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## Calcium transport in human salivary glands: a proposed model of calcium secretion into saliva

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**Abstract** Salivary calcium plays a vital role in bio-mineralization of dental enamel and exposed dentin. In order to elucidate the yet unknown cellular and molecular mechanisms of calcium secretion in human salivary glands the presence of various relevant plasma membrane transport systems for calcium were investigated. Using an RT-PCR approach, expression of the epithelial calcium channel (CaT-Like), the calcium binding protein (calbindin-2), the endoplasmic reticulum pumps (SERCA-2 and -3), and the plasma membrane calcium ATPases (PMCA-1, -2, and -4), were found in parotid and submandibular glands. Immunohistochemistry revealed that CaT-Like is located in the basolateral plasma membrane of acinar cells; while calbindin-2, SERCA-2 and SERCA-3 were found inside the acinar cells; and PMCA-2 was found in the apical membrane and in the secretory canaliculi between the cells. Based on these findings, we propose the following model of calcium secretion in human salivary glands: (1) calcium enters the acinar cell at the basolateral side via calcium channel CaT-Like (calcium influx); (2) intracellular calcium is taken up into the endoplasmic reticulum by SERCA-2 and possibly SERCA3 or bound to calbindin-2 (intracellular calcium pool); and (3) calcium is secreted by PMCA at the apical plasma membrane (calcium efflux).

**Keywords** Epithelial calcium transport · Epithelial calcium channel (CaT-Like) · Calbindin · SERCA · PMCA · Human salivary gland

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### Introduction

Dental enamel, the hardest and densest material in the body, is composed of hydroxyapatite, a complex mineral containing calcium and phosphate. This unique bio-mineralization structure is constantly renewed by remineralization, which involves the deposition of calcium and phosphate ions from saliva into the enamel subsurface areas and onto the enamel surface (Edgar and Higham 1995). Under acidic conditions apatite crystallites of the subsurface enamel dissolve into the surrounding fluid. This demineralization is one of the first steps in dental caries development (Pearce 1998). The availability of calcium and phosphate is therefore of major importance for the remineralization of initial caries lesions after acid challenge (Tanaka and Kadoma 2000). Furthermore, a low caries rate is thought to be associated with high salivary calcium concentration in children (Mass et al. 2002) and adults (Sewon and Makela 1990). Finally, superficial erosions of teeth due to acid stress are also remineralized by calcium and phosphate present in saliva (Hoyer et al. 1984).

Several studies have been performed to analyze the mechanism of calcium transport through epithelial cells. In general, two modes of transepithelial calcium transport can be distinguished, namely transcellular or paracellular. In transcellular transport calcium entry across the membrane is mediated by epithelial calcium channels such as CaT1 in small intestine (Peng et al. 1999, 2000, 2003) or CaT2 in the distal renal tubule (Hoenderop et al. 2000). Intracellular calcium is then buffered either by calcium binding proteins such as calbindin-1 in kidney and small intestine (Hoenderop et al. 2000) or taken up into the endoplasmic reticulum by active calcium pumps such as SERCA in pancreas (Petersen 2003). Efflux of calcium from the cell is achieved by primary active mechanisms such as the plasma membrane calcium ATPase (PMCA-1) and/or by the secondary active sodium-calcium exchanger (NCX1) in kidney and small intestine (Hoenderop et al. 2002). In

paracellular transport paracellin, a tight junction protein forming  $Mg^{2+}$  and  $Ca^{2+}$  pores, appears to be involved (Simon et al. 1999).

Despite several studies addressing calcium transport in salivary glands the mechanism of calcium secretion into saliva is not well characterized (Peng et al. 2003). Therefore, the aim of this study is using molecular biology and immunohistochemistry techniques to elucidate the cellular and molecular mechanisms of trans-epithelial calcium transport for human salivary parotid and submandibular glands and to propose a model for calcium secretion into saliva.

## Materials and methods

### Tissue samples

Samples of human salivary gland were obtained from resection material of adult patients undergoing surgery for various primary maxillofacial interventions. Samples were not bigger than 3 mm<sup>3</sup> and immediately after resection conserved in liquid nitrogen or in RNAlater (Qiagen, Hilden, Germany). For immunohistochemical analysis; samples were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, cryoprotected in 2.8 M sucrose, and then frozen in liquid nitrogen and stored at -70°C. Small pieces were embedded in TissueTek (Sakura, Torrance, CA, USA) and stored at -80°C. For RNA analysis, tissue samples were homogenized using the Mixer Mill MM 301 for 2 min and 200 Hz. Total RNA was isolated using the RNeasy Mini Kit (Qiagen).

