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Microglial calcium signal acts as a rapid sensor of single neuron damage *in vivo* ☆

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ABSTRACT

In the healthy adult brain microglia, the main immune-competent cells of the CNS, have a distinct (so-called resting or surveying) phenotype. Resting microglia can only be studied *in vivo* since any isolation of brain tissue inevitably triggers microglial activation. Here we used *in vivo* two-photon imaging to obtain a first insight into Ca^{2+} signaling in resting cortical microglia. The majority (80%) of microglial cells showed no spontaneous Ca^{2+} transients at rest and in conditions of strong neuronal activity. However, they reliably responded with large, generalized Ca^{2+} transients to damage of an individual neuron. These damage-induced responses had a short latency (0.4–4 s) and were localized to the immediate vicinity of the damaged neuron (<50 μm cell body-to-cell body distance). They were occluded by the application of ATPγS as well as UDP and 2-MeSADP, the agonists of metabotropic P2Y receptors, and they required Ca^{2+} release from the intracellular Ca^{2+} stores. Thus, our *in vivo* data suggest that microglial Ca^{2+} signals occur mostly under pathological conditions and identify a Ca^{2+} store-operated signal, which represents a very sensitive, rapid, and highly localized response of microglial cells to brain damage. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

1. Introduction

Microglia are the resident immune cells of the CNS. They play a critical role in defending the structural and functional integrity of the central nervous system [1,2]. In turn, the CNS microenvironment critically impacts on phenotype of resting microglia [2]. In the healthy adult brain, these cells have a small cell body with several long, highly ramified processes, which are used for an active screening/palpation of their immediate neighborhood [3,4]. Because of this ability to actively survey their microenvironment, the microglia in the healthy CNS are often referred to as surveillant microglia. The phenotype of surveillant microglia is often termed "downregulated" because of a limited number of expressed surface molecules as well as their low expression levels [2]. An appearance of a pathological agent/event rapidly initiates a process of microglial activation. When activated, microglia change their morphology, upregulate a number of surface molecules, start secreting inflammatory mediators, and, finally, acquire the features of cytotoxic, phagocytic cells [1,5,6].

Microglia strongly rely in their function on intracellular calcium signaling. *In vitro* data have identified more than twenty ligands of various ionotropic and metabotropic receptors able to evoke receptor-mediated Ca^{2+} signals in microglia (reviewed in [7,8]). In addition, increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) can be induced by activation of store-operated Ca^{2+} channels [8–11] or by Na^+/Ca^{2+} exchangers operating in a reverse mode [12]. *In vitro* studies have suggested that elevations of $[Ca^{2+}]_i$ play a central role in the process of microglial activation (for a review, see [6,8,13,14]). Such Ca^{2+} elevations can be evoked by (i) lipopolysaccharides (endotoxins of the gram-negative bacteria [15]), (ii) purines and pyrimidines, as well as (iii) glutamate and (iv) the chemokine fractalkine released from injured cells (reviewed in [6,10,14,16,17]), (v) amyloidogenic substances accumulating in the brain in the course of a neurodegenerative disease (e.g., amyloid β [18,19]), etc. These receptor-mediated Ca^{2+} signals were shown to control executive functions of activated microglia such as release of trophic factors, NO as well as proinflammatory cytokines and chemokines [1,6,8,14,15].

It remains, however, unclear to what extent the data obtained in reduced preparations (cell cultures (see above)) and brain slices [20]) reflect the properties of surveillant microglia under normal physiological conditions. In *ex vivo* preparations, microglia almost invariably appear at different stages of activation, characteristic of pathology. This is because the isolation of brain tissue is accompanied by a tissue damage, which releases a host of substances triggering microglial activation. Furthermore, cell culture procedures often utilize the tissue from early neonatal brain, where invading microglia are still amoeboid and have not yet been transformed into a differentiated form found in the mature CNS [8,21,22]. Therefore, the mechanisms underlying Ca^{2+} signaling in adult surveillant microglia remained largely unknown.

