Characterisation of K⁺ Currents in the C8-B4 Microglial Cell Line and their Regulation by Microglia Activating Stimuli

Simon Moussaud^{1,2}, Elisabeth Lamodire^{1,2}, Catherine Savage² and Henning J. Draheim²

¹University of Burgundy, INSERM U866, ²CNS Research Boehringer Ingelheim Pharma GmbH & Co KG, Biberach

Key Words

Microglia • Brain macrophage • Potassium channels
• Kv1.3 • Kir2.1 • Margatoxin • Patch-clamp

Abstract

Microglia are the intrinsic immune cells of the brain. As such, they are crucially involved in neuro-protection as well as neuro-degeneration. Their activation leads to the induction of cytokine and chemokine release, the production of reactive oxygen species and nitric oxide and an increased outward potassium conductance. In this study, we focus our interest on potassium currents and channels in the C8-B4 murine microglial cell line and compare them with those of primary cultured microglia from neo-natal mice. Using the whole cell patch-clamp technique, we have recorded prominent inward and outward rectifying voltage-dependent potassium currents but no calcium-activated potassium currents. Using pharmacological, biophysical and molecular approaches, we demonstrate that Kv1.3 and Kir2.1 channels underlie outward and inward rectifying potassium currents, respectively. In contrast to primary cultured microglia, we observe that an outward rectifying potassium current is already present in unstimulated C8-B4 cells.

However, as seen in primary microglia, this current increases after treatment with LPS, IFN- γ , TGF- β and GM-CSF and is suppressed by treatment with protein kinase inhibitors. Our study indicates that the C8-B4 cell line shows similar though not identical potassium channel properties compared to primary cultured microglia. We demonstrate that despite some differences, they are a useful tool to study potassium currents in microglial activation mechanisms by means of electrophysiological methods without the need for preparation of cells as primary culture.

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Introduction

Neuro-inflammation plays a crucial role in numerous neuro-pathologies (e.g. ischemia, hypoxia, AIDS dementia, chronic pain) and neurodegenerative diseases (e.g. Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, Creutzfeldt-Jacob disease) [1, 2].

As guardians of the CNS, microglial cells are at the front line of all these pathologies. Microglia are a cell

population of myeloid-monocytic origin that colonize the brain at different “developmental windows” [2, 3]. Microglia steadily survey their environment and can migrate and adopt different phenotypes in response to a multitude of stimuli. Microglia are usually beneficial. For example, they promote neuron survival by secreting growth factors (e.g. neurotrophin, nerve growth factor, brain-derived neurotrophic factor, glial cell derived neurotrophic factor, insulin-like growth factor 1) and anti-inflammatory cytokines (e.g. transforming growth factor beta, interleukin 1 receptor antagonist, interleukin 10). They scavenge toxins, unwanted inflammatory cells and cellular debris, and elicit neuro-plasticity by producing various proteases [4]. Yet in several pathological conditions, microglia can be detrimental due to exaggerated pro-inflammatory activity. Indeed, microglia can injure neurons through secretion of abnormal amounts of pro-inflammatory and pro-apoptotic cytokines (e.g. interleukin 1 beta, interleukin 6, tumor necrosis factor alpha) and through production of glutamate, reactive oxygen species and nitric oxide [5]. The research fields of different neuropathologies associated with inflammatory processes share the same big challenge: to modulate microglia activation in order to increase their beneficial effects whilst avoiding their damaging effects on neurons [6, 7].

Potassium (K^+) channels are involved in the regulation of membrane potential, cell volume and intracellular ion concentrations. Interestingly in immune cells, K^+ channels are also implicated in cell activation processes [8]. In microglia, K^+ current properties differ profoundly depending on the cellular state of activation [9–18]. Moreover, K^+ channels are linked to microglial activation processes like proliferation, migration, release of interleukin- 1β and production of reactive oxygen species [11, 19–23]. Thus K^+ channels are a potential target in view of modulation of microglial behaviour.

The presence of voltage-dependent and calcium-activated K^+ currents in microglia is well documented [10, 11, 20, 21, 24] but the molecular identities of the K^+ channels involved are still not clear. The Kir2.1 channel was identified as being responsible for voltage-dependent inward rectifying currents [10]. Kv1.3 and Kv1.5 voltage-gated channels are the most frequently proposed possibilities for the voltage-dependent outward K^+ current [14, 25]. But the distinction between the two channels is difficult to unravel for three reasons. Firstly, both have similar electrophysiological and pharmacological characteristics. Secondly, both were found in myeloid cells. Finally, they can form heteromers with hybrid properties [26, 27].

However, the vast majority of experiments were performed using primary cultured microglia from neonatal rodents. Strikingly, the little electrophysiological data available on microglial cell lines [16, 28] or on *in situ* microglia [25, 29, 30] shows inconsistencies in K^+ channel properties when compared to that of primary cultured microglia. Isolation of primary microglia is both difficult and time consuming and the various protocols used to prepare them are a source of discrepancy. Thus, microglial cell lines have often been used in the literature as microglial models. The C8-B4 microglia cell line is a spontaneous immortalized cell line derived from an 8-day mouse cerebellum organ culture. Consistent with primary microglia, C8-B4 cells have a similar ramified morphology and behave in a similar way in response to stimuli by producing large amounts of nitric oxide and glutamate. Furthermore, they express classical microglial markers such as F4/80, CD11b, CD16/32 and CD45 low [31–34].

