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Ultralow concentrations of bupivacaine exert anti-inflammatory effects on inflammation-reactive astrocytes

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Abstract

Bupivacaine is a widely used, local anesthetic agent that blocks voltage-gated Na⁺ channels when used for neuro-axial blockades. Much lower concentrations of bupivacaine than in normal clinical use, < 10⁻⁸ M, evoked Ca²⁺ transients in astrocytes from rat cerebral cortex, that were inositol trisphosphate receptor-dependent. We investigated whether bupivacaine exerts an influence on the Ca²⁺ signaling and interleukin-1β (IL-1β) secretion in inflammation-reactive astrocytes when used at ultralow concentrations, < 10⁻⁸ M. Furthermore, we wanted to determine if bupivacaine interacts with the opioid-, 5-hydroxytryptamine- (5-HT) and glutamate-receptor systems. With respect to the μ-opioid- and 5-HT-receptor systems, bupivacaine restored the inflammation-reactive astrocytes to their normal non-inflammatory levels. With respect to the glutamate-receptor system, bupivacaine, in combination with an ultralow concentration of the μ-opioid receptor antagonist naloxone and μ-opioid receptor agonists, restored the inflammation-reactive astrocytes to their normal non-inflammatory levels. Ultralow concentrations of bupivacaine attenuated the inflammation-induced upregulation of IL-1β secretion. The results indicate that bupivacaine interacts with the opioid-, 5-HT- and glutamate-receptor systems by affecting Ca²⁺ signaling and IL-1β release in inflammation-reactive astrocytes. These results suggest that bupivacaine may be used at ultralow concentrations as an anti-inflammatory drug, either alone or in combination with opioid agonists and ultralow concentrations of an opioid antagonist.

Introduction

Local anesthetics may interfere with opioid receptor signaling through their effects on G proteins and Ca²⁺ channels by decreasing the opioid inhibition of these channels (Komai & McDowell, 2007). Clinically used doses of such agents produce effective analgesia, by blocking voltage-gated Na⁺ channels. Bupivacaine may have other effects at much lower concentrations where the blocking of Na⁺ channels does not exist (Toda *et al.*, 2011). Doses far below those that block nerve impulse propagation, at nanomolar concentrations, may have pain-relieving actions via other targets such as neuronal G protein-coupled receptors and binding sites on immune cells (Amir *et al.*, 2006). Bupivacaine has been proposed to attenuate inflammatory responses, although the mechanisms involved and sites of action for such effects of this anesthetic are not known (Bedirli *et al.*, 2011).

Glial cells have been shown to play critical roles in neuropathic, nociceptive and inflammatory pain, which is pain that can be long-lasting and may eventually be considered as chronic (Gao & Ji, 2010; Lundborg *et al.*, 2010; Chiang *et al.*, 2012). The underlying

mechanisms of persistent/long-lasting pain involves the presence of neuroinflammation at the damaged or affected nerve(s) (Saadé & Jabbur, 2008; McMahon & Malcangio, 2009; Vallejo *et al.*, 2010), which includes the activation of glial cells.

We have recently shown that Ca²⁺ signaling in astrocytes is disturbed when the cells are influenced by inflammatory stimuli, and our previous data suggest that some receptor systems are more sensitive to inflammatory stimuli than others, especially opioid-, 5-hydroxytryptamine (5-HT)- and glutamate-receptor systems in the form of increased intracellular Ca²⁺ transients (Hansson *et al.*, 2008; Lundborg *et al.*, 2011; Block *et al.*, 2012). Astrocyte-propagating Ca²⁺ waves (Blomstrand *et al.*, 1999; Scemes & Giaume, 2006) can be evoked by transmitters released from neurons and glial cells, for example ATP and glutamate, which can activate G protein-coupled receptors on astrocytes. Inflammatory stimuli disturb the Ca²⁺ homeostasis of the astrocyte network, which results in Ca²⁺-evoked oscillations (Hansson & Rönnbäck, 2003; Hansson, 2006, 2010; Forshammar *et al.*, 2011).

The upregulation of interleukin-1β (IL-1β) secretion observed in an inflammatory environment reduces intercellular communication by affecting the passage of inositol 1,4,5-trisphosphate (IP₃) through gap junctions in the astrocyte networks (Retamal *et al.*, 2007). This results in inhibition of the intercellular neuroprotective Ca²⁺ signaling

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through gap junction channels, and increases another type of Ca^{2+} signaling providing a pathway for the release and uptake of small molecules, such as ATP, between the extra- and intracellular compartments (Froger *et al.*, 2010).

The first aim of this study was to investigate whether bupivacaine, at ultralow concentrations, $< 10^{-8}$ M, interacted with the intracellular Ca^{2+} signaling system in astrocytes. If so, the second aim was to investigate if bupivacaine, at ultralow concentrations, could interact with the opioid-, 5-HT- or glutamate-receptor systems in inflammation-reactive astrocytes through the Ca^{2+} signaling system. The final aim was to investigate whether bupivacaine could attenuate the IL-1 β secretion that is upregulated in reactive astrocytes under inflammatory conditions.

Materials and methods

In this study, an *in vitro* model involving astrocytes co-cultured with brain endothelial cells was employed (Hansson *et al.*, 2008). The biological rationale for co-culturing astrocytes with endothelial cells is that astrocytes are affected by substances released from the capillary endothelial cells of the blood–brain barrier (BBB) (Huber *et al.*, 2001). These interactions are essential for a functional neurovascular system (Abbott *et al.*, 2006; Willis & Davis, 2008). The endothelial cells are not in direct physical contact with the astrocytes *in vitro*; rather, the interaction between these two cell types is facilitated by a shared medium. Co-cultured astrocytes are morphologically differentiated from monocultured astrocytes by their long, slender processes; they also exhibit greater Ca^{2+} responses and cytokine release than monocultured astrocytes. Finally, the μ -opioid receptor is more highly expressed in co-cultured astrocytes (Hansson *et al.*, 2008), as well as the Toll-like receptor 4 (TLR4) (Forshammar *et al.*, 2011).

Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

The experimental protocols were approved by the Ethical Committee in Gothenburg, Sweden, for Laboratory Animals (Nos. 205-2010; 211-2010), and were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Primary astrocytic cultures

Primary astrocytic cultures were prepared from newborn rat cerebral cortices (Charles River, Sulzfeldt, Germany) and cultured on glass coverslips (Nr. 1, 20-mm diameter, BergmanLabora, Stockholm, Sweden) as described by Hansson *et al.* (2008).

Microvascular endothelial primary cultures

Brain capillary fragments were isolated from male Sprague–Dawley rats; cerebral cortices (Charles River, 225–250 g), and endothelial cells were cultured according to the protocol of Hansson *et al.* (2008).

