Calcium Binding Protein Immunoreactivity in Pigeon Retina

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Pigeon retina has been mapped immunocytochemically for vitamin D-dependent calcium-binding protein (D-CaBP). Immunoreactivity was found in the cones of the yellow field, but not in photoreceptors of the red field. The D-CaBP-containing cones were a subpopulation of those in the yellow field having straight fibres leading to their synaptic terminals. D-CaBP immunoreactivity was also found in horizontal cells, the amount present varying according to position along the retina, and in some amacrine cells. Immunoblots of pigeon retinal proteins separated by SDS-polyacrylamide gel electrophoresis indicated two D-CaBP forms, having apparent molecular weights of 27000 and 29000. Both these forms of D-CaBP have been found previously in rat and pigeon brain. Invest Ophthalmol Vis Sci 28: 658–664, 1987

Vitamin D-dependent calcium binding protein (D-CaBP) was discovered in intestinal mucosa, and experimental evidence linked this protein to calcium absorption.1 The development of sensitive assays for D-CaBP, however, showed that it was present in many tissues including brain tissue from birds and mammals,2 and had a wide species distribution.1 Thus a more generalized role than being solely concerned with intestinal calcium absorption is attributed to this protein.

The retina, which is an extension of the diencephalon, has recently been found to be D-CaBP immunoreactive in several vertebrate species3 including human.4 This study reports the distribution of D-CaBP immunoreactivity in pigeon retina, and reports the characterization in terms of apparent MW of the immunoreactive protein(s). Pigeon retina contains red and yellow fields with the former comprising mainly the dorso-temporal quadrant of the retina.5 The colour of these fields is due to oil droplets lying between outer and inner segments of the cones. The cones may be coloured red, yellow, or orange, and a proportion of the cones may be colourless. Red and yellow cones are particularly numerous in the red and yellow fields respectively, but are less frequently found in the rest of the retina.

Materials and Methods

Whole pigeon retinas were dissected from adult pigeon (Columbia Livia domestica), and fixed during 4 days in sublimate Bouin Hollande (Merck, Belgium) fixative. Retinas were thoroughly washed in 90% ethanol, and processed for embedding in paraffin and 5 μm sections cut for the histochemical study. The sections were oriented parallel to the pecten.

Chick intestinal D-CaBP antibody was prepared as described previously.6 Immunoperoxidase staining was carried out as follows: routinely dewaxed and hydrated sections were processed for immunochemistry according to a modified peroxidase-antiperoxidase (PAP) procedure.7 Serum dilutions were made up in Coons Veronal (Fluka, Switzerland) buffered saline (10 mM Na Veronal, pH 7.2, NaCl 0.9%) (CVBS) supplemented with 5% vol/vol normal sheep serum. The immunostaining sequence was done as follows: 1) rinse in CVBS, 2) preincubation in 5% normal sheep serum, 3) incubation with anti-chick D-CaBP at 1/6000 dilutions for 48 hr in a moist chamber, 4) incubation with sheep anti-rabbit IgG serum 1/80 dilution, 5) incubation with soluble rabbit PAP complex 1/300 dilution (DAKO, Denmark).

Each of these steps was performed at room temperature for 10 min, except for step 3, which was performed at 4°C. After a last rinse, sections were incubated with 3,3'-diaminobenzidine-HCl (Sigma, St. Louis, MO), 5 mg/10 ml dissolved in 100 mM phos-
Fig. 1. Yellow field of the pigeon retina treated with D-CaBP serum (1/6000 dilution); not counterstained. Photoreceptors, OPL, horizontal and amacrine cells (white star) are D-CaBP positive. Four immunoreactive stratifications can be distinguished in the IPL. EP = epithelium pigment; PL = photoreceptors layer; OPL = outer plexiform layer; INL = inner nuclear layer; GC = ganglion cell layer; FL = fiber layer; ILM = internal limiting membrane (PAP stain x380).

Western Blotting

The detailed procedure is published elsewhere. Retinas (200 mg of tissue/5 ml buffer) were homogenized in a buffer containing 100 U/ml Trasylol, 10 mM Tris-HCl, pH 8.0, at 4°C, and centrifuged for 2 min at 15,000 g in a microfuge. The supernatants were denatured by boiling for 2 min in a medium containing 1% SDS, 5% 2-mercaptoethanol, 2 mM EDTA, 20% sucrose, and 0.1% phenol red. The denatured proteins were separated by electrophoresis on a SDS-12.5% polyacrylamide slab gel, and were blotted onto a nitrocellulose sheet using a Biorad transblot cell (30 V, 0.9 A for 5 hr at 4°C). The molecular weights were calculated after blotting and using (14C) methylated proteins with known molecular weights (Amersham International, Belgium).

Results

Immunohistochemistry

In the receptor cell layer, D-CaBP was detectable only in the cells of the yellow field of the pigeon retina (Fig. 1) including the receptors in the area fovealis located within this field (Fig. 3). The protein could not be detected in the photoreceptors of the red field (Fig. 2), and counterstaining the sections showed that not all the photoreceptors in the yellow field were labelled. Within the photoreceptors, D-CaBP was observed in: a) the outer and inner segments, b) the cell body, c) the nucleus, and d) the synaptic pedicles. The labelled photoreceptors are identified as cones, since the region between the outer and inner segments contains a clear round zone corresponding to dissolved oil droplets (Fig. 4). Furthermore the fibres extending from their cell
Fig. 2. Red field of the pigeon retina treated with D-CaBP antiserum (1/6000 dilution); not counterstained. Photoreceptors are not D-CaBP immunoreactive. Horizontal (black star) cells are weakly labelled. Amacrine (white star) cells and their processes (arrow) are strongly labelled. Four immunoreactive stratifications can be distinguished in the IPL. Few cells are CaBP positive in GC. EP = epithelium pigment; PL = photoreceptors layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GC = ganglion cell layer; FL = fiber layer; ILM = internal limiting membrane (PAP stain X380).

