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Research report

Calbindin-D28k and calretinin as markers of retinal neurons in the anuran amphibian *Rana perezi*

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Abstract

In the present study we have analyzed the distribution of the calcium binding proteins calbindin-D28k (CB) and calretinin (CR) immunoreactive cells in the retina of the anuran *Rana perezi* using poly- and mono-clonal antibodies that were proven to be specific in the amphibian brain, without cross-reactivity. Double immunohistofluorescence techniques were used to demonstrate colocalization of both proteins in the same retinal cells. In addition, retrograde tracing experiments from the optic nerve were conducted to labeled ganglion cells and these were observed in combination with CB and/or CR immunohistochemistry. Cells containing CB were identified as all cones, scattered bipolar and amacrine cells together with cells in the ganglion cell layer. The pattern of CR immunoreactivity was strikingly different. Abundant cells contained CR in the inner retinal layers including horizontal, bipolar and amacrine cells, and cells in the ganglion cell layer. By means of double immunohistochemistry it was found that only subpopulations of amacrine cells and cells in the ganglion cell layer contained both CB and CR. Tracing from the optic nerve revealed retrogradely labeled ganglion cells with different morphologies and most of them contained CB and/or CR. All these data taken together suggest that in amphibians CB and CR are distinctly and precisely distributed in retinal neurons showing, however, peculiar features not observed previously in other vertebrates.

Keywords: Calcium binding proteins; Immunohistochemistry; Evolution; Amphibian; Eye

1. Introduction

Immunohistochemical techniques for localizing the calcium binding proteins calbindin-D28k (CB) and calretinin (CR) have been extensively used to study cell morphology in the retina of vertebrates [2,4,8,9,12–15,19–21,23]. However, the actual functional role of these proteins in the retina is not fully known but it is admitted that they are involved in calcium homeostasis and, therefore would control aspects such as phototransduction, light adaptation, photoreceptor motility and neurotransmission [16,18].

Previous studies in amphibians have yielded different results about the distribution of CB and CR in retinal cells, most likely due to the different antibodies used [7,10–12,19,20,24,25]. Since CB and CR have 50–60% sequence homology it is thought that in some of the previous works using polyclonal antibodies, cross-reactivity was obtained, in particular because the used antibodies against chick intestinal CB also recognized CR [12,19,20]. In the present study we have analyzed the distribution of CB immunoreactive (CBir) and CR immunoreactive (CRir) cells in the retina of the anuran *Rana perezi* using poly- and mono-clonal antibodies that were proven to be specific in the amphibian brain, without cross-reactivity [17]. In addition, we performed double immunofluorescence experiments to demonstrate colocalization of both proteins in the same retinal cells. Finally, in several amphibians as in representatives of almost all vertebrate classes, CB and/or CR were described in neurons of the ganglion cell layer that given their heterogeneous morphology could not be discerned as ganglion cells or displaced amacrine cells. Therefore, we conducted retrograde tracing experiments from the optic nerve that labeled ganglion cells and these were observed in combination with CB and/or CR immunohistochemistry to evaluate double or triple colocalization.

2. Materials and methods

In the present study, a total of nine green frogs (*R. perezi*) were used. The animals were obtained from the laboratory stock

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of the Department of Cell Biology, University Complutense of Madrid. The original research reported herein was performed under the guidelines established by the Spanish Royal Decree 223/1988. All animals were deeply anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz; pH 7.3) and perfused transcardially with saline followed by 200 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4). The eyecups were removed and kept in the same fixative for 2 h. They were then immersed in a solution of 30% sucrose in PB for 5 h at 4 °C, embedded in a solution of 20% gelatine with 30% sucrose in PB, and then stored for 6 h in a 4% formaldehyde solution at 4 °C. The eyecups were cut into radial sections on a freezing microtome at 20 μm thickness and collected in PB.

2.1. Single immunohistochemistry

The immunofluorescence method used included a first incubation of the sections in a rabbit anti-calbindin-D28k (CB) or rabbit anti-calretinin (CR) serum (both by Swant), diluted 1:1000 in PB containing 0.5% Triton X-100 (PBS-T), for 48–72 h at 4 °C. Subsequently, they were rinsed in PB for 10 min and incubated in the second antibody Alexa 488-conjugated goat anti-rabbit (green fluorescence; diluted 1:300 in PBS-T; Molecular Probes) for 90 min at room temperature. The specificity of the primary antibodies used in this study was previously tested for amphibians [17].

