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Immunocytochemical analysis of cyclic AMP receptor proteins in the developing rat parotid gland

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ABSTRACT

Previous studies showed that regulatory subunits of type II cyclic AMP-dependent protein kinase (RII) are present in adult rat parotid acinar cells, and are secreted into saliva. If the synthesis and intracellular distribution of RII exhibit developmental specificity, then RII can be an indicator of secretory and regulatory activity of salivary glands.

Objective: To determine the expression and distribution of RII in the rat parotid at specific ages representing defined developmental stages.

Methods: Parotid glands of fetal, neonatal and adult rats were prepared for morphologic and immunocytochemical study. The cellular distribution of RII was studied using light microscopic immunogold silver staining with anti-RII, and its intracellular distribution using electron microscopic immunogold labeling.

Results: *In utero*, parotid RII levels were low; 5–18 days after birth, labeling of secretory granules and cytoplasm rose to a peak, followed by a rapid decrease in both compartments at 25 days. At 60 days, granule labeling increased to levels near those at 18 days, whereas cytoplasmic labeling remained low. Nuclear labeling was highest during the first 3 weeks after birth, and then declined.

Conclusions: The higher nuclear and cytoplasmic labeling during the neonatal period may reflect RII involvement in acinar cell differentiation. The accumulation of RII in secretory granules is similar to the pattern of the major salivary proteins, amylase and PSP. The redistribution of RII in these compartments during development may reflect changing gene expression patterns, and may be useful for identification of genetic or metabolic abnormalities.

1. Introduction

Rat salivary glands begin their development on day 13–14 of gestation. Cytodifferentiation of the sublingual gland is relatively complete at birth (21–22 days of gestation),[1,2] whereas the submandibular and parotid glands are immature at birth and their differentiation is completed postnatally.[3–7] The development of both the submandibular and parotid glands can be divided into four distinct stages: embryonic (or prenatal), perinatal (or neonatal), transitional, and adult.[7] The embryonic or prenatal stage encompasses epithelial proliferation, ingrowth from the oral ectoderm, branching mor-

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phogenesis and formation of terminal clusters and ducts, and development of a lumen (for reviews, see[8,9]). Melnick and Jaskoll[10] and Tucker[11] have further subdivided the embryonic development of the submandibular gland into initial bud, pseudoglandular, canalicular and terminal bud stages.

During the perinatal (neonatal) stage in the parotid, from 1-day postnatal to about 9 days of age, the cells of the terminal clusters differentiate into secretory endpiece cells and begin synthesis and accumulation of some secretory proteins. During the transitional stage, from 9 days to about 25 days old, the cells of the endpieces initiate synthesis of additional secretory proteins and complete their transformation to the adult phenotype. In the adult stage, from 25 days on in the parotid, the secretory cells exhibit typical adult structure, and the glands continue to increase in size. In the submandibular gland, there are major rearrangements of cells within the endpieces and in the first segments of the ducts during the perinatal and transitional stages, as well as significant changes in secretory protein expression and cellular morphology.[12,13] In addition, the submandibular gland undergoes a hormone-dependent differentiation of the intralobular duct system to form the granular convoluted ducts. In the parotid gland, the structural changes are less marked, but changes in secretory protein expression are significant.[7]

The expression of several salivary secretory proteins, for example, some of the proline-rich proteins, is regulated, in part, by cyclic AMP.[14] Moreover, secretory protein release from the cells by exocytosis also is regulated by cyclic AMP.[15,16] Cyclic AMP exerts its effects on most tissues, including the salivary glands, by activating protein kinase A (PKA). Binding of cyclic AMP to the regulatory (R) subunits of PKA, which are cyclic AMP receptor proteins, frees the catalytic subunits to phosphorylate specific substrate proteins involved in metabolic, transcriptional and secretory events.[17] The R subunits, particularly those of type II PKA (RII), also function to localise PKA to specific subcellular sites through binding to A-kinase anchoring proteins (AKAPs).[18,19] The AKAPs form protein scaffolding that directs the specificity of downstream signal targeting.[20,21]

In rat and human parotid acinar cells, RII is a polyfunctional protein present in the nuclei, cytoplasm, and secretory granules, and is released into saliva upon granule exocytosis.[22–25] RII also is secreted by several other cell types.[26] The intracellular functions of RII include binding cyclic AMP, other proteins (e.g., PKA catalytic subunits and AKAPS) and nucleic acids; its precise extracellular functions are not known, but probably are of a similar nature.

