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Decreased expression of carbonic anhydrase isozyme II, rather than of isozyme VI, in submandibular glands in long-term zinc-deficient rats

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We previously reported that in rats, long-term Zn deficiency significantly reduced taste sensitivity and total carbonic anhydrase (CA) activity in the submandibular gland. We therefore investigated the effects of Zn deficiency on salivary secretion and the expressions of CA isozymes (II and VI) in the rat submandibular gland, since those isozymes are thought to be related to taste sensation and salivary secretion. Male Sprague–Dawley rats, age 4 weeks, were divided into three groups (Zn-def, low-Zn and pair-fed, that were fed a diet containing 2·2, 4·1 or 33·7 mg Zn/kg, respectively, for 42 d). Northern blot analysis indicated that Zn deficiency reduced CA II mRNA expression in the submandibular gland without reducing CA VI mRNA expression. In Western blot analysis, Zn deficiency significantly reduced CA II (erythrocyte CA) protein expression in the submandibular gland without reducing CA VI protein expression. Salivary secretion was lower in the Zn-def group than in the pair-fed group. These results suggest that decreased CA isozyme II expression underlies the decreased CA activity previously reported in the submandibular gland in Zn-def rats, and this may reduce regular salivary secretion.

Zinc deficiency: Carbonic anhydrase II and VI: Submandibular glands

Carbonic anhydrase (CA; EC 4.2.1.1), a Zn metalloenzyme that catalyses the reversible hydration of carbon dioxide, participates in pH regulation, and in the transport of fluid and ions in a wide range of species and tissues[1–3]. The CA present in salivary glands and saliva are thought to participate in ion transport, secretory processes and saliva production, and also in the regulation of the pH and buffering capacity of the saliva[4,5]. Moreover, CA appears to be involved in gustation because hypogeusia has been reported as a side effect by patients undergoing acetazolamide therapy[6]. A decade ago, Komai & Bryant[7] showed that the perception of carbonated water (via the lingual trigeminal nerve) by rats requires the participation of CA. Moreover, they showed that MK-927, which is a membrane-permeable type of CA inhibitor, inhibits the responses of the chorda tympani nerve to carbonated water, quinine hydrochloride and L-glutamic acid solution when applied topically to the tongue surface in rats[8]. Later, others found that CA inhibitors alter the perception of carbonated water in rats[9].

It is well known that Zn deficiency leads to hypogeusia (decreased taste acuity) in both man and rats, a symptom that can be reversed by the administration of Zn. We recently confirmed that long-term Zn deficiency significantly reduced taste sensitivity at the level of the chorda tympani nerve in rats[10]. Since we had already demonstrated that long-term Zn deficiency significantly reduces CA activity in the submandibular gland and taste epithelium[11], we hypothesised that the decreased taste sensitivity seen in long-term Zn-def rats is a consequence of a reduction in total salivary CA activity. Since it was not clear which CA isozymes might be involved in this decrease in total CA activity, we performed the present study to examine the effects of Zn deficiency on the expressions of individual CA isozymes (mRNA and protein) in the rat submandibular gland. Our decision as to which isozymes to study was informed by the following background.

To date, at least eleven isozymes of CA and CA-related proteins have been identified in mammals[2,3,12,13]. Some are expressed in almost all tissues, while others are tissue or organ specific. Four are cytosolic isozymes (I, II, III and VII), four are membrane bound (IV, IX, XII and XIV), two are present in mitochondria (VA and VB) and one is a secretory isozyme (VI). Although they all belong to the same α-class of CA[14], the isozymes show considerable divergence in DNA sequence, chromosome location and enzymic properties[12,15]. Erythrocyte CA (I and II) and VI are the ones usually reported to be expressed in salivary glands[5,16–21], although some studies have found an expression of CA III or IV in these glands[22–24]. Parkkila *et al.*[16] could find no staining specific for CA I in the serous acinar cells in human parotid and submandibular glands, while Ogawa

Abbreviations: CA, carbonic anhydrase; OD, optical density; Tris, tri(hydroxymethyl)-aminomethane.

