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The L-type voltage-gated calcium channel modulates microglial pro-inflammatory activity

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ABSTRACT

Under pathological conditions, microglia, the resident CNS immune cells, become reactive and release pro-inflammatory cytokines and neurotoxic factors. We investigated whether this phenotypic switch includes changes in the expression of the L-type voltage-gated calcium channel (VGCC) in a rat model of N-methyl-D-aspartate-induced hippocampal neurodegeneration. Double immunohistochemistry and confocal microscopy evidenced that activated microglia express the L-type VGCC. We then analyzed whether BV2 microglia express functional L-type VGCC, and investigated the latter's role in microglial cytokine release and phagocytic capacity. Activated BV2 microglia express the $Ca_V1.2$ and $Ca_V1.3$ subunits of the L-type VGCC determined by reverse transcription-polymerase chain reaction, Western blot and immunocytochemistry. Depolarization with KCl induced a Ca^{2+} entry facilitated by Bay k8644 and partially blocked with nifedipine, which also reduced TNF-α and NO release by 40%. However, no nifedipine effect on BV2 microglia viability or phagocytic capacity was observed. Our results suggest that in CNS inflammatory processes, the L-type VGCC plays a specific role in the control of microglial secretory activity.

1. Introduction

Microglial reaction is an early event which often precedes and triggers neuronal death in CNS pathological situations such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, multiple sclerosis, ischemia and brain trauma (Graeber and Streit, 2010). As a response to signals released during injury, this microglial transition from a surveillance state towards a more reactive one represents an important phenotypic change (Colton and Wilcock, 2010; Domercq et al., 2013; Kettenmann et al., 2011). As a result, reactive microglia releases pro-inflammatory cytokines and neurotoxic factors, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and reactive oxygen species, the excessive production of which may trigger or exacerbate neuronal death (Liao et al., 2012; Zhang et al., 2011). In addition, some microglial cells become increasingly dysfunctional as they age, and may participate directly in the development of secondary tissue injury and neurodegeneration (Damani et al., 2011; Liao et al., 2012). Therefore, control of microglial activation should bring therapeutic benefit for any central disorder related with neuroinflammation. A growing body of evidence suggests that ion channels expressed by microglia are modulators of cell functions, and highlights their direct involvement in these neuropathologies (Kettenmann et al., 2011; Ortega et al., 2012a, 2013; Rodríguez et al., 2013; Virgili et al., 2011, 2014).

Electrophysiological studies suggest that microglia express a voltage-gated calcium channel (VGCC) which mediates Ca^{2+} entry (Colton et al., 1994; Hegg et al., 2000; Silei et al., 1999). Structurally, all VGCC family members are organized as heteromeric complexes of five subunits: α1, α2, β, δ, and γ (Zamponi and Snutch, 2013). The α1 subunit is the pore-forming component and functions as the voltage sensor (Catterall et al., 2005). The heterogeneity of each subunit gives rise to different VGCC pharmacological and electrophysiological properties and to the classification of the P-, Q-, N-, L-, R-, and T-type channels (Catterall et al., 2005). The α_{1C} ($Ca_V1.2$) and α_{1D} ($Ca_V1.3$) genes encode the α1 subunit of the L-type Ca^{2+} channel, which was first recognized as essential for coupling excitation to contraction in skeletal, cardiac, and smooth muscle cells (Lipscombe et al., 2004). The L-type VGCC is also expressed in neurons and endocrine cells where it regulates a diversity of processes including neurohormone and transmitter secretion, gene expression,

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; DAB, diaminobenzidine; DG, dentate gyrus; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; IB4, isolectin B4; IFNγ, interferon-gamma; IL-1β, interleukin-1β; KW, Kruskal–Wallis; LPS, lipopolysaccharide; LSD, least significant difference; MDP, mean deviation product; NGS, normal goat serum; NMDA, N-methyl-D-aspartate; PBS, phosphate buffer saline; SEM, standard error of the mean; TNF-α, tumor necrosis factor alpha

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mRNA stability, synaptic efficacy, and the activity of other ion channels (Ashcroft et al., 1994; Bading et al., 1993; Bean, 1989).

