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Research report

Calmodulin inhibition regulates morphological and functional changes related to the actin cytoskeleton in pure microglial cells

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ABSTRACT

The roles of calmodulin (CaM), a multifunctional intracellular calcium receptor protein, as concerns selected morphological and functional characteristics of pure microglial cells derived from mixed primary cultures from embryonal forebrains of rats, were investigated through use of the CaM antagonists calmidazolium (CALMID) and trifluoperazine (TFP). The intracellular localization of the CaM protein relative to phalloidin, a bicyclic heptapeptide that binds only to filamentous actin, and the ionized calcium-binding adaptor molecule 1 (Iba1), a microglia-specific actin-binding protein, was determined by immunocytochemistry, with quantitative analysis by immunoblotting. In unchallenged and untreated (control) microglia, high concentrations of CaM protein were found mainly perinuclearly in ameboid microglia, while the cell cortex had a smaller CaM content that diminished progressively deeper into the branches in the ramified microglia. The amounts and intracellular distributions of both Iba1 and CaM proteins were altered after lipopolysaccharide (LPS) challenge in activated microglia. CALMID and TFP exerted different, sometimes opposing, effects on many morphological, cytoskeletal and functional characteristics of the microglial cells. They affected the CaM and Iba1 protein expressions and their intracellular localizations differently, inhibited cell proliferation, viability and fluid-phase phagocytosis to different degrees both in unchallenged and in LPS-treated (immunologically challenged) cells, and differentially affected the reorganization of the actin cytoskeleton in the microglial cell cortex, influencing lamellipodia, filopodia and podosome formation. In summary, these CaM antagonists altered different aspects of filamentous actin-based cell morphology and related functions with variable efficacy, which could be important in deciphering the roles of CaM in regulating microglial functions in health and disease.

1. Introduction

Microglia originate from bone marrow-derived myeloid precursors as a unique class of the monocyte/macrophage lineage that infiltrates the central nervous system (CNS) during its early development (Ginhoux et al., 2010; Saijo and Glass, 2011). They respond rapidly to inflammatory cues and injury by transforming from a ramified, resting state to an activated, phagocytic ameboid cell type (Kreutzberg, 1996). In their non-activated or resting state, they display a ramified morphology and subdued macrophage-like functional properties. In response to injury, infection, inflammatory or other signals, the microglia become activated and a series of morphological, molecular and functional changes take place that affect proliferation, homing and adhesion to damaged cells, phagocytosis, antigen presentation and cytotoxic and inflammation-mediating signaling (Drew and Chavis, 2000; Prinz and Priller, 2014; Saijo and Glass, 2011; Streit et al., 1999; Town et al., 2005).

Microglial functions such as motility and phagocytosis are closely associated with dynamic changes in the cytoskeleton and related to intracellular calcium (Ca^{2+}) signaling (Greenberg, 1995; Kalla et al., 2003; Mitchison and Cramer, 1996). The ubiquitous Ca^{2+}-binding proteins participate in Ca^{2+}-elicited intracellular events, either as Ca^{2+}-sensing/receptor/trigger or as

Abbreviations: Ca^{2+}, calcium ion; CALMID, calmidazolium, 1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorobenzyloxy)ethyl]-1H-imidazolium chloride; CaM, calmodulin; CNS, central nervous system; DIV, days in vitro; DMEM, Dulbecco's Modified Eagle's Medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); Iba1, ionized calcium binding adaptor molecule 1; Ki67, proliferation marker antigen identified by the monoclonal antibody Ki67; LPS, bacterial lipopolysaccharide; mRNA, messenger ribonucleic acid; PBS, phosphate-buffered saline; RT, room temperature; S.E.M., standard error of mean; subDIV, subcloned days in vitro; TBS, tris-buffered saline; TFP, trifluoperazine, 10-[3-(4-methylpiperazin-1-yl) propyl]-2-trifluoromethyl-10H-phenothiazine dihydrochloride; TI, transformation index.

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Ca^{2+}-buffering/transport proteins, by binding intracellularly stored Ca^{2+} (Ikura, 1996). They contribute to nearly all aspects of the functioning of the cell, and are important in numerous intracellular signaling processes, from the regulation of cellular homeostasis to learning and memory (Berridge et al., 2000; Clapham, 2007). Calmodulin (CaM), one of the most important intracellular Ca^{2+} receptors, exerts its biological action through its heterogenous population of target proteins, which are involved in a number of cellular regulatory processes (Kennedy, 1989; Palfi et al., 2002).

The nervous tissue is especially abundant in CaM. While its distribution has been characterized in detail for a number of neuronal cell types (Kovacs and Gulya, 2002, 2003; Palfi et al., 1999, 2001, 2005), its localization and functions in glial cells are much less known. Astrocytes express CaM protein in low quantities (Kortvely et al., 2003), but mRNA populations from all three CaM genes could still be localized both perinuclearly and in the astrocytic endfeet (Palfi et al., 2005). The expression of CaM in oligodendroglia is similarly low and has not been characterized extensively, albeit the regulatory effects of this protein on a number of membrane-bound target proteins such as the myelin basic protein (Libich and Harauz, 2008) or the 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Myllykoski et al., 2012) have been established. Of all the glial components, only the microglia seem to have a considerable amount of CaM. They express a relatively large amount of CaM when activated (Casal et al., 2001; Solá et al., 1997), and many aspects of their Ca^{2+} signaling are well documented (Färber and Kettenmann, 2006; Wong and Schlichter, 2014).

