μ-Opioid AGONISTS INHIBIT THE ENHANCED INTRACELLULAR Ca^{2+} RESPONSES IN INFLAMMATORY ACTIVATED ASTROCYTES CO-CULTURED WITH BRAIN ENDOTHELIAL CELLS

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Abstract—In order to imitate the *in vivo* situation with constituents from the blood–brain barrier, astrocytes from newborn rat cerebral cortex were co-cultured with adult rat brain microvascular endothelial cells. These astrocytes exhibited a morphologically differentiated appearance with long processes. 5-HT, synthetic μ-, δ- or κ-opioid agonists, and the endogenous opioids endomorphin-1, β-endorphin, and dynorphin induced higher Ca^{2+} amplitudes and/or more Ca^{2+} transients in these cells than in astrocytes in monoculture, as a sign of more developed signal transduction systems. Furthermore, stimulation of the co-cultured astrocytes with 5-HT generated a pronounced increase in intracellular Ca^{2+} release in the presence of the inflammatory or pain mediating activators substance P, calcitonin gene-related peptide (CGRP), lipopolysaccharide (LPS), or leptin. These Ca^{2+} responses were restored by opioids so that the δ- and κ-opioid receptor agonists reduced the number of Ca^{2+} transients elicited after incubation in substance P+CGRP or leptin, while the μ- and δ-opioid receptor agonists attenuated the Ca^{2+} amplitudes elicited in the presence of LPS or leptin. In LPS treated co-cultured astrocytes the μ-opioid receptor antagonist naloxone attenuated not only the endomorphin-1, but also the 5-HT evoked Ca^{2+} transients. These results suggest that opioids, especially μ-opioid agonists, play a role in the control of neuroinflammatory activity in astrocytes and that naloxone, in addition to its interaction with μ-opioid receptors, also may act through some binding site on astrocytes, other than the classical opioid receptor. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: astrocyte, calcium, endothelial cells, inflammatory activators, opioids, pain-transmitting peptides.

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Abbreviations: BBB, blood–brain barrier; BSA, bovine serum albumin; [Ca^{2+}]$_i$, intracellular free calcium; CGRP, calcitonin gene-related peptide; DAMGO, [D-Ala^2,NMe-Phe^4,Gly-ol^5]-enkephalin; DMEM, Dulbecco's modified Eagle's medium; DOR, δ-opioid receptor; DPDPE, [D-Pen^2,D-Pen^5]enkephalin/[D-Pen^{2,5}]enkephalin; HHBSS, Hanks' Hepes buffered saline solution; IL-1β, interleukin-1β; KOR, κ-opioid receptor; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; PBS, phosphate buffer saline; PNS, peripheral nervous system; SDS, sodium dodecyl sulfate; TLR4, Toll-like receptor 4.

Inflammation is characterized by edema formation, increased blood flow, and inflammatory cell recruitment. These signs are observed after stimulation of sensory nerves, C- and Aδ-fibers, and release of neuropeptides (Keeble and Brain, 2004). Activation of sensory fibers by inflammatory activators can cause neuropathic pain, and as a result, inflammatory models of neuropathic pain were developed (Moalem and Tracey, 2006). Inflammatory activators can affect different cell types and activate several signaling pathways. It is debated if such inflammatory agents act directly on receptors or binding sites located on the cell membrane on neurons or other cells such as mast cells or glia.