Thus, we asked whether the C8-B4 cell line is a suitable surrogate model to study ion channel activity in microglia. Using electrophysiology and molecular biology, we investigated K^+ channels in C8-B4 cells with regards to their identity, modulation and function in microglial activation. We show that this cell line displays similar though not identical electrophysiological properties to primary cultured microglia from neo-natal rodents.

Materials and Methods

Cell culture and microglia treatment

The murine microglial cell line C8-B4 (ATCC, CRL-2540, Manassas, USA) was maintained at 37°C in 10% CO₂ in Dulbecco's modified Eagle's medium with 4.5g/L glucose supplemented with 10% foetal bovine serum and 4mM L-glutamine (Invitrogen, Karlsruhe, Germany). Cells were split once a week and tested frequently for mycoplasma contamination. Primary microglia were isolated from 4-day old C57BL6 mice as described by Draheim *et al.* [9]. Purity of primary microglia was controlled by flow cytometry using antibodies against CD11b (AbDserotec, Düsseldorf, Germany) and *Griffonia simplicifolia* isolectin B₄ (Invitrogen) and was routinely in excess of 95%. For experiments, cells were seeded on glass cover-slips in 24-well plates (2x10⁴cells/ml).

16 hours before measurement, the medium was changed and cells were treated with 100 μ M 4',5,7-trihydroxyisoflavone (genistein); 100 μ M 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7); 1 μ g/ml lipopolysaccharide (LPS) from *E. coli* strain K235 (all from Sigma, Munich, Germany); 100ng/ml murine recombinant interferon gamma (IFN- γ); 10ng/ml human recombinant transforming growth factor beta 1 (TGF- β); or 100ng/ml murine recombinant granulocyte and macrophage colony stimulating factor (GM-CSF). All cytokines were pur-

Gene	Accession no.	Forward primer (5' - 3')	Reverse primer (5' - 3')	Length (bp)
GAPDH	NM_008084.2	CCATCACCATCTTCCAGGAGC	GAAGGCCATGCCAGTGAGCT	481
Kir 2.1	NM_008425.3	CAACAGTGCAGGAGCCGCTTTG	GCATTGAGTTGTCA TGGCAGTCG	812
Kv 1.1	NM_010595.3	TCCAGGTTGCTTGACCCGTGTG	GGCTTCAGAGCCAGAAGGTTTCAG	636
Kv 1.2	NM_008417.4	TAGAGCACACCTCCAGGACCTGC	TTCCAGACAGAAGCTGACGATGG	867
Kv 1.3	NM_008418.2	GTACTTCTTCGACCGCAACCGAC	TTGTCGTTACGCCAGCTCAGTGC	615
Kv 1.4	NM_021275.3	CTTAGGATCATTCGTCTGGTCCGAG	GGACAGTGGCAGGTGGAGAGAAC	661
Kv 1.5	NM_145983.2	ATTGCCATCGTGTCTGGTCTTGG	AGCCTGCTCCTCGTGGTCTGTCT	839

Table 1. Primers used in reverse transcription PCR.

chased from R&D systems, Wiesbaden-Nordenstadt, Germany which guarantees them to be endotoxin free. For electrophysiological experiments, apamin, charybdotoxin, α -dendrotoxin, margatoxin, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), 4-aminopyridine (4-AP), 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one (DC-EBIO) and barium chloride (all from Sigma) were diluted in bath solution and applied directly to the cells by superfusion.

Electrophysiology

Whole-cell membrane currents were measured according to Hamill *et al.* [35] using an EPC-9 patch clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). Data acquisition and storage were carried out with TIDA software (HEKA) on a Compaq computer. Patch electrodes of 3-8M Ω were made with a micropipette puller (P97, Sutter, Novato, USA) from borosilicate glass capillaries (outer diameter 1.5mm and inner diameter 0.84mm; WPI, Sarasota, USA) and then fire-polished with a pipette forge (List-medical, Darmstadt/Eberstadt, Germany). The electrodes were filled with the following solution: 140mM KCl, 20mM NaCl, 2mM MgCl₂, 2.5mM CaCl₂, 10mM HEPES and 5mM EGTA. In some cases, a modified pipette solution containing 4.5mM CaCl₂ was also used to reach an intracellular free Ca²⁺ concentration of 1 μ M. Both solutions were adjusted to pH 7.35 with KOH. The extracellular solution contained: 5.5mM KCl, 140mM NaCl, 1mM MgCl₂, 1.8mM CaCl₂, 10mM HEPES and 35mM glucose. The pH of the bath solution was adjusted to pH 7.4 with NaOH. All experiments were performed at room temperature (20-22°C) and results are given as mean $\pm$ S.E.M., unless otherwise stated.

