Cell Tissue Res (1983) 232:691–696

Cell and Tissue Research

Short Communication

Immunocytochemical detection of calcium-binding protein in the cochlear and vestibular hair cells of the rat

A. Rabié, M. Thomasset*, and Ch. Legrand
Laboratoire de Physiologie comparée, ERA 955 du CNRS "Neurobiologie du Développement et Endocrinologie", Université des Sciences et Techniques du Languedoc, Montpellier, France
* Unité de Recherche sur le Métabolisme hydro-minéral, INSERM U 120, Le Vésinet, France

Summary. Specific antibodies raised against human cerebellar calcium-binding protein (CaBP) intensely labelled the cochlear hair cells of the rat. The vestibular hair cells also stained weakly. In both inner and outer cochlear hair cells, the cuticular plate was the most stained area. These results suggest that CaBP may prevent excessive concentrations of intracellular calcium and thus modulate some Ca^{2+}-mediated biochemical processes, especially at the level of the cuticular plate and stereocilia; CaBP could be involved in the mechanochemical coupling of hearing or vestibular function.

Key words: Calcium-binding protein (CaBP) – Cochlear hair cells – Vestibular hair cells – Vitamin D

Vitamin D-dependent calcium-binding protein (CaBP) was first described in the chick duodenal mucosa by Wasserman and Taylor (1966). It was then demonstrated in the chick cerebellum (Taylor 1974) and other organs (Corradino et al. 1968; Christakos et al. 1979). In birds, this protein has a molecular weight of 28000 and four high-affinity calcium-binding sites per molecule. In the rat, a protein of similar size is primarily concentrated in the renal cortex and cerebellum, while a smaller CaBP of 10 000 MW is confined to the small intestine. However, many other organs and tissues contain small quantities of the 28000 MW CaBP (Thomasset et al. 1982).

Send offprint requests to: A. Rabié, Laboratoire de Physiologie comparée, Université des Sciences et Techniques du Languedoc, 34060 Montpellier Cedex, France

Abbreviations used: CaBP, calcium-binding protein; DAB, 3,3′-diaminobenzidine; PAP, unlabelled antibody peroxidase-antiperoxidase immunocytochemical complex

In the brain, this CaBP was recently found to be restricted to specific types of neurons as, for example, cerebellar Purkinje cells (Jande et al. 1981; Roth et al. 1981). The physiological function of CaBP in these neurons is still unexplained. Some of the CaBP-containing cells are known to produce voltage-dependent calcium spikes. Through its high calcium affinity, CaBP could act as a calcium buffer to avoid the appearance of excessive levels of intracellular calcium concentration during these spikes (Jande et al. 1981).

Recent studies (Flock et al. 1981; Zenner 1981) have reported the presence of actin and actin filaments as well as of tubulin and microtubules in the cochlear hair cells. They have also suggested that both actin filaments and microtubules provide the structural support for mechanochemical coupling in hearing. On the other hand, calcium has an important role to play in microtubule assembly-disassembly and in contractile mechanisms. The presence of 28000 MW CaBP in the cochlear and vestibular hair cells of young rats is reported for the first time in this paper. We used a specific antiserum against 28000 MW human cerebellar CaBP and the unlabelled antibody peroxidase-antiperoxidase immunocytochemical complex (PAP) technique.

Materials and methods

Histology. Three-day-old Wistar rats were killed under ether anesthesia by intracardiac perfusion-fixation with modified Bouin-Hollande fixative (picric acid 3.6%, cupric acetate 2.3%, formalin 3.6%). The tympanic bulla was then dissected and embedded in paraffin wax and 5-μm thick longitudinal sections were prepared.

Immunocytochemical products. Anti-28000 dalton CaBP serum was obtained from rabbits immunized against purified human cerebellar CaBP (Baimbridge et al. 1982). The specificity of the antiserum was checked by Ouchterlony's double immunodiffusion technique, by radioimmunoassay (Thomasset et al. 1982) and by immunoprecipitation (Thomasset et al. 1983). These investigations clearly established that the primary antiserum (rabbit antihuman cerebellar CaBP) was specific for human 28000-MW CaBP and cross-reacted only with CaBP having the same molecular weight in rat kidney and cerebellum. Neither calmodulin and other calcioproteins nor the 7500–10000-MW CaBP present in the intestine of the rat cross-reacted with cerebellar CaBP antibodies (Thomasset et al. 1982, 1983). Other immunocytochemical products were from Sternberger-Meyer Immunocytochemicals, Inc, USA, (goat anti-rabbit γ-globulins, normal goat serum, peroxidase-antiperoxidase complex) or from the Institut Pasteur, Paris, France (normal rabbit serum). 3,3′-diaminobenzidine (DAB) was from Touzart and Matignon (France).