The L-type VGCC, which has a role in neuronal survival and ischemia-induced axonal injury, is considered a target for therapies designed to interfere with neuropathological processes. Thus, L-type VGCC blockade with dihydropyridine or nimodipine reduces neuronal damage and improves behavioral outcome accompanied with excitotoxicity, traumatic brain injury and ischemia (Bernal et al., 2009; Mattsson et al., 1999; Zhu et al., 1999). However, calcium antagonist treatments in hypoxia-ischemia models have an effectiveness of only 50% (Horn et al., 2001) and these same VGCC blockers present contradictory results in a large number of calcium-mediated excitotoxic processes (Berg et al., 1995; Bernal et al., 2009; Frandsen et al., 1993; Fryer et al., 1999; Horn et al., 2001; Petegnief et al., 2004; Rami and Krieglstein, 1994; Small et al., 1997). Inside this framework, the characterization of microglial expression of the L-type VGCC after brain injury will help to establish why the channel blockade therapy remains so challenging in experimental and clinical assays.

In this paper we postulated that phenotypic activation of microglia during injury alters L-type VGCC expression, resulting in a modification of intracellular microglial events such as cytokine release and phagocytosis. We investigated activated microglia in our in vivo experimental model of neurodegeneration and in in vitro cultures of the BV2 microglial cell line. In our rodent model, N-methyl-D-aspartate (NMDA) is stereotaxically microinjected into the hippocampal formation to induce a neurodegenerative process involving neuronal necrosis and a potent inflammatory reaction mainly mediated by microglia (Ortega et al., 2012b; Rodríguez et al., 2013). BV2 cell culture allows the determination of the function of the L-type VGCC in microglia activity. Our results suggest a major role for the L-type VGCC in specifically controlling microglial pro-inflammatory activity in CNS pathologies.

2. Results

2.1. Reactive microglia express $Ca_v1.2$ channels after NMDA-induced hippocampal lesion

As described above, 20 nmol NMDA administration in the rat hippocampus induced a neurodegenerative process characterized at 15 days by a severe necrotic neuronal death and microglial reaction (Fig. 1). Microscope observation of Nissl-stained sections revealed major NMDA-induced layer disorganization, neuronal loss and microgliosis in all layers of the hippocampal formation, including oriens, pyramidal, lacunosum-moleculare and radial strata of CA1, as well as in granular layers of the dentate gyrus (DG) (Fig. 1A–B). This lesion covered an area of 3.2 ± 0.48 mm^2 (55% of the whole hippocampal formation area, Fig. 1G). Counting of NeuN-immunopositive neurons indicated a mean 64% neuronal loss, affecting most hippocampal subfields and part of dentate gyrus (Fig. 1C–D). A maximum of 79 ± 4% effect was reached in the CA1 pyramidal layer (Fig. 1H), whose neuronal density (neurons/mm^2), compared to the sham group, was reduced from 2154 ± 55 to 201 ± 6. In this hippocampal subfield, surviving neurons lost their characteristic pyramidal morphology and presented an abnormal shape of their soma and dendritic tree (detail in Fig. 1C,D).

IB4 immunohistochemistry showed abundant microglia with reactive morphology, hypertrophy, hyperplasia and high IB4 immunostaining distributed over the entire lesioned area (Fig. 1E–F), which evidenced a lesion-associated microgliosis. The area of this microgliosis reached 5.1 ± 0.97 mm^2 (87% of the whole hippocampal formation, Fig. 1I). Morphology of IB4-labeled cells changed with their location and proximity to the injection site. The small cell body and several ramifications of resting microglia in healthy tissue (detail in Fig. 1E) changed progressively into a mixed reactive-amoeboid morphology in the core of the lesion (detail in Fig. 1F). Counting of IB4-positive cells indicated a mean 46 ± 8% ($p = 0.03$) increase in the number of microglial cells in the lesioned hippocampus (not shown).

