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Calcium signaling in and between brain astrocytes and endothelial cells

Koen PAEMELEIRE
Department of Physiology and Pathophysiology & Department of Neurology, Ghent University & Ghent University Hospital, Belgium

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 co-culture 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^{2+}-sensitive probes (fura-2 and fluo-3) and changes of $[Ca^{2+}]_i$ were monitored using a digital microfluorimetric Ca^{2+}-imaging setup. Intercellular Ca^{2+} waves were initiated in co-cultures of astrocytes and endothelial cells using flash photolysis of caged-IP_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_3 is chemically bound IP_3 that was brought into the cytoplasm through microinjection or electroporation. Biologically active IP_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^{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^{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_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^{2+} waves, were studied. Intracellular sodium concentration ($[Na^+]_i$) and pH_i changes were measured using ion-sensitive fluorescent probes (SBFI and BCECF respectively) and the digital microfluorescence imaging setup. Extracellular potassium concentration ($[K^+]_e$) changes were measured using a K^+-selective micro-electrode.

Our results indicate that intercellular Ca^{2+} waves induced in co-cultures by flash photolysis of caged-IP_3 can propagate from astrocyte to endothelial cell and vice versa (Leybaert *et al*., 1998). The propagation of the Ca^{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^{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_3 diffusion and thus astrocyte-endothelial Ca^{2+} signaling. We studied whether in addition to the intracellular gap junction-mediated pathway also an extracellular pathway would contribute to astrocyte-endothelial Ca^{2+} signaling. We studied and compared the effects on BCECs of ATP and other extracellulary applied putative Ca^{2+} mobilizing agonists involved in astrocyte-endothelial Ca^{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^{2+} wave propagation is relevant to the native BBB endothelial cells. We demonstrated large (average radius 160 µm) intercellular Ca^{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 diffusable factor is involved in the propagation of such intercellular Ca^{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 diffusable factor is purinergic in nature as the intercellular Ca^{2+} wave propagation induced by single cell mechanical stimulation is inhibited by the presence of the P_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_3/gap junction-mediated pathway in such waves. Astrocyte-to-endothelial Ca^{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^{2+} signaling could not be tested in co-cultures of astrocytes and BCECs.

As evidence accumulated for both an intracellular IP_3/gap junction-mediated pathway and an extracellular purinergic pathway in astrocyte-endothelial Ca^{2+} signaling, we developed a model system allowing experimental 'dissection' of the two pathways to study them separately and to investigate subcellular differences between 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 Ca^{2+} signaling modes. In untransfected HeLa cells and in the absence of apyrase, cell-to-cell propagating $[Ca^{2+}]_i$ changes were characterized by initiating so-called Ca^{2+} puffs associated with the perinuclear ER. By contrast, in connexin-GFP transfected HeLa cells and in the presence of apyrase, $[Ca^{2+}]_i$ changes were propagated without initiating perinuclear Ca^{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^{2+} wave propagation.

