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POSTSYNAPTIC AND EXTRASYNAPTIC LOCALIZATION OF Kv4.2 CHANNELS IN THE MOUSE HIPPOCAMPAL REGION, WITH SPECIAL REFERENCE TO TARGETED CLUSTERING AT GABAERGIC SYNAPSES

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Abstract—Voltage-dependent potassium (Kv) channels in the CNS are involved in regulation of subthreshold membrane potentials, and thus reception and integration of synaptic signals. Although such features are particularly important for induction of hippocampal synaptic plasticity, relatively little is known about their subcellular localization. Here we analyzed the detailed distribution of Kv4.2 potassium channels in the mouse hippocampal region using confocal and electron microscopy. At the light microscopic level, the Kv4.2 immunoreactivity occurred in a punctate fashion in the whole area of the hippocampal region. In the hippocampus proper, most of the Kv4.2-positive puncta were small, and they were abundant at the dendritic compartments of pyramidal neurons. High-resolution confocal microscopy revealed that there was no apparent association between Kv4.2-positive puncta with major synaptic markers, such as vesicular glutamate transporters and glutamic acid decarboxylase. In the subicular complex and dentate gyrus, we encountered large distinct Kv4.2-positive puncta at the perimeter of somata and proximal dendrites of principal cells. These puncta were often in contact with glutamic acid decarboxylase-positive boutons, but showed no apparent association with vesicular glutamate transporters. The glutamic acid decarboxylase-positive boutons apposing to Kv4.2-positive puncta were parvalbumin-positive. Quantitative image analysis showed that approximately half of Kv4.2-positive puncta were closely apposed to glutamic acid decarboxylase-positive boutons in the parasubiculum and dentate gyrus. Electron microscopic examination substantiated the presence of large Kv4.2-positive patches at postsynaptic sites of symmetric synapses and small patches at extrasynaptic sites. No presynaptic terminals were labeled. The present findings indicate targeted clustering of Kv4.2 potassium channels at postsynaptic sites of GABAergic synapses and extrasynaptic sites, and provide some key to understand their role in the hippocampal region.

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Abbreviations: BSA, bovine serum albumin; CLSM, confocal laser-scanning microscope; DG, dentate gyrus; DPPX, dipeptidyl aminopeptidase X; FITC, fluorescein isothiocyanate; GAD, glutamic acid decarboxylase; I_A, A-type current; KchIPs, voltage-dependent potassium channel interacting proteins; Kv, voltage-dependent potassium; NCS-1, neuronal calcium sensor-1; PB, phosphate buffer; PBS, phosphate-buffered saline; PSD-95, postsynaptic-density protein of 95 kDa; PV, parvalbumin; TB, Tris buffer; VGLUT, vesicular glutamate transporter.

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A number of potassium channel subunits have been discovered in the CNS. Among the subunits, voltage-dependent potassium (Kv) channels attracted special attention because they are fundamental determinants of electrophysiological properties of neurons (Jan and Jan, 1990). In particular, fast transient (A-type) Kv channels play crucial roles in the regulation of subthreshold membrane potential and firing frequency (Hille, 1991). Although several gene families have been shown to code for A-type potassium channel subunits, recent studies have emphasized the importance of Kv4 (*shal*-type) family. The potassium channel subunit Kv4.2 is one of such key components underlying the A-type potassium current (I_A) in the CNS (Serodio et al., 1996; Song et al., 1998). In the hippocampus, immunocytochemical studies have shown that Kv4.2 subunit is highly expressed at the distal dendrites of pyramidal neurons and granule cells (Sheng et al., 1992; Rhodes et al., 2004). Electrophysiological studies have reported that I_A prevents initiation of dendritic action potentials, limits back-propagation of action potentials, and reduces excitatory synaptic events of CA1 pyramidal neurons (Hoffman et al., 1997, for review see, Johnston et al., 2000). A recent study has shown that Kv4.2 potassium channels are also responsible for confinement of dendritic plateau potentials, and act to regulate synaptic integration (Cai et al., 2004). These studies indicated an involvement of Kv4.2 potassium channels in induction of hippocampal synaptic plasticity.

Neurotransmission in the CNS is closely associated with the subcellular localization of ion channels, receptors and signaling molecules at specific sites (for review see, Sheng, 1996; Craven and Bredt, 1998). In recent years, targeted Kv4.2 potassium channel clustering has been the focus of great interest, because this phenomenon is considered to achieve neuronal plasticity. For example, some *in vitro* experiments showed the cell surface targeting and clustering of Kv4.2 (Petrecca et al., 2000; Wong et al., 2002). An *in vivo* ultrastructural study reported that Kv4.2 was present at the postsynaptic sites as well as somatodendritic plasma membrane of rat supraoptic neurons (Alonso and Widmer, 1997). On the other hand, in the rat cerebellar granule cells, Kv4.2 was concentrated on the somatodendritic plasma membrane but not at the postsyn-

aptic densities (Strassle et al., 2005). At present, relatively little is known about *in vivo* clustering of Kv4.2 potassium channels in the CNS.

The main interest of this paper is the subcellular localization of Kv4.2 potassium channels in the mouse hippocampal region (subicular complex, hippocampus proper and dentate gyrus (DG)). Although the expression of Kv4.2 potassium channels was examined in the hippocampus proper and DG at the light microscopic level (Sheng et al., 1992; Maletic-Savatic et al., 1995), the subcellular distribution has never been studied in the hippocampal region. Because the determination of the precise distribution of Kv4.2 potassium channels would be of great value in elucidating their physiological roles in the hippocampal region, we performed a series of confocal and electron microscopic experiments. Our results indicate targeted clustering of a subset of Kv4.2 potassium channels at GABAergic postsynaptic and extrasynaptic sites in the hippocampal region.