In control rats, immunohistochemistry with anti-rat α_{1C} antibody labeled the pyramidal CA1–CA3 cell layers of the hippocampus and the DG granular cell layer. Specific α_{1C}-immunoreactivity was also evident in the dendrites of the stratum radiatum and, less densely, in the stratum lacunosum-moleculare of CA1 (Fig. 1J). The intense neuronal loss and layer disorganization induced by NMDA microinjection modified the distribution of hippocampal staining with a reduction in the pyramidal CA1 and granular DG layers which almost disappeared in the radiatum stratum of CA1 (Fig. 1K). Surviving neurons that still expressed α_{1C} showed an abnormal morphology in pyramidal layers (Fig. 1K1). However, non-neuronal specific anti-rat α_{1C} immunostaining (Fig. 1K2) was observed homogeneously distributed in all lesioned hippocampal cell layers. Confocal microscopy and image analysis of double histochemistry with anti-α_{1C} antibody and IB4 revealed a clear expression of $Ca_v1.2$ channels in most amoeboid reactive microglia of the lesioned hippocampal formation (Fig. 2D–F,K). Image analysis revealed specific anti-α_{1C} antibody labeling located at the cytoplasma and the plasma membrane of 36 ± 8% IB4 positive cells (Fig. 2D–F). The mean values of co-localization parameters from MDP images were: Pearson's $r = 0.430$, overlap coefficients $R = 0{,}589$, $k1 = 0.712$, and k2 0.675 ($n = 6$ different MDP images from 3 lesioned rats) (Fig. 2G–I). Specific anti-α_{1C} staining did not co-localize IB4 staining in some reactive cells of the lesioned hippocampus. Co-localization of anti-α_{1C} antibody and IB4 staining was not found in the hippocampus of control rats (Pearson's $r = 0.065$, $R = 0.462$, $k1 = 0.699$, and $k2 = 0.304$; $n = 6$ different MDP images from 3 control rats) (Fig. 2A–C, J).

2.2. BV2 microglia express α_1 subunits of L-type VGCC

To study the functions of the L-type VGCC in microglial cells in vitro, we first analyzed the expression of $Ca_v1.2$ and $Ca_v1.3$ channels by RT-PCR analysis of total RNA extracted from three different cultures of the activated murine microglia-derived cell line BV2. We examined the expression of the L-type VGCC by analyzing distinct pore-forming α_1 subunit PCR products. The PCR products for α_{1C} ($Ca_v1.2$) and α_{1D} ($Ca_v1.3$) found in the RNA preparation of BV2 were comparable to those obtained from whole brain RNA preparations used as positive controls (Fig. 3A–B).

The presence of α_{1C} protein in microglial BV2 was studied by Western blotting of the membrane protein extracts isolated from three different cultures of BV2 cells. A positive signal was observed at 240 KDa (Fig. 3C), indicating the presence of the L-type VGCC in the microglial membrane. Compared to quiescent cells, densitometry quantification evidenced a significant 63 ± 7% increase ($n = 5$ different cultures; $p = 0.0072$) in the expression of α_{1C} protein in BV2 cells activated with LPS and IFNγ during 24 h. Immunofluorescence localized α_{1C} in BV2 cells and complemented western blotting and PCR results (Fig. 3D–F). Specific α_{1C} immunoreactivity was clearly located at the plasma membrane and characteristic membrane processes of BV2 cells stimulated with LPS + IFNγ (Fig. 3F), whereas non-activated BV2 cells presented a weak immunostaining similar to background (Fig. 3E).

2.3. L-type VGCC specific drugs modify KCl-induced intracellular calcium increase in BV2 microglia

Application of high K^+ concentrations in the culture medium has classically been used to depolarize the cell membrane and consequently to activate voltage-operated channels. To study whether cell membrane depolarization is able to produce an Intracellular calcium concentration ($[Ca^{2+}]_i$) increase in activated microglia, control and LPS + IFNγ stimulated BV2 cells were loaded with the fluorescent intracellular calcium probe Fura-2 and monitored. We used 10 μM nifedipine as specific L-type VGCC blocker and 10 μM Bay k8644 as specific opener of the channel (Fig. 4A–B).

In control cells, the basal F_{340}/F_{380} ratio was 0.223 ± 0.008 and no spontaneous intracellular calcium oscillations were observed in these