Here we combined *in vivo* two-photon imaging with several cell-labeling techniques to monitor intracellular Ca^{2+} levels in cortical microglia. Our data show that surveillant microglia rarely generate

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spontaneous Ca^{2+} transients at rest or in conditions of strong neuronal activity but utilize Ca^{2+} release from the intracellular Ca^{2+} stores to signal a focal destruction of a single cell in its vicinity.

2. Materials and methods

All experimental procedures were performed in accordance with institutional animal welfare guidelines and were approved by the government of Baden-Württemberg, Germany.

2.1. In vivo two-photon imaging

Cells in the cortical layers 1–3 (80–160 μm below the cortical surface) were imaged by means of two-photon microscopy in heterozygous *CX$_3$CR1$^{GFP/+}$*, homozygous *CX$_3$CR1$^{GFP/GFP}$*, and wild-type (C57BL/6) mice. Surgery was performed as described in Refs. [23,24]. Briefly, the mice were placed onto a warming plate (38 °C) and anesthetized by inhalation of 1.5% isoflurane (Curamed, Karlsruhe, Germany) in pure O_2. The depth of anesthesia was assessed by monitoring the tail-pinch reflex and respiration rate. After removing the skin, the skull was gently thinned under a dissecting microscope using dental drills. The custom-made recording chamber with a hole in the middle [24] was then glued to the skull with cyanoacrylic glue (UHU, Buhl-Baden, Germany). The mouse was transferred to the setup, placed onto a warming plate (38 °C), and continuously supplied with 0.8–1.2% isoflurane in pure O_2. If necessary, the concentration of isoflurane was adjusted in the course of an experiment in order to keep a rather superficial level of anesthesia (respiration rates between 90 and 120 breaths per minute [25,26]). The recording chamber was perfused with warm (37 °C) extracellular perfusion saline containing (in mM) 125 NaCl, 4.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose, pH 7.4, when bubbled with 95% O_2 and 5% CO_2. A craniotomy (~1 mm) was performed above an area devoid of big blood vessels using a thin (30 G) syringe needle.

Two-photon imaging was performed with a custom-built two-photon laser-scanning microscope based on a mode-locked laser operating at 690- to 1040-nm wavelength (MaiTai HP DeepSee, Spectra Physics, Mountain View, CA) and a laser-scanning system (Olympus Fluoview 1000, Olympus, Tokyo, Japan) coupled to an upright microscope (BX61WI, Olympus, Tokyo, Japan) and equipped with a water-immersion objective (40×, 0.80 NA or 60×, 1.0 NA, Nikon, Tokyo, Japan). Enhanced green fluorescent protein (GFP) was excited at a wavelength of 930 nm, whereas Oregon green BAPTA 1 (OGB-1), Fluo-4, and Alexa Fluor 594 (AF 594) were excited at 800 nm. To separate the GFP fluorescence, we used excitation splitting making use of the fact that GFP is very efficiently excited at 930 nm but not at 800 nm [27]. The emitted light was further separated using a beamsplitter either at 515 nm (to separate GFP from the other dyes) or at 570 nm (to separate all dyes from AF 594). Most images were collected at a sampling rate of 4 frames/s. For simultaneous imaging of microglia, astrocytes, and neurons (e.g., Fig. 2C), a sampling rate of 10 frames/s was used. For determination of rise times and latency of microglial calcium transients caused by cell damage, the sampling rate was further increased to 20 frames/s. Unless otherwise indicated, all images shown are maximum intensity projections of ±3–5 μm, taken with a 1-μm step. The microphotographs shown in Figs. 1D, 3C, and 5C represent single-plane images.

