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Electron tomographic analysis of gap junctions in lateral giant fibers of crayfish

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ABSTRACT

Innexin-gap junctions in crayfish lateral giant fibers (LGFs) have an important role in escape behavior as a key component of rapid signal transduction. Knowledge of the structure and function of characteristic vesicles on the both sides of the gap junction, however, is limited. We used electron tomography to analyze the three-dimensional structure of crayfish gap junctions and gap junctional vesicles (GJVs). Tomographic analyses showed that some vesicles were anchored to innexons and almost all vesicles were connected by thin filaments. High densities inside the GJVs and projecting densities on the GJV membranes were observed in fixed and stained samples. Because the densities inside synaptic vesicles were dependent on the fixative conditions, different fixative conditions were used to elucidate the molecules included in the GJVs. The projecting densities on the GJVs were studied by immunoelectron microscopy with anti-vesicular monoamine transporter (anti-VMAT) and anti-vesicular nucleotide transporter (anti-VNUT) antibodies. Some of the projecting densities were labeled by anti-VNUT, but not anti-VMAT. Three-dimensional analyses of GJVs and excitatory chemical synaptic vesicles (CSVs) revealed clear differences in their sizes and central densities. Furthermore, the imaging data obtained under different fixative conditions and the immunolabeling results, in which GJVs were positively labeled for anti-VNUT but excitatory CSVs were not, support our model that GJVs contain nucleotides and excitatory CSVs do not. We propose a model in which characteristic GJVs containing nucleotides play an important role in the signal processing in gap junctions of crayfish LGFs.

1. Introduction

Gap junctions in electrical synapses comprise an assembly of intercellular channels formed by connexins in vertebrates and innexins in invertebrates. The structure of connexin 26 has been studied by electron and X-ray crystallography (Maeda et al., 2009; Oshima et al., 2007). In contrast to our increasing knowledge of connexins, however, knowledge of the structure and function of innexins is very limited.

Hama (1961) performed the first electron microscopy (EM) studies of innexin-gap junctions, revealing a large number of vesicles associated with the gap junction in the lateral giant fiber (LGF) of crayfish. The crayfish LGF comprises more than a dozen neurons that are interconnected at the ganglion. The gap junctions are localized at these connecting regions and have an important role in intercellular signal processing. Subsequent studies were performed using freeze fracture EM (Peracchia, 1973b) and electrophysiology (Asada and Bennett, 1971). The crayfish has also been used to study the distribution and function of LGF-related neurons (Antonsen and Edwards, 2003; Lee and Krasne, 1993; Roberts et al., 1982) because it is an ideal animal model for studies of the neural circuit involved in escape behavior elicited by tailfin stimulation.

Studies in which the fixative condition and buffer composition were optimized for the preparation of structurally intact gap junctions from crayfish (Peracchia and Dulhunty, 1976; Peracchia and Mittler, 1972) and the disappearance of the gap junctions caused by injuries to the crayfish leg (Bittner and Ballinger, 1980; Hanna et al., 1984) demonstrated that the crayfish gap junction structure is sensitive to external stimuli. Elucidating the relationship between external stimuli and the gap junction structure at the molecular level, however, has remained a challenging problem. EM provides only projection images, which are a limited representation of a three-dimensional (3D)-object, whereas electron tomography is a very powerful method for visualizing the 3D structure of complex biologic samples (Fernández-Busnadiego et al., 2010), such as electrical synapses. Although the genes of crayfish innexins have not yet been cloned, making a genetic approach to study innexins infeasible, we chose to study crayfish gap junctions

Abbreviations: ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CSV, chemical synaptic vesicle; GJV, gap junctional vesicle; GTP, guanosine triphosphate; LGF, lateral giant fiber; OsO_4, osmium tetraoxide; PSD, post-synaptic density; EM, electron microscopy; UA, uranium acetate; VMAT, vesicular monoamine transporter; VNUT, vesicular nucleotide transporter.

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because of their distinct structure as well as the large amount of information available regarding their anatomic location (Lee and Krasne, 1993; Skinner, 1985a, 1985b).

The two-sided distribution of gap junctional vesicles (GJVs) is characteristic of crayfish gap junctions. In contrast, GJVs are present on only one side in Drosophila gap junctions (Blagburn et al., 1999), and no vesicles are observed in connexin-gap junctions (Fukuda and Kosaka, 2003) or in other innexin-gap junctions (Starich et al., 2003). We also analyzed the 3D structure of excitatory chemical synapses with excitatory chemical synaptic vesicles (CSVs) to compare the CSVs and GJVs. Aside from a clear difference in size between the two types of vesicles, GJVs had a high image density in their center, which was not observed in CSVs. The appearance of the high density in the GJVs was dependent on the fixatives used for specimen preparation, providing information on the substances located inside the vesicles. In addition, we used immunoelectron microscopy to observe the localization of the vesicular nucleotide transporter (VNUT) (Haanes and Novak, 2010; Sawada et al., 2008) on GJVs. Our findings suggest that GJVs contain nucleotides, such as ATP, that function as transmitters or modulators (Burnstock, 2007; Chen et al., 1995).

