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EXPRESSION PATTERN OF VOLTAGE-DEPENDENT CALCIUM CHANNEL SUBUNITS IN HIPPOCAMPAL INHIBITORY NEURONS IN MICE

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Abstract—Different subtypes of voltage-dependent calcium channels (VDCCs) generate various types of calcium currents that play important role in neurotransmitter release, membrane excitability, calcium transients and gene expression. Well-established differences in the physiological properties and variable sensitivity of hippocampal GABAergic inhibitory neurons to excitotoxic insults suggest that the calcium homeostasis, thus VDCC subunits expression pattern is likely different in subclasses of inhibitory cells. Using double-immunohistochemistry, here we report that in mice: 1) $Ca_v2.1$ and $Ca_v3.1$ subunits are expressed in almost all inhibitory neurons; 2) subunits responsible for the L-type calcium current ($Ca_v1.2$ and $Ca_v1.3$) are infrequently co-localized with calretinin inhibitory cell marker while $Ca_v1.3$ subunit, at least in part, tends to compensate for the low expression of $Ca_v1.2$ subunit in parvalbumin-, metabotropic glutamate receptor 1α- and somatostatin-immunopositive inhibitory neurons; 3) $Ca_v2.2$ subunit is expressed in the majority of inhibitory neurons except in calbindin-reactive inhibitory cells; 4) $Ca_v2.3$ subunit is expressed in the vast majority of the inhibitory cells except in parvalbumin- and calretinin-immunoreactive neurons where the proportion of expression of this subunit is considerably lower. These data indicate that VDCC subunits are differentially expressed in hippocampal GABAergic interneurons, which could explain the diversity in their electrophysiological properties, the existence of synaptic plasticity in certain inhibitory neurons and their vulnerability to stressful stimuli.

Key words: immunohistochemistry, GABAergic neurons, co-localization, calcium transient.

Inhibitory cells represent about 10% of the hippocampal cell population and play a critical role in regulating network oscillations, synchronization and synaptic plasticity. It has been established that inhibitory neurons are not uniform with regard to electrophysiological properties, neurochemical contents, morphology, connectivity and function. The heterogeneous interneuron population can be divided into numerous distinct subgroups. Based on their axonal arborization and site of action three major groups can be distinguished: 1) neurons that inhibit the perisomatic region of excitatory principal cells (basket and axo-axonic cells); 2) cells that terminate on dendrites (back projection cell, bistratified neuron, trilaminar cell, etc.); and 3) neurons that innervate other inhibitory neurons exclusively (calretinin (CR) and nitric-oxide synthase expressing neurons (Sik et al., 1994; Gulyas et al., 1996, 2003). The visualization of subgroups of GABAergic inhibitory neurons can be achieved most easily by immunocytochemical staining for transmitters, their synthesizing enzymes, neuropeptides,Ca^{2+}-binding proteins, receptors, or cell surface markers.

Inhibitory cells differ from the excitatory (principal) cells in the way they process calcium-mediated signals (Sik et al., 1998). For example, in contrast to the pyramidal cells, the vast majority of inhibitory neurons lack *N*-methyl-D-aspartic acid receptor (NMDAR) –mediated synaptic plasticity (Stelzer et al., 1987; Nicoll et al., 1989; Maccaferri and McBain, 1995, 1996; McMahon and Kauer, 1997; Laezza et al., 1999; Perez et al., 2001; Lamsa et al., 2005), but some inhibitory cells possess the machinery for NMDAR-independent long term potentiation and/or depression (Alle et al., 2001; Perez et al., 2001; Lei and McBain, 2002, 2004; Lapointe et al., 2004; Topolnik et al., 2005). Moreover, specific populations of hippocampal inhibitory neurons are extremely vulnerable to calcium-induced excitotoxicity, while other GABA-expressing neurons are particularly resistant (Freund and Magloczky, 1993; Magloczky and Freund, 1993; Buckmaster and Jongen-Relo, 1999) indicating differences in the Ca^{2+} entry and buffering (Sloviter, 1989; Sloviter et al., 1991; Goodman et al., 1993). However, it has been shown recently that Ca^{2+}-binding proteins do not offer protection against excitotoxicity (Freund et al., 1990; Klapstein et al., 1998; Bouilleret et al., 2000). Thus, Ca^{2+} influx into the neuronal subcompartments should have a crucial role in the Ca^{2+}-induced cell death.