Astrocytes co-cultured with adult rat brain microvascular primary cultures

Experimental astrocytes were obtained after the co-cultivation of primary brain microvascular endothelial cultures and primary astrocyte cultures. At 6 days *in vitro*, astrocyte cultures were co-cultured with newly prepared microvascular cultures. The endothelial cells were grown on inserts above the astrocyte cultures. The cells from the

two different cultures were not brought into contact, and they were grown together for 9–11 days. As a result, the experimental astrocytes were cultured for 15–17 days, including 9–11 days of co-cultivation, for the experiments in this study. The endothelial cells were removed before all experimental procedures (Hansson *et al.*, 2008).

Calcium imaging

Astrocytes co-cultured with brain microvascular endothelial cells were incubated at room temperature with the Ca^{2+} -sensitive fluorophore probe Fura-2/AM (Invitrogen Molecular Probes, Eugene, OR, USA) for 20 min [8 μL in 990 μL Hank's HEPES-buffered saline solution (HHBSS), containing 137 mM NaCl, 5.4 mM KCl, 0.4 mM MgSO_4 , 0.4 mM MgCl_2 , 1.26 mM CaCl_2 , 0.64 mM KH_2PO_4 , 3.0 mM NaHCO_3 , 5.5 mM glucose and 20 mM HEPES dissolved in distilled water, pH 7.4). The fluorophore probe was dissolved in 40 μL dimethyl sulfoxide (DMSO) and 10 μL pluronic acid (Molecular Probes, Leiden, the Netherlands) (Fig. 1). After incubation, the cells were rinsed three times with HHBSS before exposure to stimulators for 30 s. To determine the underlying Ca^{2+} source of the bupivacaine-evoked Ca^{2+} transients, internal Ca^{2+} stores were depleted either by pre-incubation with a sarcoendoplasmic reticulum Ca^{2+} -ATPase inhibitor, thapsigargin (1 μM), and caffeine (20 mM)

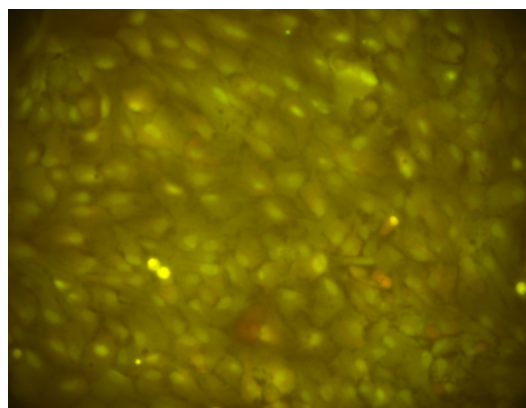


FIG. 1. Astrocytes incubated with the Ca^{2+} -sensitive fluorophore probe Fura-2/AM. See Materials and methods for further details.

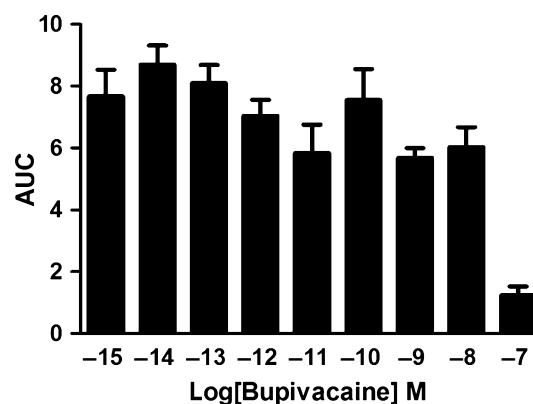


FIG. 2. Bupivacaine-induced Ca^{2+} transients. The astrocytes were stimulated with bupivacaine (10^{-15} – 10^{-7} M). All cells responded to the treatment within the 10^{-15} – 10^{-8} M concentration range. At 10^{-7} M bupivacaine, only 4/40 cells responded, and the responses were minimal. The areas under the Ca^{2+} peaks (AUC) were calculated. The data are shown as the mean \pm SEM, $n = 40$.

