Original Paper

Chloride Channel Blockers Suppress Formation of Engulfment Pseudopodia in Microglial Cells

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Key Words

Cl⁻-conductance • Hyperosmotic solution • Hypoosmotic solution • Microglial BV-2 cells • Primary microglial cells • Phagocytosis • Cell volume

Abstract

Background/Aims: Phagocytosis depends on the formation of engulfment pseudopodia surrounding the target. We tested in microglia, monocyte-derived cells in the brain, whether a swelling-activated Cl⁻-current ($I_{Cl, \text{swell}}$), required for global cell volume (CV) regulation, also contributes to local expansion and retraction of engulfment pseudopodia. **Methods:** We used scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) to visualize and quantify the uptake of polystyrene microbeads (MBs) by microglial cells. Flow cytometry was used for cell volume measurements and $I_{Cl, \text{swell}}$ was measured by whole-cell patch clamp. **Results:** We found that exposure of microglial BV-2 cells to MBs in Cl⁻-free extracellular solution attenuated MB uptake and that the Cl⁻-channel blockers DIOA, flufenamic acid, NPPB and DCPIB suppressed the uptake of MBs in BV-2 cells and in primary microglial cells. Microglial cells exposed to MBs in the presence of Cl⁻ channel blockers failed to extend engulfment pseudopodia. We observed that cells containing at least three MBs revealed an about twofold increase in current density of $I_{Cl, \text{swell}}$ compared to cells without MB. Osmotic challenges to stimulate global CV regulation before exposure to MBs modulated phagocytosis. Pre-conditioning of cells in hypo- or hypertonic medium for 12–16 hours caused a decrease in MB uptake. **Conclusion:** These findings indicate that $I_{Cl, \text{swell}}$ contributes to formation of engulfment pseudopodia and participates in engulfment and particle uptake in microglial cells.

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Introduction

Anion-conductance activation is one of the earliest events in the innate immune responses. Recognition of opsonised *Candida albicans* by human polymorphonuclear neutrophil leucocytes (PMNs) triggers the efflux of Cl^- in these cells [1]. Accordingly, inhibition of Cl^- -channel activity in human PMNs decreases the uptake of opsonized zymosan [2]. Similarly, blockade of Cl^- -conductance in rodent microglia, monocyte-derived cells in the nervous system, suppresses phagocytosis of *E. coli* [3] or engulfment of microspheres (microbeads, MBs) [4]. In contrast to uptake of particles, blockade of chloride intracellular ion channel 1 (CLIC1) increases amyloid-beta ($\text{A}\beta$) uptake in microglia and macrophages [5]. In addition to its involvement in the recognition and uptake of particles, Cl^- -channels have been associated with pathogen killing in phagolysosomes. Cystic fibrosis transmembrane conductance regulator (CFTR), acting as cAMP-regulated Cl^- -channel, and the Cl^- -channel CLC-3 are localized in the membrane of phagolysosomes in phagocytes [2, 6, 7]. Phagolysosomes from patients suffering from cystic fibrosis (CF) or from CFTR deficient mice show impaired acidification, defect chlorination of bacterial proteins and, consequently, inefficient killing of bacteria [6, 7]. Thus, anion-channels in the plasma membrane as well as in intracellular organelles (phagolysosomes) are crucially involved in phagocytosis and killing of pathogens.

Although several studies demonstrate the importance of a Cl^- -conductance in the early phase of phagocytosis, the mechanistic consequences of a change in Cl^- -conductance are not known. In the present study, we propose that activation of Cl^- -conductance is a prerequisite for the formation of engulfment pseudopodia. Following recognition of a particle, engulfment pseudopodia extend from the cell surface, tightly embrace particles and expand until entire engulfment is accomplished. The formation of engulfment pseudopodia may be envisioned as local subcellular volume changes. Similar to *global cell volume* (CV) regulation [8–10], influx or efflux of osmolytes along osmotic gradients, which is restricted to specific subcellular regions, provokes *local cell swelling* or shrinkage, respectively. In such a scenario influx of water should promote extension of engulfment pseudopodia, whereas efflux of water would accomplish the retraction phase. Applying the concept of global CV regulation to the formation of engulfment pseudopodia predicts three consequences: (i) If local volume regulation resembles global CV regulation, ion channels and transporters involved in regulatory volume increase (RVI) and decrease (RVD) are involved in formation of engulfment pseudopodia. A key ion in global CV regulation is Cl^- [11–14]. Therefore, blockade of Cl^- -conductances is expected to prevent local volume changes and suppress particle uptake. (ii) CV regulation is closely associated with osmotic challenges. Particle uptake and digestion lead to an increase in osmotic pressure, which needs to be counterbalanced by regulation of osmolytes. Exemplified by hepatocytes, glucagon not only promotes catabolism of glycogen to glucose, but also activates CV regulatory K^+ fluxes and volume-activated Cl^- -channels [15–17]. Similarly, in phagocytes the uptake of particles could promote an increase in Cl^- -conductance to prevent deleterious osmotic stress. Accordingly, an increase in the amplitude of the swelling-activated Cl^- -current ($I_{\text{Cl, swell}}$) is expected following particle uptake. (iii) If there is a mutual interaction between osmoregulation and phagocytosis, exposure of cells to osmotic challenges prior to application of particles will modulate the phagocytotic capacity.

We validated our predictions by exposing primary microglial cells as well as the microglial cell line BV-2, to MBs. We used polystyrene MBs to avoid interferences of signalling cascades induced by the MB uptake with those elicited by biological material, like pathogen associated molecular patterns (PAMP). To unequivocally distinguish between attached and internalized MBs, we used in most experiments scanning electron microscopy (SEM). Particle uptake was quantified at the single cell level in the absence of extracellular Cl^- as well as in the presence or absence of Cl^- -channel blockers. The mutual modulation of phagocytosis and osmoregulation was evaluated by analysing long term consequences of phagocytosis on $I_{\text{Cl, swell}}$ densities as well as by quantifying the consequences of long term osmotic challenges on MB uptake.

Materials and Methods

Cell culture

Primary microglial cells were isolated from forebrains of one- to three day old wild-type C57 black 6J mice [18]. Mice were killed by decapitation. After dissociation of brain tissue with trypsin for 30 min at 37 °C and mechanical dissociation by centrifugation, microglia were co-cultivated with astrocytes in poly-D-lysine (PDL)-coated 75 cm² tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco/BRL Life Technologies, MD, USA) and 1% penicillin /streptomycin (Gibco). 6 to 9 animals were used per cell culture. After 10–14 days in culture at 37 °C and 5% CO₂, primary microglial cells were harvested by vigorous shaking from an astrocyte monolayer for 3 h. The supernatant containing >97% microglia was used for experiments. Adult wild-type C57 black 6J mice were ordered from and bred in the local animal housing at the University of Salzburg. Animal handling was performed according to Austrian law and approved by local authorities.

