Abstract
Secreted carbonic anhydrase (isoenzyme VI; CA VI) was localized by immunohistochemistry in the developing postnatal rat submandibular and parotid glands using a specific monoclonal antibody to the rat enzyme. CA VI immunostaining was not detectable in the glands before birth. In the submandibular gland, granular immunostaining for CA VI was detectable in several terminal tubule cells of 1-day-old rats. At 1 week, the CA VI-positive cells were located at the periphery of the terminal tubules and appeared to be budding off the tubules. These cellular buds gradually increased, and, by 4 weeks, formed acini. CA VI was also detected in the duct lumen from day 1. The immunostaining in the parotid gland was detected sporadically in the acinar cells at 2 or 3 weeks. By 4 weeks, when the gland was almost indistinguishable from the adult one, the number of positive acinar cells had increased. Their number, however, was far smaller than in the adult gland, and the enzyme could not be detected in the duct lumen. CA II was also localized using specific antibodies to the rat isozyme. CA II was detectable in the inter- and intralobular striated ducts at 2 weeks after birth in the submandibular gland and at 3 weeks in the parotid gland. These results suggest that CA VI is secreted into saliva from soon after birth and that CA II appears in parallel with the functional maturation of the ducts. In addition, CA II was transiently expressed by the cellular buds of the submandibular gland at 2 and 3 weeks.

Introduction

Carbonic anhydrases (CAs; EC 4.2.1.1) catalyze the reversible hydration of carbon dioxide and, in mammals, are products of a gene family that encodes at least nine isozymes (Tashian 1992; Sly and Hu 1995; Opavský et al. 1996). Among the isozymes so far isolated as purified proteins, CA I, CA II, CA III, and CA VI occur in salivary gland (Hennigar et al. 1983; Kadoya et al. 1987; Parkkila et al. 1990; Spicer et al. 1990; Asari et al. 1991; Ogawa et al. 1992, 1993); CA I, CA II, and CA III are cytosolic isozymes, whereas CA VI is a secreted, higher molecular weight isozyme (for a review, see Fernley 1991). Until recently, serous acinar cells of salivary glands were thought to be the only source of CA VI, however, acinar cells of the rat lacrimal gland have also been shown recently to secrete the isozyme (Ogawa et al. 1995). The function of CA VI is most likely the regulation of pH of saliva and tear fluid (Hennigar et al. 1983; Ogawa et al. 1995). Most investigations into CA VI have been carried out on adult animals or tissues and, consequently, little is known about the isozyme in developing salivary gland. To date there has been only one investigation into the developmental changes affecting CA VI expression and this has been carried out in sheep (Penschow et al. 1997).

Acinar cell development in salivary gland has been extensively investigated using laboratory animals, mainly rat and mouse (Jacoby and Leeson 1959; Yohro 1970; Redman and Sreebny 1971; Cutler and Chaudhry 1974; Gresik and MacRae 1975; Taga and Sesso 1979). The anlagen of rat salivary glands appear at 13–14 days in utero. The submandibular gland develops during both prenatal and postnatal periods, whereas the parotid gland develops predominantly postnatally. Acinar cell differentiation in the parotid gland becomes evident postnatally and is complete approximately 25 days after birth. In the submandibular gland, the first sign of acinar cell differentiation is seen at 15–16 days in utero, however, the acinar cell is not fully differentiated until 3–4 weeks after birth (Redman and Sreebny 1971; Cutler and Chaudhry 1974). Salivary glands produce a variety of secretory proteins during development, and attempts have been made to use them as markers, not only to follow acinar cell differentiation but also to analyze the factors which...
influence this process (Yamashina and BARKA 1972; Ball et al. 1988; Moreira et al. 1991). The purpose of this study was to examine immunohistochemically the appearance of CA VI in developing submandibular and parotid glands of the rat, where the developmental changes are well documented. We also investigated the appearance of CA II by these glands. In bovine salivary gland, CA II is expressed in the duct of the immature gland but not in the mature gland (Asari et al. 1994). We have shown previously that the duct of the mature gland is stained by an antibody with dual specificity to rat CA I and CA II (Ogawa et al. 1992), however, it was not known whether the staining was attributable to CA I or CA II. In this study, we use a monoclonal antibody specific for rat CA II to investigate the appearance of the isozyme in developing rat salivary glands.

Materials and methods

All animals were used were Sprague-Dawley rats purchased from Nihon Dohbutsu (Osaka, Japan). Day-14-, 16-, 18-, and -20 embryos, 1-, 3-, and 5-day-old, 1-, 2-, 3-, and 4-week-old, and adult (4+ to 6-month-old) rats were examined. Each age group consisted of at least three animals. For postnatal examination, only male rats were used. Embryos were taken from pentobarbital sodium-anesthetized mothers, and the head and necks were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4° C. Postnatal rats were perfused through the ascending aorta with 3% formaldehyde. Sections were cut into small tissue blocks. They were further fixed in the same fixative overnight at 4° C. After fixation, the head and necks and the salivary tissue blocks were washed serially in graded sucrose solutions (10–20% in 0.01 M phosphate-buffered saline, pH 7.2; PBS), embedded in OCT compound (Miles, Elkhart, Inc., USA), and frozen in liquid nitrogen. Cryostat sections (6- to 12-µm) were cut, thaw-mounted on silane-coated glass slides, air-dried, immersed in PBS, and used for immunohistochemistry.