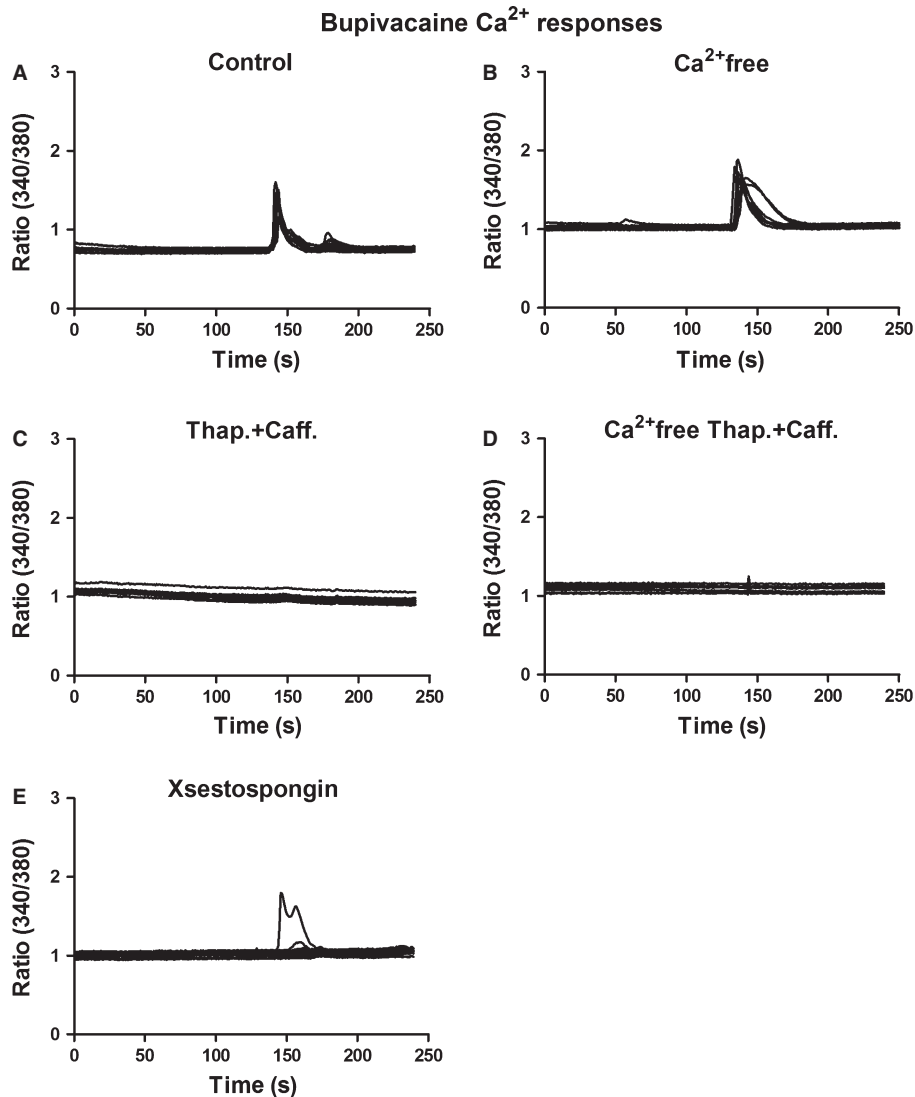


FIG. 3. Bupivacaine-induced Ca²⁺ transients were triggered via intracellular Ca²⁺ stores. The cells were stimulated and analysed using a Ca²⁺ imaging system. (A) Bupivacaine (10⁻¹² M) elicited Ca²⁺ responses in all astrocytes tested. (B) Bupivacaine administered in a Ca²⁺-free medium evoked Ca²⁺ transients in all of the cells tested. (C) To determine the source of the Ca²⁺ response, internal Ca²⁺ stores were depleted with thapsigargin followed by caffeine. Interestingly, the Ca²⁺ responses were inhibited. (D) All cells were inhibited when thapsigargin and caffeine were administered in a Ca²⁺-free buffer. (E) To examine the involvement of the IP₃-sensitive Ca²⁺ release system, the astrocytes were incubated with xestospingon C, a blocker of IP₃ receptors. Only one of the 40 cells tested responded, $n = 40$. The results shown are from a typical experiment.

(Sharma & Vijayaraghavan, 2001) or by incubation in a Ca²⁺-free buffer (CaCl₂ was replaced by MgCl₂ and 1 mM EGTA). The cells were incubated with lipopolysaccharide (LPS, *Escherichia coli* O111:B4) (10 ng/mL) for 4 or 24 h. Different combinations of bupivacaine (10⁻¹² M), naloxone (10⁻¹² M), and a combination of endomorphin-1 (10⁻⁶ M) and β -endorphin (10⁻⁶ M) were applied 3.5 min before treatment with endomorphin-1, 5-HT or glutamate (10⁻⁴ M). The experiments were performed at room temperature using a Ca²⁺ imaging system and Simple PCI software (Compix Inc., Imaging Systems, Hamamatsu Photonics Management Corporation, Cranberry Twp., PA, USA) connected to an inverted epifluorescence microscope (Nikon ECLIPSE TE2000-E) with a 20 \times (NA 0.45) fluorescence dry lens and a Polychrome V, monochromator-based illumination system (TILL Photonics, CA, USA). Various substances were applied using a peristaltic pump (Instech Laboratories, Plymouth Meeting, PA, USA) at an approximate rate of 600 μ L/min. One minute after the start of the experiment, the stimulating substance was pumped into the pump tubes for 30 s. The sub-

stance took approximately 60 s to reach the cells through the tubes. HHBSS continued to flow through the pump tubes and onto the cells throughout the experiment. The images were captured with an ORCA-12AG High Res Digital Cooled CCD Camera (C4742-80-12AG, Hamamatsu Photonics).

The total area under the curve (AUC), which reflects the amount of Ca²⁺ released (Berridge, 2007), was analysed to measure the strength of the Ca²⁺ responses. The amplitude was expressed as the maximum increase of the 340/380 ratio. The area under the Ca²⁺ peaks was calculated in Origin (Microcal Software Inc., Northampton, MA, USA). Forty cells were used for each experimental set-up; these cells were taken from four different coverslips and at two different seeding times.

Cytokine release assay

The co-cultured astrocytes were stimulated for 24 h with 10 ng/mL LPS diluted in non-supplemented minimum essential medium

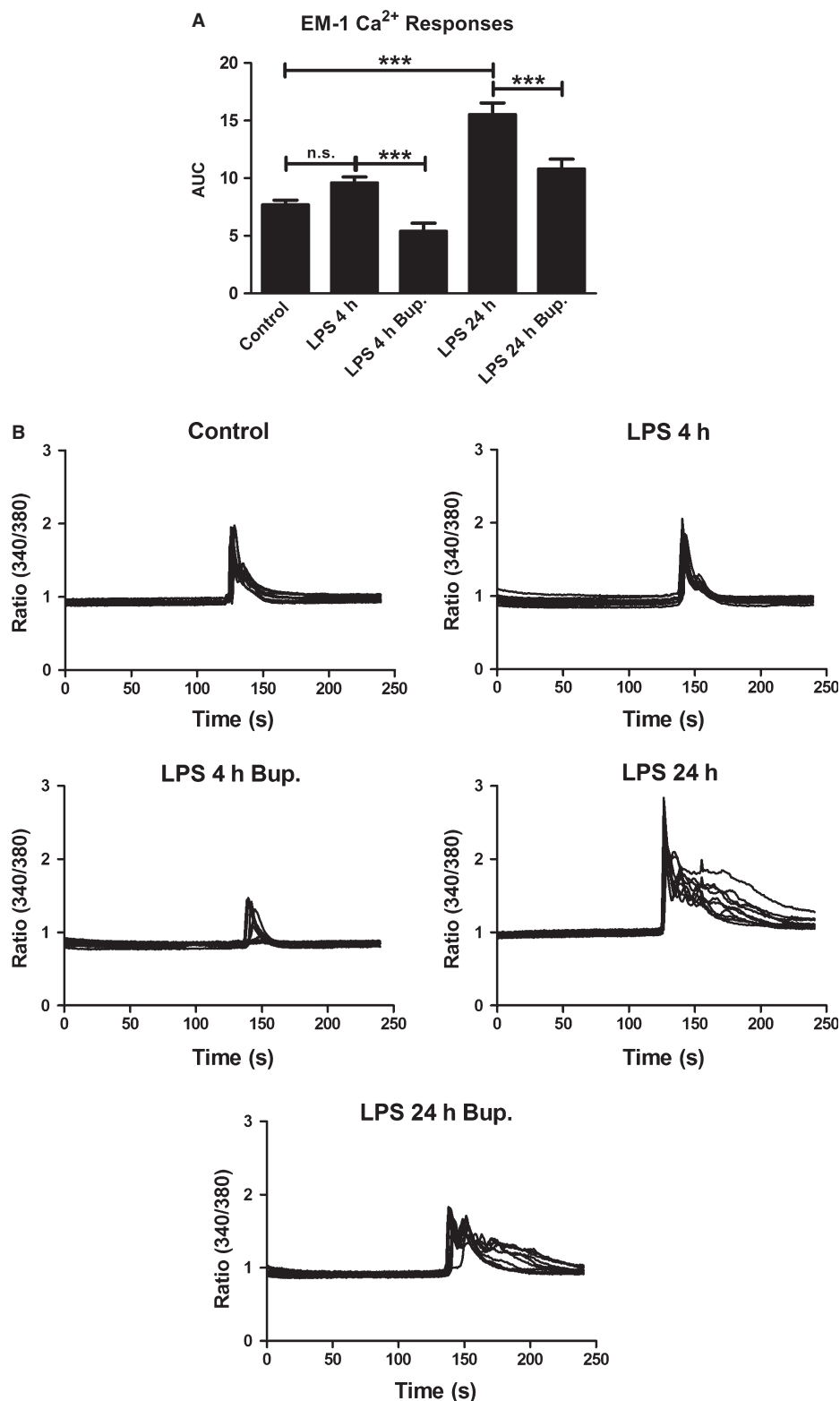


FIG. 4. Endomorphin-1 (EM-1) elicited Ca²⁺ responses in the astrocytes and was used as control. (A) Cells incubated with LPS for 4 h showed no significant changes in Ca²⁺ level compared with the control. Cells incubated with LPS for 24 h showed increased Ca²⁺ signaling. Bupivacaine (10⁻¹² M) attenuated the EM-1-induced Ca²⁺ transients that were upregulated by LPS for 4 and 24 h. The areas under the Ca²⁺ peaks (AUC) were calculated. (B) The Ca²⁺ transients were visualized. The results shown are from a typical experiment. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. ****P* < 0.001, n.s. = not significant. *n* = 40.