The murine microglial cell line BV-2 was cultured in 25 cm² tissue culture flasks in DMEM containing 2200 mg/L glucose supplemented with 10% FCS at 37 °C in a humidified atmosphere at 5% CO₂. Twice a week cells were split and supplied with new medium.

For experiments, BV-2 cells and primary microglial cells were treated with trypsin (2.5%, 1 ml for 3 min) to detach from culture flasks. Following detachment, cell suspension was transferred into a centrifugation tube containing 8 ml DMEM + 10% FCS and centrifuged at 200×g for 5 min. Cells were re-suspended in 1 ml DMEM + 10% FCS, seeded into Petri dishes (Ø 3.5 cm) on PDL-coated (0.01% for BV-2 cells; 0.1% for primary microglia) glass coverslips (Ø 12 mm) and incubated for up to 18 h to allow for adhesion. Following two washes in extracellular solution, cells were treated with different reagents in extracellular solution.

Reagents and solutions

The Cl⁻-channel blocker 4-[(Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid (DCPIB), the Cl⁻-channel- and K⁺-Cl⁻-cotransport inhibitor R(+)[(2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy] (DIOA), and the Cl⁻-channel blockers flufenamic acid (FFA) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were from Tocris (Ellisville, MO, USA).

Extracellular solution (~300 mosmol/L) contained (in mM): NaCl, 130; KCl, 5; CaCl₂, 2; MgCl₂, 1; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10; D-glucose, 8; titrated to pH 7.4 with NaOH. Cl⁻-free extracellular solution (~300 mosmol/L) contained (in mM): Na-D-gluconate, 130; K-D-gluconate, 5; Ca-D-gluconate monohydrate, 2; MgSO₄, 1; HEPES, 10; D-glucose, 8; titrated to pH 7.4 with NaOH. The measured concentration of ionized calcium in this solution was 0.31 mM. Phosphate buffered saline (PBS) contained (in mM): NaCl, 150; KH₂PO₄, 2; Na₂HPO₄, 6.4; titrated to pH 7.4 with NaOH. In a 5% CO₂ atmosphere of the incubator, the pH in the HEPES-buffered extracellular solution did not change within 15 min, but decreased from 7.4 to 7.2 within 1 h. Long-term incubation (12 h) of cells in anisoosmotic solutions was performed in DMEM containing 10% FCS. Short-term incubation (15 min, 1 h) of cells in anisoosmotic solutions was performed in extracellular solutions. Hypertonic solution (400 mosmol/L) was adjusted by addition of sucrose, and hypotonic solution (200 mosmol/L) was adjusted by dilution with Aqua bidestillata. After 12 h exposure in a CO₂ atmosphere, the pH of the diluted buffer was 7.3. Osmolarity was checked by freezing point depression (OM802; Vogel, Giessen, Germany). All chemicals were from Sigma-Aldrich (St. Luis, Missouri, USA). Red fluorescent sulfate polystyrene microspheres (2% solids, Ø 4 µm) were purchased from Invitrogen (Life Technologies, Vienna, Austria).

Flow cytometry – Cell volume (CV) regulation

CV regulation in BV-2 cells was evaluated using flow cytometry by determination of the forward scatter integral area (FSC-A). Cells were seeded in uncoated Petri dishes (Ø 35 mm) at a density of 1×10⁵ cells in DMEM with 10% FCS overnight at 37 °C and 5% CO₂. After trypsination and centrifugation at 200×g for 5 min, cells were re-suspended in 1 ml extracellular solution. After 5 min cells were exposed to hypotonic solution (200 mosmol/L) in the presence or absence of 5 µM DCPIB, or 200 µM FFA. At one minute intervals CV was measured using a FACSCanto™ II Flow Cytometry System (BD Bioscience, San Jose, CA, USA) by measuring FSC-A by counting 10,000 cells per time-point. Data were analysed using the Win MDI 2.9 software.

Scanning electron microscopy

For scanning electron microscopy (SEM), microglial cells were seeded on PDL coated glass coverslips (Ø 12 mm) at a density of 6×10^4 cells in DMEM with 10% FCS and allowed to adhere overnight at 37 °C and 5% CO₂.

For anion channel blocker experiments cells were washed twice and incubated in extracellular solution. Phagocytosis of microglial cells was assessed after incubation with MBs (3.41×10^6 MBs / 2 ml solution) in the absence and presence of the channel blockers DCPIB, DIOA, FFA and NPPB for 15 min and/or 1 h at 37 °C and 5% CO₂. Added MBs were stationary at the site of sedimentation and did not float or move within the culture dish. After incubation, cells were washed with extracellular solution and PBS and fixed in 2.5% glutaraldehyde at room temperature for 1 h and washed in PBS overnight. Cells were dehydrated in ethanol series (50–70–80–90–96–100–100–100%) for 5 min each and dried in a critical point drying apparatus (Critical Point Dryer LPD030, Baltec, Liechtenstein). After placing the coverslips on a SEM-stub, microglial cells were covered with gold (Sputter Coater/ Agar Scientific, England) and observed using a Cambridge Stereoscan 250 scanning electron microscope. Electron micrographs were digitized and analysed *via* Orion software (ORION, E.L.I. sprl, Belgium).

For experiments in Cl⁻-free extracellular solution, cells were washed twice and incubated with MBs (3.41×10^6 MBs / 2 ml solution) for 15 min and 1 h, respectively, in Cl⁻-free solution at 37 °C and 5% CO₂. After incubation times further processing followed as described above.

For long-term experiments with hypertonic or hypotonic solutions, cells were washed two times with hypertonic (400 mosmol/L) or hypotonic (200 mosmol/L) solution and incubated in it for 12 h at 37 °C and 5% CO₂. After exposure to anisotonic conditions, cells were exposed (i) for 15 min to MBs (3.41×10^6 MBs / 2 ml solution) in isotonic saline, (ii) for 15 min in isotonic saline followed by further 15 min in isotonic conditions in the presence of MBs, (iii) for 1 h in isotonic saline containing MBs, and (iv) for 15 min in isotonic saline followed by 1 h in isotonic conditions in the presence of MBs. After different incubation times, cells were treated as described for anion channel blocker experiments.

Confocal laser scanning microscopy (CLSM)

For fluorescence microscopy BV-2 cells were plated on 0.01% PDL coated glass coverslips (Ø 20 mm) at a density of 6×10^4 cells in DMEM and 10% FCS for 12–18 h. After two washing steps with extracellular solution, cells were incubated in hypertonic solutions (400, 450, 500 mosmol/L) with MBs (3.41×10^6 MBs / 2 ml solution) for 15 min and 1 h, respectively, at 37 °C and 5% CO₂. After the incubation, cells were washed with extracellular solution and PBS and fixed with methanol and glacial acetic acid (3:1) for 20 min at room temperature. Nuclei were stained with DAPI (Sigma-Aldrich, USA) for 30 min. Thereafter cells were washed with PBS and distilled water and coverslips were embedded in glycerol (DakoCytomation, USA) and stored at 4 °C until use.