### RT-PCR analysis

To evaluate the mRNA expression of genes related to calcium transport in human salivary glands RT-PCR

was performed using specific primers designed by Primer Express 2.0 Software (Applied Biosystems, Darmstadt, Germany) and obtained from MWG-Biotech AG (Ebersberg, Germany) (for sequences see Table 1). RNA (5 µg) was reverse-transcribed with Superscript II RT (Invitrogen) at 42°C for 50 min using oligo dT primers (Promega, Mannheim, Germany), control reactions did not contain RT. Hundred nanogram of cDNA or non-reverse-transcribed RNA were amplified for 35 cycles in 50 µl total volume of PCR buffer 5x-PCR (Qiagen) containing 10 µM dNTP, 1 unit *Taq* polymerase, 20 pmol of each primer. The thermal cycling program (94°C for 30 s, 55–62°C for 30 s, and 72°C for 90 s) was performed in an Eppendorf Mastercycler gradient. Amplified products were separated on a 1.2% agarose gel, all PCR-fragments were verified by sequencing. Intensities of bands from the agarose gels were quantified using the Scion Image software (National Institutes of Health, Bethesda, MD, USA), results were normalized to GAPDH.

### Western blotting

To confirm the presence of proteins at the membrane level, western blot analysis from all significantly expressed genes from calcium channel and transporters expressed in human salivary gland samples was performed. Briefly, human salivary gland samples were homogenized with the Dounce tissue homogenizer (Bellco Glass Inc., Vineland, NJ, USA) in 1 ml homogenization buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Total protein concentration was determined using the Lowry method. Protein (30 µg) were separated by electrophoresis through a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane (Millipore

**Table 1** PCR Primers used for RT-PCR analysis of human salivary gland tissues

Gene	Acc. no.	Sense primer (5'-3')	Antisense primer (5'-3')	Product (bp)
CaT-Like (TRPV6)	AF365927	AGCCTACATGACCCCTAAGGACG	GTAGAAGTGGCCTAGCTCCTCGG	448
CaT2 (TRPV5)	AF304464	GGCCTATGAGACACGTGAAGATATC	ATAGAATTGCCCCAGACTGGTTG	447
CALB1	NM004929	CACAGAAGAGAATTTCTGCTGC	CACAGATCCTTCAGTAAAGCATCC	405
CALB2	BC015484	TTCCTGTCAGACCTGCTGAAGA	GCAAGGACATGACGCTCTTTC	350
CALB3	NM004057	TCTCCTGAGGAAGTGAAGAGG	CTCCATCTCCATTCTGTCCAG	175
SERCA1	NM173201	TGAACATCTGGCTGCTGGGC	GGAAGTGAGCATCCTTTTGCTC	235
SERCA2	NM170665	AACGCCCTCAACAGCTTGTC	GGATGAGACGCTCAAGTTTGTTG	232
SERCA3	NM005173	TGGCTGTGGCCATGTCCATGG	GCCAGAAGTGAGCGCTGGGA	206
PMCA1	NM001682	CCTCAAAGAAGCTGGTCATGG	AGGACTGGAGTTACTTTTGTAGGA	331
PMCA2	NM001683	AGCTGCTCTCCACTGCAGCT	GGCTCGAGTTCTGCTTGAGC	442
PMCA3	NM021949	CCACTGTCCACAGAACAGTGGC	TTCCTCGCCTTCGGCCAGC	171
PMCA4	XM046775	TCTACATTCACCACCCAGCC	GGAAGTATGGAACGCTTTGACC	487
NCX1	NM021097	GTTGGAAGTGATCATTGAAGAATCC	CCTTCCAGAASACMGTCAGRAAGTG	227
NCX2	XM038970	TGGAGGTCATCATCGAGGAGTC	CCTTCCAGAASACMGTCAGRAAGTG	228
NCX3	NM033262	CCTCTGTCCCAGATACGTTTG	GAACCCCTTGATGTAGCAATAGG	365
GAPDH	AF261085	CTGAACGGGAAGCTCACTGG	ACCACCCTGTTGCTGTAGCC	304
β-Actin	BC014861	CTCGTCGTCGACAACGGCTC	AGTGGTACGGCCAGAGGCG	425

*CaT-Like* calcium channel, *CALB* calbindin, *SERCA* sarcoplasmic reticulum calcium ATPase, *PMCA* plasma membrane calcium ATPase, *NCX* sodium calcium exchanger, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

Corp., Bedford, MA). Antibodies against CaT-Like, Calbindin, SERCA and PCMA were used in a 1:2000 dilution, detected with anti-rabbit-IgG conjugated with horseradish peroxidase, and visualized using the ECL Western Blot Detection Kit (PerkinElmer, Boston, MA). All antibodies recognized the target proteins at the expected molecular weight. The CaT-Like immunoreaction could be blocked in the presence of the specific antigenic peptide (IINRGLEDGESWEYQI).

### Immunohistochemistry

Immunohistochemistry was used to determine the localization of the proteins involved in calcium transport. This was performed on 8  $\mu$ m thick sections of fixed frozen parotid and submandibular gland samples. Briefly, sections were washed in PBS, permeabilized with 0.1% BSA + 0.3% Triton-X 100 in PBS and blocked for 30 min in 3% milk powder (0.5% fat) in PBS. Then they were incubated with the primary antibody in PBS + 1% BSA overnight at 4°C. Primary antibodies were used against PMCA-1,-2 and -4, SERCA-2, SERCA-3, Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\beta$ -subunit (Affinity BioReagents, Golden, CO, USA); CaT-Like (kindly provided by V. Flockerzi); and calbindin-2 (Sigma, St. Louis, MS). After permeabilizing, the sections were incubated once again with the secondary antibody for 90 min at 25°C in the dark. Secondary anti-IgG and IgM antibodies (goat-anti-rabbit, donkey-anti-rabbit, and goat-anti-mouse) were conjugated with Alexa488 or Alexa555 (Molecular Probes, Eugene, OR, USA) or Cy3 (Jackson Immuno Research, West Grove, PA, USA). Nuclei were stained with DAPI. Images were made using an *Axiophot* fluo-

rescence microscope (Zeiss, Jena, Germany) equipped with a digital photo camera *AxiCam* (Zeiss).