(MEM) to measure IL-1 β release. The culture inserts containing the endothelial cells were removed immediately before incubation. The cells were incubated with 10⁻¹² or 10⁻⁶ M bupivacaine for 30 min

prior to LPS treatment, and the bupivacaine remained in the medium throughout the experiment. Control (i.e. untreated) cells were maintained in non-supplemented MEM. Culture supernatants were

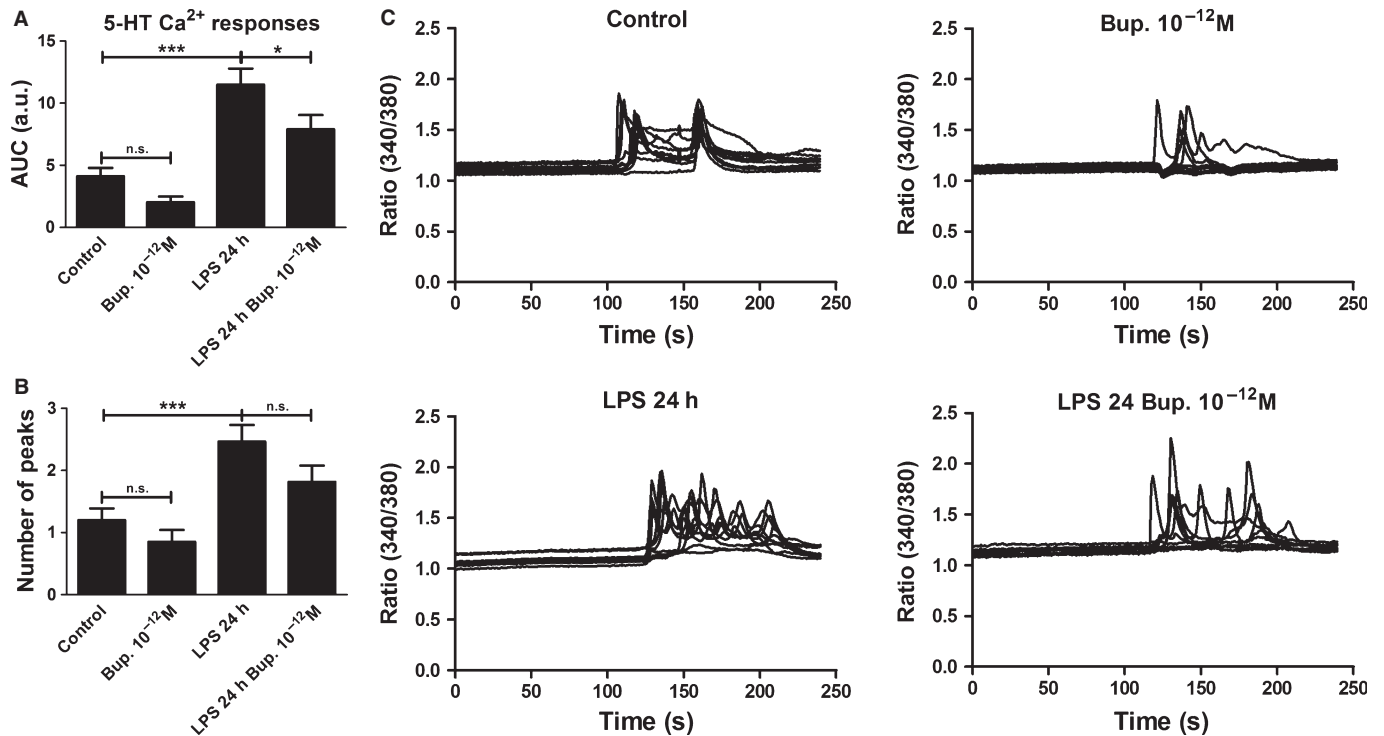


FIG. 5. 5-HT elicited Ca²⁺ responses in the astrocytes and was used as control. (A) Bupivacaine showed no significant changes in the level of 5-HT-induced Ca²⁺ signaling compared with the control. Cells incubated with LPS for 24 h showed an increased level of 5-HT-induced Ca²⁺ signaling, which were attenuated by bupivacaine (10⁻¹² M). The areas under the Ca²⁺ peaks (AUC) were calculated. (B) The number of peaks was counted for each cell. (C) The Ca²⁺ transients were visualized. The results shown are from a typical experiment. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. ****P* < 0.001, **P* < 0.05, n.s. = not significant. *n* = 40.

collected and analysed for protein concentrations. An enzyme-linked immunosorbent assay (ELISA) kit (Nordic Biosite, Täby, Sweden) was used according to the manufacturer's instructions.

Protein determination

The protein content was determined using a detergent-compatible (DC) protein assay (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's instructions, based on the method by Lowry *et al.* (1951) with minor modifications. The standards (0–4 mg/mL BSA) and samples were mixed with the reagents, incubated for 15 min at room temperature, read at 750 nm with a Versa-max microplate reader and analysed using SoftMax Pro 4.8 software (Molecular Devices, Sunnyvale, CA, USA).

Statistics

Differences across different treatments were identified using one-way ANOVA followed by Dunnett's multiple-comparisons test. Error bars represent the standard error of the mean (SEM).

Results

Bupivacaine elicits Ca²⁺ responses in astrocytes at < 10⁻⁸ M

The astrocytes were stimulated with bupivacaine (10⁻¹⁵–10⁻⁷ M), and Ca²⁺ imaging experiments were performed. All samples produced Ca²⁺ responses when treated with 10⁻¹⁵–10⁻⁸ M bupivacaine. No response was observed in cells treated with 10⁻⁷ M bupivacaine.

The amount of Ca²⁺ released by each sample was determined by calculating the AUC for each sample (Fig. 2).