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et al.[19] could detect no obvious differences in parenchymal staining between sections stained with polyclonal rabbit anti-rat erythrocyte CA (CA I and CA II) antibody and those stained with monoclonal anti-rat CA II antibody. Cytosolic CA II has a high catalytic efficiency and is widely expressed in mammalian organs. CA I has a lower catalytic efficiency than CA II and is expressed primarily in blood cells[3]. CA VI, the only secretory isozyme highly expressed in salivary glands, is expressed in the serous acinar cells of the parotid and submandibular glands, from where it is secreted into the saliva. For the above reasons, we focused on the expressions of CA II (erythrocyte CA: CA I and CA II) and CA VI (mRNA and protein) in the rat submandibular gland in our attempt to clarify the part played by Zn in the CA-related maintenance of taste sensation and saliva secretion.

Our other purpose was to investigate salivary secretion in Zn-def rats. Kondo *et al.*[25] reported that Zn constitutes an important factor in the production of secretory granules in the cells of the granular convoluted tubules in the submandibular gland. Likewise, Ishii *et al.*[26] suggested that in the submandibular gland, Zn (together with many Zn-dependent enzymes) is closely involved in the production and degranulation of secretory granules in the glandular epithelial cells, and also in the contraction of the myoepithelial cells. However, little is known about salivary secretion in Zn-def rats. The outer surface of mammalian taste receptor cells is normally covered with saliva, suggesting that the salivary environment plays an important role in the initial events of gustation. For that reason, we also examined the effects of Zn deficiency on salivary secretion rate.

Materials and methods

Animals and diets

Male Sprague–Dawley rats (age 4 weeks; weight 80–90 g at the time of delivery to our laboratory) were purchased from Japan SLC (Hamamatsu, Japan). They were fed a commercial pellet diet (F-2; Funabashi Farms Ltd, Funabashi, Japan) for 3 d before being started on the experimental diet, and they were then divided into three groups: severe Zn-def (Zn-def), marginal Zn-def (low-Zn) and pair-fed. All rats were maintained on a 12 h light–dark cycle at 22 ± 1°C with a constant humidity of 50 ± 10 %. The experimental design of the study was approved by the Animal Research–Animal Care Committee of the Graduate School of Agricultural Science, Tohoku University. The entire experiment closely followed the guidelines issued by that committee, which strictly follows government legislation in Japan (1980). Moreover, the care and use of the rats involved in the present study were under the surveillance of the above-mentioned committee.

The compositions of the basal experimental diets have been described previously[10]. Since dietary Zn levels at 2–4 mg Zn/kg diet are classified as marginal Zn deficiency[27], we set the following three types of diets with different Zn levels. The added amounts of Zn in each diet of the Zn-def, marginal Zn-def (low-Zn) and Zn-sufficient were 0, 3·84 and 33·8 mg/kg diet, respectively, while the amounts of Zn in each diet analysed by atomic absorption spectrophotometry (SAS-727; SEIKO Denshikogyo, Tokyo, Japan) were 2·2, 4·1 and 33·7 mg Zn/kg diet, respectively. Harper's mineral commercial mixture (Oriental Yeast, Tokyo, Japan) was used to provide the mineral mixture for the low-Zn diet because we have found that by feeding a diet containing this mixture, we can create a subclinical Zn deficiency in rats (although overt signs and symptoms of Zn deficiency do not appear, plasma Zn levels are reduced[11]). In fact, Harper's mineral mixture contains 0·2 g $ZnCl_2$/kg mixture[28] (added amount of Zn, 3·84 mg/kg diet), a level well under the recommended dietary requirement in the AIN-93 diet (1·65 g $ZnCO_3$/kg mixture). Dietary intake levels below 12 mg Zn/kg diet should be considered Zn deficient[29]. The mineral mixture for the Zn-def diet was prepared using the same components as in Harper's mixture but with $ZnCl_2$ excluded completely (added amount of Zn, 0 mg/kg diet). The mixture for the Zn-sufficient diet was prepared by supplementing the commercial Harper's mixture with 1·6 g $ZnCl_2$/kg (added amount of Zn, 33·8 mg/kg diet), and this diet provides an adequate amount of Zn for rats[30].

