pagepress

European Journal of Histochemistry 2025; volume 69:4159

Immunohistochemistry of carbonic anhydrases I, II and VI in the rat lingual serous salivary glands of von Ebner

Robert S. Redman

Oral Pathology Research Laboratory, Department of Veterans Affairs Medical Center, Washington, DC, USA

ABSTRACT

Carbonic anhydrase (CA) has been localized to many structures involved in ion transport including the acini and ducts of the major (parotid, sublingual and submandibular) salivary glands of humans and rodents. It also has been localized by enzyme histochemistry and by immunohistochemistry for CA isoenzyme VI (CA VI) to the acini and ducts of rat serous lingual glands of von Ebner. The purpose of this study was to explore the intracellular distribution by cell type of three CA isoenzymes in these glands. Immunohistochemistry was undertaken with antibodies to human CAs I, II and VI in paraffin sections of rat tongues that had been fixed in Helly's fluid. The density of the reaction product was scored as 0 (none) to 5 (strongest). Reactions in the acini with CA I and II antibodies were weak luminally to moderate basally and generally moderate, respectively, moderate in the intercalated ducts, and moderate basally to strong luminally in the excretory ducts. Weak to moderate CA VI reactions occurred in the acini and ducts. The stronger luminal reactions to CAs I and II in the excretory ducts suggest that they contribute to pH regulation in the saliva of von Ebner's glands *via* HCO_3^- transport.

Key words: carbonic anhydrase; immunohistochemistry; rat; salivary glands; von Ebner's glands.

Correspondence: Dr. Robert S. Redman, Oral Pathology Research Laboratory (151-I), Department of Veterans Affairs Medical Center, 50 Irving Street, NW, Washington, DC 20422, USA. Tel. +1.202.745-8490.
E-mail: Robert.Redman@va.gov

Conflict of interest: the author declares that he has no competing interests.

Ethical approval: the experimental protocol (0012) was approved by the Animal Subject Subcommittee and the Research and Development Committee of the Washington, DC, Veterans Affairs Medical Center.

Availability of data and material: a PDF of the Table of the individual density scores of each specimen and the calculated averages will be made available upon reasonable request from the author.

Funding: this study was supported by the United States Department of Veterans Affairs (Merit Review) and the Institute for Clinical Research, Inc, Washington, DC.

Introduction

Carbonic anhydrase (CA; carbonate anhydratase; E.C.4.2.1.1) isoenzymes catalyze the reversible reaction $CO_2 + H_2O \leftrightarrows HCO_3^-$.[1-3] Their function is critical to regulating ions, fluid and acid-base balance in many tissues,[3] and the cellular and intracellular location of CA enzymes provides important clues to their function. For example, CA localized to the acini and ducts relates to the CA isoenzymes with secretory and transepithelial ion transport functions.[4,5] CA isozymes I, II, and VI have been immunohistochemically (IHC) localized in both rodent,[6-8] and human[3,4,9,10] major salivary glands. CA activity also has been localized to the acini and the cells and luminal contents of ducts of serous lingual glands (von Ebner's Glands; VEG) by enzyme histochemistry of rats[11] and humans[12] and CA VI by IHC.[12] The larger (striated and excretory) ducts in rat and human major (parotid, sublingual and submandibular) salivary glands have extensive infoldings of the basolateral membranes accompanied by numerous large, elongated mitochondria.[13] These provide energy and surface area for trans-cellular movement of ions such as Na^+, Cl^- and HCO_3^-, in which their high CA activity[1,14] plays an important role.

As described by Hand,[15] the duct system in rat VEG begins at the acini as simple ducts with a single layer of cuboidal cells and a few secretory granules and continues to the orifices in the troughs around the papilla and in the foliate furrows as stratified epithelium with a basal cell layer and a short columnar luminal layer, transitioning to stratified squamous epithelium as they merge with the oral epithelium. There are no striated ducts, but the columnar cells of the excretory ducts have numerous large mitochondria. The simple duct was likened to the intercalated ducts of the major salivary glands. The terms used in the present study for the simple and more complex ducts are intercalated and excretory ducts, respectively.