To investigate whether the increase in intracellular Ca²⁺ (Fig. 3A) was due to Ca²⁺ crossing the extracellular membrane or Ca²⁺ originating from intracellular stores, bupivacaine (10⁻¹² M) was first administered in a Ca²⁺-free medium, which still evoked Ca²⁺ transients in most cells (Fig. 3B). Cells were also incubated with thapsigargin, a depletor of intracellular Ca²⁺ stores (Sharma & Vijayaraghavan, 2001), followed by caffeine, which was used to complete the store depletion (Sharma & Vijayaraghavan, 2001); interestingly, this treatment inhibited the Ca²⁺ response (Fig. 3C). Finally, no cells responded when they were treated with thapsigargin and caffeine in a Ca²⁺-free buffer (Fig. 3D). To determine the involvement of the IP₃-sensitive Ca²⁺ release pathway, astrocytes were incubated with xestospongine C (100 nM), which is a blocker of IP₃ receptors (Sharma & Vijayaraghavan, 2001). In these astrocytes, no responsive cells were observed (Fig. 3E).

Bupivacaine interacts with endomorphin-1-induced Ca²⁺ responses in LPS-treated astrocytes

Astrocytes incubated with LPS for 4 h and stimulated with endomorphin-1 did not exhibit significantly different Ca²⁺ signaling levels compared with the control. Cells incubated with LPS for 24 h showed increased endomorphin-1-induced Ca²⁺ signaling, as indicated by significantly increased AUC values (*P* < 0.001). Bupivacaine (10⁻¹² M) attenuated the endomorphin-1-induced Ca²⁺ transients in all cells, as shown by significantly decreased AUC values for the bupivacaine-treated samples (*P* < 0.001) (Fig. 4).

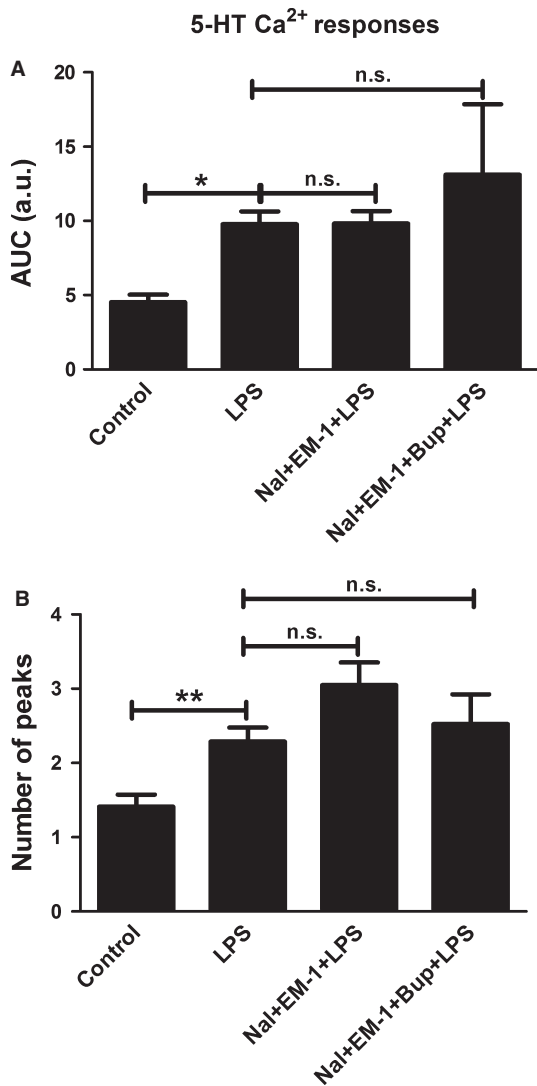


FIG. 6. 5-HT elicited Ca²⁺ responses in the astrocytes and was used as control. (A) Cells incubated with LPS for 24 h showed increased 5-HT-induced Ca²⁺ signaling compared with the control. A combination of naloxone (Nal) (10⁻¹² M) and endomorphin-1 (EM-1) (10⁻⁶) did not change the level of 5-HT-induced Ca²⁺ transients upregulated by LPS, nor did a combination of bupivacaine (Bup), Nal and EM-1. The areas under the Ca²⁺ peaks (AUC) were calculated. (B) The number of peaks was counted for each cell. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. ***P* < 0.01, **P* < 0.05, n.s. = not significant. *n* = 40.

Bupivacaine, at ultralow concentration, interacts with 5-HT-induced Ca²⁺ responses in LPS-treated astrocytes

Cells treated with bupivacaine did not exhibit significantly different 5-HT-induced Ca²⁺ signaling levels compared with the control. Cells incubated with LPS for 24 h showed increased 5-HT-induced Ca²⁺ signaling, as indicated by significantly increased AUC values (*P* < 0.001); all cells exhibited a response. Bupivacaine (10⁻¹² M) attenuated the 5-HT-induced Ca²⁺ transients that were upregulated by LPS treatment in all cells (*P* < 0.05) (Fig. 5).

However, administration of naloxone (10⁻¹² M) together with endomorphin-1 (10⁻⁶ M) did not attenuate the 5-HT-induced Ca²⁺ transients that were upregulated by LPS treatment; all cells responded (Fig. 6). No attenuation was observed either for a combination of

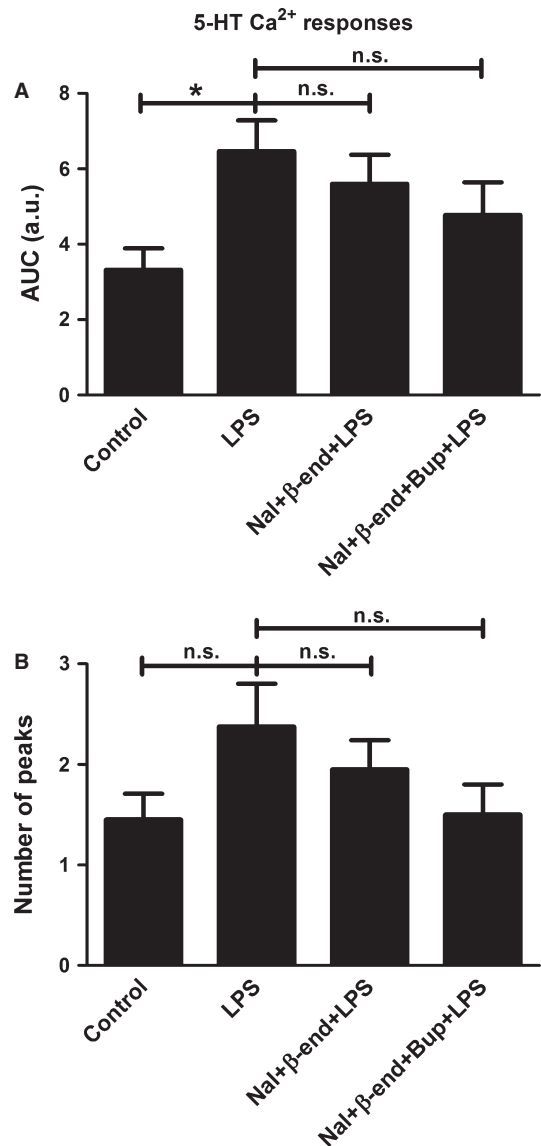


FIG. 7. 5-HT elicited Ca²⁺ responses in the astrocytes and was used as control. (A) Cells incubated with LPS for 24 h showed increased 5-HT-induced Ca²⁺ signaling compared with the control. A combination of naloxone (Nal) (10⁻¹² M) and β-endorphin (β-end) (10⁻⁶) did not attenuate the 5-HT-induced Ca²⁺ transients that were upregulated by LPS, nor did a combination of bupivacaine (Bup), Nal and β-end. The areas under the Ca²⁺ peaks (AUC) were calculated. (B) The number of peaks was counted for each cell. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. **P* < 0.05, n.s. = not significant. *n* = 40.

bupivacaine, naloxone and endomorphin-1 (Fig. 6), or a combination of bupivacaine, naloxone and β-endorphin (Fig. 7).