2. Materials and methods

2.1. Crayfish preparation

All experiments were performed in accordance with the guidelines of the Physiological Society of Japan. Adult crayfish, *Procambarus clarki*, from Hamamatsu were obtained from a commercial supplier. Crayfish were submerged in crushed ice for 15 min, then cold saline with fixative (3% glutaraldehyde [GA] and 0.1% H_2O_2 in 100 mM phosphate buffer, pH 7.4, 50 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$) was injected into the abdomen. After 5 min, the abdominal nerve cords were quickly dissected, and the dissected nerve cords were fixed overnight by immersion in the same fixative at 4 °C. The 5th ganglion was isolated from the nerve cord and washed twice at 4 °C with 100 mM phosphate buffer, pH 7.4, 50 mM NaCl, 3 mM KCl, and 2 mM $CaCl_2$ for 4 h and then overnight with fresh buffer. The 5th ganglia were then washed several times with distilled cold water, post-fixed in 2% OsO_4 for 1 h, washed again with distilled cold water, and finally block-stained with 2% uranyl acetate (UA) for 1 h. Specimens were dehydrated with 50–100% ethanol, substituted with propionic acid, and finally embedded in EPON812 resin (TAAB Laboratories Equipment Ltd.). All procedures before the ethanol dehydration were performed at 4 °C. For morphologic observations, specimens were sliced into 60-nm-thick sections. For electron tomography, 120- or 180-nm-thick sections were prepared, mounted on an EM-grid coated with a formvar film, and counterstained for 5 min each with 2% UA and 2% lead acetate.

2.2. Tailfin stimulation experiments

The tailfins of individual crayfish were stimulated with five different intensities using tweezers. The lowest-stimulation level was no touch to the tailfin. The second-level comprised six soft touches of the tailfin at approximately 1-s intervals. The third-level consisted of two strong pinches of the tailfin at approximately 2-s intervals, causing the crayfish to strongly bend its tail, which represents typical escape behavior. The fourth-level consisted of four strong pinches of the tailfin at approximately 2-s intervals, causing the crayfish to strongly bend its tail as in the third-level. The fifth-level consisted of eight strong pinches of the tailfin at approximately 2-s intervals, causing the crayfish to react strongly to the first pinch, but the reaction to additional pinches was subsequently attenuated due to habituation. In all cases, the crayfish were placed into ice-cold water for 2 min immediately after stimulation to be cooled quickly, unlike the general preparation as described in Section 2.1. They were then immersed in crushed ice for 5 min, and pre-fixed as described above. If the crayfish body is cooled by cold water for more than 2 min, it absorbs water that dilutes the fixative. Therefore, the cooling time in ice-cold water was minimized and the crushed-ice immersion step was added.

2.3. Immunoelectron microscopy

Samples for immunoelectron microscopy were prepared as described above with the following modifications: 2% paraformaldehyde (PFA) and 0.1% GA in 100 mM phosphate buffer, pH 7.4, was used for pre-fixation, and 1% OsO_4 for post-fixation. After dehydration with ethanol, the ganglia were embedded in LRWhite (London Resin Co., Ltd.) and incubated for 3 d at 55 °C. After sectioning, the sections were picked up with an EM-grid. The sections were blocked for 5 min with 4% bovine serum albumin in phosphate-buffered saline (PBS) to minimize non-specific labeling, and then incubated with primary antibody for either 4 h at room temperature or overnight at 4 °C. The primary antibodies used were a rabbit polyclonal antibody against human vesicular monoamine transporter (VMAT; catalog number: sc-15314, Medical and Biological Laboratories Co.) and a rabbit polyclonal antibody against human vesicular nucleotide transporter (VNUT; catalog number: BMP079, Santa Cruz Biotechnology, Inc.). Both antibodies were diluted 1/50 with 1% bovine serum albumin in PBS. Negative control samples were incubated in 1% bovine serum albumin in PBS without primary antibody. Sections were washed 8 times with PBS and incubated with anti-rabbit IgG-antibody conjugated to 15 or 10 nm gold particles for 4 h at room temperature or overnight at 4 °C. Sections were finally fixed with 1% GA in PBS for 5 min, washed with water, and counterstained as described above.

2.4. Electron tomography

Samples prepared as described above were coated with a carbon film and scattered 5-nm gold particles as a fiduciary marker on the carbon film. A tilt series was collected using a dose of 20 pA/cm^2 with a conventional electron microscope (JEOL1010) operated at 100 kV. The electron microscope was equipped with a 2×2 k charged-coupled device camera (Gatan). The tilt series were recorded from $-60°$ to $+60°$ with 1° angular increments by dual axes. In general, the pixel size at the specimen level was 0.74 nm, but some specimens observed with a higher magnification were recorded with a pixel size of 0.54 nm. The tilt series was aligned using the 5-nm gold particles as fiduciary markers, and 3D reconstructions were obtained using IMOD. All image-processing steps were performed with the IMOD software package (Kremer et al., 1996) from the Boulder Laboratory for 3D Electron Microscopy of Cells. Surface rendering of GJVs, gap junctional membranes, innexons, molecules on the GJV, and filaments was performed by manual tracing of each tomographic *x*–*y* plane with the IMOD software package.

2.5. Density measurements

Measurements of EM-image densities in gray scale with 8 bits per sampled pixel, i.e., 256 different intensities, were made using ImageJ (http://rsb.info.nih.gov/ij/). The density measurements at each point were performed by taking the mean density of the internal area of a circle with a diameter of approximately 9 nm centered on a specific point. First, cytoplasmic densities were measured as a fiducial level. Approximately 10 points of the cytoplasm were randomly chosen and measured. The mean of their densities was referred to as the fiducial level. The central density of the GJV