Phagocytosis of MBs was visualized with a confocal laser scanning microscope (CLSM 510 Meta, Zeiss, Germany) using an UV laser ($\lambda_{\text{ex}} = 364$ nm) to detect DAPI stained blue nuclei and a helium-neon laser ($\lambda_{\text{ex}} = 543$ nm) to visualize red MBs. Images were digitized and processing was performed with a Zeiss LSM Image Examiner.

Determination of successful particle binding, engulfment and internalisation

For the assessment of MB binding the number of particles attached to microglia cells were counted numerically. Successful MB engulfment and internalization was detected by microglia whole particle engulfment using SEM, which is clearly visible due to the spherical shape of the MBs. Using CLSM, successful particle internalization was observed when the MBs were in the same optical plane as the nuclei.

Electrophysiology

BV-2 cells were plated on PDL (0.01%)-coated glass coverslips (Ø 12 mm) and grown for at least 48 h in DMEM. BV-2 cells were pre-incubated with polystyrene MBs (Ø 4 µm) for at least 45 min up to 5 h. Electrophysiological recordings were performed on cells with three or more incorporated MBs. For long-term osmotic challenge (12–16 h), BV-2 cells were pre-incubated with hypotonic (200 mosmol/L) or hypertonic (400 mosmol/L) DMEM, respectively. Cl⁻-currents were monitored in the standard whole-cell recording mode [19, 20] with an EPC-10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) connected to a computer running Patchmaster / Fitmaster 2.65 data acquisition / analysis software. After establishing

the whole cell configuration, cells were superfused with isotonic extracellular solution containing (in mM): NaCl, 160; CaCl₂, 2; MgCl₂, 1; HEPES-FA, 5; titrated to pH 7.4 with NaOH and an osmolarity of 308–310 mosmol/L, using a gravity-flow permanent perfusion system.

$I_{Cl,swell}$ was elicited by osmotic stress in two different ways: (i) by a 85% hypoosmotic external solution, which was prepared by diluting the isotonic extracellular solution with distilled water in combination with an isotonic intracellular solution containing (in mM): CsCl, 160; MgCl₂, 2; HEPES-FA, 10; EGTA, 11; Mg-ATP, 4; titrated to pH 7.2 with CsOH and an osmolarity of 314 mosmol/L. (ii) By a hypertonic intracellular solution containing (in mM): CsCl, 160; MgCl₂, 5; HEPES-FA, 10; EGTA, 11; D-Mannitol, 30; Mg-ATP, 2; titrated to pH 7.2 with CsOH and an osmolarity of 374 mosmol/L in combination with the isotonic extracellular solution. Both salines are symmetric Cl⁻ solutions. Osmolarities were measured with a vapor pressure osmometer (Wescor, Logan, Utah, USA). Resistances of electrodes filled with the hypertonic intracellular pipette solutions ranged from 2 to 5 MΩ. Patch pipettes were pulled from WPI 1.5 mm diameter glass capillaries (WPI, Glass 1BBL W/Fl 1.5mm 3IN) in three stages and fire polished using a Narishige MF-9 microforge (NarishigeCo., Ltd, Tokyo, Japan). Immediately after forming a stable whole cell configuration, membrane (C-slow) and pipette (C-fast) capacitances were compensated using an electronic feedback *via* the patch clamp amplifier. Liquid junction potential between pipette and bath solution (< 20 mV) was not corrected. In all experiments the holding potential was kept at 0 mV to block voltage dependent currents. The Cl⁻-current was monitored either during a 500-ms voltage ramp from -100 mV to +100 mV elicited at a frequency of 10 sec, or during 500-ms voltage steps from -100 mV to +100 mV with an increment of 20 mV. All electrophysiological measurements were performed at room temperature. Further data analysis was performed using the Igor Pro 3.1 (Wave Metrics, Oregon, USA) software package and GraphPad Prism 4 (GraphPad Software, Inc.).

Statistical analysis

All data are presented as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using the SPSS 18.0 software. Each experiment was conducted at least for three times (in each experiment 100–200 cells were randomly counted), Student's double-sided t-test for independent samples and analysis of variance (ANOVA) followed by the Dunnett's post hoc test was applied to calculate levels of significance, $P < 0.05$ and $P < 0.01$. All electrophysiology data are presented as mean ± s.e.m. Statistical analyses (t-tests) were performed using GraphPad Prism 4. Sample sizes are depicted in the figures.

Results

Cl⁻-channel blockers suppress $I_{Cl,swell}$, uptake of microbeads, and formation of engulfment pseudopodia

Cell swelling induced by perfusion with a hypotonic extracellular solution or by dialyzing cells with a hypertonic intracellular solution activated an outwardly rectifying current in BV-2 cells over time ($I_{Cl,swell}$; Fig. 1A, B). This current was present in asymmetric [21] as well as in symmetric distribution of Cl⁻ in the extra- and intracellular solutions, which is reflected in a reversal potential close to 0 mV. NPPB (100 μM), DIOA (100 μM), FFA (200 μM), and DCPIB (5 μM) completely blocked the inward as well as the outward current. NPPB blocked both the inward and outward current at -100 and +100 mV, respectively, with an IC_{50} of ~4 μM (n=3) (Fig. 1C).

Formation of contact-induced engulfment pseudopodia and the following engulfment took place all over the cell body and was not restricted to the lamellipodium (Fig. 2A, B).

To test for Cl⁻-dependency of phagocytosis, BV-2 cells were incubated in nominally Cl⁻-free extracellular solution and the relative number of engulfed MBs was counted. As shown in Figure 3, MB uptake was unaffected after 15 min, but significantly attenuated by ~40% after 1 h.

