Neuroscience 131 (2005) 589–599

EFFECTS OF Na^+-Ca^{2+} EXCHANGER ACTIVITY ON THE α-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLONE-PROPIONATE-INDUCED Ca^{2+} INFLUX IN CEREBELLAR PURKINJE NEURONS

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Abstract—Variations in intracellular calcium activity ($[Ca^{2+}]_i$) play crucial roles in information processing in Purkinje neurons such as synaptic plasticity. Although Na^+-Ca^{2+} exchanger (NCX) has been shown to participate in the regulation of homeostasis and secretion in neuronal cells, the physiological role of NCX in Purkinje neurons, such as a role in cerebellar synaptic plasticity, is not well understood. NCX in acutely dissociated rat Purkinje neurons was identified by double staining with anti-calbindin D-28k antibody and anti-NCX antibody. The physiological activity of NCX was examined by measuring transient intracellular Ca^{2+} changes resulting from the Ca^{2+} influx via reverse mode of NCX (with 0 mM Na^+/2.5 mM Ca^{2+} solutions) and the efflux via the forward mode of NCX (with 140 mM Na^+/0 mM Ca^{2+} solutions). This transient increase in Ca^{2+} concentration was not elicited in the cells pretreated with NCX antisense oligodeoxynucleotides. And the Ca^{2+} influx resulting from the reverse mode of NCX was significantly reduced by 2-[2-[4-(4-nitrobenyloxy) phenyl] ethyl] isothiourea methanesulfonate, while the Ca^{2+} efflux via forward mode was inhibited by bepridil. The physiological role of NCX in synaptic function was studied by measuring Ca^{2+} transients induced by α-amino-3-hydroxy-5-methyl-4-isoxazolone-propionate (AMPA) receptor activation. This AMPA-evoked response was decreased with the inhibition of NCX forward mode and also, to less degree, with the inhibition of reverse mode. In antisense oligodeoxynucleotides pretreated cells, the AMPA-evoked response was also reduced, as was the case in NCX-inhibitor treated cells. The inhibition of NCX activity had depressant effects on Ca^{2+} transients induced by AMPA receptor activation. These results suggest that NCX plays a physiological role in modulating the activity of cerebellar Purkinje neurons, such as synaptic plasticity, via interaction with AMPA receptors in Purkinje neurons.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolone-propionate; AS-oligos, antisense oligodeoxynucleotides; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle medium; HEPES, *N*-[2-hydroxyethl] piperazine-*N*′[2-ethanosulfonic acid]; KB-R7943, 2-[2-[4-(4-nitrobenyloxy) phenyl] ethyl] isothiourea methanesulfonate; LTD, long-term depression; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide; NCX, Na^+-Ca^{2+} exchanger; NMDA, *N*-methyl-D-aspartic acid; PBS, phosphate-buffered saline; S-oligos, sense oligodeoxynucleotides.

Key words: Na^+-Ca^{2+} exchanger, AMPA receptor, Purkinje neuron, antisense oligodeoxynucleotides, bepridil, KB-R7943.

Cytosolic free calcium plays a key role in intracellular signaling in virtually all types of animal cells. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can occur by Ca^{2+} movements from the extracellular fluid via voltage-gated and receptor-operated Ca^{2+}-selective channels and Na^+-Ca^{2+} exchanger (NCX) located in the plasma membrane. And Ca^{2+} can be also released from intracellular stores by Ca^{2+}-induced Ca^{2+} release or inositol trisphosphate-activated mechanisms (Berridge, 1993; Somlyo and Somlyo, 1994; Bootman and Berridge, 1995).

NCX is expressed in the plasma membrane of virtually all cells, and transports Ca^{2+} in bi-directional modes, "the Ca^{2+} exit mode" (forward mode) and "the Ca^{2+} entry mode" (reverse mode; Blaustein and Lederer, 1999; Kang and Hilgemann, 2004). It has been demonstrated that NCX is expressed in both neurons (Yip et al., 1992; Furman et al., 1993) and glial cells (Takuma et al., 1994 and 1996). And Reuter and Porzig (1995) provided immunocytochemical evidence for the localized distribution of NCX along with the functional relevance of NCX in $[Ca^{2+}]_i$ control and synaptic vesicle recycling in the nerve terminals of cultured hippocampal cells.

