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Sigma Receptors Suppress Multiple Aspects of Microglial Activation

A. Hall Aaron, Herrera Yelenis, T. Ajmo Craig Jr, Cuevas Javier^{*}, and R. Pennypacker Keith
Department of Molecular Pharmacology and Physiology, University of South Florida College of Medicine, 12901 Bruce B. Downs Boulevard, MDC 9, Tampa, Florida, 33612

Abstract

During brain injury, microglia become activated and migrate to areas of degenerating neurons. These microglia release pro-inflammatory cytokines and reactive oxygen species causing additional neuronal death. Microglia express high levels of sigma receptors, however, the function of these receptors in microglia and how they may affect the activation of these cells remain poorly understood. Using primary rat microglial cultures, it was found that sigma receptor activation suppresses the ability of microglia to rearrange their actin cytoskeleton, migrate, and release cytokines in response to the activators adenosine triphosphate (ATP), monocyte chemoattractant protein 1 (MCP-1), and lipopolysaccharide (LPS). Next, the role of sigma receptors in the regulation of calcium signaling during microglial activation was explored. Calcium fluorometry experiments *in vitro* show that stimulation of sigma receptors suppressed both transient and sustained intracellular calcium elevations associated with the microglial response to these activators. Further experiments showed that sigma receptors suppress microglial activation by interfering with increases in intracellular calcium. In addition, sigma receptor activation also prevented membrane ruffling in a calcium-independent manner, indicating that sigma receptors regulate the function of microglia via multiple mechanisms.

Keywords

Nitric oxide; membrane ruffling; chemotaxis; neuroinflammation; stroke

INTRODUCTION

A principle aspect of the central nervous system (CNS) immune response to injury is the recruitment and activation of endogenous microglia. Microglia are the resident macrophages of the brain, which are kept in a quiescent ramified state under normal conditions. These cells respond to substances released by damaged neurons or invading pathogens by migrating to the site of injury, phagocytosing debris, and releasing pro-inflammatory mediators, such as cytokines and reactive oxygen species (Streit et al. 2004). In several pathological states of the CNS, this response contributes to the destruction of compromised neurons, enhancing neurodegeneration (Dheen et al. 2007; Rogove et al. 2002; Streit et al. 2004). Current research suggests that the inflammatory response after brain injury can be inhibited by sigma receptor activation (Ajmo et al. 2006). These receptors are neuroprotective in several animal models of stroke injury (Ajmo et al. 2006; Harukuni et al.

^{*}Corresponding Author: Dr. Javier Cuevas; Department of Molecular Pharmacology and Physiology, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., MDC-9, Tampa, FL 33612-4799, USA; Voice: (813) 974-4678; Fax: (813) 974-3079; E-mail: jcuevas@health.usf.edu.

1998), and represent a putative target for neuroprotection at delayed time points (≤ 24 hrs) following stroke onset (Ajmo et al. 2006).

Sigma receptors are membrane associated proteins found widely distributed in the mammalian brain, peripheral neurons, and visceral organs. Two subtypes of sigma receptors, sigma-1 and sigma-2, have been identified based on their pharmacological profiles (Quirion et al. 1992). Thus far, only the sigma-1 receptor has been cloned (Seth et al. 1998), and this protein is detected on the membranes of the endoplasmic reticulum and influences calcium release from this organelle (Hayashi and Su 2001).

Sigma receptors were identified in the immune system using radioligand binding studies in lymphoid tissues (Su et al. 1990). Selective sigma-1 receptor activation is associated with reduced leukocyte invasiveness and decreased levels of inflammatory cytokines *in vivo* (Zhu et al. 2003). Sigma ligands such as (+) pentazocine, haloperidol, and 1,3-di-o-tolylguanidine (DTG) were found to suppress murine splenocyte activity and polyclonal immunoglobulin production following mitogen stimulation *in vitro* (Carr et al. 1991). Systemic administration of the sigma ligand, SR 31747A, inhibits the secretion of tumor necrosis factor- α (TNF- α) and interferon gamma (IFN- γ) evoked by injection of LPS in rats (Bourrie et al. 2002). This sigma ligand has also been shown to inhibit nitric oxide (NO) production in LPS-activated macrophages (Gannon et al. 2001). In the CNS, sigma-1 receptors are expressed in microglia (Gekker et al. 2006), and our laboratory has shown that sigma receptor activation is associated with decreased reactive gliosis following stroke injury in rats (Ajmo et al. 2006).

This study investigated sigma receptor modulation of microglial activation. Specifically, experiments were done to characterize the effects of sigma receptor activation on morphological, migratory, and inflammatory responses of microglia and to determine the role of intracellular calcium concentration on the regulation of these processes. It was found that sigma receptor activation suppressed the morphological, migratory, and inflammatory aspects of microglial activation. Further studies revealed that sigma receptor activation regulates these processes by suppressing calcium increases. However, calcium-independent regulation of microglia activation by sigma receptors was also noted, indicating that sigma receptors regulate these cells by affecting various signaling pathways.