Under control conditions the overall number of BV-2 cells containing MBs was dependent on the incubation time. After 15 min and 1 h the relative number of MB containing cells was 46.8 ± 2.5 % and 81.7 ± 5.2 %, respectively (Fig. 4A). In the presence of DCPIB (20 μM), DIOA (100 μM), FFA (200 μM) and NPPB (200 μM) the phagocytotic capacity was markedly depressed or inhibited almost completely. To analyse this behaviour on the single cell level

Fig. 1. Representative current tracings of whole cell patch clamp recordings of BV-2 cells at a holding potential of 0 mV. $I_{Cl, swell}$ was evoked either by superfusion of a cell with (A) hypotonic solution (85% of normal saline; 263 mosmol/L) versus an isotonic intracellular solution (314 mosmol/L) or (B) by dialyzing the cell with a hypertonic intracellular solution (374 mosmol/L) versus an isotonic extracellular solution. Symmetrical concentrations of intra- and extracellular Cl^- were used and currents were monitored during 500 ms voltage ramps from -100 mV to +100 mV. The current was suppressed by 100 μ M NPPB (A) and 100 μ M DIOA (B). (C) Comparison of NPPB-dose-response curves in the inward (grey) and outward (black) direction of $I_{Cl, swell}$ fitted with the 4 parameter logistic equation by GraphPad Prism 4 under hypertonic intracellular conditions in control BV-2 cells. NPPB was used at 1, 3, 10, 30, 50 and 100 μ M. Current data at -100 and +100 mV were obtained from 500-ms voltage ramps and normalized to currents recorded under control conditions prior to the application of NPPB. Data are represented as mean $\pm$ s.e.m. ($n=3$ for each data point).

Fig. 2. Engulfment and attachment of hydrophobic polystyrene MBs by primary microglia and microglial cell line BV-2. (A) SEM of primary microglial cell with engulfed MBs at the soma and at the lamellipodia and attached spheres at the soma; (B) BV-2 cell with engulfed MBs at the soma of the cell and an attached sphere at the lamellipodia. Scale bars 20 μ m.

Fig. 3. Incubation in extracellular Cl^- -free solution inhibited phagocytosis of MBs by the microglial cell line BV-2 after 1 h compared to extracellular solution (CO). Number of cells with successfully engulfed MBs was counted in three independent experiments and evaluated using SEM. Data are represented as mean $\pm$ s.e.m.. Asterisks (**) indicate $P < 0.01$, when compared with control cells.

we counted distribution frequencies for BV-2 cells containing no MBs, one MB, two MBs and more than two MBs, respectively (Fig. 4B and C). After 15 min of exposure to MBs $53.2 \pm 2.5\%$ did not contain a single MB, $28.0 \pm 1.1\%$ contained one MB, $12.6 \pm 1.5\%$

Fig. 4. Blockers of $I_{Cl,swell}$ inhibit phagocytosis of MBs by primary microglial cells (primM) and the microglial cell line BV-2. (A) Incubation of BV-2 cells with MBs for 15 min (white bars) and 1 h (black bars), respectively, in the absence and presence of anion channel blockers (20 μM DCPIB, 100 μM DIOA, 200 μM FFA, and 200 μM NPPB). (B, C) Distribution frequency of MBs engulfed by BV-2 cells after 15 min (B) and 1 h (C) of incubation in the absence and presence of inhibitors, respectively. (D) Phagocytotic capacity of primary microglial cells (primM; white columns) and microglial cell line BV-2 (BV2; black columns) in the absence (CO, control) and presence of anion channel blockers (5 μM DCPIB, 100 μM DIOA, 200 μM FFA, and 100 μM NPPB) after 15 min of incubation with MBs (in D data are absent for 100 μM NPPB in BV-2 cells). (E) Frequency distribution of MBs engulfed by primM after 15 min of incubation in the absence and presence of inhibitors, respectively. (B, C and E) Number of cells with successfully engulfed MBs was counted in three independent experiments and evaluated using SEM. Data are represented mean $\pm$ s.e.m.. Asterisks (*, **) indicate $P < 0.05$, $P < 0.01$, respectively, when compared with control cells or between two groups as marked by a line.

contained two MBs, and $6.2 \pm 0.2\%$ contained more than two MBs. Incubation with 200 μM FFA revealed a distribution frequency of one or two spheres only, whereas treatment with 20 μM DCPIB or 100 μM DIOA revealed a net engulfment of only one sphere, and no uptake of microspheres was observed in microglia incubated with 200 μM NPPB (Fig. 4B). After 1 h of exposure to MBs $52.8 \pm 10.3\%$ of BV-2 cells had engulfed more than two MBs. The number of cells containing two, one, or no MBs was about 15% each (Fig. 4C). $85.0 \pm 2.4\%$ of FFA-exposed (200 μM) BV-2 cells did not contain MBs, $12.0 \pm 0.9\%$ contained

Fig. 5. The K⁺-Cl⁻-cotransporter blocker DIOA and the Cl⁻-channel blocker NPPB inhibited phagocytosis of MBs in a dose-dependent manner after incubation for 1 h in BV-2 cells. Phagocytosis at different blocker concentrations of DIOA (A) and NPPB (B) was normalized to control condition (CO), and each column represents the average of three independent experiments. (C, D) Frequency distribution of cells engulfing MBs shows that higher blocker concentrations increase the number of cells without engulfed MBs. Columns represent number of cells without phagocytosis (white columns), cells having successfully engulfed one MB (light grey columns), two MBs (black columns), and more than two MBs (dark grey columns). Number of cells with successfully engulfed MBs was counted in three independent experiments and evaluated using SEM. Data are represented as mean $\pm$ s.e.m.. Asterisks (*, **) indicate $P < 0.05$, $P < 0.01$ when compared with control conditions.

one, $2.0 \pm 1.6\%$ contained two and $1.0 \pm 0.5\%$ more than two. $99.0 \pm 0.5\%$ of cells incubated with NPPB (200 μM) did not contain MBs and the remaining one percent contained one MB only, similar to DCPIB (20 μM), where $97.6 \pm 0.8\%$ of cells did not contain MBs. The remaining $\sim 2\%$ is distributed among cells containing one and two MBs.

Primary microglial cells behaved slightly different from BV-2 cells. Under control conditions the overall number of primary microglial cells containing MBs was $64.5 \pm 4.6\%$ (Fig. 4D). Again, in the presence of the inhibitors DIOA (100 μM), FFA (200 μM) and NPPB (100 μM) the phagocytotic capacity was markedly depressed. At the single cell level only $35.5 \pm 4.6\%$ of primary microglial cells did not contain a single MB after 15 min of incubation. $27.5 \pm 8.1\%$ contained one MB, $13.5 \pm 3.2\%$ two MBs and $23.5 \pm 9.5\%$ more than 2 MBs (Fig. 4E). Incubation with DIOA (100 μM), FFA (200 μM) and NPPB (100 μM) revealed a distribution frequency of $\sim 10\%$ for cells containing one MB and $\sim 3\%$ for cells with two MBs. The majority of cells ($\sim 85\%$) did not contain a single MB (Fig. 4E). Since in primary microglia 20 μM DCPIB led to signs of apoptosis (see below), we tested its effect at the IC_{50} for $I_{Cl, \text{swell}}$, i.e. 5 μM . At this concentration DCPIB showed weak inhibitory effects: $32.5 \pm 1.1\%$ of cells contained one MB, $16.0 \pm 0.7\%$ contained two MBs and $7.8 \pm 3.2\%$ contained more than two MBs. Only $44.0 \pm 1.4\%$ of cells did not contain a MB (Fig. 4E).

