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Research report

Presence and distribution of three calcium binding proteins in projection neurons of the adult rat cochlear nucleus

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Abstract

The presence and distribution of three cytoplasmic calcium binding proteins, calbindin, calretinin, and parvalbumin, have been investigated in the projection neurons of the cochlear nucleus complex in adult rats by using immunohistochemistry in free-floating slices. Identification of the individual cell types was carried out on the basis of their intranuclear localization, morphological characteristics, and (in the cases of pyramidal and bushy neurons) by retrograde labeling with rhodamine-dextran. The most important findings were confirmed by using confocal microscopy. The data obtained in these experiments are the first to demonstrate the presence of parvalbumin in pyramidal neurons and globular and spherical bushy cells of rat cochlear nucleus, whereas octopus and giant cells did not show positivity for parvalbumin. Calretinin was not present in either Purkinje-like cells or giant neurons. According to the double immunolabeling co-localization experiments, the pyramidal neurons, Purkinje-like cells, globular bushy cells, and octopus cells express two different calcium binding proteins in their cytoplasm (although in different combinations) whereas giant cells and spherical bushy cells contain solely calbindin and parvalbumin, respectively. The presence of calretinin in globular bushy cells provides a tool for distinguishing them from spherical bushy cells. The immunolabeling of the fibers and axonal endings of the acoustic nerve in the ventral part of the cochlear nucleus indicated that these structures are also parvalbumin positive. It is concluded that the heterogenous cell composition of the cochlear nucleus is accompanied by a rather complex expression pattern of the cytoplasmic calcium binding proteins.

Theme: Sensory systems
Topic: Auditory systems: central physiology

Keywords: Calbindin; Calretinin; Parvalbumin; Immunolabeling; Confocal microscopy; Rhodamine-dextran

1. Introduction

Like other mammalian cells, neurons posses a broad variety of proteins that transiently bind Ca^{2+}. These calcium binding proteins can be classified on the basis of their molecular structure, localization, Ca^{2+} binding properties, etc. [1,24,37]. From a functional point of view, however, the most intriguing question is their involvement in the modulation of cellular activity. Some calcium binding proteins exert well-defined regulatory actions (e.g., calmodulin, calpains), whereas others have not been hitherto related directly to specific cellular functions. Nevertheless, even these proteins may affect various Ca^{2+}-dependent regulatory processes as they are able to influence the extent and duration of activity-related intracellular [Ca^{2+}] changes [1,37].

Cytoplasmic Ca^{2+} plays a rather complex role in neurons. On one hand, nerve cells posses several functions that are triggered or modulated by intracellular [Ca^{2+}] transients but, on the other hand, unusually high or long-lasting $[Ca^{2+}]_i$ elevations seem to be involved in the genesis of numerous neuronal disorders [20,27,41]. It is not surprising, therefore, that nerve cells express a considerable amount of Ca^{2+} buffer proteins, apparently as a defense tool against Ca^{2+} overload and the consequent Ca^{2+}-induced cytotoxicity. The most common calcium buffers of the neurons are parvalbumin

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(PA), calbindin-D28K (CB), and calretinin (CR) [1,6,15], all of them belonging to the EF-hand family. While PA has been described in other cell types (e.g., in skeletal muscle fibers), CB and CR seem to be exclusively present in the nervous system.

There were several efforts to describe the distribution of the aforementioned proteins in the central nervous system (CNS) of different species using various techniques [1,15]. These studies revealed similarities between the expression patterns of some neuron populations indicating common developmental origin and/or functional relationship [1]. An especially strong correlation has been established between the synaptic refinement and the expression levels of the Ca^{2+} buffer proteins in the course of ontogenesis in different parts of the CNS [15,21]. In fact, the development of the hearing function appears to be a good model for analyzing the postnatal maturation of neuronal networks, as the newborns of several species lack hearing and it takes several days or weeks until the auditory function fully develops. It is also known that impairment of the peripheral elements of the auditory apparatus leads to altered morphology and/or function of the central auditory neurons, including modifications of calcium binding protein expression [3,29,44]. Data obtained from various species agree that CB, CR, and PA are present at all parts of the auditory pathway although their expression levels show considerable cell-to-cell, developmental, and interspecies variability (see [2,19,23,40,42] and further references cited in these papers).