One of the major routes for calcium entry is via voltage-dependent calcium channels (VDCCs). VDCCs are multisubunit complexes, containing the pore forming $\alpha1$ subunit, and the auxiliary $\alpha2\delta$, β, and γ subunits, all of them conferring variable gating properties. In neurons, six types of calcium currents have been described based on their physiological and pharmacological properties (Tsien et al., 1991), which are mediated by different $\alpha1$ subunits. L-type VDCCs are composed by the $Ca_v1.2$ and $Ca_v1.3$ subunits ($\alpha1C$ and $\alpha1D$; Hui et al., 1991; Snutch et al., 1991); P/Q-, N-, and R-type VDCCs are formed by the $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ subunits, respectively ($\alpha1A$, $\alpha1B$ and $\alpha1E$;

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Abbreviations: BSA, bovine serum albumin; CB, calbindin; CCK, cholecystokinin; CR, calretinin; DG, dentate gyrus; -IR, immunoreactive; LTP, long-term potentiation; mGluR1α, metabotropic glutamate receptor 1α; NGS, normal goat serum; NHS, normal horse serum; NMDAR, *N*-methyl-D-aspartic acid receptor; PB, phosphate buffer; PV, parvalbumin; SOM, somatostatin; str., stratum, strata; TBS, Tris-buffered saline; VDCC, voltage-dependent calcium channel.

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Starr et al., 1991; Dubel et al., 1992; Soong et al., 1993); T-type VDCC contains the $Ca_v3.1$ subunit (α1G; Perez-Reyes et al., 1998).

VDCC currents have diverse functions including essential role in neurotransmitter release (P/Q-, N- and R-type; Westenbroek et al., 1992, 1995; Wu et al., 1998, 1999), in synaptic plasticity (R- and L-type; Kavalali and Plummer, 1994; Dietrich et al., 2003), in protein phosphorylation and gene regulation (L-type; Westenbroek et al., 1990) and in spike-induced calcium entry (L- and T-type; Fisher et al., 1990; McCobb and Beam, 1991; Kavalali and Plummer, 1994). Numerous electrophysiological studies have led to better understanding of the role of VDCCs in physiological processes such as rhythmic oscillations (Llinas and Yarom, 1981), calcium-dependent afterhyperpolarization (Marrion and Tavalin, 1998; Tanabe et al., 1998), synaptic plasticity (Wang et al., 1997; Cavus and Teyler, 1998; Kapur et al., 1998; Grover and Yan, 1999; Breustedt et al., 2003) and neuronal development (Jiang and Swann, 2005). VDCCs are also implicated in oscillatory responses in metabotropic glutamate receptor 1α (mGluR1α)-expressing inhibitory neurons of the hippocampal stratum (str.) oriens (Woodhall et al., 1999) and in GABA release (Jensen et al., 1999; Jensen and Mody, 2001; Bhaukaurally et al., 2005).