Cerebellar Purkinje neurons exhibit long-lasting, activity-dependent depression of synaptic efficacy, long-term depression (LTD), which has been considered as a cellular model system of information processing that underlies motor learning (Linden, 2003). Cerebellar LTD can be produced when climbing fiber and parallel fiber inputs to a Purkinje neuron are stimulated conjunctively at low frequencies and such low-frequency stimulation is expressed as an attenuation of parallel fiber-Purkinje neuron synaptic strength (Ito, 2001). During this process, synaptic attenuation can be obtained by the down-regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolone-propionate (AMPA) receptors, which mediates parallel fiber-Purkinje neuron transmission in cerebellum. It is well known that Ca^{2+} influx and protein kinase C activations are required for the induction of LTD (Linden and Connors, 1991; Wang and Linden, 2000). In this model of LTD induction, the role of NCX in Purkinje neurons is linked to AMPA receptor that produces Na^+ influx (Linden et al., 1993). The function of the AMPA receptor-mediated Na^+ flux is unclear, but it may be involved in modulating the operation of NCX. However, physiological roles of NCX in Purkinje neurons are not well understood, especially in interaction of NCX and AMPA

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doi:10.1016/j.neuroscience.2004.11.045

receptors. Thus in this study, the physiological function of NCX in interaction between NCX and AMPA receptors in the soma of Purkinje neurons was investigated. And it was found that the activity of NCX modulates AMPA-induced Ca^{2+} influx in acutely cultured cerebellar Purkinje neurons.

EXPERIMENTAL PROCEDURES

Dissociation of rat cerebellar Purkinje neurons

These experiments have been approved by the Inha University Committee for the Care and Use of Laboratory Animals. Dissociated cells were prepared with a slight modification of the protocol described by Womack et al. (2000). In brief, the cerebellum of a 10-day-old rat (Animal Breeding Laboratory of Inha University) was rapidly removed, minced and incubated in 5 ml Hanks' balanced salt solution containing 0.1% trypsin at 37 °C for 8 min. Incubated cerebellar tissues were then transferred to the solution containing 0.1% trypsin inhibitor and 0.1% bovine serum albumin (BSA) at 37 °C for 5 min. Digested cerebellar tissues were slowly triturated with fire-polished Pasteur pipettes. Thereafter dissociated cells were plated onto coverslips coated with 0.05% poly-L-ornithine and 0.01% poly-L-lysine, and incubated in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin at 37 °C under humidified 5% CO_2.

Antisense oligodeoxynucleotides (AS-oligos)

A tandem pair of chimeric phosphorothioated AS-oligos was designed to target the 3′ end region of open reading frame and 3′ untranslated region of NCX cDNA. Synthetic AS-oligos and sense oligodeoxynucleotides (S-oligos) had the following sequences: 5′-AAAAATGGTGAAGAGAGTG-3′ and 5′-GTGAACATGACAAATGTCA-3′ for AS-oligos; 5′-CACTCTCTTCACCATTTTT-3′ and 5′-TGACATTTGTCATGTTCAC-3′ for S-oligos. Oligos were dissolved in lipofectamine/plus-reagent (Invitrogen, Grand Island, NY, USA) and were incubated at room temperature for 30 min before adding to DMEM medium. Liposome-DNA complex was added to each well containing dissociated cells with DMEM medium, and then incubated for transfection at 37 °C under humidified 5% CO_2 for 4 h following a procedure provided by the manufacture. After transfection, cells were treated with Serum and antibiotics and incubated for additional 24 h. The final concentration of the AS-oligos was maintained at 1 μM. Control cells were incubated in the media containing only lipofectamine.