Neurons and astrocytes were stained either with OGB-1 (e.g., Fig. 2C) or with Fluo-4 (e.g., Fig. 2A, both from Molecular Probes, USA) using multi-cell bolus loading technique (MCBL [23]). Briefly, the dyes (in the AM (acetoxymethylester) form) were dissolved in DMSO containing 20% Pluronic F-127 (w/v) to a concentration of 10 mM. The injection pipette was pulled using a standard protocol for patch-clamp electrodes (tip diameter 1 μm) and filled with the standard pipette solution of the following composition (in mM): 150 NaCl, 2.5 KCl, and 10 HEPES, pH 7.4, supplemented with 0.5 mM of Ca^{2+} indicator dye. The pipette was placed over the craniotomy and inserted into the brain. When the pipette reached the desired depth of 200 μm, the dye was pressure-ejected into the brain by applying a positive pressure (60 kPa for 2 min). Following dye ejection, the pipette was removed and the experiment commenced about half an hour later. Under these experimental conditions, Fluo-4 predominantly accumulated in astrocytes leaving neurons virtually unstained (see also Refs. [28,29]). In contrast, OGB-1 provided good staining of both neurons and astrocytes [24,30]. In such preparations, astrocytes were identified by their bright appearance [23,30], as well as their cell-type-specific morphology [24].

2.2. In vivo staining of microglia with isolectin B4

The isolectin B4 (IB4) from *Griffonia simplicifolia* seeds has been described as a microglial marker *in situ* [31,32]. For *in vivo* labeling of microglia with IB4, we slightly modified the MCBL protocol. We used Isolectin B4 conjugated to Alexa Fluor 594 (Molecular Probes, USA). To prepare the stock solution, we diluted the dye to a concentration of 500 μg/ml in the standard pipette solution (see above) supplemented with 0.1 mM $CaCl_2$. Frozen aliquots of the stock solution were stored at −20 °C. Immediately before staining, the stock solution was further diluted in the standard pipette solution to gain the final isolectin B4 concentration of 75 μg/ml. This solution was then injected (duration of application: 1.5 min; application pressure: 60 kPa) 200 μm under the cortical surface. This protocol allowed visualization of microglial cells (e.g., Fig. 1B) up to a depth of 300 μm. Besides microglia, Isolectin B4 also labeled endothelial cells of blood vessels, but these long tubular structures were easily distinguished from the microglia (see Fig. 1B).

2.3. In vivo labeling of microglia using single-cell electroporation technique

Microglial cells were stained with OGB-1 hexapotassium salt (Molecular Probes, USA) by means of single-cell electroporation [33]. Briefly, an electroporation pipette (tip diameter <1 μm) was filled with 10 mM OGB-1 dissolved in a solution containing (in mM) 140 K-Gluconate, 14 KCl, 4 NaCl, and 10 HEPES, pH 7.3. A GFP- or AF594-conjugated IB4-labeled microglial cell was targeted by the pipette. As soon as the pipette touched the microglial cell membrane, a negative current of 600 nA was applied for 10 ms using a MVCS-C-01 iontophoresis system (NPI Electronic, Tamm, Germany). After staining, the pipette was immediately withdrawn. Please note that this electroporation protocol is more gentle than those applied *in vivo* to electroporate neurons [33–36]. It allowed clear visualization of all microglial cell somata. Sometimes, however, the OGB-1 signal was too faint to enable visualization of tiny distal processes.

2.4. Drug application

Following pharmacological substances were used in this study: ATP, ATPγS (Adenosine 5′-O-(3-thiotriphosphate)), α-β-MeATP (α,β-methylene-ATP), Bz-ATP (Benzoylbenzoyl-ATP), 2-MeSATP (2-methylthioATP), 2-MeSADP (2-methylthioADP), UDP (Uridine 5′-diphosphate), carbachol, oxotremorine, glutamate, suramin, RB-2 (reactive blue 2), PPADS (pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulfonic acid), bicuculline (all obtained from Sigma-Aldrich, St. Louis, MO, USA); trans-ACPD ((±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid), thapsigargin (obtained from Tocris, Bristol, United Kingdom); Fractalkine (Recombinant Mouse CX_3CL1 Chemokine Domain, obtained from R&D systems, Minneapolis, MN, USA); KCl (solution contained (in mM): 80 KCl, 50 NaCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 CaCl, 1 MgCl, pH 7.4 when bubbled continuously with 95% O_2 and 5% CO_2).