MATERIALS AND METHODS

Primary Cultures of Microglia

Primary cultures of microglia were prepared from postnatal (2 day) Sprague-Dawley rat pups using a protocol modified from Gottschall *et al* (Gottschall et al. 1995). Briefly, pups were decapitated, cortices dissected out and dissociated in Hank's balanced salt solution containing 0.25% Trypsin and 2.21 mM ethylenediaminetetraacetic acid (EDTA) (Mediatech, Manassas, VA). Isolated cortical cells were plated into poly-D-lysine (Sigma-Aldrich, St. Louis, MO) treated tissue culture flasks. The mixed glial cultures for the preparation of microglia were maintained in high glucose Dulbecco's Modified Eagle Media (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% horse serum, 2.5% fetal bovine serum, and an antibiotic/antimycotic cocktail containing 100 I.U. penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B. The mixed glial cultures were incubated for 8–10 days at 37 °C. Microglia were then dislodged by vigorous shaking, plated, and used for experiments the following day.

Membrane Ruffling Detection and Quantification

Microglia plated on poly-D-lysine treated glass coverslips were serum starved for four hours in DMEM then stimulated for 5 or 10 minutes with ATP (Sigma-Aldrich, St. Louis, MO) or

10 min with MCP-1 (Peprotech Inc., Norwood, MA). Compounds to be tested were incubated with the microglia in DMEM for 10 minutes prior to chemoattractant exposure. In one series of experiments Ca^{2+} -free DMEM (Invitrogen) was used. Cytoskeletal changes were visualized using phalloidin conjugated to AlexaFluor 488 (Invitrogen).

Multiple photomicrographs ($n \geq 4$) of fields containing 1–8 cells were acquired for controls (DMEM) and each test group. Morphology of the cells was evaluated by an investigator blind to the nature of the treatment and a score was given to each cell using the following criteria: “0” was given to cells which did not display any signs of membrane ruffling and had multiple apparent filopodia; “1” was given to cells which retained filopodia and lamellipodia, but which also had regions on the cell membrane where ruffling was present; and “2” was given to cells which had withdrawn all their filopodia and displayed a fully ruffled phenotype. The scores for each group were then summed and divided by the number of cell assayed in that group to yield that group’s degree of membrane ruffling in arbitrary units (A.U.). Means for each group were compared and significant differences were determined via two-way ANOVA with post-hoc Bonferroni tests.

Migration Assay

Modified Boyden chambers were used as previously described (McCord et al. 2005). Briefly, 5×10^5 freshly isolated microglia were applied to the top portion of a Boyden chamber assembled with an 8 μm pore polycarbonate membrane. Media (DMEM) containing vehicle or chemoattractant (ATP or MCP-1) with or without compounds to be tested was added to the bottom of the chamber, and the cells incubated for four hours at 37°C . The membranes were removed and microglia adhering to the top of the membrane were scraped off. The membrane was oriented on a slide such that the cells that migrated to the bottom of the membrane were facing up. Vectashield Hardset mounting media (Vector Labs, Burlingame Ca) containing 4'-6-Diamidino-2-phenylindole (DAPI) was applied to the membrane and a coverslip affixed to the slide. DAPI positive cells were illuminated at 359 nm and visualized at 461 nm using a Zeiss Axioskop 2 outfitted with a 20X objective. A minimum of 5 random fields of cells were counted and averaged per membrane, and the results of at least three experiments were averaged together.

ELISA assay

Plates (96 well) were coated with 2 ng/ml capture antibody (tumor necrosis factor- α , MAB510 anti-rat antibody; interleukin-10, MAB519 anti-rat antibody; R&D Systems, Inc., Minneapolis, MN) overnight at 4°C . The plates were washed with Tris buffered saline (TBST) consisting of: 20 mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween 20. The plates were then blocked with 1% bovine serum albumin (BSA) in TBST for 1 hour. A standard curve was prepared using recombinant protein standards. The standards as well as the unknowns were incubated for 1 hr at room temperature and then washed with TBST. Biotinylated detection antibody (tumor necrosis factor- α , #BAF510 biotinylated anti-rat antibody; interleukin-10, #BAF519 biotinylated anti-rat IL10 antibody; R&D Systems, Inc., Minneapolis, MN) was added to each well at 100 ng/ml and incubated for 2 hrs at room temperature. After incubation, the plates were washed three times with phosphate buffered saline (PBS) containing (in mM): 3.2 Na_2HPO_4 , 0.5 KH_2PO_4 , 1.3 KCl, 135 NaCl (pH 7.4). Streptavidin conjugated to horseradish peroxidase (streptavidin-HRP) was added to each well followed by 20 min incubation at room temperature. After washing, 100 μl of 3,3',5,5'-tetramethyl-benzidine (TMB) (Sigma-Aldrich, St. Louis, MO) was added and incubated for 5 minutes in the dark. Finally, 50 μl of 1 M H_2SO_4 stop solution was added to each well and optical density was measured using a microplate reader set at 450 nm.

Griess reaction

Supernatants from treated primary rat microglia cultures were collected, and clarified by centrifugation. Nitric oxide levels in the supernatant were determined using the Griess Reagent Kit (Invitrogen) according to the manufacturer's protocol.

