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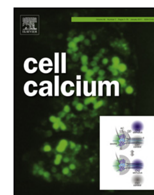

Extracellular acidosis impairs P2Y receptor-mediated Ca^{2+} signalling and migration of microglia

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A B S T R A C T

Microglia are the resident macrophage and immune cell of the brain and are critically involved in combating disease and assaults on the brain. Virtually all brain pathologies are accompanied by acidosis of the interstitial fluid, meaning that microglia are exposed to an acidic environment. However, little is known about how extracellular acidosis impacts on microglial function. The activity of microglia is tightly controlled by 'on' and 'off' signals, the presence or absence of which results in generation of distinct phenotypes in microglia. Activation of G protein coupled purinergic (P2Y) receptors triggers a number of distinct behaviours in microglia, including activation, migration, and phagocytosis. Using pharmacological tools and fluorescence imaging of the murine cerebellar microglia cell line C8B4, we show that extracellular acidosis interferes with P2Y receptor-mediated Ca^{2+} signalling in these cells. Distinct P2Y receptors give rise to signature intracellular Ca^{2+} signals, and Ca^{2+} release from stores and Ca^{2+} influx are differentially affected by acidotic conditions: Ca^{2+} release is virtually unaffected, whereas Ca^{2+} influx, mediated at least in part by store-operated Ca^{2+} channels, is profoundly inhibited. Furthermore, P2Y1 and P2Y6-mediated stimulation of migration is inhibited under conditions of extracellular acidosis, whereas basal migration independent of P2Y receptor activation is not.

Taken together, our results demonstrate that an acidic microenvironment impacts on P2Y receptor-mediated Ca^{2+} signalling, thereby influencing microglial responses and responsiveness to extracellular signals. This may result in altered behaviour of microglia under pathological conditions compared with microglial responses in healthy tissue.

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1. Introduction

Microglia are the resident macrophage of the brain and play a number of diverse and important functions during development, in health and disease [1,2]. As the only resident immune cell, microglia are also the first response unit to react to injury and assault, and initiate pro- and anti-inflammatory responses [3,4]. Under resting conditions, microglia are in a surveillance mode, in which they monitor the brain environment and enable proper brain function [5,6]. In response to an assault, however, they become activated and can assume a number of distinct phenotypes [4,7]. Many different extracellular chemicals come together to control the functional state of microglia, and it is thought that microglial activity is controlled by "on" and "off" signals [7]. "On" signals are extracellular messengers, which are normally not present, and whose presence triggers activation of microglia; these can be further divided into "eat me" and "help me" signals [3]. "Off" signals, on the other hand, are extrinsic factors that are present under resting conditions and promote the surveying microglial phenotype; they can be divided into "resting" and "do not eat me" signals [3].

Purines such as ATP, its break-down product ADP, as well as UTP and UDP, are key signalling molecules for microglia that are released from injured and dying cells and are seen as "on" signals by microglia. Activation of different types of purinergic G protein coupled (P2Y) receptors stimulates distinct signalling cascades, including G_q-mediated Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx into microglia (P2Y1, 2, 4, 6, 11 receptor subtypes), as well as G_s/G_i-induced increases/decreases in cAMP (G_s: P2Y11 receptor subtype; G_i: P2Y12, 13 and 14 receptor subtype) [8], which in turn trigger distinct responses in microglia, including phagocytosis and migration [7].

Acidification of extracellular pH occurs in the brain under physiological and pathological conditions. Activity-dependent pH changes in brain interstitial fluid were first reported over 60 years

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ago and occur over varying time courses [9,10]. Pathologically, extracellular acidosis occurs as a direct consequence of oxygen deprivation (e.g. in stroke/ischaemia, neurodegenerative diseases), cell death (e.g. stroke, neurodegeneration, trauma) and (altered) cell metabolism (e.g. cancer, immune cell activity) [11–14]. Hence, microglia are exposed to changes in extracellular pH, yet very little is known about how this affects microglial function.

This study addresses the impact that extracellular acidosis has on P2Y receptor-mediated intracellular Ca^{2+} signalling in microglia and functional consequence of extracellular acidosis on P2Y receptor-mediated migration of these cells. We find that extracellular acidosis impairs P2Y receptor mediated Ca^{2+} signalling and migration, providing a mechanistic explanation how microglia can be concentrated at sites of cell injury and death.

2. Materials and methods

2.1. Cell culture

C8B4 cells were obtained from ATCC and cultured in DMEM (2 mM L-glutamine), 10% foetal calf serum and 20 U/ml penicillin + 100 μg/ml streptomycin in 75 mm^3 cell culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. Medium was changed every 3–4 days and cells were subcultured when confluency reached 70%. For imaging experiments, cells were plated at 30–50% confluency on glass coverslips (13 mm diameter; four coverslips per 3.5 cm cell culture dish). C8B4 cells are murine cerebellar microglia that retained microglial markers and behaviour and are therefore an excellent model for studying microglial physiology (http://www.lgcstandards-atcc.org/products/all/CRL-2540.aspx?geo_country=gb#characteristics).