Previously my colleagues and I explored the distribution of CA isoenzymes I, II and VI in the major salivary glands of rats during postnatal development.[16,17] The objective of this study was to see if CA might be a useful marker for the *functional* (pertaining to ion transport) differentiation of the striated and excretory ducts in these glands. Although CA VI, the lone secretory CA isoenzyme, had been localized to the acini in rat salivary glands,[8] in preliminary tests with a mature (80 days old) rat submandibular gland, the antibody that we obtained localized mainly to the ducts. However, we found no published reports of CAs I or II and only one of CA VI in VEG.[12] To fill this void, sections of the posterior portions of tongues were included on the slides with the intention of determining the postnatal ontogeny of these three CAs in VEG. Too many of the VEG specimens from rats at the earlier ages were unsatisfactory, so that a manuscript on the developing glands was not pursued. However, all three CAs are represented in specimens from all 6 rats at the oldest age (42 days) of the study, when the acini and ducts of rat VEG are histologically mature.[15,18,19] Therefore, the purpose of this presentation is to determine the extent to which the IHC localization of CAs I, II and VI in the excretory ducts of the rat VEG may be similar to that of the striated ducts of the major glands, *i.e.*, denser in the luminal cytoplasm.

Materials and Methods

Many of the previously published experimental procedures[16] are repeated here almost verbatim, and others unique to von Ebner's glands are added, in order to provide a comprehensive narrative.

Experimental animals

A breeding colony of Sprague Dawley rats was housed under controlled conditions of temperature (22°C) and humidity (40-60%), a daily cycle of lights on at 08.00 and off at 20.00 h. and unlimited access to a commercial pelleted diet. Pups were considered 0 days old on the day of birth. They were weaned at age 21 days and separated by sex at 30 days.

Rats were selected from five litters (B, C, D, E and F) to make up the sample of one rat of each sex from each of three different litters (n=6) at representative postnatal ages (1, 7, 14, 21, 28 and 42 days). For example, at age 14 days, the sample was one of each sex from litters D, E and F, and at age 42 days it was one of each sex from litters B, C and E. Rats were killed by cervical section and the parotid, sublingual and submandibular glands and the portion of the tongue harboring von Ebner's glands were excised.

Immunohistochemistry

All procedures were carried out at ambient (~22°C) temperature. Immediately after dissection, tissues were immersed in Helly's fluid (fixative) for 3 hr, then in 2 % $K_2Cr_2O_7$ for 2 hr, washed overnight in tap water, processed routinely through ethanol and xylene, and embedded in paraffin. Sections were cut at 6 μm and mounted on Superfrost Plus™ slides (Fisher Scientific, Pittsburgh, PA, USA). Specimens were oriented in the cassettes such that sections would include the parotid, sublingual and submandibular glands, and a cross (transverse) section of the tongue to display the VEG associated with both the vallate papilla and the foliate furrows, all in the same slides from each animal.

Polyclonal (pc) sheep antibodies to human CA I and II, (CA I ab, CA II ab), conjugated to horseradish peroxidase, were purchased from Biodesign International (Kennebunk, ME, USA). Pc rabbit anti-human CA VI IgG fraction (CA VI ab) was purified at the University of Umeà and its specificity confirmed by methods similar to those of Murikami and Sly[20] and modified by Parkkila *et al.*[3] Pc goat anti-rabbit antibodies conjugated to horseradish peroxidase (Goat ab) and normal sheep serum (NSS) were purchased from Dako (Carpinteria, CA, USA).

The specificity of the antibodies was further analyzed by

Table 1. Immunohistochemistry of carbonic anhydrase isozymes in rat von Ebner's glands.

Antibody	Acini		Intercalated ducts		Excretory ducts	
	B	L	B	L	B	L
CA I	1	0	2	2	3	5
CA II	2	2	2	2	3	4
CA VI	1	0	1	2	1	2

Mean scoring range was 0 (none) to 3 (moderate) to 5 (strong), rounded to the nearest whole number, for reactions in the basolateral (B) and luminal (L) aspects of the structures in sections from 6 rats.

Western blots of human saliva, and extracts of human and adult (80 days old) rat red blood cells (rbcs) and adult rat salivary glands (Figure 1). Reactions of the antibodies occurred in bands of molecular weight consistent with those that are well established for the three CA isoenzymes, except that none reacted with the rat red blood cells lysate. No bands were marked when primary antibodies were omitted. The CA VI ab also reacted strongly with the serous acini and demilunes and no other structures except weakly with some of the larger ducts in a human submandibular gland in the prior paper.[16]

The sequence of IHC procedures was as follows. i) $HgCl_2$ deposits were removed from sections with Grams Iodide solution followed by $Na_2S_2O_3$ (Luna, 1968). ii) Incubate in NSS. iii) Rinse in phosphate buffered saline (PBS). iv) Incubate in the CA antibody. v) Rinse in PBS, and for CA VI, only, incubate in Goat ab, followed by two rinses in PBS. vi) Incubate in 3,3' diaminobenzidine (DAB) freshly mixed with H_2O_2. Rinse with distilled water and counterstain with Harris's hematoxylin. vii) Dehydrate with ethanol and xylene and mount with Pro-Texx™. Negative control slides were included in which the primary antibodies were omitted.