Immunocytochemical procedures. Staining was performed using the peroxidase-antiperoxidase (PAP) method of Sternberger (1979). Briefly, the sections were flooded with diluted anti-CaBP serum in phosphate buffer (0.1 M, pH 7.4) for 16 h at 4° C (1:200–1:6000 dilutions were tested; we used 1:2000 which gave the clearest staining). They were then carefully washed with phosphate buffer and flooded with goat anti-rabbit γ-globulin (diluted 1:40 in buffer) for 30 min. After washing in buffer, the sections were covered for 30 min with a solution containing the PAP complex (0.033 mg of antiperoxidase/ml of phosphate buffer containing 1% of normal goat serum). The slides, incubated for 10–15 min with 0.05% DAB, 0.01% H_2O_2 in Tris saline 0.05 M buffer, pH 7.6, were then washed several times and mounted in Permount (Fisher Scientific Company, USA). For the control sections, we replaced the specific antibody by normal non-immune rabbit serum (dilution 1:2000) or specific antiserum preabsorbed with excess of rat cerebellar CaBP (500 μg of purified rat cerebellar CaBP per ml of undiluted antiserum). Rat cerebellar CaBP was purified to electrophoretic homogeneity

by successive gel filtrations and ion-exchange chromatographies according to Hitchman et al. (1973).

Results and discussion

The use of the immunocytochemical procedure involving antiserum to human 28000-MW CaBP intensely labelled rat cochlear hair cells, and especially the inner hair cells, throughout the cochlear duct. The CaBP reaction product was localized throughout the cytoplasm of the hair cells. The nucleus was poorly labelled, especially in the outer hair cells. In both inner and outer hair cells, the cuticular plate was the most stained organelle (Fig. 1a). The vestibular hair cells were also stained but the labelling was rather weak compared to that of the cochlear hair cells (Fig. 1c). Lastly, changes in antiserum dilution always gave the same difference in staining between the three types of hair cells observed.

No staining was observed on cochlear hair cells (Fig. 1b) or on vestibular hair cells (not shown) when the specific antiserum was preabsorbed by antigen excess.

Our data on 3-day-old rats demonstrate that specific cell populations found in the inner ear (the inner and outer cochlear hair cells and the vestibular hair cells) contain a protein with an antigenic determinant common to cerebellar 28000-MW vitamin D-dependent CaBP in the same species. At 3 days, i.e. about one week before the onset of cochlear potentials (Crowley and Hepp-Reymond 1966), the structure and the innervation of these cells are still immature (Lenoir et al. 1980; Sans and Chat 1982). However, the cells have already developed the complex structure of stereocilia anchored to the cuticular plate. This structure is supposed to be at the origin of the mechanochemical coupling in hearing or vestibular function. The precise mechanism of this coupling however is still unknown. Flock and Cheung (1977) and Flock et al. (1981) have reported that actin is present in the stereocilia of vestibular and cochlear hair cells. Zenner (1981) has shown that tubulin and actin are also present in the stereocilia and the cuticular plate of cochlear hair cells. He proposed that microtubular proteins and actin form the cytoskeletal network which maintains the shape and stiffness of cochlear sensory hairs and that the contractile capacity of these hairs could be at the origin of mechanochemical coupling in hearing. Furthermore, Macartney et al. (1980) have demonstrated the presence of myosin in the same structures. Thus, the presence of putative high concentrations of CaBP suggests an important role of calcium in the cochlear hair cells and supports the hypothesis of the contractile capacity of the sensory hairs. A comparable situation has already been described in the intestine. Actin (Bretscher and Weber 1978), as well as calcium-dependent contractile activity (Mooseker 1976), have been demonstrated in microvilli. Furthermore, Glenney et al. (1980) have shown that the highly-ordered cytoskeletal structure of intestinal microvilli disassembles when the calcium level is greater than 10^{-6} M. They proposed that calmodulin, with about a 10^{-6} M affinity