Fig. 3. Section of pigeon retina through the fovea treated with D-CaBP (1/6000 dilution) and counterstained with haematoxylin. Red field (RF) is to the left of the section; the fovea (F) is the middle and the yellow field (YF) is on the right of the section. Observe the variation of the labelling of the photoreceptors (PL). (PAP stain X60).
Fig. 4. Photoreceptors in the yellow field of pigeon retina treated with D-CaBP (1/6000 dilution) and counterstained with haematoxylin. Outer segments (arrow), inner segments (is), cell body (cb) and synaptic pedicles (sp) are CaBP positive. Observe the dissolved oil droplet between the inner and the outer segment (star) and the straight pedicles of these photoreceptors. Labelled horizontal cells (h). A labelled bipolar cell is distinguishable (two arrows) (PAP stain ×60).

bodies to their synaptic terminals are straight and perpendicular to the retina (Figs 1 and 4); and all their pedicles extend to the same stratum of the outer plexiform layer (OPL).

In the OPL, two D-CaBP-positive stratifications can be distinguished (Fig. 5), the outer one consisting of the photoreceptors synaptic pedicles, and the inner one composed of cell processes from horizontal cells (Fig.

Fig. 5. Photoreceptors, OPL and INL in the yellow field of pigeon retina. Section treated with D-CaBP antiserum (1/6000 dilution); counterstained with haematoxylin. PL = photoreceptor layer. OPL (outer plexiform layer) with two stratifications: a) outer one (one arrow) is synaptic pedicle of photoreceptors; b) inner one (two arrows) is processes from horizontal cells (H) (PAP stain ×760).
6). Both stratifications are strongly labelled in the yellow field, whereas the labelling is weak in the redish area. The inner nuclear layer contains both horizontal and bipolar cells. Immunoreactivity is found in the perikaryons and processes of the horizontal cells in both the yellow and red fields, although the intensity of the reaction product was greater in the yellow area (compare Figs. 1 and 2). An indication that some of the bipolar cells are labelled is given by the presence of reaction product in the small perikaryons in the outer and middle sections of the inner nuclear layer (INL). These cell bodies also have long, thin processes perpendicular to the retina and reaching the IPL (Fig. 4). Muller cells are D-CaBP negative in both the pigeon and chick retina. In the inner part of the INL, amacrine cells are strongly labelled in both the red and yellow fields (Figs. 1, 2). In the inner plexiform layer, amacrine cell processes and four horizontal sublayers are D-CaBP positive, but ganglion cell dendrites are not labelled. In the ganglion cell layer, few cells contain D-CaBP, and both the fiber layer and the inner limiting membrane are negative.

**Western Blotting**

Among the numerous proteins of pigeon homogenates are two that cross react with a-D-CaBP (Fig. 7). Their apparent molecular weight (27,000 and 29,000 daltons) are identical to the two D-CaBP found in pigeon and rat brain.
Discussion

D-CaBP has been previously localized in cells of chick, frog, and human retina, and its presence established in the photoreceptor layer of these tissues, but it could not be clearly shown in the photoreceptors of mouse or rat retina. Recently, we showed that in humans, it is only cones and not rods which contain CaBP. Since the mouse and rat contain very few cones, it seems likely that D-CaBP is present in the cones of other species as well as human. In birds, cones can be identified relatively easily because of the oil droplets, which may be coloured. In this study, CaBP immunoreactivity in the photoreceptor layer was located only in cells of the yellow field, and never in the red field. The immunoreactivity was also present in a subpopulation of cones having straight pedicles, known as straight cones. Three different straight cones have been described for both chick and pigeon differing by their color oil droplets. The yellow field of pigeon retina is similar in cone composition to the chicken retina, being dominated by double cones. In the red sector, there is a majority of red and orange cones. Further characterizations are needed to demonstrate which cone subtypes contain D-CaBP in different species, and reasons why only particular cone population do express D-CaBP gene need to be explored.

In horizontal cells, D-CaBP is also present, but with different intensities along the retina. According to the classification made by Cajal, soma and dendrites of type I "brush shaped" horizontal cells are D-CaBP positive. It is less clear if axons terminals from type I and type II horizontal cells contain D-CaBP.

This study confirms previous observations on D-CaBP distribution in tissues with a complex cell pattern. It seems that this protein has a specific cellular distribution in tissues containing cells with different physiological characteristics. This is the first demonstration that cones can be divided into separate groups according to their biochemical, as well as their histological, appearance. Presumably, the different groups of cones have different physiological roles or characteristics, and they may be a good model for identifying the role of D-CaBP in the retina and the brain.

Amacrine cells are known to contain various neuropeptides including TRH, somatostatin, enkephalin, vasointestinal polypeptide, glucagon, cholecystokinin, neurotensin, CRF and LRF. In the retina, the stimulated release of TRH, somatostatin, and substance P is calcium-dependent, and in another study, it was also demonstrated that the K+-stimulated increase of substance P release was inhibited by the absence of Ca2+ in the normal K+-rich physiological solution. We propose that Ca2+ translocation and its buffering by D-CaBP play a role in the neuromodulator and/or neurotransmitter action of amacrine cell.

Key words: calcium binding protein, pigeon retina, immunohistochemistry, photoreceptor cells, western blotting

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References


