechanical damage of astrocytes produces intercellular propagating increases of [Na⁺]. We hypothesize these are involved in promoting the survival of neural tissue following neurotrauma because an increase of astrocytic [Na⁺] is associated with increased production of lactate in these cells that acts as energy substrate for the neurons (Pellerin et al., 1998).

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REFERENCES

Complete list of references and a copy of the doctoral thesis are available upon request.


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