Calcium Imaging

Intracellular calcium ($[Ca^{2+}]_i$) was measured using the ratiometric Ca^{2+} sensitive dye, fura-2 as previously described (Katnik et al. 2006). Microglia, plated on coverslips, were incubated for 1 hour at 37°C in DMEM (Mediatech, Manassas, VA) containing 5 μ M fura-2, acetoxymethylester (fura-2 AM; Invitrogen) and 0.1 % dimethyl sulfoxide (DMSO). The coverslips were washed in physiological saline solution (PSS) consisting of (in mM): 140 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 glucose, and 25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH adjusted to 7.4 with NaOH) prior to the experiments being carried out. A DG-4 high speed wavelength switcher (Sutter Instruments Co., Novato, CA) was used to apply excitation light of alternating wavelength (340/380nm). Fluorescent emission was captured using a Sensicam digital CCD camera (Cooke Corporation, Auburn Hills, MI) and recorded with Slidebook 3.0 software (Intelligent Imaging Innovations, Denver, CO). Changes in $[Ca^{2+}]_i$ were calculated using the Slidebook 3 software.

DAF Imaging

Nitric oxide concentration was measured using the NO sensitive dye, 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM). Microglia plated on coverslips were incubated for 1 hour at 37°C in DMEM (Mediatech) containing 5 μ M DAF-FM (Invitrogen) and 0.1 % DMSO. The coverslips were washed in PSS prior to NO measurements. DAF-FM loaded cells were illuminated at 488 nm and visualized at 510 nm, using the same instrumentation described for calcium imaging experiments. Background fluorescence was determined by measuring fluorescent intensities in areas between cells and was subtracted from the raw intensity values. Fluorescent intensity was expressed as arbitrary units.

Data Analysis

Analysis of measured intracellular $[Ca^{2+}]$ responses was conducted using Clampfit 9 (Molecular Devices, Union City, CA). Statistical analysis was conducted using SigmaPlot 9 and SigmaStat 3 software (Systat Software, Inc., San Jose, CA). Two-way ANOVA procedures with appropriate post-hoc tests were used to analyze data from the calcium imaging and migration experiments, while a three-way ANOVA procedure with post-hoc Tukey test was used to analyze data from the NO imaging experiments.

Reagents

The sigma ligands DTG and metaphit were obtained from Tocris Bioscience (Ellisville, MO). The microglial activator MCP-1 was purchased from Peprotech Inc., (Norwood, MA), ATP and LPS from Sigma-Aldrich (St. Louis, MO), thapsigargin from Alomone Labs (Jerusalem, Israel), and ionomycin from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma-Aldrich and were of analytical grade or higher.

RESULTS

Sigma receptor activation suppresses changes in microglia morphology in response to chemoattractant stimulation

ATP released from degenerating neurons activates microglia during brain injury (Davalos et al. 2005). ATP binds purinergic receptors on microglia and causes these cells to change

shape and migrate to the site of injury (Honda et al. 2001). To study these phenomena, microglia were treated with ATP and labeled using phalloidin, which binds to the actin cytoskeleton. Unstimulated microglia exhibited discrete, actin rich filopodia (Figure 1A and 1D). Upon stimulation with ATP (100 μ M), for either 5 min (Figure 1B) or 10 min (Figure 1E) microglia retracted their filopodia and aggregated actin along the leading edge of the cell membrane in a manner consistent with membrane ruffling. Administration of the sigma receptor agonist DTG (100 μ M) 10 min prior to and during ATP exposure decreased the aggregation of actin in the leading edge and promoted the retention of filopodia (Figure 1C and 1F). DTG treatment alone resulted in cells which were morphologically indistinguishable from control (data not shown). Since ATP-induced retraction of filopodia is an extracellular Ca^{2+} -independent process, experiments were also carried out in nominal extracellular Ca^{2+} . In the absence of extracellular Ca^{2+} , microglia exhibited normal morphology, and filopodia were readily observed (Figure 1G). Upon application of ATP (5 min), filopodia were retracted and membrane ruffling occurred (Figure 1H), and this membrane ruffling was still inhibited by the addition of DTG (Figure 1I).

Sigma receptor activation significantly decreases membrane ruffling induced by ATP

The changes in membrane structure were quantified to determine if activation of sigma receptors altered the morphological response to ATP. ATP treatment significantly increased the degree of membrane ruffling relative to the DMEM control (Figure 2). Furthermore, while in the control group no cells were found to show complete membrane ruffling ($n = 63$), 62% of cells in the ATP treatment group showed this phenotype ($n = 53$). DTG treatment alone had no effect on membrane morphology, with 11 of the 12 cells examined showing no evidence of membrane ruffling. However DTG pretreatment reduced the response to ATP, as evident by the significant reduction in the degree of membrane ruffling induced by the purine (Figure 2). The percentage of cells which exhibited membrane ruffling decreased to 11% when DTG was co-applied ($n = 44$).