### Statistical analysis

Data are expressed as means  $\pm$  SD. Statistical analysis was performed using Student's unpaired *t*-test. *P* values less than 5% were considered statistically significant. All analyses were performed using Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA).

## Results

### RNA expression of calcium transport genes

The expression of genes coding for calcium entry channel (CaT family), intracellular calcium binding (calbindin family), calcium sequestration (SERCA family), and calcium efflux (PMCA and NCX family) was investigated in human parotid and submandibular gland. Fragments obtained were characterized by their molecular weight and by sequence homology with the expected product. As shown in Table 2, CaT-Like was expressed in 91% of the parotid samples and 78% of the submandibular samples, while CaT2 mRNA was not detected. From the members of the calcium binding protein family only calbindin-2 was detected in the parotid as well as in the submandibular gland, with comparable high frequencies (94% vs. 83%, respectively). Expression of endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) was found in both, parotid and submandibular glands. SERCA-2 expression was found in parotid (89%) and

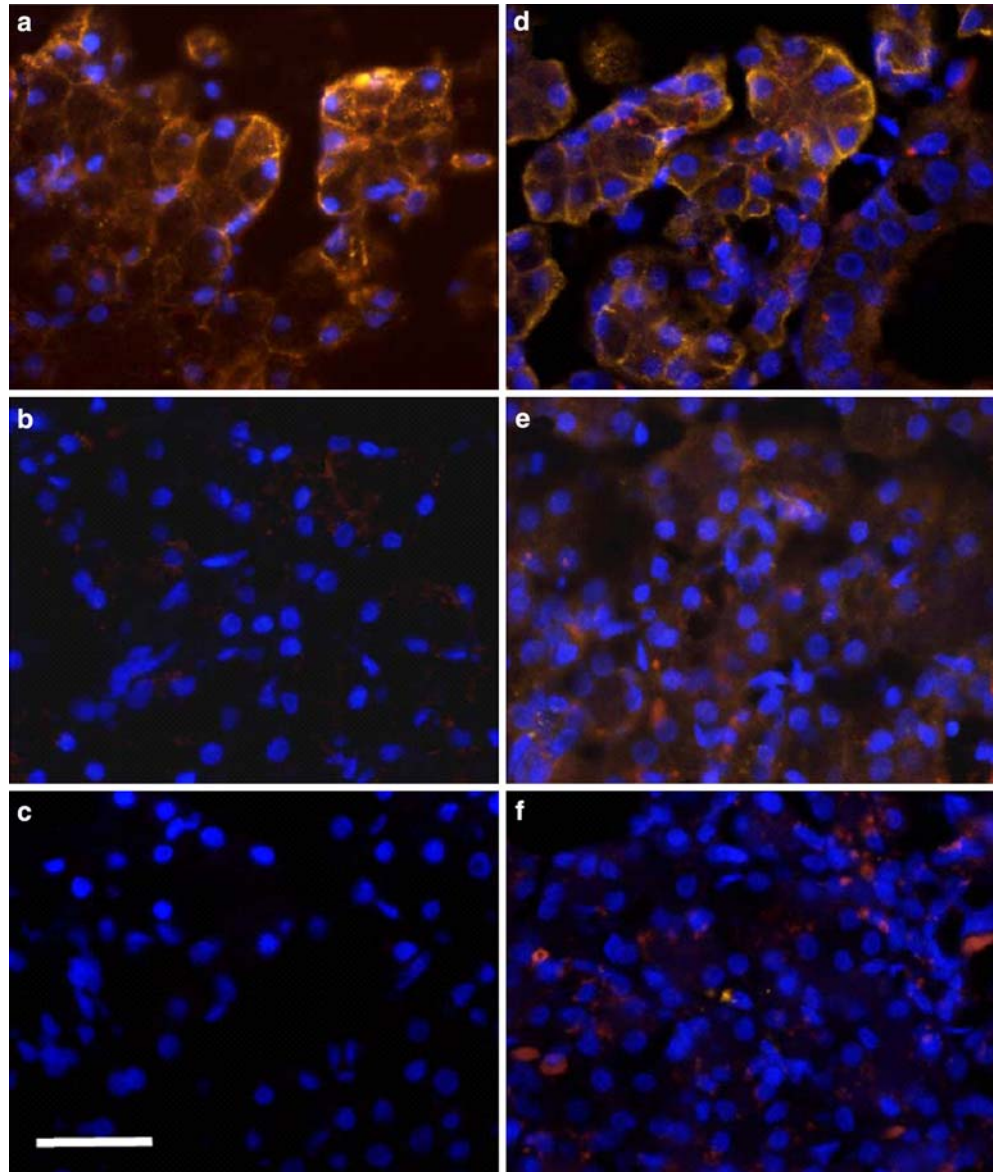
**Table 2** Expression of calcium transport genes in human salivary gland: RT-PCR analysis and relative expression