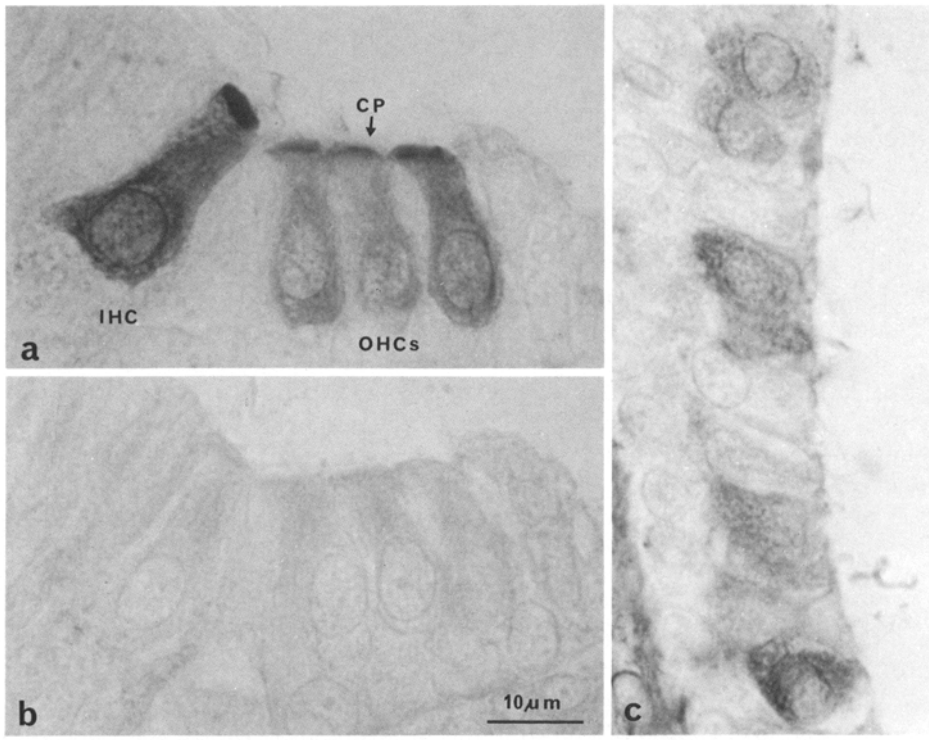

Fig. 1 a–c. Immunocytochemical detection of calcium-binding protein in inner ear receptor cells. **a** Staining is more intense on the inner hair cells (*IHC*) than on the outer hair cells (*OHCs*) of the cochlea. In both cell types, the cuticular plate (*CP*) appears particularly labelled. **b** Control section of cochlear hair cells made by using specific antiserum preabsorbed by rat cerebellar 28000 MW CaBP excess. **c** Staining is weak in the vestibular hair cells

for calcium, could exert Ca^{2+}-buffering activity. With its high affinity for calcium (about $2 \cdot 10^{-6}$ M, Bredderman and Wasserman 1974), CaBP might prevent ionic calcium concentration from increasing above 10^{-6} M (Baimbridge et al. 1982). Therefore at the level of the terminal web, where it is present in high concentrations (Marche et al. 1980), CaBP may exert this buffering activity. By acting as a calcium buffer in the inner ear, CaBP may also contribute to the protection of the cytoskeleton of the hair cell, especially in its apical part near the cuticular plate where CaBP concentration seems to be very high. By thus protecting the cell structure, CaBP would favour the existence of calcium-regulated contractile mechanisms involving actin, myosin and perhaps other proteins, such as calmodulin, related to calcium regulation.

It must also be noted that the concentration of CaBP in a localized area like the cuticular plate suggests that this protein, usually considered as cytosolic, may sometimes be tightly bound to subcellular structures. The cochlear hair cells would be good material for studying this property.

Lastly, the demonstration of the presence of 28000 MW CaBP in the cochlear and vestibular hair cells of the rat inner ear suggests that target tissues of the vitamin D endocrine system extend beyond classical sites such as intestine, kidney, bone, mammary gland and skin (Colston et al. 1980).

Acknowledgements. We wish to thank Pr. J. Legrand for his advice and encouragement, C.O. Parkes for kindly supplying the specific 28000 MW CaBP antiserum and A. Uziel for helpful discussion. The expert technical assistance of C. Aimar, F. Caruso and M.C. Clavel is also gratefully acknowledged. This work was supported by INSERM (Prc. N° 135 016).

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Accepted March 19, 1983