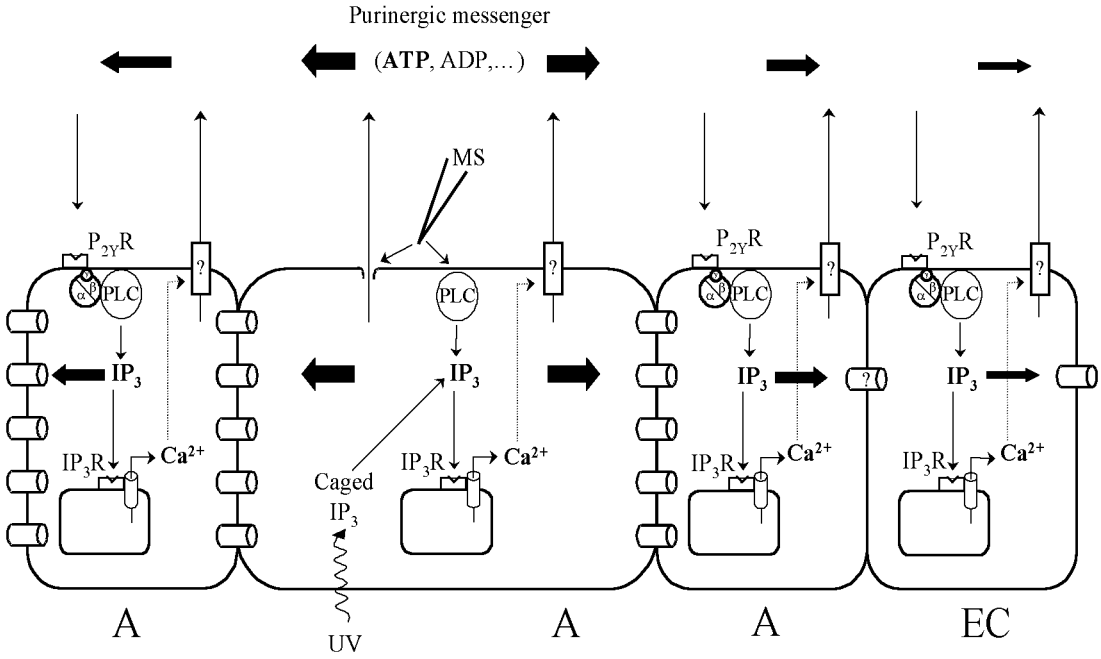

FIG. 1. — Proposed mechanisms of astrocyte-endothelial intercellular Ca^{2+} signaling. Astrocytic intercellular Ca^{2+} waves can be induced by a mechanical stimulus (MS) applied to a single cell or by the photolytical release in a single cell of the second messenger IP_3 from its caged precursor and consist of $[Ca^{2+}]_i$ increases propagating between consecutive rows of cells. The MS is thought to activate membrane-associated phospolipase C (PLC) leading to intracellular production of IP_3. IP_3 acts on the IP_3 receptor (IP_3R) which releases Ca^{2+} from intracellular stores (mainly the endoplasmic reticulum) by opening a Ca^{2+} channel. Astrocytes (A) show extensive gap junctional coupling allowing intercellular IP_3 diffusion. Intercellular Ca^{2+} waves mediated by such intracellular IP_3/gap junction pathway can propagate towards endothelial cells (EC) through a small number of gap junctions coupling adjacent astrocytes and endothelial cells. An extracellular purinergic messenger also mediates astrocyte-endothelial Ca^{2+} signaling. Cellular nucleotides (ATP, ADP,…) may be released by the MS and recent evidence indicates that Ca^{2+} by itself can trigger purinergic messenger release (by an at present unknown mechanism) suggesting that regenerative ATP release takes place in the course of a propagating Ca^{2+} signal. The extracellular messenger acts on P_{2Y} purinoceptors ($P_{2Y}R$), which are both present on the astrocytic and endothelial cell plasmamembrane. The purinergic messenger action increases the cytoplasmic IP_3 and Ca^{2+} concentration by a G-protein and PLC signaling cascade. This figure does not show the subcellular differences observed between the extracellular purinergic messenger pathway, characterized by initial perinuclear Ca^{2+} puffs, and the intracellular IP_3/gap junction pathway, characterized by initial Ca^{2+} changes that occur at the mouth of the gap junctions. For reasons of clarity endothelial-to-astrocyte intercellular Ca^{2+} signaling has not been shown.

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^{2+} waves, propagating changes in other intra- and extracellular ions (Paemeleire and Leybaert, 2000b). We observed a significant $[Na^+]_i$ increase in cells located up to 66 µm away from the stimulated cell. The kinetics of these changes suggest the $[Na^+]_i$ changes are secondary to the Ca^{2+} wave event. $[Ca^{2+}]_i$ changes appear to be preceded by $[K^+]_e$ changes. The origin of the $[K^+]_e$ changes is unclear but a likely explanation is that they derive from K^+ released from the stimulated and damaged cell.