	PG	SMG	PG (RE)	SMG (RE)
<b>Epithelial Ca<sup>2+</sup> channels</b>				
CaT-Like	91% (32)	78% (27)	65% (9)	75% (7)
CaT2	0% (32)	0% (27)	–	–
<b>Ca<sup>2+</sup> binding proteins</b>				
CALB1	0% (19)	0% (24)	–	–
CALB2	94% (19)	83% (24)	48% (7)	32% (4)
CALB3	0% (19)	0% (24)	–	–
<b>Sarcoplasmic reticulum Ca<sup>2+</sup>-ATPases</b>				
SERCA1	0% (19)	0% (21)	–	–
SERCA2	89% (19)	86% (21)	99% (7)	82% (7)
SERCA3	68% (19)	81% (21)	59% (6)	64% (5)
<b>Plasma membrane Ca<sup>2+</sup>-ATPases</b>				
PMCA1	97% (32)	85% (27)	53% (9)	72% (8)
PMCA2	75% (32)	67% (27)	38% (7)	59% (6)
PMCA3	0% (32)	0% (27)	–	–
PMCA4	78% (32)	67% (27)	41% (9)	45% (6)
<b>Na<sup>+</sup>/Ca<sup>2+</sup> exchangers</b>				
NCX1	0% (29)	0% (31)	–	–
NCX2	0% (29)	0% (31)	–	–
NCX3	0% (29)	0% (31)	–	–

RT-PCR was performed using the primers listed in Table 1. Results are shown as percentage of tissue samples tested. The relative gene expression (RE) of calcium transport systems is shown as percentage of GAPDH expression. The number of glands analyzed is given in parenthesis

PG parotid gland, SMG submandibular gland

**Fig. 1** CaT-Like labeling in human parotid (a–c) and submandibular (d–f) salivary glands. **b** and **e** shows the competition analysis using the specific antigenic peptide and **c** and **f** show controls without primary antibody. Magnifications:  $\times 630$ . Scale bar 20  $\mu\text{m}$

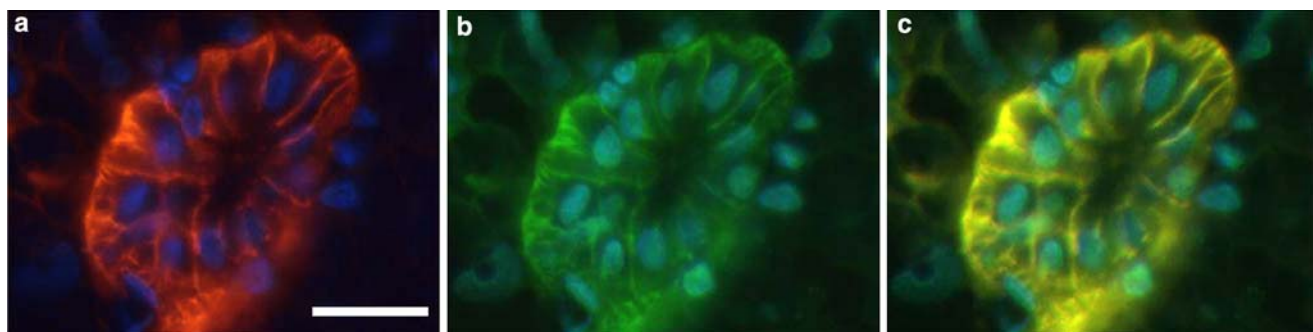


submandibular (86%) glands, while SERCA-3 expression in these salivary glands was 68 and 81%, respectively. None of the samples expressed SERCA-1. PMCA-1, PMCA-2 and PMCA-4 were expressed in most tissue samples. The frequency varied between 97 and 67%. No significant difference was observed between parotid and submandibular glands. Interestingly, in none of the salivary gland samples mRNA for members of the sodium/calcium exchanger (NCX) family was found. Relative expression of all transport channels and transporters did also not show any difference between parotid gland and submandibular gland.

#### Immunohistochemical localization of epithelial calcium transport proteins

Figure 1 shows the immunoreactions observed with a specific antibody against CaT-Like isoform. Immun-