Western Blot analysis

C8-B4 cells were lysed and the protein concentration of each lysate was determined using a BCA protein quantification kit (Pierce, Rockford, USA). Proteins (30 μ g per lane) were separated on 4-12% polyacrylamide minigels (Invitrogen) and subsequently transferred to nitrocellulose membranes using a semi-dry transfer system (Bio-Rad, Munich, Germany). After blocking in buffer containing 20mmol/L Tris, pH 7.4, 500mmol/L NaCl, 0.1% Tween 20, and 5% bovine serum albumin for 90min, the membranes were incubated overnight at 4°C with rabbit polyclonal antibodies against Kv1.3 or Kv1.5 (1:500; Alomone Labs, Jerusalem, Israel) and with a mouse monoclonal antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Sigma) for the loading control. Specificity of the antibodies used was challenged with pre-incubation with

the control antigen. Furthermore, as positive control for Kv1.5 immuno-reactivity, 20ng of epitope-GST fusion protein was loaded in parallel to the samples. Infrared fluorescent dye-conjugated secondary antibodies (1:5000; Li-Cor, Bad Homburg, Germany) and the Odyssey infrared imaging system were used for two-colour multiplexed detection.

Immuno-fluorescence

Cells were fixed using a 4% formaldehyde solution in phosphate-buffered saline. After washing, cells were permeabilized and non-specific binding sites were blocked using 0.005% saponin and 1% bovine serum albumin in phosphate-buffered saline. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1h at room temperature. The following primary antibodies were used; rabbit anti-Kv1.3 (1:100; Alomone Labs), rabbit anti-Kir2.1 (1:100; Abcam, Cambridge, UK) and rat anti-CD11b/Mac1 (1:100; AbDserotec). Alexa 488 or 647 fluorophore-conjugated anti-immunoglobulin G were used as secondary antibodies (1:1000; Invitrogen). After a final washing step, the cover-slides were mounted and visualized using a DM IRBE inverted confocal microscope (Leica, Wetzlar, Germany).

Reverse transcription polymerase chain reaction

Total RNA was extracted from C8-B4 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). To avoid genomic DNA (gDNA) contamination, RNA was treated with DNase (Qiagen). Moreover, as a control, untranscribed RNA was also tested by PCR. Complementary DNA (cDNA) was synthesised with reverse transcriptase and random hexamer primers p(dN)₆ (Applied Biosystems, Foster City, USA). The cDNA was then used as a template for PCR reactions using gene-specific primers (Table 1). Amplification was carried out in a thermocycler (PeqLab Biotechnologies, Erlangen, Germany) using Taq-DNA polymerase (New England Biolabs, Frankfurt am Main, Germany). The amplified DNA fragments were loaded in 1.2% agarose gels containing 0.5 μ g/ml ethidium bromide (Invitrogen).

Data analysis and statistics

Data analysis and statistics were performed with GraphPad Prism 5.0 software (San Diego, USA). The statistical significance of differences between experimental groups was calculated by one-way analysis of variance test followed by Dunnett's post test. Differences were considered to be statistically significant with *P< 0.01.

Fig. 1. Comparison of voltage-dependent K^+ currents in primary microglia and the C8-B4 cell line. (A) Cells were patched in whole cell configuration and held at a holding potential of -60mV. Voltage-dependent currents were elicited with 50ms voltage steps from -150mV to +30mV in 10mV increments. (B) Representative recording of K^+ currents from a single C8-B4 cell. (C) 20 independent current measurements were averaged to construct current-to-voltage curves. K^+ currents in resting primary microglia ($\circ$) in comparison to primary microglia treated for 16h with 1 μ g/ml LPS ($\triangle$) or to unstimulated C8-B4 cells ($\blacksquare$). Values are given as mean $\pm$ S.E.M.; $n=20$.

Results

C8-B4 cells show microglia-like K^+ currents

Using the patch clamp technique, K^+ fluxes across the cell membrane of a single cell can be measured. C8-B4 cells were patched in whole cell configuration and held at a holding potential of -60mV. The K^+ current intensity was recorded at voltage steps from -150mV to +30mV in 10mV increments (Fig. 1A). Under these conditions, two prominent voltage-dependent K^+ currents were observed (Fig. 1B).

Between a holding potential of -150mV and -80mV, a strong inward K^+ current was measured. Then, between the equilibrium potential of K^+ and approximately -30mV, the cells had no measurable current. Finally from -30mV to +30mV, a prominent outward K^+ current was registered (Fig. 1C).

The inward rectifying K^+ current seen in C8-B4 microglia was similar to the one seen in primary cultured microglia from neo-natal mice (Fig. 1C). But in comparison to resting primary microglia, unstimulated C8-B4 cells also display a significant voltage-dependent outward K^+ current. It is noteworthy that a similar outward K^+ current was observed in primary microglia treated for 16 hours with 1 μ g/ml LPS (Fig. 1C).

Calcium-activated K^+ currents

Calcium-activated K^+ currents were investigated in order to determine whether C8-B4 cells have functional calcium-activated K^+ channels. To block SK and IK channel activities, TRAM-34 (3 μ M) or apamin (100nM) were used. Calcium-activated currents were triggered by challenging the cells with 1 μ M intracellular free calcium and/or 100 μ M DC-EBIO application. Neither DC-EBIO application nor 1 μ M intracellular free calcium stimulation were able to activate K^+ currents (data not shown).