The microglial migratory response to chemoattractant application is suppressed by sigma receptor activation

A modified Boyden chamber was used to measure the chemotaxis of freshly isolated microglia in response to ATP, with and without DTG present. ATP (100 μ M) promoted a tenfold increase in the number of microglia that migrated across the membrane relative to control (Figure 3A). This increase in migration was statistically significant ($p < 0.001$) and similar to that previously reported (Honda et al. 2001). DTG (300 μ M) alone had no effect on microglial migration ($p = 0.47$), but co-application of DTG with ATP significantly ($p < 0.0001$, $n = 3$) depressed the ATP-evoked migration of microglia (Figure 3A) by $72.68 \pm 3.55\%$ relative to control (ATP and no DTG). In the presence of DTG, ATP failed to evoke a statistically significant increase in migration above that observed when DMEM was applied ($p = 0.076$).

The chemokine MCP-1, which binds the CCR2 receptor (Ogilvie et al. 2004), was also tested to determine whether sigma receptor mediated inhibition of migration was limited to the effects elicited by purinergic receptors. Application of MCP-1 (10 nM) caused a five-fold increase in the number of microglia that migrated through the membrane compared to control (Figure 3B). This increase in migration was less than that observed with ATP, but consistent with the 3- to 4-fold increase in macrophage and microglial migration reported for this concentration of MCP-1 (Dzenko et al. 2001; Peterson et al. 1997). Co-treatment with 300 μ M DTG significantly depressed MCP-1-induced microglial migration relative to the control group ($p < 0.001$, $n=4$). Furthermore, there was no statistical significance between MCP-1 and DMEM-induced migration in the presence of DTG ($p = 0.56$, Figure 3B).

The microglial inflammatory response is suppressed by sigma receptor activation

To ascertain whether sigma receptors modulated the pro-inflammatory response of microglia, cytokine, and NO production evoked by LPS were examined after pretreatment with DTG. Lipopolysaccharide activates microglia and causes robust increases in cytokine production (Hoffmann et al. 2003). Microglial cultures were preincubated with varying concentrations of DTG (50–1000 μ M) for 30 minutes and the cells were then activated with LPS (1 μ g/ml). TNF- α and IL-10 levels in the supernatant were measured using ELISA and NO levels were quantified with the Greiss reaction. DTG suppressed both cytokine and NO production in a concentration dependent manner (Figure 4). Fits of the data determined IC₅₀ values of 338.9, 109.6, and 166.0 μ M for DTG inhibition of TNF α , IL10, and NO release, respectively (n = 4 for all groups tested).

Transient intracellular calcium signaling is suppressed by sigma receptor activation

Changes in intracellular calcium are a critical component of the signaling cascade triggering the immune response of microglia. Given that sigma receptors regulate intracellular calcium concentrations ([Ca²⁺]_i) in neurons (Katnik et al. 2006), it seemed prudent to examine the effects of sigma receptor activation on changes in [Ca²⁺]_i following microglia stimulation. Focal application of 300 μ M ATP (10 sec) onto microglia produced intracellular calcium increases of 140.4 $\pm$ 16.2 nM in ~90% of cells tested (Figure 5A). Following pretreatment (10 min) with 100 μ M DTG, the ATP-elicited increases in [Ca²⁺]_i were reduced by 82 $\pm$ 16.4% (n=39), which was statistically significant (p < 0.001, Figure 5C). To confirm that DTG was acting via sigma receptors, cells were preincubated with the irreversible sigma receptor antagonist metaphit. Pretreatment with metaphit (50 μ M, 1 hr, room temperature) abolished the inhibitory effects of DTG on ATP-induced [Ca²⁺]_i increases (Figure 5B), such that no statistically significant difference (p = 0.52) existed between the ATP-induced [Ca²⁺]_i responses observed in the absence (ATP + Met) and presence of DTG (ATP + DTG + Met) (Figure 5C).

Basal intracellular calcium increases are suppressed by sigma receptor activation

To determine whether DTG could inhibit the sustained increase in [Ca²⁺]_i associated with the LPS-induced inflammatory response (Boddeke et al. 1999; Hoffmann et al. 2003), microglia were treated with LPS for 24 hours and then the basal intracellular calcium was measured. Treatment with LPS evoked a 46.1 $\pm$ 11.0% increase in [Ca²⁺]_i relative to control cells and this increase was statistically significant (p < 0.05, n = 31 Figure 6). In contrast, in cells pretreated with 300 μ M DTG, LPS failed to evoke increases in [Ca²⁺]_i (Figure 6). This inhibition of basal [Ca²⁺]_i by DTG was statistically different from LPS treatment alone (p < 0.05, n = 31). These results are consistent with the hypothesis that sigma receptors inhibit the inflammatory response elicited by LPS via the inhibition of LPS-induced increases in intracellular calcium.