Immunoperoxidase histochemistry

Characterization of the monoclonal anti-rat CA VI antibody, polyclonal rabbit anti-rat erythrocyte CA (CA I and CA II) antibody, and monoclonal anti-rat CA II antibody have been described previously (Ogawa et al. 1992; Toyosawa et al. 1996). Monoclonal antibodies were used in the form of immunoglobulin, which was purified from ascites by protein A affinity chromatography (Affi-Gel Protein A MAPS II kit; Bio-Rad, Richmond, Calif., USA). The ascites were generated in adult female Balb/c mice (6 weeks old) according to the method of Harlow and Lane (1988).

Detailed procedures of the immunoperoxidase histochemistry have been described previously (Ogawa et al. 1992, 1995). Some sections were incubated first with normal rabbit serum and then with one of the monoclonal antibodies, others with normal swine serum first and then with the polyclonal antibody. Monoclonal anti-CA VI was used at a dilution of 1:5000, anti-CA II at 1:2000, and polyclonal anti-CA I and CA II at 1:5000.

Submandibular gland sections of 3-week-old rats were incubated with 3% normal goat serum for 10 min at room temperature and then with polyclonal anti-CA I and CA II antibody (1:5000) overnight at 4° C. After washing in PBS, the sections were incubated with monoclonal anti-CA VI antibody (1:20000) overnight at 4° C. They were washed with PBS, and sequentially incubated with biotinylated horse anti-mouse IgG (1:100; Vector, Burlingame, Calif., USA), streptavidin–Texas Red (1:50; Amersham, UK), and FITC-conjugated donkey anti-rabbit IgG (1:500; Amersham). Each of these incubations was carried out for 1.5 h at room temperature and followed by at least a 30-min wash with PBS. The sections were coverslipped with a mixture of PBS:glycerol (1:9) containing 0.1% p-phenylenediamine and observed with a Nikon fluorescence microscope with an appropriate excitation filter (B excitation filter for FITC and G excitation filter for Texas Red).

Negative controls

Negative controls for immunostaining were performed by substituting the primary antibodies with PBS, normal mouse IgG (Miles Scientific, Naperville, Ill., USA) or preimmune rabbit IgG. Normal and preimmune IgGs were used at the same protein concentrations as the primary antibodies. The protein concentration was determined by the bicinchoninic acid procedure (Smith et al. 1985).

Results

The anatomical nomenclature of Jacoby and Leeson (1959) and Redman and Sreebny (1971) for developing rat salivary glands has been used for this study.

CA VI in the developing submandibular gland

CA VI immunostaining was not seen in the rudimentary gland before birth (Fig. 1a). All subsequent ages refer to postnatal ages. At 1 day after birth, when the gland consisted of interlobular ducts, intralobular ducts, and terminal tubules and appeared as if they were budded off from the luminal surface of striated duct cells (arrowheads). Bars a 100 µm, b–h 50 µm, e 25 µm.

Double immunofluorescence histochemistry

Submandibular gland sections of 3-week-old rats were incubated with 3% normal goat serum for 10 min at room temperature and then with polyclonal anti-CA I and CA II antibody (1:5000) overnight at 4° C. After washing in PBS, the sections were incubated with monoclonal anti-CA VI antibody (1:20000) overnight at 4° C. They were washed with PBS, and sequentially incubated with biotinylated horse anti-mouse IgG (1:100; Vector, Burlingame, Calif., USA), streptavidin–Texas Red (1:50; Amersham, UK), and FITC-conjugated donkey anti-rabbit IgG (1:500; Amersham). Each of these incubations was carried out for 1.5 h at room temperature and followed by at least a 30-min wash with PBS. The sections were coverslipped with a mixture of PBS:glycerol (1:9) containing 0.1% p-phenylenediamine and observed with a Nikon fluorescence microscope with an appropriate excitation filter (B excitation filter for FITC and G excitation filter for Texas Red).

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After fixation, the head and necks and the salivary tissue blocks were washed serially in graded sucrose solutions (10–20% in 0.01 M phosphate-buffered saline, pH 7.2; PBS), embedded in OCT compound (Miles, Elkhart, Inc., USA), and frozen in liquid nitrogen. Cryostat sections (6- to 12-µm) were cut, thaw-mounted on silane-coated glass slides, air-dried, immersed in PBS, and used for immunohistochemistry.

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After inactivation of endogenous peroxidase with 3% H2O2, the sections were incubated with peroxidase-conjugated rabbit anti-mouse IgG (1:200; Dako, Glostrup, Denmark) or peroxidase-conjugated swine anti-rabbit IgG (1:200; Dako). They were then incubated with 3,3′-diaminobenzidine tetrahydrochloride–H2O2 solution to visualize immunoreaction sites, counterstained with methyl green, dehydrated, and coverslipped with Permount.