Neuroscience Letters 510 (2012) 99–103

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Variety of horizontal cell gap junctions in the rabbit retina

Jiook Cha[a], Hong-Lim Kim[a,b], Feng Pan[c], Myung-Hoon Chun[a], Stephen C. Massey[c], In-Beom Kim[a,*]

[a] Department of Anatomy, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea
[b] Integrative Research Support Center, College of Medicine, The Catholic University of Korea, Republic of Korea
[c] Department of Ophthalmology and Visual Science, University of Texas Medical School at Houston, Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 10 November 2011
Received in revised form
11 December 2011
Accepted 4 January 2012

Keywords:
Gap junction
Cx50
Cx57
Electron microscopy
Horizontal cells
Rabbit retina

ABSTRACT

In the rabbit retina, there are two types of horizontal cell (HC). The axonless A-type HCs form a coupled network *via* connexin 50 (Cx50) gap junctions in the outer plexiform layer (OPL). The axon-bearing B-type HCs form two independently coupled networks; the dendritic network *via* gap junctions consisted of unknown Cx and the axon terminal network *via* Cx57. The present study was conducted to examine the localization and morphological features of Cx50 and Cx57 gap junctions in rabbit HCs at cellular and subcellular levels. The results showed that each gap junction composed of Cx50 or Cx57 showed distinct features. The larger Cx50 gap junctions were located more proximally than the smaller Cx50 gap junctions. Both Cx50 plaques formed symmetrical homotypic gap junctions, but some small ones had an asymmetrical appearance, suggesting the presence of heterotypic gap junctions or hemichannels. In contrast, Cx57 gap junctions were found in the more distal part of the OPL but never on the axon terminal endings entering the rod spherules, and they were exclusively homotypic. Interestingly, about half of the Cx57 gap junctions appeared to be invaginated. These distinct features of Cx50 and Cx57 gap junctions show the variety of HC gap junctions and may provide insights into the function of different types of HCs.

1. Introduction

Gap junctions composed of connexins (Cxs) form electrical synapses between cells and they are widely distributed throughout the central nervous system. All retinal neurons make gap junctions and four different neuronal Cxs have been found in the mammalian retina [2,16], where gap junctions are thought to play important roles in visual processing.

Horizontal cells (HCs) provide negative feedback to cones and thus contribute to surround inhibition in bright light [1]. HCs are well coupled *via* gap junctions and thus form extensively coupled networks [17,30]. This is the basis for the property that the HC receptive field is considerably larger than the dendritic field and the strength of coupling in the HC network determines the spatial extent of the feedback signal to photoreceptors [3,5]. In the rabbit retina, there are two morphological types of HC. The A-type HC is an axonless cell with thick dendrites that contacts only cones. In contrast, the B-type HC is axon bearing. The somatic dendrites of B-type HCs contact cones, whereas the axon terminals contact rods exclusively [5,24]. Together, the two HC types make three different coupled networks: the axonless A-type HC network, the somatodendritic network of B-type HC and the B-type axon terminal network in the outer plexiform layer (OPL) [17,18,22].

Our previous work using confocal microscopy has shown that two Cxs, Cx50 and Cx57 contribute to the HC coupled networks in the rabbit retina. The extensively coupled A-type HCs express Cx50 gap junctions whereas B-type HC axon terminals are coupled *via* Cx57 gap junctions [19,21]. Neither Cx was reported to be at the tips of dendrites or axon terminals, the presumed sites of feedback to photoreceptors. Rather, Cx50 and Cx57 were located in the HC neuropil. These findings are not consistent with the ephaptic hypothesis of HC feedback [13] based on the electron microscopic findings that hemichannels were located on the dendritic tips of horizontal cells in the goldfish retina.

Recently, in the mouse retina, confocal microscopic observations suggested that Cx57 may be localized at the tips of HC axon terminals where they contact rod spherules [4]. However, in another report, electron microscopic observation did not provide any evidence for the localization of Cx57 at the tips of HC axon terminals [11]. Therefore, in this study, we have examined the location and morphological features of both Cx50 and Cx57 gap junctions in the OPL of the rabbit retina at electron microscopic level.

Abbreviations: CX, connexin; GCL, ganglion cell layer; HC, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

* Corresponding author at: Department of Anatomy, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea. Tel.: +82 2 2258 7263; fax: +82 2 536 3110.
E-mail address: ibkimmd@catholic.ac.kr (I.-B. Kim).

0304-3940/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2012.01.010

2. Materials and methods

Five Adult New Zealand Albino rabbits were anesthetized with urethane (1.5 mg/kg, i.p.). The eyes were removed and hemisected, and the retina was isolated while immersed in bicarbonate-based Ames' medium (Sigma–Aldrich). The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul and Institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston Medical School, which conform to the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The isolated retina was fixed for 1 h with 4% formaldehyde in 0.1 M phosphate buffer (PB: pH 7.4) for vertical vibratome sections. Alternatively, for intracellular dye injection, wholemount pieces were submerged with 5 μM 6-diamino-2-pheylindole (DAPI) to prelabel HC nuclei and then fixed for 20 min with 4% formaldehyde in PB.

Pieces of retina prelabeled with DAPI were visualized on an Olympus BX-50WI microscope equipped with epifluorescence. HCs were impaled under visual control by using pipettes whose tips were filled with 4% Neurobiotin (Vector Laboratories,) and 0.5% Lucifer yellow-CH (Molecular Probes) in ddH2O, then back-filled with 3 M LiCl. Electrode resistance was ~100 MΩ. The impaled cells were injected with a biphasic current (±1.0 nA, 3 Hz) for 10 min. After injection, the retinal pieces were fixed in 4% paraformaldehyde.