Fig. 2. Characterisation of the voltage-dependent inward rectifying K^+ current. The inward rectifying K^+ current of C8-B4 cells was measured after application of increasing barium concentrations (0, 0.01, 0.1, 1, 10 and 100 μ M). (A) Example of a K^+ inward rectifying current measurement performed on a C8-B4 cell by voltage clamp before ($\circ$) and after application of barium at 1 μ M ($\blacktriangle$), 10 μ M ($\blacksquare$) and 100 μ M ($\blacklozenge$). (B) Dose-response curve comprising of the average of 13 independent measurements at a holding potential of -140 mV. The half maximal inhibitory concentration of barium was measured at 14 μ M.

We only observed that DC-EBIO and TRAM-34 at high concentrations slightly decreased voltage-gated K^+ current amplitudes. Thus, no significant calcium-activated K^+ current was observed in C8-B4 microglia.

The voltage-dependent inward rectifying K^+ current

The inward rectifying K^+ current was found in all measured unstimulated C8-B4 cells. This current was elicited by hyperpolarisation and started at a threshold potential of approximately -80mV (Fig. 1C). The density of this current had a mean value of $-10.5 \pm 2\text{pA/pF}$ ($n=20$) at a holding potential of -140mV . Thus, in unstimulated C8-B4 cells, the inward rectifying K^+ current was weaker than in resting primary microglia but larger than in activated primary microglia.

To better characterise the inward rectifying K^+ current, the K^+ channel blocker barium chloride was applied in the bath solution at the following concentrations: 0, 0.01, 0.1, 1, 10 and $100\text{ }\mu\text{M}$ (Fig. 2A). The inward rectifying K^+ current of C8-B4 cells showed a high sensitivity to barium ions. The blockade was fully reversible and dependent on the barium concentration. At a concentration of $100\text{ }\mu\text{M}$, the inhibition was nearly complete and a half maximal inhibitory concentration (IC_{50}) of $14\text{ }\mu\text{M}$ was determined (Fig. 2B). All these characteristics are in line with the assumption that $\text{Kir}2.1$ channels underlie the inward rectifying K^+ current of C8-B4 cells.

Pharmacological characterisation of the voltage-dependent outward rectifier K^+ channels

All measured C8-B4 cells displayed an outward K^+ current even without any prior treatment. This current was voltage-dependent and started at a threshold potential of approximately -30mV . The density of this current had a mean value of $14.7 \pm 2\text{pA/pF}$ ($n=20$) at a holding potential of $+20\text{mV}$ in unstimulated cells (Fig. 1C).

To identify the nature of the involved channel, a pharmacological profile of the outward K^+ current was established. Different classical channel blockers were applied via the bath solution to single cells patched in the whole cell configuration. The outward K^+ current was measured before and after application to calculate the relative inhibition as a percentage (Fig. 3A).

The outward K^+ current of C8-B4 microglia showed no significant sensitivity to apamin (100nM) and TRAM-34 ($3\text{ }\mu\text{M}$), excluding the implication of calcium-activated K^+ channels of the SK or IK family. Addition of 4-aminopyridine at a concentration of 1mM almost completely blocked the outward K^+ current ($91 \pm 1\%$).

Fig. 3. Pharmacological characterisation of the voltage-dependent outward K^+ current. The outward K^+ currents were elicited by depolarisation of the membrane from -60mV to $+20\text{mV}$ for 50ms. (A) The outward K^+ current density was measured before and after application of bath solution alone (control) or with the following: 100nM apamin, $3\text{ }\mu\text{M}$ TRAM-34, 1mM 4-aminopyridine, 300nM α -dendrotoxin, 100nM charybdotoxin or 100nM margatoxin. Values are given as mean $\pm$ S.E.M.; $n=5$; * $P < 0.01$. (B) Outward K^+ current density was measured before and after application of increasing concentrations of margatoxin (0.01 , 0.1 , 1 , and 10nM). The results of 11 independent experiments were averaged and the half maximal inhibitory concentration value of margatoxin on C8-B4 was determined to be 110pM .

Therefore the outward K^+ current of C8-B4 cells can be attributed to a voltage-gated channel of the shaker-related family ($\text{Kv}1.x$) [36]. α -Dendrotoxin (300nM), a potent $\text{Kv}1.1$, $\text{Kv}1.2$ and $\text{Kv}1.6$ channel blocker, had no effect on the outward K^+ current and thereby excluded the implication of the aforementioned three channels. Charybdotoxin and margatoxin, both at a concentration of 100nM , were able to block the K^+ outward current by $87 \pm 1\%$ and $96 \pm 1\%$, respectively. All these results are a good hallmark for the implication of either $\text{Kv}1.3$ and/or $\text{Kv}1.5$ channels.

Fig. 4. The outward K^+ current of C8-B4 cells is cumulatively inactivated. (A) K^+ currents of single cells were recorded at depolarizing voltage steps of +20mV (duration 1s). This voltage step was repeated 20 times and each record was spaced from the next by a 5s pause at a holding potential of -60mV. (B) Example of a current measurement. The peak amplitude of the outward K^+ current decreased pulse after pulse. (C) Extinction kinetic of the outward K^+ currents of C8-B4 microglia in response to a train of 20 depolarizing pulses. Values are given as mean $\pm$ S.E.M.; $n=30$.