2.2. Fluorescence experiments

Solutions and fluorescence experimental setup were as described in Ref. [15]. Experiments in the absence of extracellular Ca^{2+} were performed in the additional presence of 0.1 mM EGTA. Fluorescence ratios were measured every 2 s.

MRS 2365, MRS 2693, MRS 4062, PSB 1114, MRS 2768 and NF 546 were purchased from R&D Systems, dissolved/diluted as required and stored in aliquots at −20 °C. Fresh aliquots were used for each new experimental day. Fura-2 AM was bought from Molecular Probes, Invitrogene. ATP (Mg^{2+} salt) was bought from Sigma Aldrich, dissolved in water and buffered with HEPES to pH7.2. BTP2 and synta66 were kindly provided by Prof. A. Parekh's lab; cells were exposed to these drugs following Fura 2 AM incubation for at least 30 min prior to the experiment and the drug of interest was present throughout the experiment. To test impact of P2Y receptor agonists at a given extracellular pH, agonists were dissolved in buffer at the required pH value and applied to cells following 100 s baseline recording to ensure stable read-out of baseline fluorescence levels. Control and test experiments were carried out on the same day and individual graphs only contain data obtained on the same experimental days.

2.3. Migration assay

Radius™ 24-well cell migration assay kit (Cell Biolabs, Inc.) was used as manufacturer's protocol. Extracellular pH was adjusted by adding 22, 5.5 or 2.2 mM sodium bicarbonate to culturing medium, yielding pH values of 7.4, 6.8 or 6.4 in 5% CO_2, respectively. Cells were allowed to migrate for 22 h before images were taken. Areas of migrated cells were analysed by Image J software.

To assess impact of store-operated calcium channel blocker BTP2 on P2Y receptor mediated cell migration, cells were plated into 6-well cell culture plate and allowed to grow to confluence. A scratch was made across the cell layer using a sterile pipette tip. After washing with PBS twice, DMEM medium containing 1 μM P2Y1 agonist MRS 2365 or 1 μM P2Y6 agonist MRS 2693 in the presence or absence of 10 μM BTP2 was added. Plates were photographed at 0 h and 24 h at the identical location of initial image. Results were analysed with software Image J.

2.4. RT-PCR experiments

Total RNA from C8B4 cells was extracted using RNeasy MiniKit (Qiagen) according to manufacturer's protocols; three distinct C8B4 preparations were used. First-strand complementary DNA (cDNA) was prepared from 1 μg of total RNA with the Superscript III Kit (Invitrogen, Carlsbad, California) in the presence of 1 μg of Oligo(dT). PCR was performed with primers (forward and reverse) specific for the mRNA encoding each of the following: P2Y1, P2Y2, P2Y4, and P2Y6 receptors and GAPDH.

For murine P2Y1, forward (5′-TTATGTGCAAGCTGCAGAGG-3′) and reverse (5′-CGGAGAGGAGAGTTGTCCAG-3′) primers were used to amplify a 381-bp fragment.

For murine P2Y2, forward (5′-TCCTCTTCCTCACCTGCATC-3′) and reverse (5′-GCGAAGACGGCCAGTACTAA-3′) primers were used to amplify a 400-bp fragment.

For murine P2Y4, forward (5′-CATCAACCTGGTGGTGACTG-3′) and reverse (5′-ACACATGATACGGCCTGTGA-3′) primers were used to amplify a 391-bp fragment.

For murine P2Y6, forward (5′-AGCATCCTGTTCCTCACCTG-3′) and reverse (5′-CTGCTACCACGACAGCCATA-3′) primers were used to amplify a 400-bp fragment.

For murine GAPDH, forward (5′-GTGCAGTGCCAGCCTCGTCC-3′) and reverse (5′-TTCAAGTGGGCCCCGGCCTT-3′) primers were used to amplify a 362-bp fragment.

The following protocol was used: (1) 95 °C for 5 min. (2) 20 cycles for GADPH and 30 cycles for the other genes using the following settings for each cycle: 95 °C for 30 s, 56 °C for 30 s and then 72 °C for 30 s. (3) 72 °C for 5 min. The RT-PCR products were then electrophoresed through a 8% polyacrylamide gel and subsequently visualised by ethidium bromide staining.

2.5. Data analysis

Waves containing fluorescence ratios (356 nm/380 nm) were analysed offline using Igor Pro and Microsoft Excel (2010). Cells were divided into responders (those with increases in fluorescence following exposure to agonist) and non-responders; these groups were used to calculate percentage of responding cells. Fluorescence signals in responding cells were analysed in terms of peak response (peak fluorescence ratio–basal fluorescence ratio just before application of drug) and time to peak (time lapse between application of drug and peak fluorescence response); in some cases, time to peak was further divided into time to respond (time lapse between drug application and start of fluorescence change following drug application) and rise time of peak (time lapse between start of fluorescence change and peak fluorescence change following drug application). Error bars represent SEM; n represents number of cells. Statistical analysis was carried out using InStat 2.03 (Macintosh) and Excel (Windows), and either ANOVA (for comparison of more than two mean values) or unpaired Student *t* test (for comparison of two mean values) were used.

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