Bupivacaine, at ultralow concentration, interacts with glutamate-induced Ca²⁺ responses in LPS-treated astrocytes

Bupivacaine (10⁻¹² M) reduced glutamate-induced Ca²⁺ signaling when compared with the control (*P* < 0.05), which consisted of cells treated with glutamate alone. All cells exhibited a response. Cells incubated with LPS for 24 h showed increased glutamate-induced Ca²⁺ signaling, as indicated by a significantly increased AUC value (*P* < 0.01). Again, all cells responded. Bupivacaine did

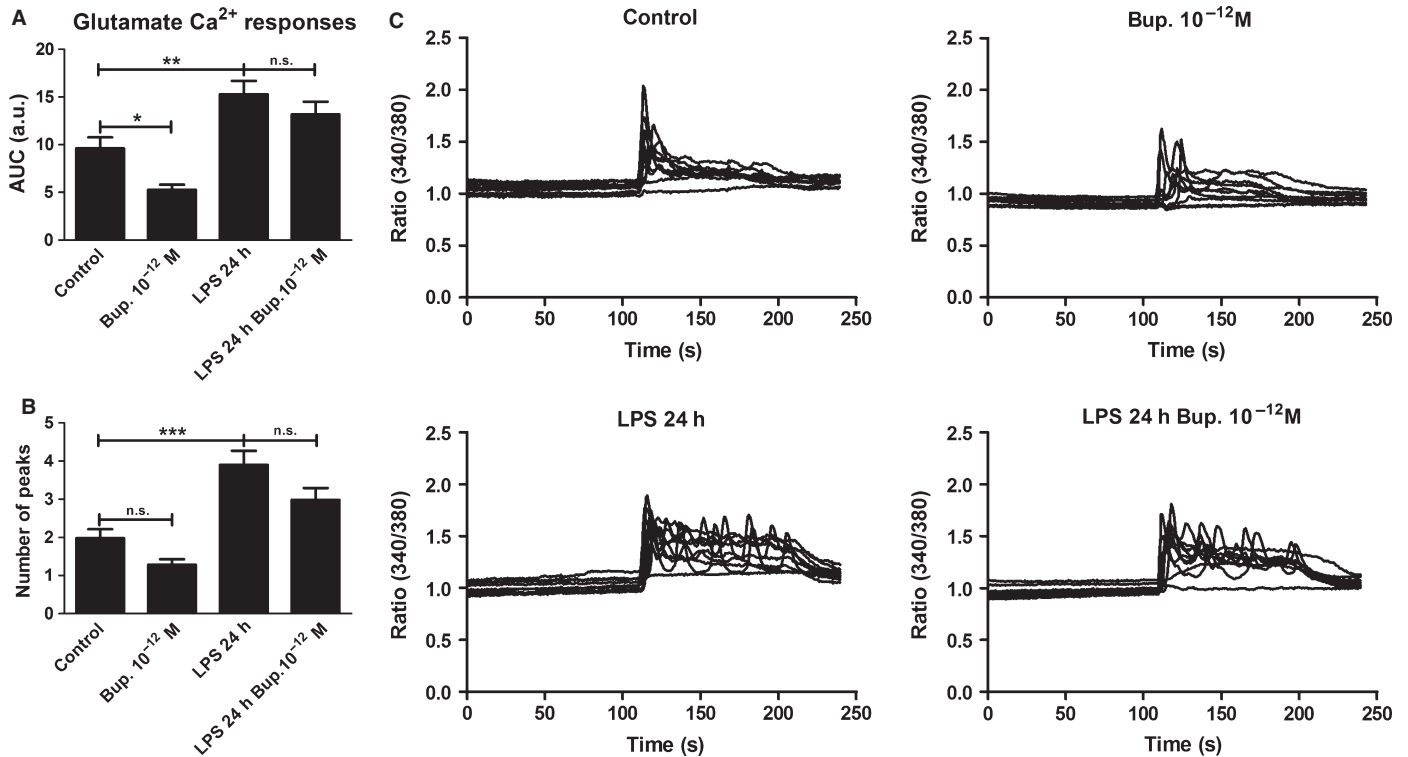


FIG. 8. Glutamate elicited Ca^{2+} responses in the astrocytes and was used as control. (A) Bupivacaine (10^{-12} M) attenuated the glutamate-induced Ca^{2+} signaling compared with the control. Cells incubated with LPS for 24 h showed increased glutamate-induced Ca^{2+} signaling. Bupivacaine did not attenuate the glutamate-induced Ca^{2+} transients that were upregulated by LPS. The areas under the Ca^{2+} peaks (AUC) were calculated. (B) The number of peaks was counted for each cell. (C) The Ca^{2+} transients were visualized. The results shown are from a typical experiment. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, n.s. = not significant. $n = 40$.

not restore the glutamate-induced Ca^{2+} transients that were upregulated by LPS treatment to the control level (Fig. 8); all cells responded to these treatments.

Cells treated with naloxone (10^{-12} M) and endomorphin-1 (10^{-6}) did not restore the glutamate-induced Ca^{2+} transients that were upregulated by LPS treatment, but a combination of bupivacaine, naloxone and endomorphin-1 did ($P < 0.001$); all cells responded to these treatments (Fig. 9). Furthermore, a combination of bupivacaine, naloxone and β -endorphin attenuated the glutamate-induced signaling that was upregulated by LPS ($P < 0.05$); again, all cells responded (Fig. 10).

Ultralow concentrations of bupivacaine reduce IL-1 β release

Astrocytes incubated with LPS for 24 h secreted IL-1 β . Other astrocyte samples were treated with bupivacaine (10^{-12} or 10^{-6} M) 30 min prior to incubation with LPS and bupivacaine for 24 h. In these cells, the ultralow concentration of bupivacaine (10^{-12} M) reduced the LPS-induced increase in IL-1 β secretion (Fig. 11).

Discussion

Bupivacaine is clinically used at concentrations that inhibit neuronal voltage-gated Na^+ channels, which results in the blockage of both nerve membrane excitation and action potential generation (Hildebrand *et al.*, 2001). We found that bupivacaine at lower concentrations, $< 10^{-8}$ M, evoked intracellular Ca^{2+} transients that were IP_3 receptor-dependent in astrocytes. The concentration-dependent curve for bupivacaine did not follow a Gaussian curve. For peptides a dose-response curve does not seem to exist in an ordinary way.