To distinguish between Kv1.3 homomeric, Kv1.5 homomeric and Kv1.3/5 heteromeric channels, margatoxin sensitivity of C8-B4 cells was studied [9, 26, 27, 37].

The dose-response inhibition of margatoxin on the outward K^+ current was determined by sequential application of increasing concentrations of margatoxin (Fig. 3B). The density of the outward K^+ current was measured after each application. In response to increasing margatoxin concentrations, the outward K^+ current decreased. The inhibition described a sigmoidal curve with an IC_{50} value of 110pM which corresponds to the value of a pure Kv1.3 homomere channel [38].

Biophysical characterisation of the voltage-dependent outward K^+ current

Kv1.3 and Kv1.5 channels have different inactivation kinetics. Whereas Kv1.1, Kv1.2 and Kv1.5 channels are constantly open in response to a long depolarisation, Kv1.3 channels present a time-dependent inactivation.

Furthermore, this inactivation recovers slowly.

Therefore Kv1.3 activity presents a use dependent decrease in response to repeated depolarizing pulses called cumulative inactivation [16, 25, 27, 36].

To examine the inactivation kinetics of the outward K^+ current of C8-B4 microglia, cells were patched in whole cell configuration. The holding potential was set from -60mV to +20mV for 1s (Fig. 4A). In response to this voltage step, voltage-dependent K^+ channels opened, generating a large K^+ efflux (Fig. 4B). Less than 30ms after the onset of the stimulation, maximal amplitude was reached. Current amplitude then decreased slowly following an inverse hyperbolic time course. Thus, the involved channel has a very fast opening kinetic followed by a slow time-dependent inactivation.

After the 5s pause at physiological holding potential, the second electrical pulse failed to produce the same current amplitude as the first one and, pulse after pulse, the peaks became progressively smaller (Fig. 4B). The K^+ current decay fit a two-phase exponential curve. After a rapid decrease phase, the intensity of the current reached a plateau corresponding to 25% of its initial value (Fig. 4C). Taken together, these data show that the outward K^+ current of C8-B4 cells has a cumulative inactivation kinetic in accordance with the biophysical characteristics of Kv1.3 channels but not of Kv1.5 channels [38].

Molecular characterisation of K^+ channels

To compare our electrophysiological results and verify the presence of K^+ channels, molecular experiments were conducted on C8-B4 microglia. First of all, the expression of the Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5 and Kir2.1 channels was tested by reverse transcription PCR on unstimulated C8-B4 cells (Fig. 5A) using gene-specific primers (Table 1). Bands at the expected size were found for Kv1.3 and Kir2.1 demonstrating the expression of both genes in C8-B4. In contrast, no expression of Kv1.1, Kv1.2, Kv1.4 and Kv1.5 genes was seen.

Subsequently, a closer look at the protein level was taken using antibodies against Kir2.1, Kv1.3 and Kv1.5 channels. Western blot analysis revealed the presence of Kv1.3 protein and the absence of Kv1.5 protein in both unstimulated and stimulated C8-B4 cells (Fig. 5B). Following on from this, confocal immuno-fluorescence pictures confirmed the presence of Kv1.3 and Kir2.1 channels in C8-B4 cells and gave us information about their cellular localisation (Fig. 5C and D). Both channels had similar localisation, being predominantly in the cell body and partially co-localised with CD11b at the plasma mem-

Fig. 5. Molecular characterisation of K⁺ channels in C8-B4 microglia. (A) Ethidium bromide-stained gel showing reverse transcription PCR amplified products of GAPDH, Kir2.1, Kv1.1, Kv1.2, Kv1.3, Kv1.4 and Kv1.5, from 2 μ g of mRNA extracted from unstimulated C8-B4 microglia. GAPDH expression was used as a positive control (lane GAPDH) and gDNA contamination was excluded by performing reverse transcription PCR on untranscribed mRNA with the GAPDH specific primers (lane GAPDH-RT). (B) Western blot analysis of C8-B4 cell lysates. Prior to lysis, cells were incubated for 16 hours with fresh medium alone (unstimulated), or with fresh medium supplemented with the following: 1 μ g/ml LPS, 100ng/ml IFN- γ , a combination of both, 100ng/ml GM-CSF or 10ng/ml TGF- β . In the left panel, GAPDH was labelled in red and Kv1.5 in green. In the right panel, GAPDH is labelled in red and Kv1.3 in green (bands are marked with the arrow). (C and D) Confocal immuno-fluorescence pictures of unstimulated C8-B4 microglia. The red stain indicates anti-CD11b antibodies whilst the green stain indicates anti-Kv1.3 antibodies and anti-Kir2.1 antibodies, respectively. The cells display the characteristic ramified shape seen in resting microglia.

brane. Furthermore a punctate staining of the cytoplasm was observed suggesting vesicle localisation.

