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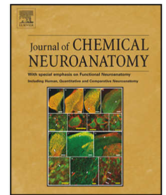

Differential expression of the calcium-sensing receptor in the ischemic and border zones after transient focal cerebral ischemia in rats

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ABSTRACT

G-protein-coupled calcium-sensing receptor (CaSR) has been recently recognized as an important modulator of diverse cellular functions, beyond the regulation of systemic calcium homeostasis. To identify whether CaSR is involved in the pathophysiology of stroke, we studied the spatiotemporal regulation of CaSR protein expression in rats undergoing transient focal cerebral ischemia, which was induced by middle cerebral artery occlusion. We observed very weak or negligible immunoreactivity for CaSR in the striatum of sham-operated rats, as well as in the contralateral striatum of ischemic rats after reperfusion. However, CaSR expression was induced in the ischemic and border zones of the lesion in ischemic rats. Six hours post-reperfusion there was an upregulation of CaSR in the ischemic zone, which seemed to decrease after seven days. This upregulation preferentially affected some neurons and cells associated with blood vessels, particularly endothelial cells and pericytes. In contrast, CaSR expression in the peri-infarct region was prominent three days after reperfusion, and with the exception of some neurons, it was mostly located in reactive astrocytes, up to day 14 after ischemia. On the other hand, activated microglia/macrophages in both the ischemic and border zones were devoid of specific labeling for CaSR at any time point after reperfusion, despite their massive infiltration in both regions. Our results show heterogeneity in CaSR-positive cells within the ischemic and border zones, suggesting that CaSR expression is regulated in response to the altered extracellular ionic environment caused by ischemic injury. Thus, CaSR may have a multifunctional role in the pathophysiology of ischemic stroke, possibly in vascular remodeling and astrogliosis.

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Introduction

Calcium-sensing receptor (CaSR), which is coupled to G-protein, was initially associated with the maintenance of calcium homeostasis by regulating the secretion of the parathyroid hormone (Brown et al., 1993; Brown and MacLeod, 2001). However, the identification of calcium as an external ligand has generated a special interest in the function of CaSR, unrelated to systemic calcium homeostasis (see as reviews Conigrave and Ward, 2013; Smajilovic and Tfelt-Hansen, 2007; Ward et al., 2012). Particularly, cumulative evidence has shown that CaSR is involved in modulating wide-ranging aspects of cellular function in the central nervous system, by sensing changes in the extracellular Ca^{2+} levels (Bandyopadhyay et al., 2010; Bouschet and Henley, 2005). CaSR is present in almost all brain areas, with high expression in the subfornical organ, olfactory bulb, and hypothalamus, suggesting a role for CaSR in region-specific neuronal functions (Bandyopadhyay et al., 2010; Bouschet and Henley, 2005; Chen et al., 2010; Mudo et al., 2009; Ruat and Traiffort, 2013; Yano et al., 2004). In addition, expression of CaSR in nerve terminals suggests its involvement in synaptic plasticity and neurotransmission, while its presence in glial cells (i.e., oligodendrocytes, astrocytes, and microglia), suggests a role for CaSR in local ionic homeostasis in the brain (Bandyopadhyay et al., 2010; Bouschet and Henley, 2005; Ruat and Traiffort, 2013). However, the role of CaSR in glia and neurons remains unclear.

There have been several reports regarding the regulation of CaSR expression in a variety of pathological conditions, including epileptic seizures (Mudo et al., 2009), Alzheimer's disease (Armato et al.,

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2012; Chiarini et al., 2009; Conley et al., 2009; Dal Pra et al., 2014), and traumatic brain injury (Kim et al., 2013). In addition, Kim et al. (2011, 2013) showed that after traumatic and ischemic brain injury, CaSR overexpression and concurrent down-regulation of metabotropic γ-aminobutyric acid receptor ($GABA_BR$) occurred before apparent neurodegeneration, suggesting that alteration of CaSR expression contributes to brain injury. Considering that calcium overload due to an excitotoxic mechanism contributes to neuronal injury induced by cerebral ischemia (Candelario-Jalil, 2009; Mehta et al., 2013), the induction of CaSR in the ischemic brain is of great interest. However, the temporal regulation and identification of the precise cell phenotypes expressing CaSR in the ischemic brain remain to be established.

In the present study, we determined the spatiotemporal expression pattern of CaSR in response to a disruption of ionic homeostasis caused by ischemic injury. For this purpose, we used a rat model of focal cerebral ischemia-reperfusion, induced by the occlusion of the middle cerebral artery (MCA). Our results clearly showed that CaSR expression in the ischemic zone (which is destined for tissue destruction) presented a different pattern than in the peri-infarct border zone (which has the potential for full recovery). Thus, we focused our attention on identifying the phenotypes of CaSR-positive cells in the ischemic and border zones using double-labeling techniques for various cell type-specific markers.

Materials and methods

Animal preparation

All experimental procedures were conducted in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Survival Surgery, and were approved by the IACUC (Institutional Animal Care and Use Committee) in the College of Medicine, The Catholic University of Korea (Approval Number: CUMC-2014-0006-01).