Increases in intracellular calcium are sufficient to induce membrane ruffling in microglia

The calcium ionophore ionomycin was used to increase intracellular calcium in microglia via a pathway distinct from the sigma receptor-sensitive influx pathways activated in response to ATP, MCP-1 and LPS. Ionomycin treatment alone altered the morphology of microglia by promoting retraction of filopodia and inducing membrane ruffling when compared to control (Figure 7A and 7B). Unlike the results obtained when ATP was used to stimulate actin rearrangement, DTG was unable to inhibit ionomycin-induced membrane ruffling (Figure 7C). Quantification of the results obtained with ionomycin and DTG treatment is summarized in Figure 7D. Ionomycin evoked a statistically significant increase in the degree of membrane ruffling both in the absence and presence of DTG preincubation, and no significant interaction was noted between sigma receptor stimulation and the

response to the ionophore. These data show that increasing $[Ca^{2+}]_i$ is sufficient to promote such changes in microglial morphology. Moreover, the inability of DTG to decrease these ionomycin-evoked changes to the membrane suggest that sigma receptors are acting at the level of $[Ca^{2+}]_i$ and not at downstream targets of this molecule.

Microglial migration is a calcium dependent process which is not restored by ionomycin treatment

We next investigated whether ionomycin could overcome the blockade of microglial chemotaxis observed following sigma receptor activation. The membrane ruffling evoked by ionomycin was not associated with an increase in chemotaxis, and the number of migrating cells observed in response to ionomycin treatment was similar to that observed with vehicle alone (Figure 8A). Chemotaxis was observed as before in response to ATP, and it was significantly inhibited by co-administration of DTG. However, microglial migration in response to ATP was disrupted by ionomycin treatment alone (Figure 8A). In contrast to the observed effects on the microglial morphological response, the addition of ionomycin failed to promote microglial chemotaxis in the presence of ATP and DTG, instead significantly further decreasing migration relative to that observed in the ATP+DTG group.

Microglial migration is independent of calcium release from internal stores but requires calcium influx

To determine the source of increased $[Ca^{2+}]_i$ necessary for microglial migration, calcium release from intracellular stores and influx from the extracellular media were selectively blocked prior to ATP-stimulation. The sarcoplasmic-endoplasmic calcium ATPase inhibitor thapsigargin was used to deplete calcium from the ER by blocking reuptake. Pretreatment with 10 μ M thapsigargin alone, failed to suppress the migratory response to ATP (Figure 8B). In thapsigargin treated samples, 100 μ M ATP stimulated cell migration averaged 747.0 ± 190 cells per field compared to 683 ± 187 cells per field in control samples ($p = 0.67$, $n = 4$). The pan-selective calcium channel blocker La^{3+} was employed to block calcium channel mediated influx through the plasma membrane. Lanthanum alone did not promote a significant change in migration when compared to control ($p = 0.4897$, $n = 5$). However, co-application of 50 μ M La^{3+} with 100 μ M ATP significantly suppressed microglial migration from 2069 ± 121 cells per field (ATP alone) to 1636 ± 124 cells per field (ATP + La^{3+}) (Figure 8C, $p = 0.022$, $n = 5$). Figure 8D summarizes these results, showing that under control conditions ATP induces a 320 ± 67 % increase in cell migration which is reduced to 183 ± 32 % in the presence of 50 μ M La^{3+} . Thus, inhibition of Ca^{2+} influx to the cell, such as that produced by sigma receptor activation, can prevent microglia chemotaxis.

Calcium influx restores nitric oxide production following sigma receptor activation in LPS stimulated microglia cells

Increases in basal $[Ca^{2+}]_i$ are integral to NO production in mouse microglia (Hoffmann et al. 2003). Experiments were conducted to determine if ionomycin facilitated Ca^{2+} influx would overcome the inhibitory effects of DTG on LPS-induced NO production. Primary microglia were preincubated (30 min) with vehicle (0.1% DMSO in DMEM), DTG (300 μ M), ionomycin (1 μ M), or ionomycin + DTG in the presence and absence of LPS (1 μ g/ml) for 24 hours. Nitric oxide levels were measured using the intracellular NO indicator DAF-FM (Figure 9). LPS stimulation alone increased NO levels $138.7 \pm 10.87\%$ compared to vehicle ($p < 0.001$, $n = 455$). Co-application of DTG with LPS resulted in a statistically significant suppression of NO levels ($p < 0.001$, $n = 461$), which were $22.2 \pm 6.36\%$ higher than that of the DTG control group. Application of LPS in the presence of ionomycin produced an increase in NO similar to that observed when LPS was administered alone, such that NO levels were $129.9 \pm 10.36\%$ greater than that of the ionomycin control group ($p < 0.001$, $n = 446$). In contrast to the results observed for ATP-induced migration, addition of ionomycin

to the LPS + DTG group was able to overcome the inhibitory effects of sigma receptor activation. When ionomycin was co-applied with LPS and DTG (LPS+DTG+Iono) intracellular NO levels were increased by $86.1 \pm 8.43\%$ from DTG+Iono control ($p < 0.001$, $n = 418$). These values were significantly greater than those observed for the LPS + DTG group ($p < 0.001$, $n = 418$), and similar to those elicited by the LPS + Iono group ($p = 0.562$, $n = 446$).

DISCUSSION

The salient finding of this study is that sigma receptor activation can suppress multiple aspects of microglial activation, including the morphological, migratory and inflammatory responses to several known microglial activators. The results of this study provide insight into the role of sigma receptors in the regulation of immune system function. Sigma receptor activation can inhibit membrane ruffling and migration of microglia, both of which are novel findings to the best of our knowledge. As these processes are critical to the microglial response to injury, these findings reveals a novel aspect of the modulation of microglial activation by sigma receptors.