Conclusion

We have presented evidence for bidirectional astrocyte-ECV304 Ca^{2+} signaling in co-culture, involving both an extracellular purinergic pathway and an intracellular IP_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 cocultures of astrocytes with primary endothelial cells isolated from brain capillaries, we showed astrocyte-to-BCEC Ca^{2+} signaling in an *in vitro* model of the rat BBB. The existence and role *in vivo* of the two Ca^{2+} signaling pathways mentioned, is unclear. The extracellular purinergic pathway seems most relevant to the native BBB, as ATP induces a Ca^{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^{2+} signaling could exert its paracrine effect by diffusion across the basement membrane at the BBB. Purinergic Ca^{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_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 IP_3-mediated Ca^{2+} 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 Ca^{2+} 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 $[Na^+]_i$. We hypothesize these are involved in promoting the survival of neural tissue following neurotrauma because an increase of astrocytic $[Na^+]_i$ 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.

1. Bradbury M. W. The blood-brain barrier. *Exp. Physiol*., 1993, **78** : 453-472.
2. Braet K., Paemeleire K., D'Herde K., Sanderson M. J., Leybaert L. Astrocyte-endothelial cell calcium signals conveyed by two signaling pathways. *Eur. J. Neurosci*., 2001, **13** : 79-91.
3. Charles A. Intercellular calcium waves in glia. *Glia*, 1998, **24** : 39-49.
4. Dermietzel R., Spray D. C. Gap junctions in the brain : where, what type, how many and why ? *Trends Neurosci*., 1993, **16** : 186-192.
5. Doberstein C. E., Hovda D. A., Becker D. P. Clinical considerations in the reduction of secondary brain injury. *Ann. Emerg. Med*., 1993, **22** : 993-997.
6. Guthrie P. B., Knappenberger J., Segal M., Bennett M. V., Charles A. C. *et al*. ATP released from astrocytes mediates glial calcium waves. *J. Neurosci*., 1999, **19 :** 520-528.
7. Kimelberg H. K., Norenberg M. D. Astrocytes. *Sci. Am*., 1989, **260** : 66-72, 74, 76.
8. Leybaert L., Paemeleire K., Strahonja A., Sanderson M. J. Inositol-trisphosphate-dependent intercellular calcium signaling in and between astrocytes and endothelial cells. *Glia*, 1998, **24** : 398-407.
9. Paemeleire K., de Hemptinne A., Leybaert L. Chemically, mechanically, and hyperosmolarity-induced calcium responses of rat cortical capillary endothelial cells in culture. *Exp. Brain Res*., 1999, **126** : 473-481.
10. Paemeleire K., Leybaert, L. ATP-dependent astrocyte-endothelial calcium signaling following mechanical damage to a single astrocyte in astrocyte-endothelial co-cultures. *J. Neurotrauma*, 2000a, **17** : 345-358.
11. Paemeleire K., Leybaert L. Ionic changes accompanying astrocytic intercellular calcium waves triggered by mechanical cell damaging stimulation. *Brain Res*., 2000b, **857** : 235-245.
12. Paemeleire K., Martin P. E., Coleman S. L., Fogarty K. E., Carrington W. A. *et al*. Intercellular calcium waves in HeLa cells expressing GFP-labeled connexin 43, 32, or 26. *Mol. Biol. Cell*, 2000, **11** : 1815-1827.
13. Pellerin L., Pellegri G., Bittar P. G., Charnay Y., Bouras C. *et al*. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev. Neurosci*., 1998, **20** : 291-299.
14. Sanderson M. J. Intercellular calcium waves mediated by inositol trisphosphate. *Ciba Found. Symp*., 1995, **88** : 175-189.

Dr. Koen Paemeleire,
Ghent University Hospital,
Department of Neurology (1K12 IA),
De Pintelaan 185,
B-9000 Ghent (Belgium).
E-mail : koenpaemeleire@yahoo.com.