In Situ Hybridization and Monoclonal Antibody Analysis of Plasma Membrane Ca-Pump mRNA and Protein in Submandibular Glands of Rabbit, Rat and Man

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IN SITU HYBRIDIZATION AND MONOCLONAL ANTIBODY ANALYSIS OF PLASMA MEMBRANE Ca-PUMP mRNA AND PROTEIN IN SUBMANDIBULAR GLANDS OF RABBIT, RAT AND MAN

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Abstract

The degree of supersaturation of saliva with calcium (Ca) is related to the mineral phase of enamel in erupted teeth, the incidence of caries, and the formation of calculus. The mechanisms for regulating salivary Ca concentration are therefore of relevance to dentistry. Sections of rabbit, rat and human submandibular gland (SMG) were processed for immuno-histochemistry with a specific anti-plasma membrane Ca-pump antibody, 5F10. Western blots confirm that the molecular weight of the proteins identified by our antibody (135 kDa) is consistent with an appropriate molecular weight for PMCA antigen (135-150 kDa). Tissue sections were also processed for in situ hybridization to study the distribution of the PMCA mRNA isoforms. In mammals, the PMCA1 gene is reported to code for a PMCA protein with a role in maintaining the intracellular Ca levels in both epithelial and non-epithelial cells. Other genes including the PMCA2 and PMCA4 genes may code for PMCA proteins specific to Ca transporting tissues. Our studies demonstrate cytoplasmic labeling of PMCA mRNA with hPMCA-1 and hPMCA-4 specific cDNA probes in humans, and rPMCA-1 and rPMCA-2 specific oligonucleotide probes in rats. Labeling of PMCA protein and all mRNA isoforms was found in the cytoplasm of the interlobular and intralobular ducts (except for intercalated ducts). The demonstrated presence of PMCA in SMGs of rabbit, rat, and man, may suggest a role for PMCA in the regulation of intracellular Ca and in a mechanism for regulating and maintaining the high concentration of Ca in saliva.

Key Words: Submandibular gland, Ca-Pump, Ca^{++}-Mg^{++}ATPase, in situ hybridization, immunohistochemistry.

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Introduction

The supersaturation of saliva with calcium and phosphate in association with dental plaque, creates a mineral-rich microenvironment at the enamel-plaque interface which may contribute to maturation and remineralization of enamel [9]. In addition, this microenvironment may act as a buffer between enamel and the acids produced by some oral microorganisms. Such buffering action assists in the prevention of acid dissolution of the enamel and the onset of dental caries [17]. Conversely, a disturbance in the relationship between salivary proteins and the high calcium and phosphate concentrations in saliva, may promote the precipitation of calcium salts in the form of calculus [11]. A basic understanding of the mechanisms that affect the calcium concentration of saliva are, therefore, fundamentally important to preventive and restorative dentistry.

In a typical transport epithelium, Ca enters the cells through Ca channels down chemical and electrical gradients. Cell Ca then is taken up by organelles such as mitochondria and endoplasmic reticulum or it is bound to cellular calcium binding proteins. Whether the SMG interlobular and intralobular ducts contain the same or similar Ca^{++} sequestering mechanisms has yet to be determined. For Ca to be transported out of the cell, it must again cross the plasma membrane against electrical and chemical gradients, by means of a Na/Ca exchanger or a plasma membrane Ca-pump [8].

The present study will describe the distribution of plasma membrane Ca-pumps in SMGs from rabbit, rat, and man. Plasma membrane Ca-pumps are a family of proteins derived from at least five different genes. The PMCA1 gene in rats and humans is believed to code for plasma membrane Ca-pumps with primary roles in the regulation of intracellular Ca levels [14]. The PMCA2 gene in rats is believed to code for plasma membrane Ca-pumps in epithelial tissues involved primarily in vectorial Ca transport [14]. The PMCA4 gene in humans, like the PMCA2 Ca-pump gene in rats, is believed to code for plasma membrane Ca-pumps specifically in transport epithelia [14]. In this study, we show that

plasma membrane Ca-pumps of both types are found in highest concentration in the cells of the SMG known to play a role in the regulation of electrolyte concentration of saliva.

Materials and Methods

Preparation of SMGs for immunohistochemistry and *in situ* hybridization

Rabbit SMGs were obtained from young adult albino New Zealand rabbits of both sexes. Rat SMGs were obtained from weanling female Sprague-Dawley rats. All animals were euthanized by pentobarbital overdose. Human SMG tissue was obtained from autopsy. Tissue samples were excised as rapidly as possible, prior to immersion in 4% paraformaldehyde in 0.1 M cacodylate buffer. After fixation, tissues were dehydrated through a series of ethanol and xylene baths before embedding in paraffin and serial sectioning.