Rabbit anti-Cx40 antibody that cross-reacts with Cx50 C-terminus was purchased from Chemicon (dilution 1:1000). This antibody specifically stains Cx50 gap junctions in A-type HCs of the rabbit retina [19]. Rabbit anti-Cx57 antibody (dilution 1:200) was used. In our recent work [21], the specificity of antibody was tested and it specifically stained Cx57 gap junctions in B-type HC axon terminal network of the rabbit retina.

Wholemount pieces of retina after intracellular dye injection or vibratome sections were blocked in 5% donkey serum in PB for 2 h to reduce nonspecific labeling. The tissue was incubated in primary antibody (anti-Cx40/50 or anti-Cx57) in the presence of 1% donkey serum/PB with 0.5% Triton X-100/0.1% sodium azide overnight for vertical vibratome sections or for up to 1 week for wholemounts. The vertical vibratome sections were washed with PB and incubated in peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch) for 2 h. Washed with PB and 0.1 M Tris buffer (TB: pH 7.6), the sections were preincubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in TB for 10 min, followed by incubation in the same solution containing 0.05% hydrogen peroxide for an additional 10 min. The wholemounts were washed with PB and incubated in Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) and Cy3-Streptavidin (Jackson ImmunoResearch). Wholemount preparations were mounted with Vectashield (Vector Laboratories) and viewed with Zeiss LSM 510 Meta confocal microscope equipped with a krypton/argon laser.

For immunoelectron microscopy, retinal vertical sections were prepared and immunostained, as described above for light microscopy but without Triton X-100 and sodium azide. Stained sections were post-fixed in 1% glutaraldehyde in PB for 1 h and in 1% OsO_4 in PB for 1 h. They were washed and dehydrated in a graded series of alcohol. During the dehydration procedure, they were stained *en bloc* with 1% uranyl acetate in 70% alcohol for 1 h, then transferred to propylene oxide, and flat embedded in Epon 812. After curing at 60 °C for 3 days, well-stained areas were cut out and attached to an Epon support for further ultrathin sectioning (Reichert-Jung). Ultrathin sections (90 nm thick) were collected on one-hole grids coated with Formvar, and 180 sections were examined using an electron microscope (Jeol 1200EX).

3. Results

Immunohistochemistry performed on vertical sections of the rabbit retina revealed that there are two types of Cx50-immunoreactive plaques in the outer plexiform layer (OPL) (Fig. 1A). One type was large, often string-like, and localized to the proximal part of the OPL. The second type was small or dot-like and localized to the distal OPL. These two Cx50 plaque types could be distinguished in wholemount preparations (Fig. 1B) where A-type HCs were dye injected with Neurobiotin to visualize the dendritic matrix (Supplementary Fig. S1A). Essentially all the Cx50 plaques were confined to the A-type HC dendritic matrix. Large plaques or strings were found at large A-type HC dendritic crossings whereas small plaques or dots were observed in terminal clusters, each of which is formed by the convergence of fine dendrites from several A-type HCs. These findings suggest that large Cx50 gap junction plaques may be found at the crossings of A-type HC proximal dendrites and the small ones may be localized at distal fine dendrites.

Using preembedding immunoelectron microscopy, Cx50 gap junctions were easily identified as electron-dense DAB products. Unfortunately, we were unable to observe the typical pentalaminar ultrastructure of gap junctions, probably due to the dense staining. However, the two labeled cell membranes were more closely apposed than other intercellular spaces formed between neighboring unlabeled cell membranes. A total of 166 putative Cx50 gap junctions were observed in the OPL. They could be divided into two types. The first type ($n = 75$) was the large gap junctions (>2 μm in length) which were exclusively localized in the proximal half of the OPL (Fig. 1C). They were formed between two putative A-type HC proximal dendrites (large one in Fig. 1C) and between somata and passing dendrites of putative A-type HCs (Supplementary Fig. S2). The second type ($n = 91$) was smaller (<2 μm in length) and they were mainly found in the middle of the OPL (small one in Fig. 1C). All large Cx50 gap junctions and most of the small Cx50 gap junctions appeared to be homotypic, *i.e.* symmetrically labeled on both sides of the gap junction (Fig. 1C and Supplementary Fig. S2). However, in some interesting cases ($n = 12/91$) Cx50 immunoreactivity was restricted to only one dendritic membrane (Fig. 1D and Supplementary Fig. S3), suggesting the presence of heterotypic gap junctions or hemichannels. These putative Cx50 hemichannels or heterotypic gap junctions were found in the distal OPL, beneath rod spherules or cone pedicles, but we never observed contact or penetration of the photoreceptor terminal.

In vertical sections, Cx57 immunoreactivity was found exclusively in the OPL as small plaques. Cx57-immunoreactive plaques were relatively constant in size and localized in the middle to distal part of the OPL (Fig. 2A). In wholemounts, the B-type HC axon terminal matrix was visualized by filling with Neurobiotin (Supplementary Fig. S1B). The resulting network of fine processes could be identified as the axon terminal matrix by the absence of B-type HC somas and the presence of many fine branch tips matching the density of the rod spherules. When this preparation was processed for Cx57 immunoreactivity, the Cx57 plaques were confined to the B-type HC axon terminal matrix (Fig. 2B). Essentially all the Cx57 plaques were colocalized with the axon terminal matrix and therefore appear yellow. Most plaques were distributed along the axon terminal branches but it was difficult to determine whether Cx57 plaques were located at branch crossings, due to the limitation of resolution. Cx57 plaques were not found at the fine branch endings which enter individual rod spherules. These results suggest that Cx57 gap junctions may be localized on axon terminal branches which form a densely coupled matrix beneath the level of rod spherules.

As previously for Cx50, preembedding immunoelectron microscopy with specific antibodies against Cx57 was performed to examine the exact localization and the ultrastructural features