Substance P, calcitonin gene-related peptide (CGRP), lipopolysaccharide (LPS), and leptin are inflammatory activators released in the periphery and could also activate cells within the CNS. Substance P has proinflammatory actions both in peripheral tissue and in the CNS after tissue injury (Mantyh, 1991), and can enhance immunoregulatory and neurotrophic astrocytic functions (Palma et al., 1997). It is mainly synthesized and released by neurons in the CNS and peripheral nervous system (PNS), but astrocytes also express this peptide, and its receptor, neurokinin 1 (NK-1) (Palma et al., 1997). It is commonly referred to as a pain-transmitting or pain-modulating peptide (Lee and Kim, 2007). CGRP is distributed throughout the CNS and PNS (Brain and Grant, 2003). The peptide which is synthesized in sensory neurons in the dorsal horn region of the spinal cord, is co-localized with glutamate and substance P, and is released from nociceptive fibers in response to noxious stimuli (Trang et al., 2005). CGRP is one of the most potent vasodilator agents of peripheral and cerebral vessels (Moreno et al., 2002a). Its receptor, CRLR, was identified in cerebral vasculature (Oliver et al., 2002), and in astrocytes (Moreno et al., 2002b). It is also known as a pain-transmitting or pain-modulating peptide (Lee and Kim, 2007). The endotoxin LPS is a cell wall component of Gram-negative bacteria and a very potent inducer of inflammation (Nakamara, 2002). It stimulates the Toll-like receptor 4 (TLR4), which is present on astrocytes and microglia (Kielian, 2006). TLR4 contributes to the initiation of CNS neuroimmune activation (Tanga et al., 2005) and is considered to be involved in the initiation of neuropathic pain (Moalem and Tracey, 2006). Leptin is secreted by fat cells and enters the brain via a saturable transport system. It was shown that epinephrine enhances the transport of leptin across the blood–brain barrier (BBB) (Banks, 2001). The leptin

receptor, OB-R, is found in many tissues and cross-talk was observed in the main signaling pathways such as protein kinase C, mitogen-activated protein kinases, and phospholipase C (Frühbeck, 2006). Leptin was found to be involved in response to infection and inflammation (Hosoi et al., 2000) but it also acts as a marker for stress (Jürimäe et al., 2003).

The molecular transport across the BBB makes it possible for the brain to be supplied with essential nutrients and mediates efflux of waste products (Abbott et al., 2006). Both astrocytes and endothelial cells respond to a variety of stimuli with an increase in intracellular free calcium ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ serves as an essential intracellular messenger. The intracellular Ca^{2+} level is regulated by Ca^{2+} transport across the cell membrane and Ca^{2+} release and/or binding to intracellular structures, resulting in free $[Ca^{2+}]_i$ in mammalian cells of about 100 nM. This value is 10,000 times lower than extracellular $[Ca^{2+}]$. Different soluble factors, some of them released upon neuronal activity, induce Ca^{2+} mediated signaling in astrocytes, and also propagation of Ca^{2+} fluctuations into other astrocytes via gap junctions, in which connexin 43 and purinergic receptors are shown to be basic elements (Cotrina et al., 1998; Blomstrand et al., 1999a,b; Guthrie et al., 1999; Arcuino et al., 2002). Astrocytes express receptors for many neurotransmitters and neuroactive substances (Hansson and Rönnbäck, 2004), and the cells act as sensors for ions and neuroactive substances in the extracellular milieu, as well as sensors for pH and osmotic balance. Astrocytic $[Ca^{2+}]_i$ waves in the form of transients or oscillations cause release of transmitters like glutamate, D-serine and ATP (Bezzi et al., 2004; Haydon and Carmignoto, 2006). These transmitters, released from astrocytes, can affect and enhance neuronal excitability (Pasti et al., 1997, 2001; Kang et al., 1998). Astrocyte Ca^{2+} signaling, especially Ca^{2+} oscillations, can also induce dilation of brain microvessels via Ca^{2+} dependent cyclooxygenase activation and prostaglandin E2 production (Zonta et al., 2003a,b), which can in turn regulate the neuronal activity and brain microcirculation (Morita et al., 2005).

Regulation of Ca^{2+} dynamics by transmitters and other soluble factors is thus a possible mechanism by which the astrocyte networks detect changes in the CNS micro-environment and regulate brain activities such as inflammatory processes, chronic pain, regeneration and memory formation under various physiological and pathophysiological conditions.