Endomorphin-1, nicotine and pituitary adenylate cyclase-activating peptide show similar patterns (Delbro *et al.*, 2009; Hansson *et al.*, 2009; Block *et al.*, 2012). For monoamines and amino acids, ordinary dose-response curves are obtained. Therefore, we did not expand this curve to lower concentrations especially as 10^{-12} M bupivacaine interacted significantly with astroglial endomorphin-, 5-HT- and glutamate-receptor systems. At higher concentrations, $> 10^{-8}$ M, bupivacaine blocked the Ca^{2+} release. The absence of a response at higher concentrations of bupivacaine may be due to the inhibition of Na^+ channels.

The complexity of Ca^{2+} signaling has made it difficult to determine the underlying physiology of this phenomenon; however, it probably reflects an important function of astrocytes (Hansson & Rönnebeck, 2003; Hansson, 2010; Forshammar *et al.*, 2011). In addition, we found that ultralow concentrations of bupivacaine decreased the LPS-induced increase in IL-1 β release, which is also upregulated under inflammatory conditions. It seems important that bupivacaine has the ability to prevent an increase of IL-1 β release under inflammatory conditions.

Given these results, we hypothesized that bupivacaine may have anti-inflammatory properties when used at ultralow concentrations. Under these conditions, bupivacaine could potentially restore the astrocyte network, which is influenced and reorganized by inflammatory stimuli (Hansson, 2006, 2010). The dysregulation of these inflammatory pathways can lead to pathogenic and chronic neuroinflammation and neurodegeneration, which can have deleterious effects on adjacent neurons (Farina *et al.*, 2007).

Previous studies have indicated that opioids, alone or in combination with the μ -opioid antagonist naloxone and either 5-HT or glutamate,

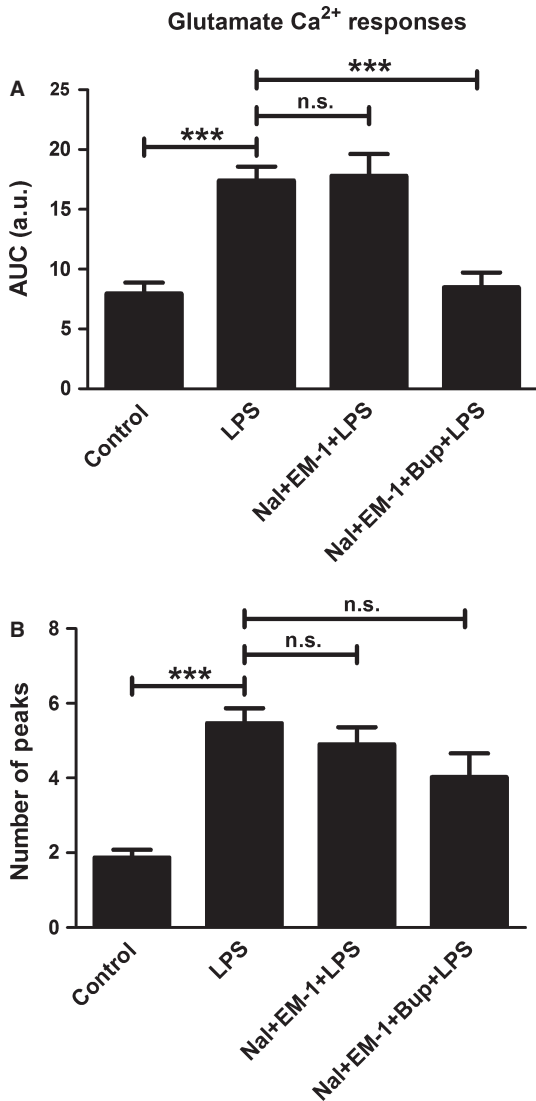


FIG. 9. Glutamate elicited Ca²⁺ responses in the astrocytes and was used as control. (A) Cells incubated with LPS for 24 h showed increased glutamate-induced Ca²⁺ signaling compared with the control. A combination of naloxone (Nal) (10⁻¹² M) and endomorphin-1 (EM-1) (10⁻⁶) did not attenuate the glutamate-induced Ca²⁺ transients that were upregulated by LPS. However, a combination of bupivacaine (Bup), Nal and EM-1 attenuated the LPS-upregulated glutamate-induced signaling. The areas under the Ca²⁺ peaks (AUC) were calculated. (B) The number of peaks was counted for each cell. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. ****P* < 0.001, n.s. = not significant. *n* = 40.

affect the astrocytes and the astrocyte network when these cells are inflammation-reactive (Hansson *et al.*, 2008; Forshammar *et al.*, 2011; Block *et al.*, 2012; Gérard & Hansson, 2012).

A neuroinflammatory state is often induced when LPS is used both *in vitro* and *in vivo*. LPS is an endotoxin and a potent inflammatory activator (Nakamura, 2002) that stimulates the TLR4 of astrocytes and microglia (Kielian, 2006). The changes associated with neuroinflammation include the downregulation of Na⁺ transporters, a change in Ca²⁺ signaling in the astrocyte network and a release of cytokines (Morita *et al.*, 2003; Schmidt *et al.*, 2007; Hansson *et al.*, 2008; Delbro *et al.*, 2009; Vallejo *et al.*, 2010).

In this study, the astrocytes were incubated with LPS, and a resulting increase in intracellular Ca²⁺ release was observed when