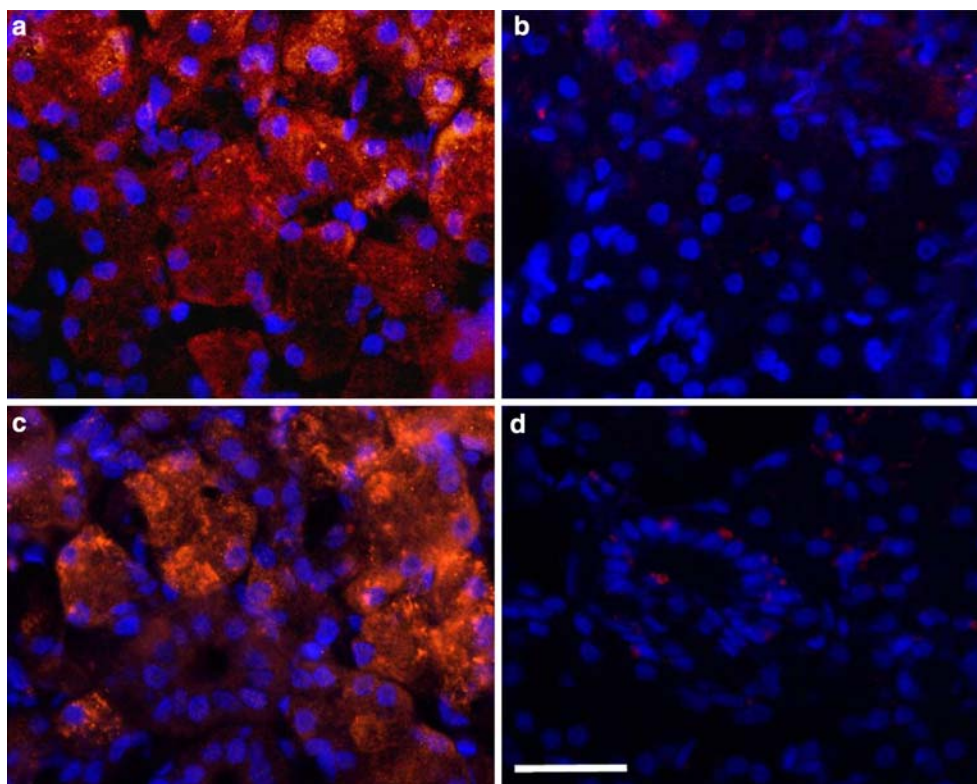
histochemistry using antibodies directed against other CaT1 isoforms did not show any reaction. As shown in Fig. 1a and d, the specific antibody against CaT-Like stains the basolateral membrane of parotid and submandibular acini. The specificity of the antibody used was confirmed by competition analyses with the antigenic peptide (Fig. 1b, e) in which CaT-Like staining was completely abolished. Figure 2 shows colocalization studies with the marker for basolateral membranes,  $\text{Na}^+/\text{K}^+$ -ATPase. The overlay in Fig. 2c clearly shows identical localization within the cell. The localization of calbindin-2 is shown in Fig. 3. A rather strong fluorescence is seen in parotid and submandibular glands, it fills the whole cell in a cloudlike pattern. Figure 4 illustrates the reaction of acini with an antibody against SERCA-2. The fluorescence signal observed is arranged in dense clusters in the cytosol of the basal cell pole. Preliminary results using a SERCA-3 antibody revealed colocalization of the staining with



**Fig. 2** Costaining of CaT-Like and  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit in human parotid salivary gland. **a** CaT-Like (red), **b**  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit (green) and **c** overlay (yellow). The overlay

shows identical localization within the cell. Magnification: 630 $\times$ . Scale bar 20  $\mu\text{m}$

**Fig. 3** Calbindin-2 labeling in human parotid (**a**, **b**) and submandibular (**c**, **d**) salivary glands. **b** and **d** show controls without primary antibody. Magnifications:  $\times 630$ . Scale bar 20  $\mu\text{m}$