The Zn-def and low-Zn rats were given free access to the appropriate diet, but the pair-fed rats, which were fed the Zn-sufficient diet, were pair-fed against the Zn-def rats (1 d later)[10]. After feeding on the experimental diet for 42 d, the rats were killed under anaesthesia (ethyl ether inhalation), then deprived of blood. The submandibular gland was immediately excised from twelve rats (Zn-def, *n* 4; low-Zn, *n* 4; pair-fed, *n* 4) and frozen in liquid N_2. In order to evaluate the mRNA and protein expression levels for CA II and CA VI in the submandibular gland on day 42 of the experimental period, Northern and Western blot analyses were performed.

Ribonucleic acid extraction

Total RNA was isolated from the submandibular gland using ISOGEN (Nippon Gene Co. Ltd, Tokyo, Japan) according to the manufacturer's protocol. One part of frozen tissue (80–100 mg) was immediately homogenised in 1 ml ISOGEN. The RNA in the samples was quantified using optical density (OD) 260 and the quality of the mRNA samples was assessed using the OD260:OD280 ratio.

Cloning of rat carbonic anhydrase II and carbonic anhydrase VI cDNA

First-strand cDNA was synthesised from the total RNA isolated from the rat submandibular gland using SuperScript II RNase H^- RT (Invitrogen, Cergy Pontoise, France). The amplification of rat CA VI cDNA was carried out using TaKaRa Taq DNA polymerase (Takara Biomedicals, Kyoto, Japan). The primers were referred to the nucleotide sequence of expressed sequence tag (EST) clone estimated mouse CA VI (GenBank accession no. AF079835: forward, TAAGGAATGTGG; reverse, TGAAAATCTCCAAAATCACTTTA). A PCR product of approximately 380 bp was separated by agarose-gel electrophoresis, isolated using Gene Clean (Qbiogene, Irvine, CA, USA), ligated into the pT7blue T plasmid vector (Novagen Inc, Madison, WI, USA) and transformed using *Escherichia coli* DH5α. The nucleotide sequences of the CA VI cDNA inserts in the plasmid vector and EST clone containing CA II cDNA (GenBank accession no. AW524377) were determined using a sequencing kit (Applied Biosystems, Foster City, CA, USA). The cDNA

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fragments for the hybridisation probe were prepared from both plasmids digested with *Eco* RI and *Hin*dIII.

Northern blot analysis

Total RNA, 25 μg, from the submandibular gland was electrophoresed through 1·2 % agarose gel containing formaldehyde (2·2 mol/l). After the electrophoresis, the RNA were transferred to Hybond-N^+ nylon membranes (Amersham Biotech, Little Chalfont, Bucks, UK), and immobilised by UV cross-linking. The membrane was prehybridised at 68°C for 30 min in ExpressHyb hybridisation solution (Clontech Laboratories Inc., Palo Alto, CA, USA). Rat CA II and CA VI cDNA fragments were labelled with [^{32}P]dCTP using a Random Primer DNA Labelling Kit (Takara Biomedicals, Kyoto, Japan). The labelled DNA probes were purified by gel filtration, and hybridised to the membranes at 68°C for 90 min in ExpressHyb hybridisation solution. After hybridisation, the membranes were washed twice for 30 min at 50°C in 2 × saline sodium citrate (SSC), 0·05 % SDS, and twice for 40 min at 50°C in 0·1 × SSC, 0·1 % SDS. The membranes were then exposed to an imaging plate (Fujifilm, Tokyo, Japan) for image detection. Signal intensity on the imaging plate was quantified using FLA-2000 (Fujifilm, Tokyo, Japan) and analysed using Image Reader VI 4J and Image Gauge version 3.0 software (Fujifilm, Tokyo, Japan). Quantitative analyses of CA II and CA VI mRNA level were normalised with respect to the abundance of 18S rRNA.