Inhibition of particle engulfment as well as block of $I_{Cl, \text{swell}}$ showed similarities in their concentration dependencies. DIOA and NPPB potently inhibited $I_{Cl, \text{swell}}$ and effectively suppressed particle uptake in our study. For example, NPPB in the concentration range from 30 to 50 μM suppressed MB uptake to $\sim 50\%$ compared to control conditions and the IC_{50} for

Fig. 6. Short-term hypertonicity inhibited phagocytosis of MBs by BV-2 cells. (A) BV-2 cells incubated with different concentrations of hypertonic solutions (400, 450 and 500 mosmol/L) decreased phagocytosis of MBs after 15 min and 1 h, respectively, compared to incubation in normotonic solution (CO). Hypertonicity decreased the frequency of MBs engulfed by microglial cells for 15 min (B) and 1 h (C), respectively. Columns represent the relative number of cells without MBs (white columns), cells having successfully engulfed one MB (light grey columns), two MBs (black columns), and more than 2 MBs (dark grey columns). Cells with successfully engulfed MBs were counted in three independent experiments and evaluated using CLSM. (D–F) Representative SEM images of BV-2 cells incubated with extracellular hypertonic solutions: 400 mosmol/L (D), 450 mosmol/L (E), and 500 mosmol/L (F). Scale bars 20 μ m (E, F) and 40 μ m (D). Data are represented as mean $\pm$ s.e.m.. Asterisks (*, **) indicate $P < 0.05$, $P < 0.01$, respectively, when compared with control conditions or between two groups marked by a line.

$I_{Cl,swell}$ is $\sim 4 \mu$ M. 100 μ M DIOA and 100 μ M NPPB completely suppressed $I_{Cl,swell}$ (Fig. 1) and effectively blocked uptake of MBs (Figs. 4 and 5). DCPIB blocked $I_{Cl,swell}$ at concentrations of about 5 μ M (data not shown), but significantly suppressed particle uptake only at higher concentrations of about 20 μ M (Fig. 4). In the frequency distribution a significant inhibition of cells able to phagocytose more than two beads can be observed at an NPPB concentration of 0.1 μ M (Fig. 5D). A similar observation was made for DIOA (Fig. 5C).

In electrophysiological experiments Cl⁻ currents with biophysical and pharmacological properties similar to $I_{Cl,swell}$ but induced by increased intracellular Ca²⁺ or cAMP in contrast to cell swelling, are terminated by superfusion with hyperosmotic extracellular solutions [21]. In our engulfment experiments we used a similar approach; BV-2 cells were exposed to hyperosmotic conditions (400, 450 and 500 mosmol/L, respectively) for 15 min or 1 h and uptake of MBs was quantified using CLSM. Suppression of MB uptake increased with the osmolarity of the extracellular solution (Fig. 6A). Under control conditions, the frequency distribution of cells containing none, one, two, or more than two MBs differed between cells exposed to MBs for 15 min and 1 h, respectively. Cells exposed for 15 min showed a frequency distribution pattern in which cells containing no MB dominated (Fig. 6B), but after 1 h most cells contained more than two MBs. The number of cells containing at least one MB decreased with increasing extracellular osmolarity, but did not differ between cells exposed to 450 or 500 mosmol/L for 15 min or 1 h. On the other hand, comparing frequency distribution of cells incubated with the lowest hypertonic osmolarity

Fig. 7. The Cl⁻-channel blocker DCPIB delays RVD. After incubation of cells in normotonic saline (CO), cells were exposed to hypotonic solution (200 mosmol/L) in the absence (CO) or presence of 5 μ M DCPIB or 200 μ M FFA, respectively. Cells exposed to DCPIB showed a higher cell volume (CV) increase and a delayed RVD compared to cells exposed to hypotonic solution alone. CV was evaluated by flow cytometry as FSC-A for three independent experiments. Data are represented as mean $\pm$ s.e.m..

(400 mosmol/L) revealed a significant decrease in cells without MBs and a significant increase in cells containing two MBs when compared between 15 min and 1 h, respectively (Fig. 6B, C). Although hyperosmotic extracellular solutions significantly suppressed uptake of MBs, SEM revealed that the cells did not exhibit a shrunken morphology (Fig. 6D, E, F), indicating efficient volume regulatory processes in these cells. Thus, similar to the suppression of Cl⁻-currents induced in normotonic solutions by intracellular Ca²⁺ or cAMP, hyperosmotic extracellular solutions suppressed engulfment of MBs. These experiments suggest that ion conductances required for CV regulation, like the Cl⁻-conductance, are also involved in particle uptake.

Next, we evaluated whether the low number of engulfed MBs in cells exposed to Cl⁻-channel blockers was due to inhibition of engulfment or due to less accessibility to MBs, like, e.g., by a decrease in cell migration. We assumed that no change or even an increase in the number of attached MBs, but a decrease in the number of engulfed MBs, indicated an inhibition of the engulfment process. We added 3.41×10^6 MBs per dish ($\emptyset$ of dish: 3.5 cm). Assuming a statistically even distribution of MBs, ~ 2 MBs were present per 500 μm^2 . Accordingly, a cell with a length of 60 μm had about 45 MBs within an area with a diameter twice of its length as potential targets. The ratio of engulfed to attached particles was balanced in control conditions to 1:0.9. Microglial cells incubated with Cl⁻-channel blockers had more particles attached than engulfed. The relative distribution of engulfed to attached particles in cells treated with DCPIB (20 μM), DIOA (100 μM) and FFA (200 μM) was 1:2.2, 1:4.4 and 1:1.5, respectively. Cells treated with NPPB (200 μM) did not contain MBs, however, on average 1.6 MBs were attached to the cell surface. These observations support the assumption that Cl⁻-channel blockers interfere with the engulfment process and do not limit accessibility of the cell to the MBs. Furthermore, Cl⁻-conductance inhibition does not prevent MB attachment, but suppresses extension of engulfment pseudopodia.

Hypotonic challenges of $\sim 70\%$ (extracellular 200 mosmol/L) induced cell swelling in BV-2 cells. Under control conditions CV increased within 1 min by $\sim 46\%$ and cells re-gained their initial CV within 13 min by RVD. In the presence of DCPIB (5 μM) CV increased by $\sim 63\%$, i.e. $\sim 15\%$ more than in absence of the inhibitor; and RVD was incomplete within 15 min. In the presence of FFA (200 μM) CV increased by $\sim 33\%$ and cells regulated back to their initial CV within 13 min (Fig. 7).