It should be noted that Helly's fluid extinguished all native peroxidases in these sections. Several sections also were pre-treated for 10 min with 3% H_2O_2 in absolute methanol to quench the pseudoperoxidase reaction of rbcs.

The density of reaction product in the slides was considered an indication of the amount of CA reacting with the particular antibody in particular sites relative to other samples. The density was subjectively quantified on a scale of 0 (no reaction) to 5 (strongest reaction) as previously described.[16]

Results

Photomicrographs representative of the reactions obtained with the CA ab are presented in Figure 2. The scores for the density of reactions to the antibodies are listed in Table 1. Reactions in the acini with CA I ab (Figure 2A) were weak basally to none luminally, moderate in the intercalated ducts, and moderate basally to strong luminally in the excretory ducts. CA II ab reactions were moderate in the acini and intercalated ducts (Figure 2C) and moderate basally to moderately strong luminally in the excretory ducts (Figure 2D). The CA VI ab reactions were none in the acini, weak to moderate in the intercalated ducts (Figure 2E), and weak basally to moderate luminally in the excretory ducts (Figure 2F). In the striated muscles of the tongue, reactions were moderate to strong with the CA I and II ab and weak to moderate with the CA VI ab. The intensity of reaction also varied considerably among the different groups of muscle fibers. No reactions were seen in any tissues or structures when the primary antibody was omitted (Figure 2B).

Discussion

Specificity of the antibodies

The specificity of the CA antibodies used here was discussed previously.[16] In particular, the failure of the anti-human CA I and II ab to react with rat rbc and homogenates raised concern regarding which rat CA I and II isoenzymes were labeled. It was pointed out that the homology as well as the diversity in the α-family of CA protein sequences provides a rationale for cross-reactivity among antibodies to CA isoenzymes [22] For example, it is well established that CA VI is stored in acinar secretory granules and secreted into saliva.[4,20] However, the antibody to human salivary