Regulation of inward and outward rectifying K⁺ currents and their role in cell activation

To investigate the regulation of inward and outward rectifying K⁺ currents, C8-B4 cells were activated *in vitro*. Classical microglial activators known to influence the inward and outward K⁺ currents in primary cultured microglia were tested [10]. As pro-inflammatory stimuli, the lymphokine IFN- γ and the potent immune reaction elicitor LPS from *E. coli* K 235 were used. Furthermore, a combination of LPS and IFN- γ was also used for maximal pro-inflammatory stimulation. Two other cytokines were also tested on C8-B4 cells. The first, GM-CSF, is a key growth factor of myeloid lineage which strongly induces microglial proliferation. The second, TGF- β , is known for its neuro-protective and anti-inflammatory

effects. Finally, to investigate the effect of protein kinases on the regulation of inward and outward K⁺ currents, C8-B4 cells were exposed to the tyrosine kinase inhibitor genistein, and the serine/threonine kinase inhibitor H-7.

To compare unstimulated and stimulated C8-B4 microglia, cells were incubated for 16 hours with the previously mentioned substances or with fresh medium alone as a control (Fig. 6). The cells were patched to measure their K⁺ currents. Similar to previous observations in primary cultured microglia [9-19], C8-B4 cells changed their voltage-gated inward and outward rectifying currents in response to various activating stimuli (Fig. 6). Activated C8-B4 cells displayed larger outward K⁺ currents than unstimulated cells (Fig. 6A). Treatment with LPS, IFN- γ or both together led to a 59%-73% increase in amplitude of the outward K⁺ current. In contrast, GM-CSF and TGF- β treatments induced a more than 2-fold increase in amplitude of the outward K⁺ current (Fig. 6A).

Fig. 6. Inward and outward rectifying K⁺ currents are modulated in response to cell activation and protein kinase activity. Cells were incubated for 16 hours with fresh medium alone (control) or with fresh medium supplemented with the following: 1 μ g/ml LPS, 100ng/ml IFN- γ , a combination of both, 100ng/ml GM-CSF, 10ng/ml TGF- β , 100 μ M H-7 or 100 μ M genistein. After treatment, C8-B4 cells were patched in the whole cell configuration. The results of 70 measurements were averaged in these histograms. (A) The outward K⁺ current density was measured during a 50ms voltage step to +20mV. (B) The inward K⁺ current density was measured during a 50ms voltage step to -140mV. Values are given as mean $\pm$ S.E.M.; n=70; * P< 0.01.

The regulation of the inward rectifying K⁺ current regulation was more complex (Fig. 6B). TGF- β was the only substance to increase the amplitude of the inward rectifying K⁺ current. On the other hand, LPS or IFN- γ treatment provoked a 23% and 34% decrease, respectively. Interestingly, when LPS and IFN- γ were combined, their effect on inward rectifying K⁺ currents appeared to be cumulative, with a larger decrease of 52% being observed.

To determine whether the increase of outward K⁺ current seen after cell activation was due to additional activity of another K⁺ channel or to an increase of Kv1.3

channel activity, the pharmacological and biophysical characterisations previously performed on unstimulated cells were repeated for each stimulatory condition (Table 2).

Unstimulated and activated microglia showed virtually identical pharmacological profiles. They were both insensitive to apamin (100nM), TRAM-34 (3 μ M) and α -dendrotoxin (300nM) application. Additionally they were both sensitive to 4-aminopyridine (1mM), charybdotoxin (100nM) and margatoxin (100nM). Furthermore, the margatoxin IC₅₀ values were all in the same range and close to those of Kv1.3 homomeric channels [38]. Finally, the outward K⁺ currents in all measured cells had the same cumulative inactivation kinetic. Thus, the properties of the outward K⁺ current of activated C8-B4 cells can also be attributed to a pure Kv1.3 homomeric channel activity.

To clarify whether the increase of outward K⁺ current seen after cell activation was due to an increase of Kv1.3 channel quantity, a western blot analysis was carried out. Unexpectedly, western blot analysis revealed no significant difference in Kv1.3 protein expression between the different conditions (Fig. 5B).

Given that Kv1.3 channel quantity stayed constant, regulation through phosphorylation mechanisms were investigated. Treatment with two protein kinase inhibitors, namely genistein and H-7, drastically reduced the outward K⁺ currents by 81% and 97%, respectively (Fig. 6A). Protein kinase inhibition also affected the inward rectifying K⁺ current, though to a lesser extent (Fig. 6B). These results are in line with phospho-regulation of Kv1.3 activity.

Discussion

As the resident immune cells of the brain, microglia are the focal point of many neuro-pathologies. K⁺ channels have been ascertained to be involved in microglial activation mechanisms. Thus far they have been studied mostly in primary cultured microglia and never been observed in the microglial cell line C8-B4.

The first aim of this study was to examine the electrophysiological characteristics of the C8-B4 cell line in comparison with primary microglia, with the focus of our interest being the identification of K⁺ channels present in these cells. Additionally, we investigated the regulation of these K⁺ channels in response to microglial activation.