Resting microglia exhibit a ramified morphology *in vivo* characterized by fine processes expressing chemosensitive receptors (Glenn et al. 1992). Studies using GFP-labeled microglia *in vivo* demonstrated that these processes are highly motile and quickly move towards ATP released by injured neurons (Davalos et al. 2005). Upon further activation *in vivo*, these processes are withdrawn and the microglia take on an amoeboid phenotype which is associated with an inflammatory state (Kreutzberg 1996; Lyons et al. 2000). *In vitro*, membrane ruffling describes the cytoskeletal re-arrangements critical for the movements of microglial filopodia. Our results demonstrate that sigma receptor activation blocks the formation of membrane ruffles in response to ATP application. Metabotropic P2Y₁₂ receptors, which evoke increases in $[Ca^{2+}]_i$ in microglia (Ohsawa et al. 2007), are critical to the induction of membrane ruffling by ATP exposure (Honda et al. 2001). ATP also elicits a transient increase in intracellular calcium via the activation of ionotropic P2X₄ receptors expressed in microglia. Intracellular calcium is an important co-factor in the process of membrane ruffling, since either depression of intracellular Ca^{2+} with BAPTA or deletion of the Ca^{2+} -binding domain of Iba1, which is involved in ATP-induced membrane ruffling in microglia (Kanazawa et al. 2002; Ohsawa et al. 2000), inhibits agonist-elicited actin rearrangement in these cells (Ohsawa et al., 2000). Elevations in intracellular calcium have also been implicated in macrophage-colony stimulating factor (M-CSF)-evoked membrane ruffling in microglia (Ohsawa et al., 2000). Our studies show that increasing $[Ca^{2+}]_i$ in microglia with ionomycin treatment is sufficient to trigger membrane ruffling. Similarly, in rat microglia/astrocyte co-cultures, the calcium ionophore calcimycine/A21837 evoked a loss of ramifications in microglia (Kalla et al. 2003). Sigma receptor activation suppressed both the ATP mediated calcium transient and membrane ruffling which suggests that the calcium increase is linked to membrane ruffling. Sigma receptor activation was unable to inhibit the ionomycin-induced membrane ruffling, indicating that sigma receptors are acting upstream of the calcium elevation in this pathway.

A second pathway which is $[Ca^{2+}]_i$ -independent is able to induce membrane ruffling in microglia. Previous studies have shown that purinergic receptor mediated increases in intracellular calcium are necessary for migration but not membrane ruffling in these cells (Honda et al. 2001; Ohsawa et al. 2007). Sigma receptor activation depressed membrane ruffling in the absence of extracellular Ca^{2+} and in conditions under which the Ca^{2+} -independent membrane ruffling induced by ATP is observed (Honda et al. 2001). Thus, sigma receptor activation is able to disrupt membrane ruffling both by preventing $[Ca^{2+}]_i$

increases which may stimulate membrane ruffling and by blocking the Ca^{2+} -independent pathway.

While both membrane ruffling and migration in response to ATP are dependent on P2Y₁₂ receptor activation and intracellular calcium signaling, migration must also incorporate chemoattractant sensing and directionality. In our migration studies we saw robust migration in response to ATP and MCP-1, both of which induce migration through a pertussis toxin sensitive Gi/o-protein (Dzenko et al. 2001; Honda et al. 2001; Sozzani et al. 1994). Migration induced by both chemoattractants was inhibited by sigma receptor activation, suggesting that sigma receptors inhibit migration either by direct interaction with the Gi/o-protein or by interfering with downstream calcium signaling. Interestingly, unlike membrane ruffling, increasing intracellular calcium levels with ionomycin failed to restore microglial migration in the presence of sigma receptors. In fact, inclusion of ionomycin effectively suppressed ATP induced migration. This effect may be due to ionomycin-induced decreases in purinergic receptor signaling, which has been previously reported (Hoffmann et al. 2003). Alternatively, the homogeneous elevations in $[\text{Ca}^{2+}]_i$ produced by ionomycin treatment may saturate and disrupt any directional effects produced by the purinergic signaling. Similarly, the Ca^{2+} ionophore, A23187, disrupts microglial migration in response to complement 5a (Nolte et al. 1996). In contrast, depletion of intracellular calcium stores with thapsigargin had no effect on migration, whereas the calcium channel inhibitor lanthanum partially suppressed microglial migration in response to ATP. This latter finding is consistent with ionotropic P2X₄ receptor signaling being essential to ATP induced microglial activation, which has been reported (Ohsawa et al. 2007). Given that sigma receptor activation decreased the calcium signaling known to be essential for microglial migration, this is the likely mechanism by which sigma receptor activation suppresses movement of the cells.