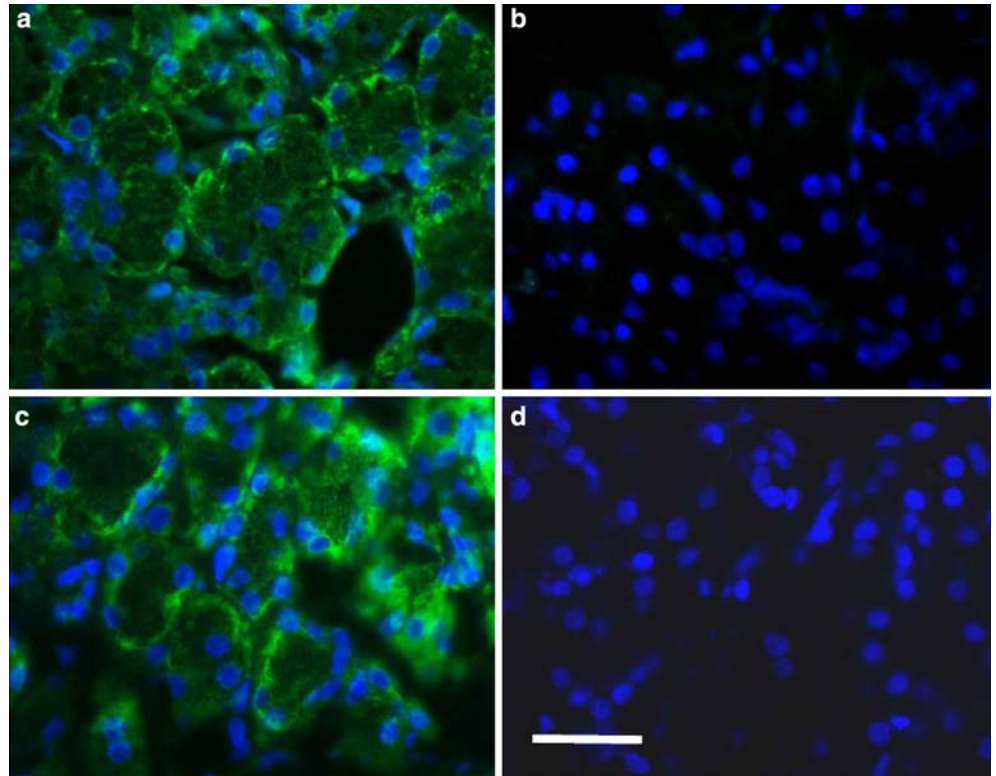


SERCA-2 distribution (data not shown). PMCA-1, -2 and -4 are located on the apical membrane. PMCA-2 shows a starfish like structure in the centre of the acini, the “arms” reaching deep between the cells (Fig. 5). To confirm the apical localization of PCMA-2, staining with an antibody against  $\text{Na}^+/\text{K}^+$ -ATPase, a marker of basolateral membranes, was used. As shown in Fig. 6, PMCA-2 is located in the apical membranes and the secretory canaliculi between the acinar cells and does not colocalize with the  $\text{Na}^+/\text{K}^+$ -ATPase, which as expected is observed on the basolateral membrane.

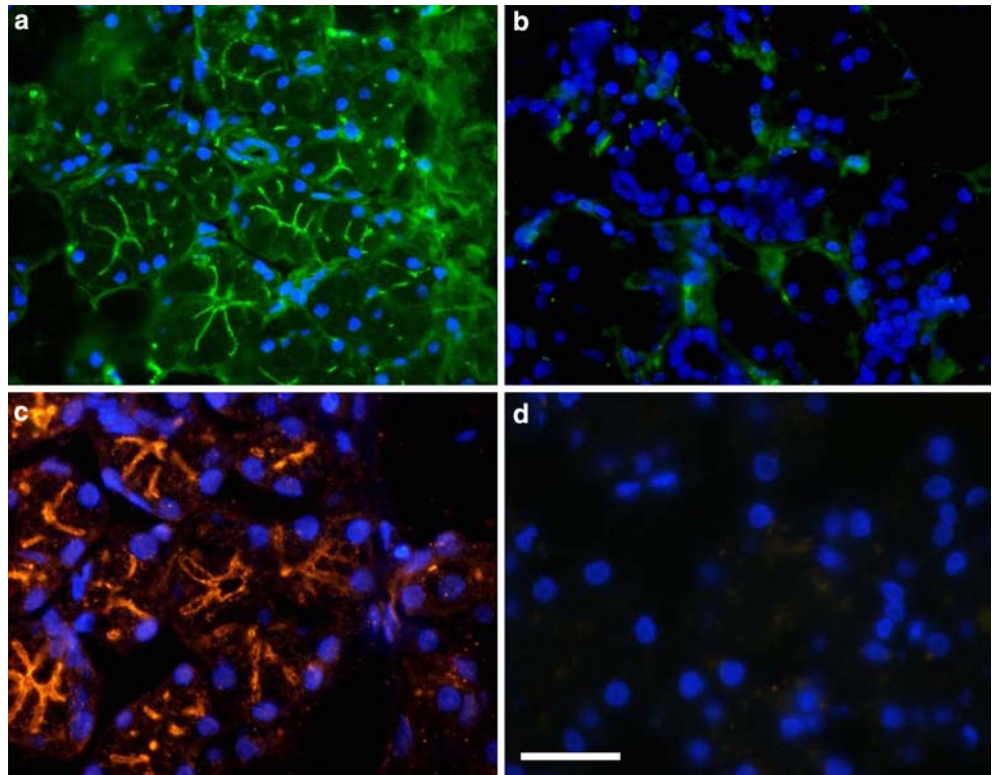
#### *Proposed model of calcium secretion in human salivary glands*

Figure 7 summarizes the distribution of the calcium channels and transport systems obtained in the present study. Since no significant differences in expression or cellular location were found between parotid and submandibular glands, the scheme presented here for the transepithelial transport of calcium applies to both of them. The proposed model can be summarized as follows: (1) calcium enters the acinar cell at the basolateral site via calcium channel CaT-Like

**Fig. 4** SERCA-2 labeling in human parotid (**a, b**) and submandibular (**c, d**) salivary glands. **b** and **d** show controls without primary antibody. Magnifications:  $\times 400$ . Scale bar 20  $\mu\text{m}$