Several previous reports described the presence of calcium-activated K⁺ currents in primary cultured micro-

	Unstimulated	LPS	IFN- γ	LPS + IFN- γ	TGF- β	GM-CSF
Cumulative inactivation	yes	yes	yes	yes	yes	yes
Apamin (100nM) inhibition in %	14 $\pm$ 6	8 $\pm$ 2	7 $\pm$ 6	9 $\pm$ 5	12 $\pm$ 5	12 $\pm$ 5
TRAM-34 (3 μ M) inhibition in %	15 $\pm$ 10	19 $\pm$ 5	26 $\pm$ 5	23 $\pm$ 4	25 $\pm$ 3	26 $\pm$ 6
4-Aminopyridine (1mM) inhibition in %	91 $\pm$ 1	92 $\pm$ 2	94 $\pm$ 1	93 $\pm$ 0.5	93 $\pm$ 1	93 $\pm$ 0.5
Dendrotoxin (300nM) inhibition in %	- 4 $\pm$ 7	2 $\pm$ 4	3 $\pm$ 4	-1 $\pm$ 4	8 $\pm$ 6	5 $\pm$ 2
Charybdotoxin (100nM) inhibition in %	87 $\pm$ 1	91 $\pm$ 2	92 $\pm$ 2	89 $\pm$ 1	90 $\pm$ 1	92 $\pm$ 1
Margatoxin (100nM) inhibition in %	96 $\pm$ 1	97 $\pm$ 0.5	97 $\pm$ 1	98 $\pm$ 0.5	98 $\pm$ 1	97 $\pm$ 1
Margatoxin IC50	110 pM	107 pM	104 pM	106 pM	99 pM	115 pM

Table 2. Comparison of activated and unstimulated C8-B4 microglia. % inhibition of the outward K⁺ current at +20mV after substance application, $\pm$ S.E.M.

glia, which were attributed to SK or BK channels [11, 20, 21] while others did not [9]. Intriguingly, in acute hippocampal slices, Schilling *et al.* did not detect calcium-activated K⁺ currents in primary murine microglia. However when the brain slices were cultured, calcium-activated K⁺ currents appeared [30]. In this study, C8-B4 microglia did not show any calcium-activated K⁺ currents.

Consistent with what has been described in primary cultured microglia [9-19, 24, 39], C8-B4 cells functionally express voltage-dependent K⁺ currents. Two distinct voltage-activated currents, inward and outward rectifying K⁺ currents, were recorded. Both currents appeared to be identical to the ones described in primary microglia. However, in contrast with primary cultured microglia, the outward K⁺ current in C8-B4 microglia was already present in unstimulated cells. As a control, we incubated primary cultured microglia from neo-natal mice with C8-B4-conditioned medium. C8-B4-conditioned medium did not induce the appearance of an outward K⁺ current (data not shown). Thus, the presence of a constitutive outward K⁺ current can not be explained by artefact stimulation such as a contamination or the presence of endotoxins or paracrine and autocrine stimulation. It is noteworthy that similar constitutive outward K⁺ currents were described in bone-marrow macrophages and in the J774.1, and RAW 264.7 cell lines [26, 27, 39, 40] and that C8-B4 cells were described to be “pre-activated” [32, 34].

Our assumption is that this constitutive outward K⁺ current might be linked to the continual proliferation of the C8-B4 cell line. This idea correlates with the fact that outward K⁺ currents are induced by proliferation. It is well known that primary cultured microglia patched on an astrocyte-layer [41] or incubated with astrocyte-conditioned medium also display an outward K⁺ current [12,

17]. Indeed, astrocyte-conditioned medium contains GM-CSF and TGF- β , both cytokines that are involved in microglial proliferation and deactivation, respectively, and are known to up-regulate the outward K⁺ current of primary cultured microglia [12, 16].

More recently, Schilling *et al.* demonstrated that murine microglia patched in acute brain slices displayed both inward and outward rectifying voltage-dependent K⁺ currents [30]. Furthermore, Draheim *et al.* noticed differences in the occurrence of inward and outward rectifying voltage-dependent K⁺ currents between embryonic and neo-natal as well as between rat and mouse cultured microglia [9, 15].

By using electrophysiological, pharmacological, biophysical and molecular methods, we identified the channels underlying the K⁺ currents observed. Concerning the inward rectifying current, its electrophysiological and pharmacological properties were close to the Kir2.1 voltage-gated channel [10]. Furthermore, Kir2.1 transcript and protein expression were detected in C8-B4 cells.

The outward K⁺ channel identification was more complex due to the number of potential candidates. The pharmacological profile of the outward K⁺ current gave evidence for voltage-gated K⁺ channels of the shaker-related family. Indeed, the outward K⁺ current was 4-AP, charybdotoxin and margatoxin sensitive. But α -dendrotoxin, at high concentration, failed to have a significant effect. These results exclude Kv1.1, Kv1.2 and Kv1.6 as the channels underlying the outward currents.

To distinguish between pure Kv1.3 homomeric channels, pure Kv1.5 homomeric channels, and Kv1.3/5 heteromeric channels, margatoxin sensitivity and the channel closing kinetic were investigated. Margatoxin had an IC50 value of 110pM which is in accordance with the

sensitivity of pure Kv1.3 homomeric channels [9, 26, 36, 38]. Further evidence for the presence of Kv1.3 channels in C8-B4 cells was the fast, time-dependent inactivation and the cumulative inactivation kinetic of the outward K⁺ current. Thus, from a functional point of view, we provide strong evidence for pure Kv1.3 channels being responsible for the outward K⁺ currents of unstimulated C8-B4 microglia.