Another crucial component of microglial function is the induction of the inflammatory response. Activation of sigma receptors in the peripheral immune system potently suppresses the inflammatory response to a variety of stimuli (Bourrie et al. 2002). It was proposed that these anti-inflammatory effects were mediated by sigma receptor-induced interleukin-10 (IL10) production (Zhu et al. 2003). However, studies on sigma receptor-mediated immunosuppression in RAW 264.7 macrophages suggested that IL10 is not always involved in these responses (Gannon et al. 2001). This latter observation is consistent with our finding that IL10 production in response to LPS treatment was decreased with similar kinetics to reductions of TNF- α and NO production. This observation implies that sigma receptor activation affects signaling upstream of protein synthesis-dependent processes in the inflammatory response.

Sustained increases in basal intracellular calcium levels are integral to the induction of the inflammatory response (Hoffmann et al. 2003). Sigma receptor activation reduces the capacity of microglia to generate this sustained increase in calcium in response to LPS. This is associated with a decreased release of cytokines and NO. Reintroduction of calcium with ionomycin overcame the sigma receptor activation induced blockade of NO production.

Our results showing that activation of sigma receptors suppresses ATP-induced $[\text{Ca}^{2+}]_i$ in microglia suggest that sigma receptors are inhibiting P2X receptors in these cells, since these channels account for most of the increase in $[\text{Ca}^{2+}]_i$ observed in our experiments (data not shown). Previous studies, including works from our laboratory, have shown that sigma receptors couple to both voltage-gated and ligand-gated ion channels (Aydar et al. 2002; Herrera 2007; Zhang and Cuevas 2002; Zhang and Cuevas 2005). Thus, this observation is consistent with the hypothesis that sigma receptors couple to a diverse group of ion channels. While the regulation of these plasma membrane ion channels will affect $[\text{Ca}^{2+}]_i$, sigma receptors also affect other proteins which influence $[\text{Ca}^{2+}]_i$, including the IP₃ receptor

of the endoplasmic reticulum and the BiP chaperone found in the mitochondrion-associated ER membrane (Hayashi and Su 2001; Hayashi and Su 2007). However, the fact that sigma receptors affect membrane ruffling in the absence of Ca^{2+} signaling indicates that the influence of sigma receptors on microglial function is not limited to the regulation of Ca^{2+} signaling alone. Thus, the mechanisms by which sigma receptors regulate the activity of microglia remain to be fully elucidated.

Activated microglia have been associated with neurodegenerative disease progression (Streit et al. 2004), and the presence of activated microglia is linked to increased neuronal damage. In contrast, ablation of microglia is also associated with increased damage (Lalancette-Hebert et al. 2007), which shows that microglia play a complex part in the etiology of neurodegenerative disease. Our findings support and expand upon the theory that sigma receptors have potent immunoregulatory properties (Bourrie et al. 1995; Bourrie et al. 2002). Prior to our work, the relationship between sigma receptors and microglia had only been studied in an in vitro HIV-1 infection model, where it was shown that sigma receptor activation increases HIV-1 expression (Gekker et al. 2006). Our studies shed light on the mechanism by which sigma receptors modulate the function of microglia in response to brain injury. Accordingly, sigma receptors are excellent pharmacological targets to suppress neurodegeneration evoked by activated microglia responding to a neuropathological state.

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Figure 1. Sigma receptor activation suppresses changes in microglia morphology in response to chemoattractant stimulation

Photomicrographs of primary rat microglia cultures exposed to vehicle (0.1% DMSO in DMEM) (A, D, G), 100 μ M ATP for 5 min (B, H) and 10 min (E), or 100 μ M ATP for 5 min (C, I) and 10 min (F) in the presence of 100 μ M DTG. Microglia in A–F were treated in normal DMEM, whereas microglia in G–I were treated in Ca^{2+} -free DMEM. All insets represent magnified image (2.7x). Scale bar = 2 μ m.

Figure 2. Sigma receptor activation significantly decreases membrane ruffling in microglia stimulated with ATP

Degree of membrane ruffling assessed as described in the methods section. Microglia were preincubated with vehicle, or DTG (100 μ M DTG) for 5 minutes then stimulated with DMEM or ATP (100 μ M). Bars represent means $\pm$ SEM. Asterisks indicate significant differences between DMEM and ATP within Control (no DTG, $p < 0.001$) and DTG groups ($p < 0.001$). Pound symbol indicates significant difference between Control and DTG within the ATP group ($p < 0.001$). Significance was determined via two-way ANOVA with post-hoc Tukey Test.

Figure 3. The microglial migratory response to chemoattractant application is suppressed by sigma receptor activation

Microglial migration was assayed using a Boyden chamber fitted with a polycarbonate membrane containing 8 μ m pores. Microglia (500,000 cells) were placed in the upper chamber and control media, 100 μ M ATP (A) or 10 nM MCP-1 (B) were added in the absence and presence of 300 μ M DTG to the bottom chamber. Microglia were allowed to migrate for 4 hrs at 37 $^{\circ}$ C, then stained with DAPI and counted. Asterisks indicate a significant difference between ATP (A; $p < 0.001$, $n = 6$) and MCP-1 (B; $p < 0.01$, $n = 4$) from DMEM within the Control group (no DTG). Pound symbols indicate significant difference between Control and DTG within the ATP (A; $p < 0.001$) and MCP-1 (B; $p < 0.01$) groups, respectively. In both cases significance was determined by two-way ANOVA followed by post-hoc Bonferroni tests.