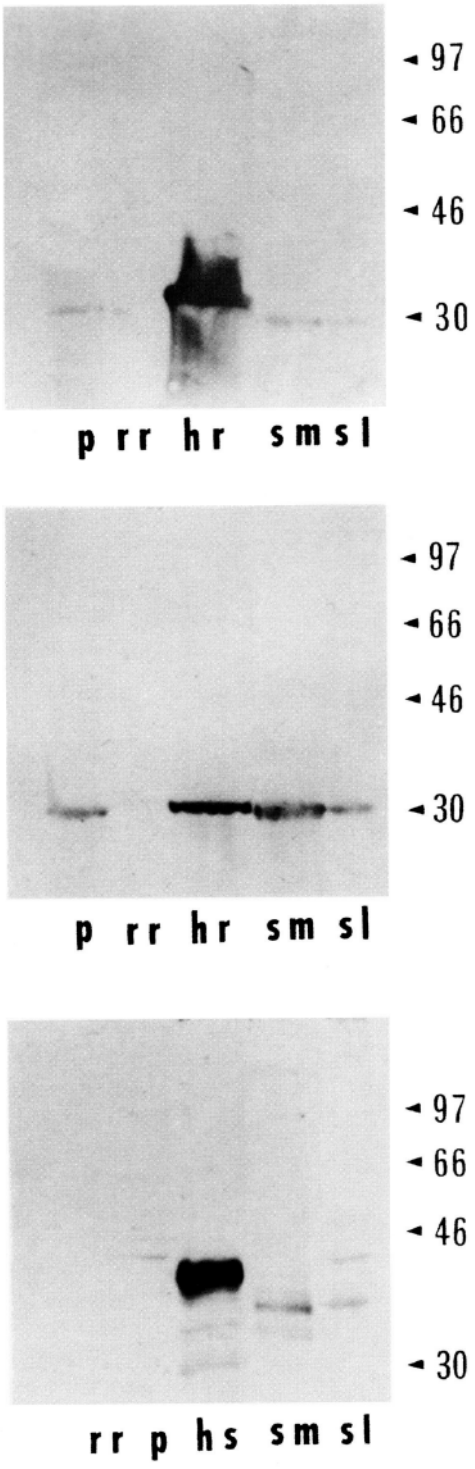

Figure 1. Western blot, demonstrating reactions of the CA isoenzymes to their cell lysate or fluid of antigenic origin, rat salivary gland homogenates, and a rat red blood cell lysate. Panels: Top, CA I; center, CA II; bottom, CA VI. Molecular weights (kiloDaltons) of representative standards are on the right of each panel. The total amount of protein (µg) placed in each lane was: molecular weight markers, one lane per panel (*not shown*), 49; for CA I and II, all lanes, 30; for CA VI, hs, 3; p and sm, 5; rr, 10; sl, 30. hr, red blood cells; rr, red blood cells; hs, human whole saliva; p, rat parotid glands; sl, sublingual glands; sm, submandibular salivary glands. Reproduced from Figure 1 in Peagler *et al.*[16] (Anat Rec 1998;250:190-8; with permission).

CA VI in the present study reacted with duct, not acinar cells, in rat parotid and submandibular glands, and not only the ducts, but also the serous demilunes in rat sublingual gland.[16,17] The purpose of these two previous reports was to document the functional differentiation of the ducts in the rat major salivary glands as related to the physiological roles of CAs in salivary gland ducts. To this end, the choice of fixative and CA antibodies that preferentially labeled the ducts was essential. The extent to which this may have been affected by cross-reactions among CA isoenzymes might better be considered fortuitous rather than flawed.

CA III is the dominant CA of skeletal muscles in the adult rat.[23] The reactions to CA I, II and VI ab in the tongue muscles around the VEG again raised the possibility that cross-reactions to other CAs may have been an important confounding source in the present study. However, at age 42 days, rats are in the early post-pubertal period of growth, when the fast-twitch muscles, rich in CAs I and II, have just begun being replaced by the CA III rich slow-twitch type of muscles.[24] This accounts for the immunolocalization of CAs I, II and III in various skeletal muscles of the 200 g (~45 days old) male rats by Jeffery *et al*.,[25] and to a considerable extent, therefore, the CA antibodies in the present study.

Significance

Major functions of the CAs in the striated and excretory ducts of salivary glands include maintaining not only intracellular physiological conditions of pH, CO_2 and HCO_3, as with most cells and tissues, but also the special conditions of the saliva as related to the activities of salivary enzymes, mucins and other proteins in the oral cavity and digestive tract. For VEG, these include rapidly renewing environmental taste items around the taste buds by rinsing the troughs around the circumvallate papillae and in the foliate furrows[15] and providing an optimal salivary pH[26] for the activities of VEG salivary enzymes such as α-amylase.[27] More specifically, it has been shown that dietary zinc deficiency reduces taste acuity by impairing the activity of salivary CAVI,[28] which directly affects the taste bud receptors.[11,12] Experiments with isolated segments of striated ducts *in vitro* have demonstrated passage of water and HCO_3^- into the lumen.[29] However, there are no striated ducts in rat[15] or human[30,31] VEG. Nonetheless the stronger reactions to CA I, II and VI in the luminal cytoplasm of the VEG ducts in the present study suggest that these ducts also may have a role in pH regulation of saliva *via* HCO_3^- secretion. To my knowledge, VEG saliva or ductal contents have not been analyzed for electrolytes. In this regard, it is interesting that reduction of CA II expression was more than that of CA VI in submandibular glands of zinc-deficient rats.[32]

Acknowledgments

I thank Mr. Rodney McNutt for technical assistance, Dr. Ingegerd Johansson for the CA VI antibody, the late Dr. Frederick D. Peagler for his invaluable participation, and Dr. Bidhan C. Bandyopadhyay for reviewing the manuscript.