Immunofluorescence

To visualize NCX in cerebellar Purkinje neurons, cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde at 4 °C for 20 min, and permeabilized with 0.2% Triton X-100 in PBS at room temperature for 2 min. And then the permeabilized cells were washed twice with PBS containing 0.05% Tween 20 and blocked with 1% BSA for 30 min. Cells were double-labeled using a mouse anti-calbindin D-28k monoclonal antibody (1:200; Sigma, St. Louis, MO, USA) and a rabbit anti-NCX polyclonal antibody (1:200; Swant, Bellinzona, Switzerland). FITC-conjugated goat anti-mouse secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and rhodamine-conjugated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch) were used for calbindin D-28k and NCX, respectively.

Confocal microscopy

A confocal laser-scanning microscope (Radiance 2100; Bio-Rad, Hercules, CA, USA) equipped with a Nikon ECLIPSE E600 fluorescence microscope was used to identify fluorescence-processed Purkinje neurons. Specimens were viewed with a 60×1.4 NA oil immersion Plan-Apochromat objective lens and illuminated with a 488 nm 50 mW argon laser and a 543 nm 1.4 mW Green-He laser. For the data analysis, Laser Sharp 2000 program (Bio-Rad) was used. Fluorescence of FITC and rhodamine was measured through HQ 515/30 and HQ 590/70 filters, both with the pinhole 10–30% open. Fluorescence intensities and transferred images were displayed in a split configuration as 512×512-pixel images (pixel size: 0.2 μm).

Ca^{2+} imaging

Dissociated Purkinje neurons were loaded with 3 μM fura-2AM and 0.02% pluronic F-127 (Molecular Probes, Eugene, OR, USA) in the Tyrode's solution at 37 °C for 1 h. Incubated cells were superfused with the Tyrode's solution for 30 min to wash away the remaining extracellular dye and to allow fura-2 AM hydrolyzed. The excitation wavelengths filtered through 340 and 380 nm filters were supplied by high pressure xenon UV lamp. Fluorescence intensity through 510 nm wavelength filter was collected using a cooled CCD digital camera (PXL-37; Photometrics, Tucson, AZ, USA). $[Ca^{2+}]_i$ was presented as the ratio of collected fluorescence intensities of fura-2 excited at 340 and 380 nm, $R_{340/380}$, and processed using Axon imaging workbench 2.2 (Axon Iinstruments, Union City, CA, USA) as described earlier (Park et al., 2001). Experiments were done at 37 °C.

Solutions

The composition of Tyrode's solution was 140 mM NaCl, 2.5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM HEPES, and 5.5 mM glucose at pH 7.4. In the 0 mM Na^+/2.5 mM Ca^{2+} solution (Na^+-free solution), NaCl was isosmotically replaced by *N*-methyl-D-glucamine and 140 mM Na^+/0 mM Ca^{2+} solution (Ca^{2+}-free solution) was made by omitting $CaCl_2$. To isolate NCX activity from other Ca^{2+} pathways, 1 μM thapsigargin (ER Ca^{2+}-ATPase inhibitor), 5 mM caffeine (ryanodine receptor inhibitor), and 250 μM La^{3+} (plasma membrane Ca^{2+}-ATPase inhibitor) were added to the superfusing solutions.

RESULTS

The expression of NCX in Purkinje neurons

Acutely dissociated Purkinje neurons were identified by double staining as described in the experimental procedures. The soma (about 20 μm) of Purkinje neuron, detected by FITC-conjugated anti-calbindin antibody, was relatively larger than that of other cells (Fig. 1A). And rhodamine-conjugated anti-NCX antibody activity was extensively detected at the soma of the same Purkinje neuron (Fig. 1B).

The expression of NCX in Purkinje neurons was further confirmed by the treatment of NCX AS-oligos. For this experiment, control cells were pretreated with lipofectamine while AS-oligos cells were pretreated with fluorescein-tagged AS-oligos dissolved in lipofectamine. Those cells were then incubated with a primary rabbit anti-NCX polyclonal antibody (1:200) and a goat anti-rabbit IgG secondary antibody (1:200) conjugated to rhodamine. Transfection of AS-oligos into the cells was confirmed by the fluorescence of fluorescein-tagged AS-oligos (Fig. 2B). Fluorescence intensity of NCX antibody in NCX AS-oligos pretreated cells was lower than that of control cells (Fig. 2).