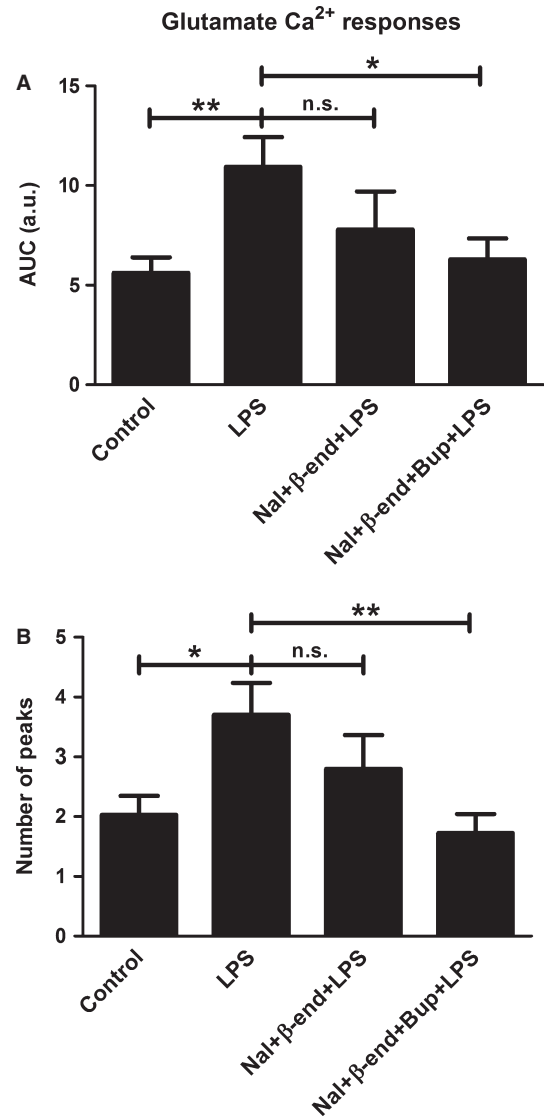


FIG. 10. Glutamate elicited Ca²⁺ responses in the astrocytes and was used as control. (A) Cells incubated with LPS for 24 h showed increased glutamate Ca²⁺ signaling compared with the control. A combination of naloxone (Nal) (10⁻¹² M) and β-endorphin (β-end) (10⁻⁶) did not attenuate the glutamate-induced Ca²⁺ transients that were upregulated by LPS. However, a combination of bupivacaine (Bup), Nal and β-end attenuated the LPS-upregulated glutamate-induced signaling. The areas under the Ca²⁺ peaks (AUC) were calculated. (B) The number of peaks was counted for each cell. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. ***P* < 0.01, **P* < 0.05, n.s. = not significant. *n* = 40.

the μ-opioid receptor agonist endomorphin-1 was used as a stimulator. Bupivacaine attenuated these Ca²⁺ oscillations and intracellular Ca²⁺ release. The mechanisms behind this interaction are currently unknown as it is unclear which cellular systems are affected by bupivacaine. Endomorphin-1 interacts with the G_{i/o} protein, which stimulates or activates the sodium transporter Na⁺/K⁺-ATPase (Masocha *et al.*, 2002). Bupivacaine may also interact with this system, and it is likely that bupivacaine acts at the receptor and/or ion level in the plasma membrane, given that these reactions occur within seconds. It has been reported that local anesthetics can modulate different subunits of G proteins. G protein-mediated release of IP₃-sensitive Ca²⁺ stores was mediated by the G_{αq} subunit in *Xenopus* oocytes

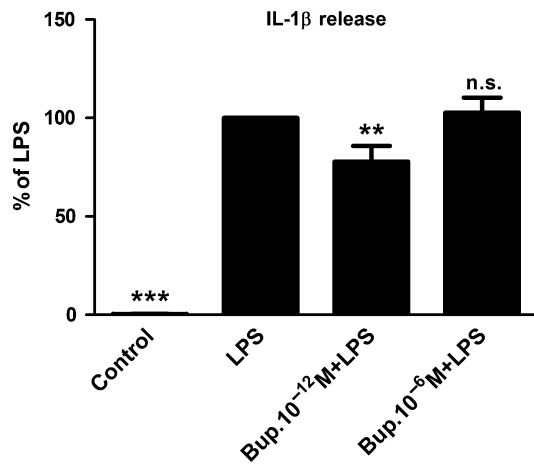


FIG. 11. Ultralow concentrations of bupivacaine reduced the amount of IL-1 β secreted. The astrocytes were treated with bupivacaine (10^{-12} or 10^{-6} M) 30 min prior to incubation with both LPS and bupivacaine for 24 h. The administration of bupivacaine at an ultralow concentration (10^{-12} M) reduced the LPS-induced IL-1 β secretion. Statistical analysis: the level of significance was analysed using one-way ANOVA followed by Dunnett's multiple-comparisons test. *** $P < 0.001$, ** $P < 0.01$, n.s. = not significant. $n = 6$.

(Hollmann *et al.*, 2001). $G_{\alpha i}$ -mediated inhibition of cAMP induced by activation of adenosine A1 receptors was expressed in CHO cells (Benkowitz *et al.*, 2003). Furthermore, interactions between opioid receptors and $G_{\alpha o}$ subunits have been reported as well as GTP hydrolysis by G_i and G_o proteins (Hageluken *et al.*, 1994), and decreases in opioid inhibition of Ca^{2+} channels (Komai & McDowell, 2007).

Astrocytes exhibit an increase in intracellular Ca^{2+} response to a variety of stimuli, including glutamate, γ -amino butyric acid (GABA) and 5-HT (Nilsson *et al.*, 1991; Hansson *et al.*, 1994; Hagberg *et al.*, 1998), which can result in the release of gliotransmitters, such as glutamate, D-serine and ATP (Parpura *et al.*, 1994; Araque *et al.*, 1998, 1999; Fiacco & McCarthy, 2004; Mothet *et al.*, 2005), that bind to pre- and/or postsynaptic neuronal receptors to modulate synaptic transmission and activity (Dani *et al.*, 1992; Nedergaard, 1994; Parri *et al.*, 2001).

In the current study, we investigated whether bupivacaine influenced 5-HT- and/or glutamate-induced intracellular Ca^{2+} responses. LPS treatment resulted in increased Ca^{2+} oscillations and intracellular Ca^{2+} release. This increased Ca^{2+} signaling activity was attenuated when the astrocytes were also treated with bupivacaine in the 5-HT system. Furthermore, a combination of bupivacaine, ultralow concentrations of naloxone, and either endomorphin-1 or β -endorphin attenuated the LPS-induced upregulation of Ca^{2+} signaling activity in the glutamate system.

Both 5-HT and glutamate stimulate metabotropic receptors, which stimulate the $G_{q/11}$ protein. As bupivacaine interacts with both 5-HT and glutamate, it is possible that bupivacaine interacts with these systems as well. It is also possible that bupivacaine interacts with G proteins that then interfere with the cAMP system and other G proteins that can affect the IP_3 system. Currently, there are no blocking agents available that can interact with or block these different systems. It would be very interesting to determine whether different G protein systems can be selectively blocked.

Based on these results, we conclude that low concentrations, $< 10^{-8}$ M, of bupivacaine evoked Ca^{2+} transients that are IP_3 receptor-dependent. These results suggest that bupivacaine interacts with the astrocyte Ca^{2+} signaling system. Bupivacaine also interacts with

the μ -opioid-, 5-HT- and glutamate-receptor systems in inflammation-reactive astrocytes, and has the ability to restore cellular parameters that are altered by inflammation to their normal, non-inflammatory states. In addition, bupivacaine attenuates the IL-1 β secretion that is upregulated in response to LPS. This result indicates that ultralow concentrations of bupivacaine exert anti-inflammatory astrocyte effects. Bupivacaine, when used at ultralow concentrations, might, through such mechanisms, be clinically useful as an anti-inflammatory drug, either alone or in combination with opioid agonists and an opioid antagonist at ultralow concentrations.

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Abbreviations

5-HT, 5-hydroxytryptamine; AUC, area under the curve; HHBSS, Hank's HEPES-buffered saline solution; IL-1, β interleukin-1 β ; IP_3 , inositol 1,4,5-trisphosphate; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4.

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