Western blot analysis

One part of the submandibular gland (200–300 mg) was immediately homogenised in six parts by volume of cold tri(hydroxymethyl)-aminomethane (Tris)-HCl buffer (50 mM; pH 7.2). Each homogenate was centrifuged at 1000 ***g*** for 15 min (4°C), then the supernatant fraction was used for Western blot analysis. Protein concentration was measured by the method of Lowry *et al.*[31]. Protein (10 μg) was loaded on to a 12·5 % polyacrylamide gel, then transferred to an Immobilon-PPVDF membrane (Millipore Japan, Tokyo, Japan) for 2 h at 250 mA using transfer buffer containing 48 mM-Tris-HCl, 39 mM-glycin and 10 % methanol. The blot was washed three times in Tris-buffered saline Tween 20 (TBS-T) buffer (20 mM-Tris-HCl, pH7.6, 137 mM-NaCl, 0·1 % Tween 20). The membrane was then blocked with 10 % skimmed milk powder (Snow Brand, Tokyo, Japan) in TBS-T buffer for 1 h at room temperature. After blocking, the membrane was incubated in the presence of the primary antibodies for CA II and CA VI for 1 h at room temperature. The primary antibodies used were as follows: (1) polyclonal rabbit anti-rat erythrocyte CA (CA I and CA II) antibody (1:5000) and (2) monoclonal mouse anti-rat CA VI antibody (1:5000). Both antibodies were generous gifts from Dr Yuzo Ogawa (Osaka University, Osaka, Japan). The membrane was then washed and incubated with the secondary antibody. The secondary antibodies used were as follows: (1) anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Pierce Chemical