2.2. Double immunohistochemistry

For the CB/CR colocalization analysis a two-step protocol with antibody cocktails was used as follows: (1) incubation was for 72 h at 4 °C in a mixture of primary mouse anti-CB (Swant) and rabbit anti-CR antibodies (both diluted 1:1000 in PBS-T); (2) second incubation was for 90 min at room temperature in a mixture of Alexa 488-conjugated goat anti-mouse (green fluorescence; diluted 1:300 in PBS-T; Molecular Probes) and Alexa 594-conjugated goat anti-rabbit (red fluorescence; diluted 1:500 in PBS-T; Molecular Probes).

2.3. Dextran amine tracing combined with immunohistochemistry

In three deeply anesthetized frogs, biotinylated dextran amine (BDA 10 kDa, Molecular Probes) was applied unilaterally to the optic nerve. After a survival period of 5–7 days, the retinas were prepared as described above. In all cases, the tracer was observed in combination with immunohistochemistry for CB and CR.

3. Results

The distinct patterns of distribution for CB and CR observed in the retina of *R. perezi* were consistent from animal to animal. Moreover, the two anti-CB (monoclonal and polyclonal) antibodies gave essentially identical results, although staining intensity was slightly lower with the monoclonal antibody.

3.1. CB immunolabeling

The retina of *R. perezi* shows thick and well-organized layers in which CB was found in subpopulations of many retinal neuron types (Fig. 1a–c). The most outstanding CBir cells were photoreceptors. As observed in Nissl-stained retina, the outer nuclear layer (ONL) is organized into two rows where rod nuclei are generally more scleral than cone nuclei (Fig. 1a). Thus, CBir photoreceptors corresponded to the inner row of cones that were strictly aligned above the narrow outer plexiform layer (OPL) (Fig. 1b and c) and were strongly stained in their cell bodies and inner segments, whereas the outer segments lacked immunoreactivity. A few scattered bipolar cells, which are the most numerous in the inner nuclear layer (INL), were CBir. Their perikarya were located in the outer part of the INL and showed short dendrites that arborized in the OPL beneath the cones and axons that crossed the INL and arborized in the outer part (off laminae) of the thick inner plexiform layer (IPL) (Fig. 1b and c). Among the CBir cell bodies located immediately below the OPL no CBir horizontal cells could be identified. A scattered population of CBir neurons was observed in the inner portion of the INL that judging from their morphology appeared to be amacrine cells, although dendritic morphology was not very evident (Fig. 1b and c). Finally, CBir cells also included a few sparse neurons in the ganglion cell layer (GCL). Because small and large CBir cells were found in the GCL, they could correspond to both ganglion cells and/or displaced amacrine cells, known to be present in the GCL of amphibians.

3.2. CR immunolabeling

The pattern of CR immunoreactivity observed was strikingly different from that of CB, supporting the lack of cross-reactivity of the used antibodies. All photoreceptors were CR immunonegative, whereas abundant cells were CRir in the deeper retinal layers (Fig. 1d and e). Three different cell types were CRir in the thick INL of *R. perezi*. First, horizontal cell somata were labeled in the margin of the INL, just beneath the OPL. These cells formed a scattered population that often showed flattened bodies and multiple processes that arborized in the OPL. The most numerous CRir cell population was constituted by several rows of bipolar cell perikarya located in the outer half of the INL (Fig. 1b and d). The inner half of the INL was almost devoid of CRir bipolar cells allowing the clear distinction of the third CRir cell population, the amacrine cells. Their perikarya were of different morphologies, i.e. some of them were directly found in the inner margin of the INL, whereas others were located more into the INL (Fig. 1b and d). Strikingly, the processes of CRir neurons (bipolar, amacrine and ganglion cells) arranged into 5–6 distinct sublaminae in both the off and on sublayers of the thick IPL (Fig. 1b). Finally, intensely CRir neurons were found in the GCL. This abundant cell population was heterogeneous and was constituted by large, medium-sized and small neurons. In addition, CRir fibers were observed in the optic fiber layer.