EXPERIMENTAL PROCEDURES

Tissue preparations

Every experimental procedure was approved by the Committee of the Ethics on Animal Experiment in Graduate School of Medical Sciences, Kyushu University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Fifteen adult male C57BL/6J mice (22–25 g body weight, 8–11 weeks old) were used in this study. Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg body weight) and perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by two kinds of fixatives: (A) 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4); (B) 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB. Eleven mice were fixed with fixative A for light microscopic analysis, and four mice were fixed with fixative B for electron microscopic analysis. The brains were left *in situ* for 1–2 h at room temperature and then were removed from the skull. Caudal halves of the brain hemispheres containing the hippocampal region were cut horizontally into 50-μm-thick serial sections on a vibrating microtome (VT1000S; Leica Microsystems, Heidelberg, Germany).

Immunohistochemistry

After overnight incubation in 1.0% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 and 0.05% sodium azide at room temperature, sections were incubated in mouse monoclonal anti-Kv4.2 antibody (1:5000, gift from Dr. Trimmer; Rhodes et al., 2004) for 5 days at 20 °C. Then, sections were processed according to the avidin–biotin–peroxidase complex method (Hsu et al., 1981) by using Vector kits (Vector Laboratories, Peterborough, UK). After rinses in PBS and then in 50 mM Tris buffer (TB, pH 7.6), they were incubated in 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA) in 50 mM TB containing 0.01% H_2O_2 for 5–15 min at room temperature. Sections were treated with 0.04% OsO_4 in 0.1 M PB for 30 min at room temperature to enhance the reaction products, and then dehydrated in graded series of ethanol, infiltrated in propylene oxide, and flat-embedded in Epon-Araldite. Stained sections were examined under a light microscope (Axioskop 2; Zeiss, Oberkochen, Germany) equipped with Nomarski optics. The observed images were captured by a digital camera (AxioCam; Zeiss) attached to the microscope. To prepare illustrations for this study, the contrast and brightness of selected images were processed using the Adobe Photoshop 6.0 image-editing software package (Adobe Systems, San Jose, CA, USA).

Generation and characterization of the monoclonal antibody against Kv4.2 was described in a previous paper (Rhodes et al., 2004). Here we performed control experiment using the anti-Kv4.2 antibody preabsorbed with its antigenic peptide before immunocytochemical processing. Sections incubated with the preabsorbed anti-Kv4.2 antibody showed only negligible background staining of the mouse hippocampal region (data not shown).

Immunofluorescent multiple labeling

Sections were incubated overnight with 1.0% BSA in PBS containing 0.3% Triton X-100 and 0.05% sodium azide at room temperature. In some cases, Triton X-100 was omitted from the solutions, because the detergents disturbed glutamic acid decarboxylase (GAD) immunostaining of somatic profiles. Then, they were incubated for 5 days at 20 °C in mixtures of following primary antibodies raised in different species: mouse monoclonal anti-Kv4.2 antibody (1:5000, gift from Dr. Trimmer), rabbit polyclonal GAD65/67 antibody (1:10,000; Sigma), rabbit polyclonal anti-parvalbumin (PV) antibody (1:10,000; gift from Dr. Heizmann; Kägi et al., 1987), rabbit polyclonal anti-synaptophysin antibody (1:5000; Zymed, San Francisco, CA, USA), sheep polyclonal antibody S3 for GAD (1:2000; Oertel et al., 1981), guinea pig polyclonal anti-vesicular glutamate transporter (VGLUT)1 antibody (1:25,000; Chemicon, Temecula, CA, USA) and guinea pig polyclonal anti-VGLUT2 antibody (1:25,000; Chemicon). Then, sections were incubated with a mixture of Rhodamine Red-conjugated donkey anti-mouse antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody (1:1000; Jackson ImmunoResearch Laboratories) or FITC-conjugated donkey anti-guinea pig antibody (1:1000; Jackson ImmunoResearch Laboratories), and indodicarbocyamine-conjugated donkey anti-goat antibody (1:500; Jackson ImmunoResearch Laboratories) for 3 h. Some sections were also incubated with a RNA/DNA marker, YOYO-1 (1:10,000; Molecular Probes, Eugene, OR, USA) for cytoplasmic staining. The sections were rinsed briefly in PBS, mounted in Vectashield (Vector Laboratories) and observed under a confocal laser-scanning microscope (CLSM; TCS-SP2; Leica Microsystems).

Digital image analysis and quantification

Eight-bit black and white CLSM optical section stacks were captured in layers II and III of the parasubiculum, stratum radiatum of the CA1 region and granule cell layer of the DG. High-resolution images (pixel size=0.05×0.05 μm) were obtained under an oil immersion objective lens (100×, NA 1.40). The images taken from different sections were compared under constant conditions by adjusting the CLSM parameters, such as pinhole size, laser power and detector voltage using the equivalent anatomical structures.

Kv4.2-positive puncta size analysis was performed with the ImageJ 1.33 image-analysis software (NIMH, Bethesda, MD, USA). Six sections from three mice (two sections per animal) were used in this analysis. For measurement of puncta areas, single representative optical sections were selected from each stack. We used an automatic particle define procedure incorporating threshold and background subtract paradigms of the ImageJ 1.33 software (NIMH). Lower threshold value was set at 33% of maximum intensity of the gray scale, and puncta below 0.1 μm^2 were eliminated considering the resolution limit of CLSM. Data were collected and statistically analyzed by the Excel 2000 software package (Microsoft, Redmond, WA, USA).

The relationship between GAD-positive boutons and Kv4.2-positive puncta was analyzed using eight double-labeled sections from four mice. Representative CLSM images were captured and processed as mentioned above, and then automatically defined