Since Cl⁻-channel blockers may have toxic effects, we evaluated cell blebbing, a hallmark of apoptosis, using SEM. About 300–600 cells in three independent experiments were evaluated in the presence or absence of Cl⁻-channel blockers. In the absence of Cl⁻-channel blockers we did not observe cells with blebs. Similarly, exposure of cells for 15 min to DCPIB (5 μM), FFA (up to 200 μM), and NPPB (up to 200 μM) did not reveal cells containing blebs. However, DIOA (100 μM) and DCPIB (20 μM) increased the number of blebbing cells to $10.6 \pm 4.1\%$ and $19.9 \pm 2.4\%$ within 15 min and to $60.7 \pm 12.5\%$ and $47.0 \pm 14.8\%$ within

Fig. 8. (A, B) Representative cells treated with DCPIB (20 μ M) showing blebbing, a hallmark of apoptosis visualized by SEM. Arrows point to cells with blebbing. Scale bars 100 μ m (A) and 20 μ m (B).

Fig. 9. Scanning electron microscopy of microglial BV-2 cells with successive engulfment of a hydrophobic polystyrene MB at the soma of a cell. (A) The MB is attached to the soma of a microglial cell. (B) Numerous fine protrusions extend from the cell surface at the contact area between the microglial cell and the MB. These nascent protrusions are tightly fitting the MB. (C) Protrusions augment on the base to flat, broad extensions, engulfing the MB. (D) Processes extend continuously, fusing together to an area with fine protrusions distally, engulfing more than 2/3 of the MB. Simultaneously internalization can be observed. (E) The MB is completely enclosed by the cell membrane. At the area of process contact, engulfing processes overlap and melt together. (F) Engulfment and internalization is accomplished. The MB is subsided into the cell and only the shape of it can be seen on the cell surface. Scale bars 2 μ m (C–E) and 4 μ m (A, B, F).

1 h, respectively. Thus, among the Cl^- -channel blockers used, DIOA and DCPIB considerably increased the number of cells showing blebs. Figures 8A and B visualize representative cells with blebbing DCPIB (20 μ M). Since DIOA blocks Cl^- -channels [3, 22] as well as KCC [3, 22–26], these may cause an imbalance in Cl^- or intracellular pH homeostasis, because Cl^- -channels and transporters are also permeable to HCO_3^- [9, 27].

Cl⁻-channel blockers suppress formation of engulfment pseudopodia

As mentioned above, pseudopodia formation and the following particle engulfment take place all over the cell body and are not restricted to the lamellipodium (Fig. 2A, B). SEM images visualized two modes of MB uptake: (i) Contact of a particle with the plasma membrane initialized formation of an engulfment pseudopodium. This tight fitting pseudopodium extended along the particle and fused at the top of the MB. In parallel to fusion of engulfment pseudopodia the particle was descended into the cell soma (Fig. 9). (ii) Delicate, filopodia-like protrusions extended from the cell surface and transformed to

Fig. 10. Scanning electron microscopy of microglial BV-2 cells showing successive engulfment of hydrophobic polystyrene MB at the lamellipodia of a cell. (A) The MB is attached to a lamellipodia of a microglial cell. (B) Fine protrusions from the cell surface contact the MB, augmenting to a broad, flat engulfing area between the cell and the MB. (C) Engulfing extensions envelop tightly half of the MB. (D) Almost the whole MB is engulfed by the cell. Extending engulfing areas laterally overlap; only a blank space between the engulfing processes is left. (E) The MB is completely engulfed by the cell. Overlapping engulfing processes melt together on area of contact. (F) The MB is internalized by microglial cell surface. The shape of the MB can be seen on the lamellipodia of the microglial cell. Scale bars 2 μm (D, E) and 4 μm (A–C, F).

Fig. 11. Attachment of hydrophobic polystyrene MBs by microglial BV-2 cell. Scanning electron microscopy of microglial cells exposed to different anion channel blockers, i.e., 5 μM DCPIB (A), 100 μM DIOA (B), 200 μM FFA (C), and 200 μM NPPB (D). Scale bars 4 μm .

flat engulfing pseudopodia. These extending engulfing areas followed the contour of the particle, overlapped at the area of contact and fused with each other. In contrast to mode one, particles were not invaginated, but protruded above the cell surface (Fig. 10).

Cells exposed to Cl^- -channel blockers behaved differently (Fig. 11); in contrast to control conditions, under these conditions cells failed to extend engulfment pseudopodia. Accordingly, fewer MBs are engulfed (Fig. 4). However, cells exposed to MBs in nominally Cl^- -free conditions revealed formation of lamellipodia and filopodia similar to control conditions despite their attenuated phagocytotic capacity (Fig. 3). This may indicate that swelling-activated Cl^- -channels are not selective to Cl^- , but are also permeable for other anions.

Fig. 12. Whole cell patch clamp technique was used to characterize anion currents evoked by dialyzing BV-2 cells with hypertonic (374 mosmol/L) internal solution *versus* an external isotonic solution (308 mosmol/L). (A) Maximally activated $I_{Cl,swell}$ of a cell containing at least three MBs (cell with MB, closed circles) and a cell without MB (open circles) in response to voltage-steps from -100 to +100 mV with 20-mV increments as shown in (B) and (C), respectively. Note the higher current amplitudes in the cell containing MBs. The current of the cell with MBs could be completely blocked with 33.3 μ M NPPB (closed diamonds). (D) Activation curves of the inward (-100 mV) and outward (+100 mV) current over time obtained from 500-ms voltage ramps from -100 to +100 mV at 10-sec intervals in cells with (rectangles; n = 5–9) and without MB (triangles; n = 2–8). Time point zero is the start of the superfusion with external isotonic solution (308 mosmol/L). (E) Maximally activated current of BV-2 cells without and with MBs. Control cells without MB are denoted as 'cells without MB'. (F) Long-term osmotically stressed cells were cultured for 12–16 h in hypertonic (400 mosmol/L) or hypotonic (200 mosmol/L) culture medium plus serum and are denoted as '12 h hyper' and '12 h hypo', respectively. Data are represented as mean $\pm$ s.e.m.. Asterisks (***) indicate $P < 0.001$. The numbers of individual experiments are depicted in the figure.