While a wealth of information is available about the structure (i.e.: Curtis and Catterall, 1984; Takahashi and Catterall, 1987; Ahlijanian et al., 1990; Witcher et al., 1993; Liu et al., 1996a; Letts et al., 1998), and biophysical properties of VDCCs (i.e.: Hagiwara and Naka, 1964; Hagiwara et al., 1964; Eckert and Chad, 1984; Sala, 1991; Patil et al., 1998) and their functions in hippocampal excitatory cells (i.e.: Jones et al., 1989; Ozawa et al., 1989; Fisher et al., 1990; Westenbroek et al., 1990; Chen et al., 2005; Moosmang et al., 2005), little is known about their localization in hippocampal inhibitory neurons. Using pharmacological approach, studies have already determined the existence of some VDCC subtypes in certain hippocampal inhibitory neurons (Fraser and MacVicar, 1991; Lambert and Wilson, 1996; Poncer et al., 1997), but detailed analysis of VDCCs expression in different hippocampal layers, regions as subgroups has not be carried out. Determining the precise cellular expression pattern of VDCCs in GABAergic cells is essential to understand the mechanism of synaptic plasticity, the control of network synchronization and the calcium-induced neurodegeneration under pathological conditions (Magloczky and Freund, 1993, 1995; Lie et al., 1999). Therefore we used immunohistochemical approach to determine the expression pattern of various VDCCs in defined subtypes of hippocampal inhibitory neurons.

EXPERIMENTAL PROCEDURES

Tissue preparation

Adult male C57 mice (Charles River Laboratories, Raleigh, NC, USA) were used in this study (seven animals for each VDCC immunolabeling). Animals were housed by groups of three to four in polycarbonate cages with *ad libitum* access to food and tap water and maintained under a 12-h light/dark cycle in a controlled ambient temperature. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by Laval University Committee on Ethics and Animal Research. All efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were anesthetized with ketamine/xylazine (100 mg/kg), intracardially perfused with 0.9% NaCl followed by ice-cold 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.4) containing 0.05% glutaraldehyde. Brains were removed and post-fixed for 1 h in the fixative then cut into 25- and 50-μm coronal sections using a VT-1000S Vibratome (Leica, Wetzlar, Germany).

Double fluorescence immunohistochemistry

Sections were washed several times in Tris-buffered saline (TBS, 0.05 M, pH 7.4, Sigma-Aldrich, Seelze, Germany) and blocked for 45 min with 10% normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA) containing 0.5% Triton X (Sigma-Aldrich). Sections were then incubated overnight at 4 °C with one of the primary antibodies directed against VDCC subunit and one of the primary antibody serving as an interneuron marker (see Table 1) in TBS containing 5% NGS and 1% bovine serum albumin (BSA, Sigma-Aldrich). Sections were washed in TBS, incubated for 3 h with Alexa-488 goat anti-rabbit (1:2000, Molecular Probes, Eugene, OR, USA) and CY3-conjugated goat anti-mouse secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA, USA) in TBS. Sections were washed, mounted onto slides and coverslipped with home-made Mowiol. To confirm the specificity of the VDCC antibodies, antisera were incubated overnight at 4 °C with 1 μg of the peptide against which the antibody was generated. Sections were treated with these complexes followed by the same protocol described above.

Double fluorescence immunohistochemistry with cholecystokinin (CCK)

Because all VDCC antibodies and CCK antiserum are raised in rabbit, we used the protocol developed by Jackson Immuno-

Table 1. List of the antibodies used in this study

VDCC antibody	Source	Working dilution	Inhibitory cell markers	Source	Working dilution
$Ca_v2.1$ (R)	Alomone	1:100	PV (M)	Sigma	1:4000
$Ca_v2.2$ (R)	Alomone	1:50	CR (M)	Chemicon	1:4000
	Dr. Elise Stanley	1:200			
$Ca_v1.2$ (R)	Sigma	1:100	CB (M)	Sigma	1:2500
	Alomone	1:50			
$Ca_v1.3$ (R)	Sigma	1:60	mGlur1α (M)	Dr. T. Gorcs	1:50
	Alomone	1:50			
$Ca_v2.3$ (R)	Alomone	1:50	SOM (M)	Biomeda	1:50
$Ca_v3.1$ (R)	Chemicon	1:200	CCK (R)	Sigma	1:10,000

M, mouse source; R, rabbit.