Figure 4. The microglial inflammatory response is suppressed by sigma receptor activation

Primary microglial cultures were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 hrs and TNF α , IL10, and NO levels in the supernatant were measured by ELISA and the Greiss reaction, respectively. Data points represent mean $\pm$ SEM for TNF α , IL10, and NO levels as a function of DTG concentration ($n=4$ for all groups). Lines indicate best fits to the data using a Langmuir-Hill equation and yielded IC_{50} values of 338.9, 109.6, and 166.0 μM with Hill coefficients of 1.90, 1.94, and 1.4 for TNF α , IL10, and NO release, respectively.

Figure 5. Transient intracellular calcium signaling evoked by ATP was suppressed by sigma receptor activation

Representative traces of $[Ca^{2+}]_i$ as a function of time recorded during ATP (300 μ M) stimulation in the absence (Control) and presence of DTG (100 μ M), without (A) or with (B) Metaphit (50 μ M, 1 hr, room temperature). C, Mean change in peak $[Ca^{2+}]_i$ ($\pm$ SEM) measured in the above treatment groups. Asterisk denotes significant difference ($p < 0.05$, $n = 25$), as measured by one-way ANOVA with post-hoc Dunn's test using ATP as control.

Figure 6. Basal intracellular calcium increases are suppressed by sigma receptor activation Microglia, plated on poly-D-Lysine coverslips, were stimulated for 24 hours with LPS (1 μ g/ml) in the absence (Vehicle) and presence of 300 μ M DTG (DTG). Mean basal calcium levels ($\pm$ SEM) measured using fura-2 calcium imaging. Values represent measurements from three pooled experiments each having a minimum of 30 cells per group. Asterisk denotes statistical difference ($p < 0.05$, $n = 31$) between Media and LPS treatment groups within the Vehicle group, and pound symbol denotes significant difference between Vehicle and DTG within the LPS treatment group ($p < 0.05$, $n=31$). Statistical difference was determined using a two-way ANOVA with post-hoc Bonferroni test.

Figure 7. Increases in intracellular calcium induce membrane ruffling in microglia

Micrographs of phalloidin stained microglia treated with vehicle (DMSO, A) or 1 μ M ionomycin for 5 minutes (B). C, Bar graph of degree of membrane ruffling determined for the indicated conditions. Bars represent means $\pm$ SEM. Asterisks indicate a significant difference between DMEM and Ionomycin treatment groups ($p < 0.001$). Significance was determined via two-way ANOVA with post-hoc Tukey Test. All insets represent magnified image (2.7x). Scale bar = 2 μ m.

Figure 8. Microglial migration is independent of calcium release from internal stores but requires La^{3+} -sensitive calcium influx

A, Microglial chemotaxis in response to ATP (100 μ M) in the absence and presence of DTG (300 μ M) and DTG with ionomycin (1 μ M). Data were analyzed via two-way ANOVA with post-hoc Tukey test. Asterisk indicates a statistical difference from respective controls ($p < 0.001$ for all), pound symbol indicates a statistical difference from ATP alone ($p < 0.001$ for all), and dagger indicates statistical significance from ATP+DTG ($p < 0.05$ for all). B, Microglia migration induced by 300 μ M ATP without and with pretreatment of the cells with 10 μ M thapsigargin for 30 min (37 $^{\circ}$ C) (Thapsigargin). Asterisk indicates a statistical difference from control ($p < 0.05$). Bar represent means $\pm$ SEM, $n = 3$. C, Microglia migration evoked by 300 μ M ATP in the absence (Control) and presence of 50 μ M La^{3+} . Asterisk indicates a statistical difference from Control ($p < 0.001$), pound symbol indicates a statistical difference from ATP alone ($p < 0.01$). Bar represent means $\pm$ SEM, $n = 3$. Statistical significance was determined by two-way ANOVA followed by post-hoc Bonferroni multiple comparison tests. D, Relative ATP induced migration in the absence (Control) and presence of 50 μ M La^{3+} . Asterisk denotes statistical difference ($p < 0.05$ by student's T test).

Figure 9. Calcium influx restores nitric oxide production following sigma receptor activation in LPS stimulated microglia

Relative levels of NO produced by microglia under control condition (DMEM), in the presence of 300 μ M DTG (DTG), and following application of 1 μ M ionomycin alone (Iono) and with 300 μ M DTG (Iono + DTG). Microglia were also treated for 24 hrs with LPS (1 μ g/ml) alone (LPS), LPS and 300 μ M DTG (LPS+DTG), LPS and 1 μ M ionomycin, and with a combination of the three compounds (LPS+DTG+IONO). Bars represent mean fluorescent intensities from DAF-FM loaded cells. Asterisk indicates statistical difference from DMEM treated cells, pound symbol denotes statistical significance from LPS treated cells, and dagger indicates significance from LPS+DTG treated cells. In all cases $p < 0.001$, as determined by three-way ANOVA with post-hoc Tukey test ($n \geq 400$ cells for all samples).