Particle uptake causes an increase in Cl^- -conductance

In hepatocytes, glucagon-induced catabolic processes are associated with an increase in Cl^- -conductance [15]. Accordingly, we expected an increase in $I_{Cl,swell}$ in microglial cells, because phagocytes degrade internalized particles, or because of an increase in the cell's volume if non-digestible particles accumulate within the cell. In our study, uptake of each MB with a diameter of 4 μ m added $\sim 34 \mu m^3$ to the CV. BV-2 cells were exposed to MBs for ~ 300 min, and $I_{Cl,swell}$ was subsequently measured. As shown in Figure 12, the kinetic of current development (Fig. 12D) and the maximum current density (Fig. 12E) measured in cells containing at least three MBs significantly differed from control cells without MBs. The

Fig. 13. Long-term hypertonicity and hypotonicity inhibited phagocytosis of MBs by microglial cell line BV-2. (A) Timetable as schematic illustration of cell procedure in anisotonic conditions. After culture of microglia for 12 h in anisotonic conditions (black bars), cells were exposed for 15 min (15') or 1 h (1h) in isotonic saline in the presence of MBs (white bars with black spheres). Another group of cells was exposed for 15 min in isotonic saline alone (grey bars) before addition of MBs to isotonic saline for 15 min (15'15') or 1 h (15'1h). Microglial cells cultured in hypertonic solution (B) and hypotonic solution (D) decreased phagocytosis of MBs after 15 min and 1 h, respectively, compared to incubation in extracellular solution (CO). Control groups were cultured in isotonic saline for 12 h and incubated for 15 min (15'CO) with MBs. Frequency distribution of microglial cells with phagocytosed MBs after culture in hypertonic (C) or hypotonic (E) solution are shown as columns representing the number of cells without MBs (white columns), cells successfully engulfed 1 MB (light grey columns), 2 MBs (black columns), and more than 2 MBs (dark grey columns). The number of cells with successfully engulfed MBs was counted in 3 independent experiments and evaluated using SEM. Data are represented as mean $\pm$ s.e.m.. Asterisks (*, **) indicate $P < 0.05$, $P < 0.01$ when compared with appropriate control groups.

slope of inward and outward current activation was steeper and the current density after ~ 9 min was approximately 2-fold higher in phagocytizing cells. Similar to $I_{Cl, swell}$ in control cells, the current in cells containing MBs was outwardly rectifying. Current densities of maximally activated $I_{Cl, swell}$ were 136.8 ± 15.4 and -40.7 ± 5.5 pA/pF at +100 and -100 mV in cells containing MBs versus 38.0 ± 4.9 and -11.0 ± 1.6 pA/pF in cells without MBs. The Cl^- current in cells containing MBs was sensitive to NPPB (Fig. 12 A), DIOA, FFA and DCPIB (data not shown). These findings indicate that particle engulfment up-regulates ion channels for $I_{Cl, swell}$ in the plasma membrane of BV-2 cells.

Pre-conditioning of BV-2 cells in hypotonic or hypertonic medium attenuates microsphere uptake

If the observed up-regulation of $I_{Cl,swell}$ is a consequence of an increase in CV or, comparably, an increase in intracellular osmotic pressure following degradation of a digestible particle, long-term osmotic challenges might affect phagocytotic capacity. Accordingly, we assumed that anisotonic pre-conditioning, which has been reported to alter the set point for CV regulation in different cell types [8], might modulate particle uptake. Figure 13A shows the schedule of our experiments. Cells were exposed to hypo- (200 mosmol/L) or hypertonic (400 mosmol/L) conditions for 12 h. Then they were allowed to undergo post-RVD RVI and post-RVI RVD, respectively, in normotonic conditions for 15 min. BV-2 cells were exposed to MBs in normotonic conditions for 15 min or 1 h. In some experiments, cells were first adapted to normotonic conditions for 15 min and then exposed to MBs for 15 min or 1 h. We did not observe significant alterations in $I_{Cl,swell}$ magnitude in BV-2 cells pre-exposed to hypotonic or hypertonic conditions (Fig. 12F). However, a significant decrease of MB uptake upon hypo-, or hypertonic pre-conditioning was detectable (Fig. 13). Cells cultured for 12 h in hypotonic and hypertonic conditions, respectively, revealed a decrease in phagocytosis of MBs. Cells conditioned in hypertonic medium showed a decrease by about 21% after incubation with MBs for 15 min and 1 h, respectively (Fig. 13B). At the single cell level, the decrease in MB uptake was visualized by an increase in the number of cells without MBs and a decrease in the number of cells containing MBs (Fig. 13C). Cells conditioned in hypotonic culture medium showed a decrease by ~30% after 15 min or by ~65% after 1 h compared to cells cultured in control medium (Fig. 13D). There was no difference between phagocytosis of cells exposed directly to isotonic solution or cells pre-incubated in isotonic solution for 15 min before addition of MBs. The frequency distribution revealed that after 15 min about 60% of cells did not contain a single MB, ~30% contained one and less than 10% two or more than two MBs. After 1 h, the majority of cells contained more than two MBs, and the minority contained no MB in control conditions (Fig. 13E).

Discussion

Our findings indicate changes in Cl^- -conductance in an early phase of phagocytosis as well as hours after particle uptake. In an early phase, the increase in Cl^- -conductance is essential for formation of engulfment pseudopodia. The late phase changes in Cl^- -conductance may be related to similarities between phagocytosis and osmoregulation. Both rely on the activation of a Cl^- -conductance and osmotic challenges modulate particle uptake.

Early consequences of $I_{Cl,swell}$ activation on phagocytosis

The earliest morphological change following contact between the phagocyte and a potential target is the formation of an engulfment pseudopodium. In the present study, we tie changes in Cl^- -conductance to engulfment pseudopodium formation. Changes in ion conductance in phagocytes following contact with a particle are a common phenomenon [28–31]. Cell-attached recordings from human macrophages during particle ingestion reveal inward and outward single channel currents, carried by Cl^- and K^+ -ions, respectively [30]. Since an inward Cl^- -current represents an efflux of Cl^- , K^+ as well as Cl^- leaves the cell. Similarly, in human PMNs, phagocytosis of opsonized *Candida albicans* induces a Cl^- -efflux [1]. Furthermore, in microglial cells and PMNs, application of Cl^- -channel blockers suppresses engulfment of polystyrene MBs, *E. coli*, *S. aureus*, or *C. albicans* [2–4, and present study]. As shown in Figure 3, phagocytosis in microglia is a chloride dependent process. Since phagocytosis leads to cell swelling, we focused in the present study on swelling activated Cl^- -current ($I_{Cl,swell}$). Activation of $I_{Cl,swell}$ is due to an osmotic imbalance, like hypotonic extracellular or hypertonic intracellular conditions [8, 32]. However, a Cl^- -conductance with a similar electrophysiological and pharmacological signature is also activated in normotonic conditions via an increase in intracellular Ca^{2+} or cAMP and elevated PKA activity or

stimulation of exchange proteins directly activated by cAMP (Epac) [15, 21, 33–36]. Thus, contact between a particle and a phagocyte could trigger an intracellular signalling cascade, which activates Cl⁻-channels in microglial cells, even at normotonic conditions. We found that formation of engulfment pseudopodia is prevented by blockade of $I_{Cl,swell}$. Thus, the most important function of $I_{Cl,swell}$ activation in the early phase of phagocytosis is its contribution to the formation of engulfment pseudopodia. Failure of engulfment pseudopodia formation prevents uptake of particles.