To confirm our electrophysiological experiments and to look at the presence of non functional K⁺ channels, molecular methods were also used. Reverse transcription PCR analysis provided details on expression of K⁺ channels. Kv1.3 and Kir2.1 but no Kv1.1, Kv1.2, Kv1.4 and Kv1.5 transcripts were detected in unstimulated C8-B4 microglia. Western blot analysis and confocal microscopy immuno-fluorescence measurements confirmed the presence of Kv1.3 and Kir2.1 channels in C8-B4 microglia. The confocal immuno-fluorescence analyses gave clues to the localisation of Kv1.3 and Kir2.1 channels. Both were co-localised with CD11b at the plasma membrane. Some Kv1.3 and Kir2.1 fluorescence was also found in the cytoplasm in vesicle-like punctate staining.

The fact that Kv1.5 activity is not recorded by patch-clamping and not found by molecular techniques is an interesting though not surprising discovery. The presence and the activity of Kv1.3 and Kv1.5 channels in primary cultured microglia are well documented and discussed. Kv1.5-like currents have been measured in rat microglia [25] but not in murine microglia [9, 16, 30]. Furthermore, even if Kv1.5 was detected in cultured rat microglia by molecular methods, no Kv1.5-like currents were measured [19, 21]. Kotecha and Schlichter observed transitory Kv1.5 activity *in situ* in rat microglia and demonstrated that when the cells were maintained in culture, this Kv1.5 activity disappeared with time and was replaced by Kv1.3 activity in proliferating microglia [25]. Recently, Kv1.1 and Kv1.2 channels were also detected at the molecular level in the BV-2 cell line and in primary rat microglia [19, 22, 23]. In our opinion, these apparent differences reflect the discrepancies between the models used and the Kv channel redundancy.

In order to compare C8-B4 cells with primary cultured microglia, we also investigated the regulation of inward and outward rectifying currents with regard to the microglial activation state. As has been described in primary microglia [9-19], both voltage-dependent K⁺ currents were modulated in response to cytokines or bacterial components. Meanwhile the inward rectifying K⁺ current was reduced in presence of IFN- γ and/or LPS.

LPS, IFN- γ , TGF- β and GM-CSF treatments provoked prominent increases in amplitude of the outward K⁺ current in C8-B4 cells. Interestingly, the outward K⁺ current seemed to be regulated on a gradient. LPS and/or IFN- γ treatments turned the outward K⁺ current to its intermediate level whereas GM-CSF or TGF- β treatments turned it to the maximal level.

In addition, we demonstrated that the up-regulation of the outward K⁺ current amplitudes seen in stimulated C8-B4 cells was due to increased Kv1.3 homomeric channel activity and not to the activation of a different channel type. Strikingly, the increased amplitude of the outward K⁺ currents seen in stimulated C8-B4 were not linked to global channel quantity up-regulation. These results are in apparent contradiction with a transcriptional regulation of the Kv1.3 channel. Paradoxically, in LPS activated rat primary microglia, Fordyce *et al.* observed an increased amplitude of the outward K⁺ current and an enhanced Kv1.3 transcript quantity without any change in Kv1.3 protein expression [19].

Finally, we demonstrated that the tyrosine kinase inhibitor genistein and the serine/threonine kinase inhibitor H-7 strongly down-regulated the outward K⁺ current of C8-B4 cells. In current publications, H-7 but not genistein was found to down-regulate the outward K⁺ current of primary cultured microglia [16, 18]. This difference in kinase inhibitor sensitivity might reflect a signalling pathway difference in the regulation of the K⁺ currents in C8-B4 cells compared to primary cultured microglia. Nonetheless, the fact that the outward K⁺ current is abolished by protein kinase inhibitors and that Kv1.3 was also found in the cytoplasm of C8-B4 cells in vesicle-like structures suggests that Kv1.3 activity modulation is not only transcriptional but also linked to post-translational regulation processes such as phosphorylation and sequestration.

All our observations question the role of the outward K⁺ current occurrence as marker of "activated" microglia and reinforce the role of outward K⁺ currents in microglia activation and proliferation. Nevertheless, more investigations are needed to better understand the function of this current. It will be interesting to further investigate the differences between *in situ* and *in vitro* microglia, as well as between different species and developmental stages.

However, in the brain, Kv1.3 channels are essentially found in microglia and their inhibition dramatically blocks microglial pro-inflammatory activities and proliferation. Here, we have shown that the C8-B4 cell line has similar electrophysiological properties to *in vivo* and

in situ primary microglia. In comparison to these other models, C8-B4 cells are much simpler to use. Furthermore, due to their lack of calcium-activated K⁺ currents, C8-B4 microglia may be a useful tool for research on Kv1.3 or Kir2.1 in microglia. In consequence, C8-B4 cells may be a suitable surrogate model in screening scenarios that use automated patch-clamp, fluorescence or impedance assays to identify small molecules interacting with the Kv1.3 channel.

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