Adult male Sprague-Dawley rats (250–300 g) were used in this study. Transient focal ischemia was induced by the intraluminal thread method described previously (Longa et al., 1989). Briefly, the right external carotid artery was ligated with a 4-0 silk suture, and a 3-0 rounded tip nylon suture was introduced into the right common carotid artery. The suture was advanced through the internal carotid artery to occlude the MCA. Reperfusion was performed by withdrawing the surgical suture from the common carotid artery after 60-min ischemia. Interruption of blood flow distal to ligation and the restoration of blood flow in the right common carotid artery, external carotid artery, and internal carotid artery were confirmed under a dissecting microscope. Body temperatures (measured rectally) were maintained at 37.5 ± 0.3 °C with a heating lamp during and after ischemia. Sham-operated rats underwent the same experimental procedure except that the MCA was not occluded. In this model, ischemia for 60 min consistently resulted in a large infarct confined to the territory of the right MCA, whereas no morphological ischemic injury was detected in the territory of the left MCA.

Animals were allowed to live for 6 h, or 3, 7, or 14 days after reperfusion. At each of the four time points following reperfusion, animals ($n = 5$ per time point for ischemic group; $n = 3$ per time point for the sham-operated group) were deeply anesthetized with 16.9% urethane (10 mL/kg i.p.) and sacrificed by transcardial perfusion with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). In addition, at 3 ($n = 7$) and 7 ($n = 7$) days following MCA occlusion (MCAO), ischemic animals were killed for quantitative analysis. For western blot analysis, rats from 4 groups (sham controls, experimental rats 6 h, or 3, 7, or 14 days after reperfusion ($n = 5$ per group) were killed by decapitation under anesthesia (16.9% urethane; 10 mL/kg i.p.). The cortical and striatal tissues from the ipsilateral (ischemic) and contralateral hemispheres were carefully dissected under stereoscopic microscope and immediately frozen in liquid nitrogen. Brain samples were stored at −70 °C until further processing.

To evaluate tissue injury in animals subjected to 1 h MCAO, rats ($n = 3$) were deeply anesthetized with 16.9% urethane (10 mL/kg i.p.) at 3 days after reperfusion. Following decapitation, brains were quickly removed and were sliced at 1-mm thickness. Brain slices were incubated at 37 °C for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA).

Histochemistry and immunohistochemistry

To simultaneously detect apoptotic cells and the CaSR protein, we performed immunostaining for CaSR and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Free-floating sections (25-μm thick) were incubated at 37 °C for 60 min with biotinylated 2′-deoxyuridine-5′-triphosphate (dUTP), according to the manufacturer's protocol (Roche Diagnostics Corporation, Indianapolis, IN, USA). The sections were then immunostained with monoclonal mouse anti-CaSR antibody (Sigma-Aldrich; 1:100), followed by 1-hr incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:50) for CaSR staining and Cy3-conjugated streptavidin (Jackson ImmunoResearch; 1:1500) for the TUNEL method. The specificity of immunoreactivity was confirmed by the absence of immunohistochemical reaction in sections from which primary or secondary antibodies were omitted. Counterstaining of cell nuclei was carried out with DAPI (4′,6-diamidino-2′-phenyindole, Roche, Mannheim, Germany; 1:1000) for 10 min. Slides were viewed with a confocal microscope (LSM 700; Carl Zeiss Co. Ltd., Oberkochen, Germany) equipped with four lasers (Diode 405, Argon 488, HeNe 543, HeNe 633). Images were converted to the TIFF format, and contrast levels were adjusted using Adobe Photoshop v. 7.0 (Adobe System, San Jose, CA, USA).

Immunohistochemistry and double labeling

For double-immunofluorescence histochemistry, sections were incubated at 4 °C overnight with a mixture of monoclonal mouse anti-CaSR antibody (Sigma-Aldrich; 1:100) and one of following antibodies: polyclonal rabbit antibody to glial fibrillary acidic protein (GFAP; Millipore, Temecula, CA, USA; 1:1500), ionized calcium-binding adaptor molecule 1 (Iba1; Wako Pure Chemical Industries, Ltd., Osaka, Japan; 1:500), NG2 chondroitin sulfate proteoglycan (NG2; Millipore; 1:500), laminin (Sigma-Aldrich; 1:100), von Willebrand factor (Sigma-Aldrich; 1:200), Ki67 (Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK; 1:1000), and biotin-conjugated mouse monoclonal anti-neuronal nuclei (NeuN; Millipore; 1:500). Antibody staining was visualized using the following secondary antibodies: Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch; 1:2000), Alexa Fluor 488 goat anti-mouse (Molecular Probes, Eugene, OR, USA; 1:300), Alexa Fluor 488 goat anti-rabbit (Molecular Probes; 1:300), and Cy3-conjugated streptavidin (Jackson ImmunoResearch; 1:1500). Control sections were prepared as described above. Counterstaining of cell nuclei was carried out by incubating the sections with DAPI for 10 min. Slides were viewed with a confocal microscope.

Cell counting

To count the number of CaSR-positive cells and CaSR/GFAP or CaSR/NeuN double-labeled cells in the border zone at days 3 and

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