CaM immunoreactivity or CaM gene-specific transcripts are often colocalized with those of the target enzymes of CaM within the same cytoplasmic compartments (Erondu and Kennedy, 1985; Sanabria et al., 2008; Seto-Ohshima et al., 1983; Strack et al., 1996). For example, actin is accompanied by CaM in the cell cortex, helping to remodel the actin-based cytoskeleton in accordance with the actual (patho) physiological signals (Mitchison and Cramer, 1996; Psatha et al., 2004). Ionized calcium-binding adaptor molecule 1 (Iba1) is another intracellular Ca^{2+}-binding protein with actin-binding capability that is expressed in macrophages and microglia, and is widely used to detect both resting and activated microglial phenotypes (Imai et al., 1996). CaM and Iba1 proteins share a number of molecular structural variants that are related to either their Ca^{2+} binding or their target protein recognition (Yamada et al., 2006). In contrast with the wide-ranging regulatory roles of CaM, Iba1 plays a much more restricted role in microglial functions, e.g. remodeling the actin cytoskeleton during migration (Siddiqui et al., 2012; Vincent et al., 2012).

The modulatory action of Ca^{2+}-bound CaM on multiple target proteins can be regulated by a number of compounds. Calmidazolium (CALMID; 1-[bis(4-chlorophenyl) methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorobenzyloxy)ethyl]-1H-imidazolium chloride) and trifluoperazine (TFP; 10-[3-(4-methylpiperazin-1-yl)propyl]-2-trifluoromethyl-10H-phenothiazine dihydrochloride) are potent inhibitors of CaM-related cellular activities (Borsa et al., 1986; Sunagawa et al., 2000). It is presumed that, apart from binding to the CaM protein (Matsushima et al., 2000; Vandonselaar et al., 1994; Vertessy et al., 1998), they can also exert their effects on some of the CaM-regulated targets directly (Sunagawa et al., 2000).

In contrast with the extensive studies on the involvement of CaM in a number of neuronal phenomena, only limited information is available on its role in the development and maintenance of the microglial phenotype and its specific functions. Relatively little is known, for example, as concerns the possible involvement of CaM mediation in such important microglial functions as phagocytosis and the cellular functions associated with it, e.g. dynamic cytoskeletal reorganization. Thus, in view of the importance of CaM-mediated cell functions and the paucity of data on specific microglial functions related to and possibly regulated by CaM, we set out to investigate the localization and intracellular distribution of CaM in pure microglial cell populations derived from rat primary mixed forebrain cultures by using immunocytochemical and Western blot techniques. Selected CaM inhibitors such as CALMID and TFP, previously reported to have different modes of action (Matsushima et al., 2000; Sunagawa et al., 2000), were quantitatively tested for their ability to modify the microglial morphology, lamellipodia, filopodia and podosome formation, and specific functions such as cell proliferation and survival, protein expression and phagocytosis in unchallenged (control) and lipopolysaccharide (LPS)-challenged cells. Stimulation with LPS was used to evaluate the ability of microglial cells to respond to activation (Fricker et al., 2012; Song et al., 2014; Tokes et al., 2011).

2. Material and methods

All animal experiments were carried out in strict compliance with the European Council Directive (86/609/EEC) and EC regulations (O.J. of EC No. L 358/1, 18/12/1986) regarding the care and use of laboratory animals for experimental procedures, and followed the relevant Hungarian and local legislation requirements. The experimental protocols were approved by the Institutional Animal Welfare Committee of the University of Szeged (I-74-11/2009/MÁB). The pregnant Sprague-Dawley rats (180–200 g) were kept under standard housing conditions and fed ad libitum.

2.1. Antibodies

The antibodies used in the immunocytochemical and Western blot studies are listed in Table 1. For a thorough characterization of different microglial phenotypes developed in vitro, an antibody against Iba1, an intracellular actin- and Ca^{2+}-binding protein expressed in the CNS specifically in macrophages and microglia (Imai et al., 1996; Ahmed et al., 2007), was used in our immunocytochemical and Western blot analyses. An anti-CaM monoclonal antibody was used to detect both Ca^{2+}-bound and Ca^{2+}-free forms of the antigen (Sacks et al., 1991). The anti-Ki67 antibody was used to detect proliferating cells. Ki67 is a nuclear protein expressed in all active phases of the cell cycle from the late G1 phase through the end of the M phase but is absent in non-proliferating and early G1 phase cells (Scott et al., 2004). The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as an internal control in Western blot experiments (Wu et al., 2012). Dilutions of primary and secondary antibodies, and also incubation times and blocking conditions for each antibody used were carefully tested for both immunocytochemistry and Western blot analysis. To detect the specificities of the secondary antisera, omission control experiments (staining without the primary antibody) were performed. In such cases, no fluorescent or Western blot signals were detected.

2.2. Preparation of primary mixed cortical cell cultures

Mixed primary cortical cell cultures were established from embryonic day 18 (E18) wild-type rat embryos by the use of the methods described previously (Szabo and Gulya, 2013). Briefly, 6–8 fetal rats under deep ether anesthesia were surgically decapitated and the frontal lobe of the cerebral cortex was removed, minced with scissors, and incubated in 9 ml Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 1 g/l D-glucose, 110 mg/l Na-pyruvate, 4 mM L-glutamine, 3.7 g/l $NaHCO_3$, 10,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate and 25 μg/ml amphotericin B, and supplemented with 0.25% trypsin (Invitrogen) for 10 min at 37 °C, then centrifuged at 1000 × *g*