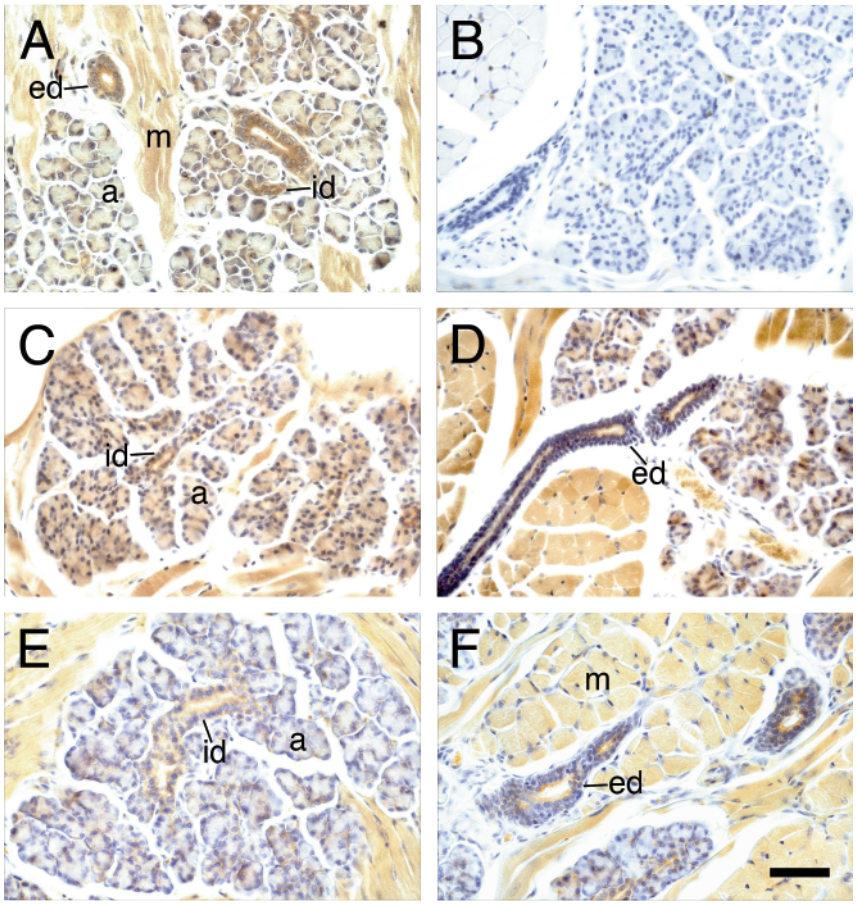

Figure 2. Photomicrographs of immunohistochemically localized carbonic anhydrase (CA) isozymes (yellow to brown deposits of reaction product) in lingual serous (von Ebner's) glands of 42 days old rats. **A**) CA I. **B**) Negative control. **C,D**) CA II. **E,F**) Ca VI. a, acini; id, intercalated ducts; ed, excretory ducts; m, skeletal muscle. Hematoxylin counterstain. Scale bar: 50 µm.