Previously, we have described the membrane properties of two projection neurons (bushy and pyramidal cells) of the rat cochlear nucleus (CN) [7,11,30,31]. As the functional properties of these cells might be strongly influenced by intracellular modulatory mechanisms (including $[Ca^{2+}]_i$ changes), the characterization of the Ca^{2+} homeostasis has also been attempted. The results of these experiments on pyramidal cells [12,13,35] necessitated, however, a better characterization of their cytoplasmic Ca^{2+} buffer capacity. Moreover, to be able to compare the Ca^{2+} handling of the different types of neurons, such data from other CN cells are also required. In order to gain a more complete understanding of the presence and distribution of the three main Ca^{2+} buffer proteins in the rat CN, double immunolabeling experiments using both fluorescence microscopy and confocal microscopy were carried out in adult, fully matured animals. Some of the results presented here are in good accordance with earlier findings. Moreover, our results also provide new information about the co-expression of the calcium binding proteins in the primary projection neurons of the CN.

2. Materials and methods

2.1. Preparation of cochlear nucleus

Free-floating sections were prepared from 30-day-old Wistar rats following the steps of an earlier detailed description [36] (the protocol was authorized by the Ethical Committee of the University of Debrecen). Briefly, after decapitation of the animal, the brain was prepared, the meninges and the major blood vessels were removed, then the brain was cut in half by making a sagittal section in the midline. When retrograde tracing was performed (see below), the last step was omitted. The cerebellum and the telencephalon were removed from the brain-stem, and the cochlear nuclei were cleared from the chorioid plexus and the remnants of the meninges. All steps of the preparation were performed in an ice-cold (approximately −2 °C) low-Na^+ artificial cerebrospinal fluid (aCSF) that was made of (in mM): sucrose, 250; KCl, 2.5; glucose, 10; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgCl_2$, 1; myo-inositol, 3; ascorbic acid, 0.5; Na-pyruvate, 2 (pH was set to 7.2; tonicity was 320 mosM).

2.2. Immunohistochemistry

In order to investigate the co-localization of the calcium binding proteins, double immunolabelings were performed; whereas single immunolabelings were employed for the confocal microscopy. The isolated brain-stems were first fixed in 4% paraformaldehyde (4 °C, 4 h), rinsed three times in 0.1 M phosphate buffer (PB; 0.1 M $Na_2HPO_4 \times 2H_2O$, 0.1 M $NaH_2PO_4 \times H_2O$; pH 7.4) for 10 min, then 50–60 μm thick sagittal slices were cut using a vibrating slicer (Campden Instruments, Loughborough, UK). The tissue sections were washed in PB for 10 min then rinsed with Tris-buffered saline (TBS; 8 mM Tris-base, 42 mM Trizma HCl, 150 mM NaCl; pH 7.4; 3 × 10 min). Blocking was performed with 10% normal Horse Serum (Sigma-Aldrich, St. Louis, MO, USA) dissolved in TBS containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature, followed by incubation with the primary antibodies overnight (room temperature). The combinations and dilutions of the antibodies applied for the double labelings are listed in Table 1.

On the following day, the slices were rinsed in TBS (3 × 10 min) and incubated with fluorochrome-conjugated secondary antibodies (3 h at room temperature, see Table 1). This was followed by further washes in TBS (3 × 10 min). Finally, the slices were mounted using Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA) to stain the cell nuclei in the sections.

Control experiments were also conducted. The validity of the calbindin-specific immunolabeling was confirmed by preincubating the primary antibody with its specific blocking peptide, provided by the manufacturer of the antibody (Santa Cruz Biotechnology; the preadsorbtion experiments were carried out according to the manufacturer's instructions). The application of the blocking peptide completely prevented the immunolabeling of the expected structures.