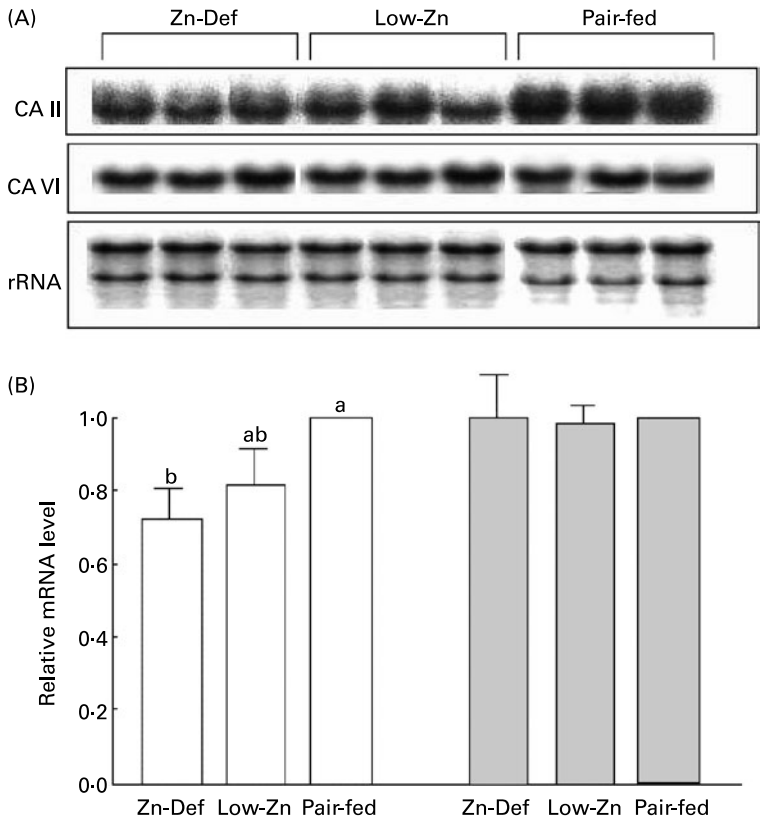

Fig. 1. Northern blot analysis for measurement of the expressions of carbonic anhydrase (CA) II and CA VI mRNA in rat submandibular glands on day 42 of the experimental period. (A) Total RNA isolated from Zn-def, marginally Zn-def (low-Zn) and pair-fed rats was loaded in the various lanes, with the corresponding rRNA being used as the control. (B) mRNA levels for CA II (□) and CA VI (▨) are shown relative to those in pair-fed rats. Values are means (*n* 4), with their standard errors represented by vertical bars. [ab] Mean values with unlike letters were significantly different ($P<0·05$).

Company, Rockford, IL, USA) and (2) anti-mouse IgG conjugated to horseradish peroxidase (1:5000; Amersham Biotech). The immune complexes were detected with the aid of ECL + Plus Western blotting detection reagents (Amersham Biotech) using the manufacturer's protocol. The bands were visualised by an enhanced chemiluminescence method. The intensity of the bands was quantified using LAS-2000 (Fujifilm, Tokyo, Japan) and analysed using Image Reader VI 4J and Image Gauge version 3.0 software (Fujifilm).

Measurement of salivary secretion rate

An additional twelve rats (Zn-def, *n* 4; low-Zn, *n* 4; pair-fed, *n* 4) were used after they had been fed the experimental diets for 42 d. They were anaesthetised with urethane (100 mg/kg body weight) plus α-chloralose (50 mg/kg body weight) at 3 h after the end of feeding, and the trachea was cannulated to facilitate breathing. The whole saliva was collected for 1 min using absorbent cotton wool (a small cotton bud), the weight of each bud being measured before and after collection.

Statistical analysis

The results are expressed as mean values with their standard errors. All data were analysed using a one-way ANOVA. In all experiments, *post hoc* multiple comparisons were made using the Bonferroni–Dunn test. The StatView program (StatView J-4.5; Abacus Concepts, Berkeley, CA, USA) was used for the analysis in each case.

Results and discussion

Our Zn-def rats displayed features typical of Zn deficiency, as described both by other investigators and in our previous work[11]. Anorexia and growth retardation, as well as a depletion of the plasma (serum) Zn concentration, were observed. After 21 d feeding on the Zn-def diet, severe signs of Zn deficiency were evident (epilation, hyperkeratosis of oral mucosal tissues and scaly encrustation of the paws). In contrast, none of these severe signs was seen in the low-Zn or pair-fed rats. Plasma Zn concentrations of the Zn-def rats were significantly reduced compared with the pair-fed rats after 4 d feeding. In the case of the low-Zn rats, none of these severe signs was seen, although plasma Zn levels were reduced (data not shown).

Northern blot analysis was performed to examine the effects of Zn deficiency on the abundance of the mRNA for CA II and CA VI (Fig. 1). The CA II mRNA expression in the submandibular gland was lower in the Zn-def rats than in the pair-fed rats, and it was at an intermediate level in the low-Zn rats (Fig. 1 (B)).