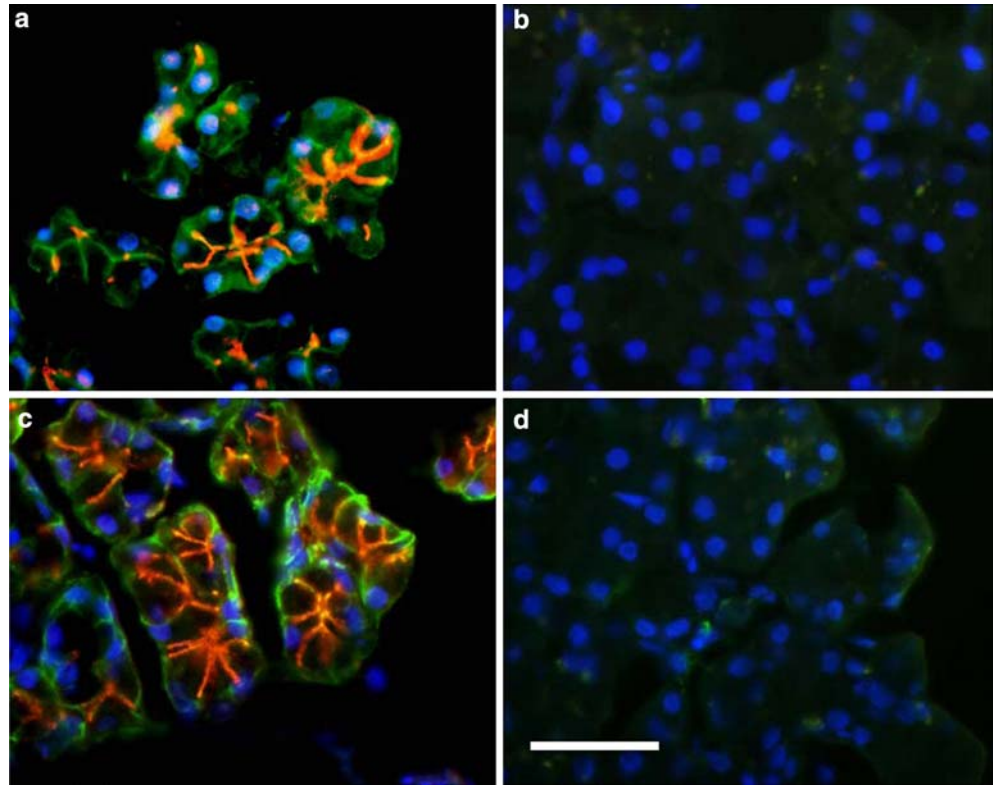
**Fig. 5** PMCA-2 staining in human parotid (**a, b**) and submandibular (**c, d**) salivary glands. Labeling of the apical membrane and secretory canaliculi is present in the acini. Secondary antibody labeled with AlexaFluor488 (*green*) or Alexa Fluor555 (*red*). **b** and **d** show controls without primary antibody. Magnifications:  $\times 400$ . Scale bar 20  $\mu\text{m}$



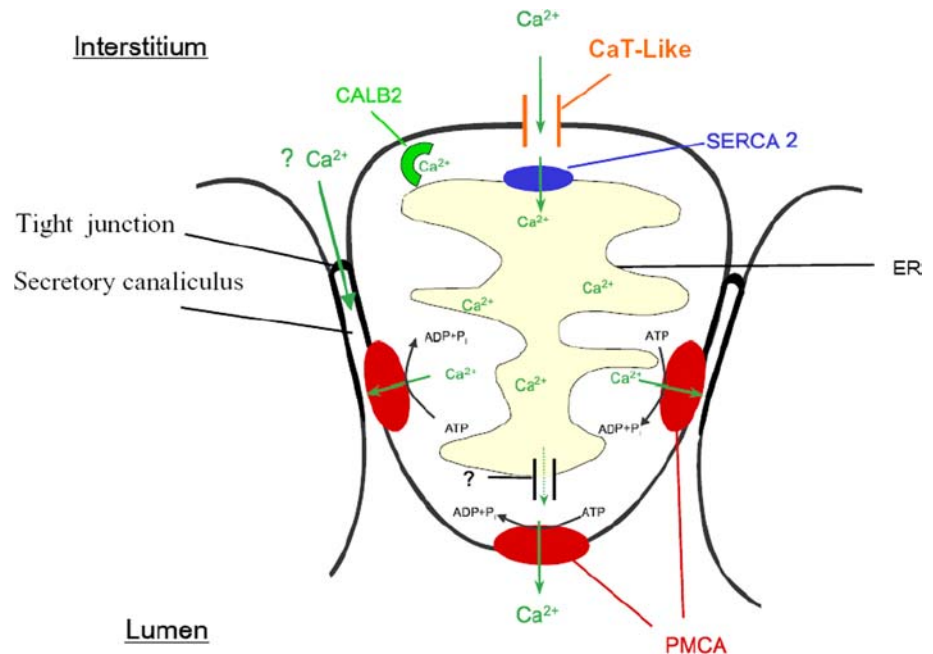
(calcium influx); (2) intracellular calcium is taken up into the endoplasmic reticulum by SERCA-2 and possibly SERCA3 or bound to calbindin-2 (intracel-

lular calcium pool); and (3) calcium is secreted by PMCA2 on the apical plasma membrane (calcium efflux).

**Fig. 6** Costaining of PMCA-2 and  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit in human salivary glands. **a** and **b** parotid gland and **c** and **d** submandibular gland. Labeling of  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit is seen in basolateral membrane in both glands (*green*), PMCA2 staining is shown in the apical membrane and secretory canaliculi (*red*). **b** and **d** show controls incubated without primary antibody. Magnification: 630 $\times$ . Scale bar 20  $\mu\text{m}$



**Fig. 7** Proposed model of calcium secretion in human salivary gland acinar cells. Expression of calcium channels and transport proteins were analyzed by RT-PCR. Its localization was assessed by immunohistochemistry. *CaT-Like*, calcium channel 1; *CALB-2*, calbindin-2; *SERCA-2*, sarcoplasmic reticulum calcium pump; *PMCA*, plasma membrane calcium ATPase