5-HT has for years been associated with e.g. pain processing and modulation (Eide and Hole, 1993). This amine is released by mast cells and platelets after tissue injury (Sommer, 2004; Theoharides and Cochrane, 2004) and can increase the axonal excitability of C-fibers (Moalem et al., 2006). Various 5-HT receptors are localized on C-fibers, neurons, astrocytes, and endothelial cells (Hagberg et al., 1998; Cohen et al., 1999; Sommer, 2004). Pronounced Ca^{2+} oscillations are elicited in the astrocytes upon stimulation with 5-HT in cells co-cultured with endothelial cells (Hansson et al., 2004).

The aims of the present study were to:

1) investigate whether or not astrocytes co-cultured with brain microvascular endothelial cells from adult rat brain are more differentiated, especially from a morphological point of view, than astrocytes in monoculture. Preliminary results supporting a more differentiated morphology are already published (Hansson et al., 2004).
2) examine effects of different opioids on Ca^{2+} mobilization elicited by 5-HT as the primary activator in the presence of classic inflammatory and pain mediating activators in co-cultured astrocytes.

EXPERIMENTAL PROCEDURES

Astroglial primary cultures

The primary astroglial cultures were prepared from newborn rat cerebral cortices (Charles River, Sulzfeldt, Germany) and cultivated on glass coverslips as described earlier (Hansson et al., 1984).

Microvascular endothelial primary cultures

Brain capillary fragments were isolated, and endothelial cells cultured, using a modified version of the method used by Abbott and co-workers (1992). In short, three male Sprague–Dawley rats, 225–250 g, gave sufficient capillary fragments for plating 48 inserts with a diameter of 12 mm and a pore size of 0.4 mm (Transwell-clear, tissue culture treated polyester membrane, Corning Costar, Cambridge, MA, USA). All chemicals were obtained from Sigma (St. Louis, MO, USA), if not otherwise indicated. The inserts were precoated with a mixture of 0.33 mg/ml collagen IV from human placenta and 20 μg/ml fibronectin from rat plasma. The cerebral cortices were dissected and the meninges and choroid plexus peeled off. The tissue was chopped and spun at 600×g, 4 °C, for 5 min in BSA buffer (calcium- and magnesium-free Hanks' balanced saline solution, buffered with 10 mM Hepes (Research Organics Inc., Cleveland, OH, USA), pH 7.3, containing 1% penicillin–streptomycin (PEST, Invitrogen Corporation, Paisley, Scotland, UK) and 0.5% bovine serum albumin (BSA 30% solution, Serologicals Corporation, Norcross, GA, USA)). The preparation involved a two step enzymatic dissociation to degrade extracellular matrix. The cells were first treated with 10 ml 0.1% collagenase II solution (containing 50 μg/ml gentamicin and 2 mM L-glutamine) at 37 °C for 2 h on a thermomixer with gentle shaking every 10 min. The suspension was spun at 600×g, 4 °C, for 15 min. The supernatant was removed and 20% BSA buffer was added. The solution was mixed and centrifuged at 1000×g for 20 min at 4 °C, to separate capillary fragments from myelin, neurons, astrocytes, and other single cell contaminations. The top layer was removed to eliminate traces of myelin. The capillary pellet was resuspended in 5 ml 0.1% collagenase/dispase solution (Roche Diagnostic GmbH, Penzberg, Germany, in Dulbecco's modified Eagle's medium, low glucose (1000 mg/l) (DMEM) containing 50 μg/ml gentamicin and 2 mM L-glutamine, incubated at 37 °C for 1.5 h on a thermomixer with gentle shaking every 10 min. The suspension was centrifuged at 600×g for 5 min at 4 °C. The supernatant was removed and the pellet resuspended in 1 ml DMEM solution and put on a prepared Percoll gradient. After centrifugation for 20 min at 1000×g and 4 °C, the white−greyish band of endothelial cell clusters above the red blood cells was aspirated and added to 10 ml of DMEM solution. The tube was spun at 600×g for 5 min at 4 °C, and the pellet was resuspended in DMEM solution. After the last spinning step (600×g for 5 min at 4 °C) the supernatant was aspirated and the pellet mixed with