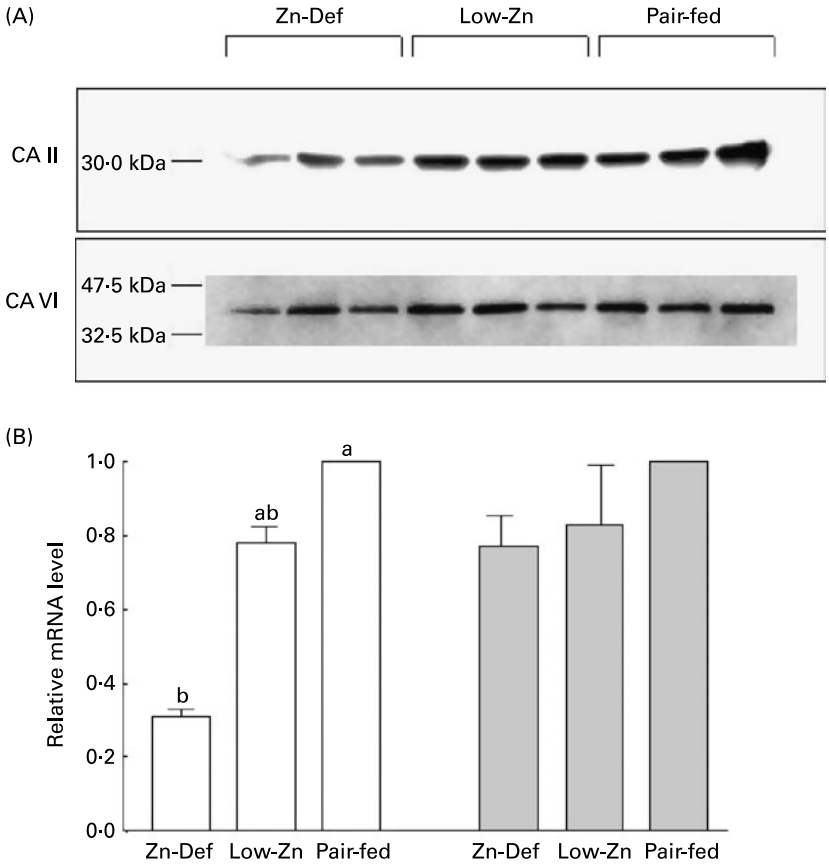

Fig. 2. Western blot analysis for measurement of the expressions of carbonic anhydrase (CA) II and CA VI in rat submandibular glands from Zn-def, marginally Zn-def (low-Zn) and pair-fed rats on day 42 of the experimental period. (A) CA II was detected using polyclonal rabbit anti-rat erythrocyte CA (CA I and CA II) antibody, while CA VI was detected using monoclonal mouse anti-rat CA VI antibody. (B) Amounts of CA II (□) and CA VI (■) expressed relative to those in pair-fed rats. Values are means (*n* 3), with their standard errors represented by vertical bars. [ab] Mean values with unlike letters were significantly different ($P<0·05$).

The relative level of CA II mRNA (normalised with respect to rRNA) was 0·72 (SEM 0·09) in the Zn-def rats ($P=0·031$ *v.* that in the pair-fed rats), while that in the low-Zn rats was 0·81 (SEM 0·11) ($P=0·137$). On the other hand, CA VI mRNA expression in both the Zn-def and low-Zn rats was at the same level as in the pair-fed rats.

The reactions of the primary antibodies in the Western blot analysis are illustrated in Fig. 2 (A). No obvious differences in parenchymal staining are reportedly seen between those sections stained with polyclonal rabbit anti-rat erythrocyte CA (CA I and CA II) antibody and those stained with monoclonal anti-rat CA II antibody[17,19]. On this basis, we assumed that the reaction of the polyclonal antibody to CA I plus CA II indicated the reaction to CA II. The antibodies we used have been shown to respond to the corresponding rat CA II and CA VI[19]. Although Hennigar *et al.*[5] have demonstrated CA I and/or CA II immunoreactivity in the serous acinar cells of several rodent species, including the rat, it is necessary to take into consideration the cross-reaction of their anti-human antibodies with other isozymes in these animals[17]. In the present study, CA II protein expression in the submandibular gland was significantly lower in the Zn-def rats than in the pair-fed rats, and it was at an intermediate level in the low-Zn rats (Fig. 2 (B)). The relative amount of CA II protein in the Zn-def rats was 0·31 (SEM 0·02) ($P=0·011$ *v.* that in the pair-fed rats), while that in the low-Zn rats was 0·78 (SEM 0·04) ($P=0·280$). This confirmed that CA II (erythrocyte CA) protein expression was affected by dietary Zn content. On the other hand, CA VI protein expression in both the Zn-def and low-Zn rats was at the same level as in the pair-fed rats.