Because of its significant functions intracellularly as well as in saliva, the expression of RII during development of the rat parotid gland was determined using immunocytochemistry at the light and electron microscopic levels. The possibility that PKA is compartmentalised and undergoes intracellular redistribution during differentiation of secretory function was evaluated by quantitative assessment of RII expression in different subcellular compartments of the parotid acinar cells. The basic hypothesis is that the expression pattern of the RII subunits during salivary gland development follows that of other secretory proteins. Secondly, determining the expression pattern of RII would give insight into the development of related signalling and regulatory events involved in gene expression and protein secretion. As rat (and mouse) parotid glands are structurally[27–29] and functionally[30,31] similar to the human parotid, their use in studies of events that result in structure/function modification may yield information applicable to human salivary physiology and disease.

2. Methods

Timed-pregnant female Sprague–Dawley rats were purchased from Harlan Industries (Indianapolis, IN) and housed in microisolator cages in the Center for Laboratory Animal Care, University of Connecticut Health Center (UCHC). All animal procedures were approved by the UCHC Animal Care Committee and carried out according to NIH guidelines.

Parotid glands were obtained from rats aged 20 days *in utero*, and 1, 5, 9, 14, 18, 25 and 60 days after birth, as described previously.[7] Briefly, the rats were anesthetised with ketamine/xylazine, the glands were dissected, and small pieces ($\sim$1 mm^3) were fixed in either 4% paraformaldehyde (overnight at 4 °C) or 1% glutaraldehyde (Polysciences, Warrington, PA) (1 h at room temperature) in 0.1 M sodium cacodylate buffer, pH 7.4. The tissue pieces were rinsed in 0.1 M cacodylate buffer, dehydrated in cold methanol, embedded in LR Gold or Lowicryl K4M resin (Polysciences), and polymerised under UV light at −20 °C. Randomly selected tissue blocks from two animals at each time point were used to prepare sections for light and electron microscopic study.

A polyclonal antibody reagent, rabbit antiserum to rat parotid RII,[32] was used to determine the localisation of RII in the developing parotid glands. Western blotting was carried out as described previously.[33,34] Briefly, a sample (12 μg) of commercial RII standard (Sigma Chemical Co., St. Louis, MO) and proteins of the soluble fraction of rat parotid gland tissue (20 μg total protein per lane) were separated by polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose. The membrane was treated with a blocking agent (1% bovine serum albumin (BSA)), incubated with anti-RII antibody diluted 1:1000 with phosphate-buffered saline (PBS), then with horseradish peroxidase-labeled goat anti-rabbit IgG, and developed with diaminobenzidine-H_2O_2. A reactive band with the same relative mobility as the commercial RII standard was present in the parotid extract lane (Fig. 1). A faint faster moving component of approximately 30 kDa size has been identified previously by photoaffinity labeling as an RII fragment.[23]

For light microscopic immunohistochemistry, 1 μm sections on glass slides were labeled with anti-RII diluted 1:10,000–1:40,000 in PBS containing 1% BSA and 5% normal goat serum. Bound anti-RII was detected with goat anti-rabbit IgG labeled with 5 nm gold particles (Amersham, Arlington Heights, IL), followed by silver enhancement (BB International, Cardiff, UK). Controls included omission of the primary antibody or the use of non-immune or pre-immune serum. The sections were stained with methylene blue-azure II, examined in a Leitz Orthoplan microscope, and colour images were recorded on 35 mm Ektachrome film (Eastman Kodak, Rochester, NY). The slides were scanned in a film scanner (Agfa DuoScan, Agfa Gevaert, Mortsel, Belgium), selected images were converted to grayscale and contrast and brightness adjusted in Adobe Photoshop version 6.0.1 (Adobe Systems Incorporated, San Jose, CA).