$I_{Cl,swell}$ is more sensitive to Cl⁻-channel blockers than the phagocytotic activity. For example, in our experiments ~4 μ M NPPB were required to block half of $I_{Cl,swell}$ (Fig. 1), whereas about ~40 μ M were necessary to suppress the overall MB uptake to 50% (Fig. 5B). However, a significant inhibition of cells able to phagocytose more than two MBs can be already observed at an NPPB concentration of 0.1 μ M and the estimated IC_{50} for this phenomenon is ~10 μ M (Fig. 5D), which corresponds to the IC_{75} for NPPB of $I_{Cl,swell}$ (Fig. 1). This indicates on the one hand the exquisite dependency of phagocytosis on Cl⁻-channels. On the other hand it raises the interesting question about the number of Cl⁻-dependent pathways, like Cl⁻-channels, which are required to initiate and maintain phagocytosis. Although we cannot answer this question it is tempting to speculate that a surplus of Cl⁻-channels is required for multiple uptake of MBs or for counterbalancing the increase in CV or osmotic pressure (see below).

Lamellipodia of migrating cells and engulfment pseudopodia of phagocytes share morphological and structural similarities, which may be rooted to similarities in their genesis. Specifically, in both structures swelling-activated Cl⁻ channels may contribute to local ion movements establishing local changes in CV. In migrating cells, ion pathways serving for local cell shrinkage (RVD), like e.g. K⁺-, Cl⁻- and Ca²⁺-channels are confined to the rear of the cell, whereas ion pathways leading to local cell swelling (RVI), like e.g. Na⁺/H⁺-exchangers, Cl⁻/HCO₃⁻-exchangers and Na⁺/K⁺/2Cl⁻-cotransporters are sorted to the leading edge. Because blockers of $I_{Cl,swell}$ may block Cl⁻-channels as well as KCCs, as DIOA does [3, 22, 26, 37], and Cl⁻-free conditions affects several Cl⁻-dependent transport proteins like KCCs, NKCCs [22, 38], the contribution of these different ion channels and transporters to engulfment pseudopodium formation remains to be characterized. Since all blockers used in the present study suppress at least Cl⁻-channel activity [3, 22, 37, 39] and lamellipodium formation, we conclude that Cl⁻-channels contribute significantly in lamellipodium formation. In addition, these ion transport mechanisms are tightly linked to the cytoskeleton and hence regulate the dynamics for adhesion and pseudopodia protrusion [40–42]. Whether a similar polarized distribution of ion channels and transporters is established during the formation of engulfment pseudopodia is, however, not known yet.

Late consequences of $I_{Cl,swell}$ on phagocytosis

Long-term consequences of phagocytosis could include enhancement of volume regulatory mechanisms and/or down-regulation of excess uptake of particles to prevent mechanical rupture of the cell membrane and/or threatening increases in intracellular osmotic pressure following degradation of phagocytosed particles. We assume that phagocytosis promotes long-term activation of $I_{Cl,swell}$ to counterbalance increases in intracellular osmolytes due to degradation and metabolism of phagocytosed material by CV regulatory ion fluxes. In liver cells, a catabolic signal is associated with water loss and shrinkage and an anabolic signal with water influx and swelling. In hepatocytes, a glucagon-cAMP-mediated pathway not only promotes glycogenolysis and gluconeogenesis, but also cell shrinkage, whereas insulin not only facilitates glucose uptake and glycogenesis but also cell swelling [43]. Interestingly, glucagon activates Cl⁻-channels in hepatocytes *via* a cAMP-Epac-dependent signalling pathway [15]. Similarly to hepatocytes, digestion of engulfed material by phagocytes may increase the intracellular osmotic pressure, which causes an

influx of water and cell swelling if not counterbalanced by fluxes of osmolytes across the plasma membrane. In the present study we found that uptake of MBs causes an increase in $I_{Cl, swell}$ density. This may reflect a long-term response of phagocytes to counterbalance an increase in CV or intracellular osmolytes by an increase in ion fluxes across the plasma membrane.

Long-term consequences of osmotic challenges on phagocytosis are documented. Warskulat and co-workers observed that 12 h exposure of Kupffer cells to hypoosmotic conditions increases phagocytosis of latex MBs, whereas exposure to hyperosmotic conditions decreases phagocytosis [44]. In contrast to our study, these authors exposed Kupffer cells to latex MBs in hypoosmotic and hyperosmotic conditions, respectively, whereas in the present study, BV-2 cells were pre-conditioned in hypoosmotic and hyperosmotic environment, but exposed to MBs in normotonic conditions. Neither condition induced a significant change in $I_{Cl, swell}$ density, indicating that the cells have adequately adapted $I_{Cl, swell}$ to the prevailing anisotonic condition and are able to adjust their set point volume. Nonetheless, the phagocytotic capacity is reduced after both hyper- and hypotonic preconditioning. From the present data we cannot give an explanation for this phenomenon. However, from a hermeneutic point of view it may be assumed, that a cell chronically exposed to anisotonic stress down-regulates processes, which pose further osmotic burden on it, like e.g. phagocytosis.

Most studies on phagocytosis focus on the cytoskeleton. The present study indicates that volume regulation due to $I_{Cl, swell}$ also significantly contributes to the uptake of particles. Since ion channels and transporters are linked to the actin cytoskeleton [45], ion channels in the plasma membrane and the cytoskeleton below the plasma membrane may cooperate to engulf particles.

In conclusion, our observations indicate that phagocytotic uptake of polystyrene microspheres by microglial cells is associated with activation of Cl^- -currents, because (i) under Cl^- -free extracellular conditions the uptake of microspheres is attenuated, (ii) Cl^- -channel blockers suppress the extension of engulfment pseudopodia as well as the uptake of microspheres, and (iii) engulfment of microspheres up-regulates $I_{Cl, swell}$ (iv). In addition, preconditioning of the cells to hypo- or hyperosmotic conditions decreases microsphere uptake.

Abbreviations

CLSM (confocal laser scanning microscopy); CV (cell volume); MB (microbead, microsphere); $I_{Cl, swell}$ (swelling-activated Cl^- -current/conductance); KCC (K^+/Cl^- -cotransporter); NKCC ($Na^+/K^+/2Cl^-$ -cotransporter); RVD (regulatory volume decrease); RVI (regulatory volume increase); post-RVD RVI (normotonicity-induced RVI under following hypotonicity-induced RVD); post-RVI RVD (normotonicity-induced RVD following hypertonicity-induced RVI); SEM (scanning electron microscopy).

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