As an overall tendency, the results indicated that Zn deficiency leads to a clear reduction both in mRNA abundance and in protein level of CAII, but not in CA VI. This seems to indicate the complexity of the effects of Zn deficiency in transcriptional and translational regulation of gene expression. However, at this point, we do not know how Zn deficiency affected differently in these two isozymes.

In our previous report[11], CA activity in the submandibular gland of the Zn-def rats was 36·4 % of that shown by the pair-fed rats, while that of the low-Zn rats was 66·3 % of the level shown by the pair-fed rats. The reduction in CA activity in the submandibular gland seems to correspond to the level of the reduction in CA II protein expression. These results suggest that a reduction in CA II expression may underlie the reduction in total CA activity previously reported in the submandibular gland in Zn-def rats[11].

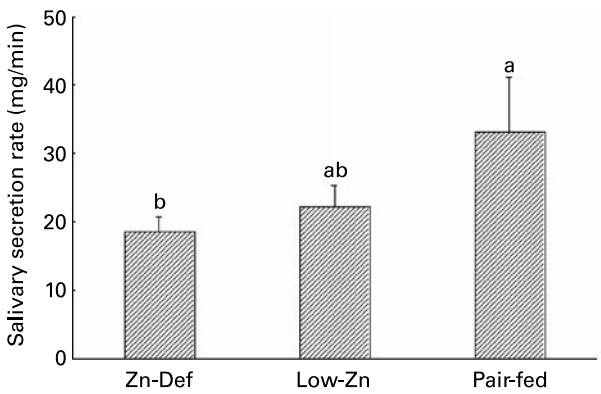

Fig. 3. Salivary secretion rate in Zn-def, marginally Zn-def (low-Zn) and pair-fed rats on day 42 of the experimental period. Values are means (n 4), with their standard errors represented by vertical bars. [ab] Mean values with unlike letters were significantly different ($P<0·05$).

Fig. 3 shows that the salivary secretion rate was lower in the Zn-def rats (18·53 (SEM 2·19) mg/min) than in the pair-fed rats (33·18 (SEM 8·12) mg/min) ($P=0·046$) after 42 d feeding, and that it was at an intermediate level in the low-Zn rats (22·23 (SEM 3·05) mg/min). The salivary secretion rate of the Zn-def rats was 55·8 % of that shown by the pair-fed rats, while that of the low-Zn rats was 67·0 % of the level shown by the pair-fed rats. This confirmed that salivary secretion rate is affected by the dietary Zn content.

Thus in the present study we detected a reduction in CA II expression in the submandibular gland in Zn-def rats, although CA VI expression was not reduced. Since cytosolic CA II has been proposed both to catalyse the production of salivary bicarbonate[16] and to play a role in transacinar water movement[32], a reduction in CA II expression in the submandibular gland in Zn-def rats seems to be in accord with the decreased salivary secretion observed in such rats. Consideration of the experimental data presented here leads us to suppose that CA II might need to be present if a sufficient amount of saliva is to be produced. It has been reported that CA VI secretion occurs independently of salivary secretion in human subjects[33], although it is not clear whether or not CA VI is involved in salivary secretion.

In summary, in the present investigation we confirmed that Zn deficiency reduces CA II (protein and mRNA) expression in the rat submandibular gland without a reduction in CA VI expression (protein or mRNA). These results suggest that a decreased CA II expression (a) underlies the decreased total CA activity seen in the submandibular gland in Zn-def rats, and (b) may reduce regular salivary secretion and also reduce taste sensitivity.

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