References

1. Carpentier P, Chetail M, Fournie J. [Mise en evidence de deux anhydrases carbonique au niveau du complexe salivare sous-mandibulare-sublingual principal chez le rat].[Article in Fench]. J Biol Buccale 1980;8:33-47.
2. Ogawa Y, Fernley T, Ito R, Ijuhin N. Immnunohistochemistry of carbonic anhydrase isozymes VI and II during development of the rat salivary glands. Histochem Cell Biol 1998;110: 81-8.
3. Parkkila S, Kaunisto K, Rajaniemi L, Kumpulainan T, Jokinen K, Rajaniemi H. Immunohistochemical localization of carbonic anhydrase isoenzymes VI, II and I in human parotid and submandibular glands. J Histochem Cytochem 1990;38:941-7.
4. Piras M, Tandler B, Barbarossa IT, Piludu M. Immunogold labeling of carbonic anhydrase isozyme (CA-VI) in secretory granules of human parotid glands. Acta Histochem 2012;114: 406-8.
5. Ohana E. Transepithelial transport across duct cells of the salivary gland. Oral Dis 2015;21:826-35.
6. Hennigar RA, Schulte BA, Spicer SS. Immunolocalization of carbonic anhydrase isozymes in rat and mouse salivary and exorbital lacrimal glands. Anat Rec 1983;207:605-14.
7. Noda Y, Sumitomo S, Orito T, Mori M. Histochemical localization of carbonic anhydrase I and II in the submandibular glands of mouse, rat, hamster and guinea pig. Arch Oral Biol 1986;31:795-800.
8. Ogawa Y, Chang C-K, Kuwahara H, Hong SS, Toyosawa S, Yagi T. Immunoelectron microscopy of carbonic anhydrase isozyme VI in rat submandibular gland: comparison with isozymes I and II. J Histochem Cytochem 1992;40:807-17.
9. Noda Y, Sumitomo S, Hikosaka N, Mori M. Immunohistochemical observations on carbonic anhydrase I and II in human salivary glands and obstructive sialadenitis. J Oral Pathol 1986;15:187-90.
10. Ogawa Y, Hong SS, Toyosawa S, Kuwahara H, Shimakazi M, Yagi T. Immunoelectron microscopy of carbonic anhydrase isozyme VI in human submandibular gland: comparison with isozymes I and II. J Histochem Cytochem 1993;41:343-51.
11. Brown D, Garcia-Segura LM, Orci L. Carbonic anhydrase is associated with taste buds in rat tongue. Brain Res 1984;324:346-8.
12. Leinonen, Parkkila S, Kaunisto P, Rajniemi H. Secretion of carbonic anhydrase VI from human and rat lingual von Ebner's glands. J Histochem Cytochem 2001;49:657-82.
13. Tandler B. Ultrastructure of the duct system in mammalian salivary glands. Micros Res Tech 1993;28:57-74.
14. Carpentier P, Chetail M, Fournie J. Carbonic anhydrase in the rat salivary glands: distribution and ultrastructural localization. Biol Cell 1985;54:241-50.
15. Hand AR. The fine structure of von Ebner's gland of the rat. J Cell Biol 1970;44:340-53.
16. Peagler FD, Redman RS, McNutt RL, Kruse DH, Johansson I. Enzyme histochemical and immunohistochemical localization of carbonic anhydrase as a marker of ductal differentiation in the developing rat parotid gland. Anat Rec 1998;250:190-8.
17. Redman RS, Peagler FD, Johansson I. Immunohistochemical localization of carbonic anhydrases I, II and VI in the developing rat sublingual and submandibular glands. Anat Rec 2000;258:69-76.
18. Hamosh M, Hand AR. Development of secretory activity in serous cells of the rat tongue. Cytological differentiation and accumulation of lingual lipase. Dev Biol 1978;65:100-13.
19. Kock K, Bläker M, Schmale M. Postnatal development of von Ebner's glands: accumulation of a protein of the lipocalin family in taste papillae of rat tongue. Cell Tissue Res 1992;267: 313-20.
20. Murakami H, Sly WS. Purification and characterization of human salivary carbonic anhydrase. J Biol Chem 1987;262: 1382-8.
21. Luna LG. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd ed., New York: McGraw-Hill; 1968.
22. Hewett-Emmett D, Tashian RE. Functional diversity, conservation, and convergence of the α, β and γ carbonic anhydrase gene families. Mol Phylogenet Evol 1996;5:50-77.
23. Carter N, Jeffery S, Shiels A. Immunoassay of carbonic anhydrase III in rat tissues. FEBS Lett 1982;139:265-6.
24. Riley DA, Ellis S, Bain J. Carbonic anhydrase activity in skeletal muscle fiber types, spindles, and capillaries of rat soleus and extensor digitorum longus muscles. J Histochem Cytochem 1982;30:1275-88.
25. Jeffery S, Carter ND, Smith A. Immunocytochemical localization of carbonic anhydrase isozymes I, II and III in rat skeletal muscle. J Histochem Cytochem 1986;34: 13-6.
26. Bernfeld B. Amylases, α & β. In: Colowick SP, Kaplan NO (eds.), Methods in Enzymology, vol. 1. New York: Academic Press; 1955. pp. 149-58.
27. Field RB, Hand AR. Secretion of lingual lipase and amylase from rat lingual serous glands. Am J Physiol 1987; 253: G217-25.
28. Henkin R, Martin BM, Agarwal RP. Efficacy of exogenous oral zinc in treatment of patients with carbonic anhydrase VI deficiency. Am J Med Sci 1999;318:392-405.
29. Ueno K, Hirono C, Kitagaw M, Shiba Y, Sugita M. Different rate-limiting activities of intracellular pH regulators for HCO3- secretion stimulated by forskolin and carbachol in rat parotid intralobular ducts. J Physiol Sci 2016;66:477-90.
30. Azzali G, Bucci G, Gatti R, Orlandini G, Ferrari G. Fine structure of the excretory system of the deep posterior (Ebner's) salivary glands of the human tongue. Acta Anat (Basel) 1989;136:257-68.
31. Testa Riva F, Cossu M, Lantini MS, Riva A. Fine structure of human deep posterior lingual glands. J Anat 1985;142:103-15.
32. Goto T, Shirakawa H, Furukawa Y, Komai M. Decreased expression of carbonic anhydrase isozyme II, rather than of isozyme VI, in submandibular glands in long-term zinc-deficient rats. Brit J Nutr 2007;99:248-53.

Received: 7 November 2024. Accepted: 17 February 2025.

European Journal of Histochemistry 2025; 69:4159
doi:10.4081/ejh.2025.4159