## Discussion

The present study delineates for the first time a mechanism of transepithelial calcium transport in human salivary parotid and submandibular glands using immunohistochemistry techniques and molecular biology. Comparable RT-PCR data expression of all genes

investigated has already been reported in rat salivary glands (Melvin et al. 2005), we confirm their presence for human salivary glands. The experiments performed here faced several obstacles. First of all, saliva contains a high concentration of RNases, which, if not the necessary precautions are taken, can destroy mRNA very rapidly. Thus, several glands gave no result at all due to the fact

that the time span between resection, mincing and immersion in the appropriate RNase inhibitor containing solutions was too long. Another point of concern was the state of the tissue adjacent to the diseased tissue. In some cases, although the tissue looked macroscopically normal, the expression pattern for the transport systems was changed dramatically, whereas the expression of the housekeeping genes GAPDH,  $\beta$ -actin and  $\text{Na}^+/\text{K}^+$ -ATPase was very reproducible. Such alterations may be the reason for the scatter observed for the amount of RT-PCR products generated and that the frequency of positive results was less than 100%.

One of the most striking findings in the immunohistochemical investigations is the basolateral localization of CaT-Like. CaT-Like is specifically expressed in placenta, pancreatic acinar cells, prostate and salivary glands (Peng et al. 2000; Wissenbach et al. 2001; Zhuang et al. 2002). Until now the role of CaT-Like in salivary glands has not yet been studied. Basolateral calcium influx in rat salivary glands has been functionally characterized as store-operated calcium entry (Ambudkar 2000). However, the molecular basis of this channel was not identified. Later the molecule was proposed to be member of the transient receptor potential (TRP) gene family (Petersen et al. 1999; Petersen and Fedirko 2001; Hoenderop et al. 2003; Nijenhuis et al. 2003). CaT-Like belongs to the same family (Hirnet et al. 2003). It codes for a calcium-selective channel (TRPV6\_Human, GenBank NM\_018646). Interestingly, CaT-Like is abundantly expressed in tissues with exocrine functions but not in absorptive tissues, such as intestine and kidney (Wissenbach et al. 2001). This study shows that in human salivary gland this protein is localized on the basolateral membrane, suggesting an important physiological role for calcium influx into the acinar cells.

The sarcoplasmic reticulum calcium pump (SERCA) has been found to be expressed in salivary gland cells (Lee et al. 1997; Meehan et al. 1997). The primary function of SERCA appears to maintain an intracellular calcium pool. In addition, it is assumed that SERCA-2 mediates a kind of transcellular tunneling of calcium (Petersen 2003). This evidence has been derived from the functional data obtained in pancreatic acini and in isolated salivary glands (Ambudkar 2000). In the salivary gland, however, calbindin also appears to assume a role in intracellular calcium shuttling. The calbindin pathway has been reported in small intestine and kidney (Hoenderop et al. 2005). These data are in concordance with the results obtained in the present study, suggesting an important role of SERCA and calbindin in the regulation of the intracellular calcium pool in human salivary glands.

Another important finding of this study is the localization of PMCA-1, -2 and -4 in salivary glands. The localization of PMCA on the apical plasma membranes of the acini, but not on the basolateral membranes, leads to the asymmetry of transport properties of the two membrane areas required for transepithelial transport (Kinne 1991). Our data demonstrate that PMCA constitutes the major calcium efflux pathway in human

parotid and submandibular salivary glands. The absence of NCX expression correlates with the findings of other authors (Ambudkar 2000; Melvin et al. 2005). In addition, apical membrane localization of PMCA-2 has also been reported in other exocrine cells, such as the mammary gland (Reinhardt and Horst 1999; Reinhardt et al. 2000) and pancreas (Lee et al. 1997). Our results suggest that calcium transport across human salivary gland acinar cells is an active transcellular process that plays an important role in salivary calcium secretion.

The potential clinical relevance of the data shown in the present study is worth considering in more detail. It is evident that in other organs the transport systems found in the acinar cells are subjected to hormonal regulation. Such control is clearly established for vitamin D in intestine (Wood et al. 2001) and kidney (van Baal et al. 1999; Hoenderop et al. 2001), and for prolactin in the mammary gland (Shennan and Peaker 2000) to give a few examples. If the appropriate receptors were present in the salivary gland a variation in salivary calcium secretion depending on the hormonal status might be expected. Furthermore, local factors—such as presence of diseased tissue in the neighborhood—appear to influence the expression of the transporters and thus might also affect calcium secretion into saliva.

In conclusion, we propose a model of calcium transport and calcium secretion in human salivary glands. This model can be summarized as follows: (1) calcium enters the acinar cell at the basolateral site via calcium channel CaT-Like (calcium influx); (2) intracellular calcium is taken up into the endoplasmatic reticulum by SERCA-2 and possibly SERCA3 or bound to calbindin-2 (intracellular calcium pool); and (3) calcium is secreted by PMCA on the apical plasma membrane (calcium efflux). In closing, a detailed analysis of the various gene expressions described here, regarding calcium secretion into saliva and the development of dental caries should be pursued further. Similarly, physiological investigations on intact salivary glands are required to confirm the proposed model in vivo.

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