Materials

Reagents for labeling and detection of cDNA probes, oligonucleotide probes, and kits for immunohistochemistry were obtained from Vector Laboratories (Burlingame, CA). Restriction enzymes for preparation of the probes were obtained from USB (Cleveland, OH). Other reagents were obtained from Sigma (St. Louis, MO).

Monoclonal antibody to plasma membrane Ca-pump

Human erythrocyte plasma membrane Ca-pump was purified by calmodulin-affinity chromatography as previously described [16]. Monoclonal antibodies directed against the plasma membrane Ca-pump have been characterized and were prepared as described previously [1, 2]. One of the monoclonal antibodies, 5F10, was found to recognize Ca-pump molecules in diverse tissues and species, and was therefore used for this study [3, 4, 5, 6].

Immunohistochemistry

SMG tissues were processed for immunohistochemical localization of Ca-pump protein using an avidin-biotin-peroxidase technique [12]. With this technique, sections of SMG tissue were found to bind optimally to monoclonal antibody 5F10 at a dilution of 1:1000 after a 60 minute incubation at room temperature. The site of 5F10 binding to SMG tissues was determined by linkage of a peroxidase enzyme to the site of antibody binding in the tissue through a series of connecting molecules. In the presence of diaminobenzidine (DAB) and hydrogen peroxide, peroxidase produces a brown colored precipitate over areas of the tissue containing Ca-pump. Negative controls were prepared by processing serial sections without the 5F10 antibody. Sections of rat kidney known to contain plasma membrane Ca-pump were processed with each test run as positive controls. Sections were counter-stained with hematoxylin, or Periodic Acid-Schiff reagent (PAS) for identification of tissue morphology.

Western blot analysis of rabbit and rat Ca-pump protein

Specimen preparation for Western blots. Fragments of rabbit and rat SMGs were rapidly excised and frozen in liquid nitrogen. Frozen specimens were stored at -70°C until processed for gel electrophoresis.

Polyacrylamide gel electrophoresis. Fragments of frozen rabbit and rat SMGs were homogenized with a OMNI 1000 tissue homogenizer (OMNI International, Waterbury, CT). These fragments were homogenized on ice in 1 ml of phosphate buffered saline (PBS), pH 7.4, containing 0.5 mM dithiothreitol (DTT), 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), and 0.5 mM benzamidine for 60 seconds in 12 second bursts. Total protein content of the homogenate was determined by the bicinchoninic protein assay (BCA) (Pierce, Rockford, IL). The protein content of each homogenate was equilibrated. An equal concentration of each homogenate was denatured by heating in a boiling water bath (100°C for 5 minutes) in the presence of 50 mM Tris-HCl pH 6.8 containing 5% beta-mercaptoethanol, 2% SDS, 10% glycerol and 0.002% bromophenol blue. Twenty micrograms of each sample were loaded in wells of 7% SDS-PAGE gels prepared by the methods of Laemmli [13].

Protein transfer to nitrocellulose. Following gel electrophoresis, the separated proteins were transferred from the gels to nitrocellulose by electro-blotting [21]. Nitrocellulose-bound proteins were placed in 0.1% sodium azide plus 3% hydrogen peroxide in PBS for 10 minutes to block peroxidase activity endogenous to the tissues. To block non-specific binding of proteins to the blot, a solution of PBS (pH 7.4) containing 10 mg/ml bovine serum albumin (BSA) was applied to the blot for one hour. Protein bands representing plasma membrane Ca-pump were identified on the nitrocellulose by immunochemistry with a 1:1000 dilution of antibody 5F10 using an avidin-biotin-peroxidase technique essentially as described for localization of Ca-pump in tissue sections. The molecular weights of proteins transferred to nitrocellulose were estimated by measuring the migration of the protein bands relative to marker proteins of known molecular weight.

In situ hybridization of SMG mRNA with cDNA and oligonucleotide probes to plasma membrane Ca^{++}-pump isoforms

Specific double-stranded cDNA probes were designed to identify regions unique to the human plasma mem-

brane Ca-pump hPMCA-1 and hPMCA-4 mRNA transcripts. These probes were prepared by Drs. Anil Verma and Hugo Adamo in the laboratory of Dr. John T. Penniston at the Mayo Clinic in Rochester, MN. Both hPMCA-1 and hPMCA-4 probes have been previously characterized and represent sequences in the translated regions of the Ca^{++}-pump genes [20, 22].

Specific probes were also prepared to study rat plasma membrane Ca-pump rPMCA-1 and rPMCA-2 mRNA distribution. Rat single-stranded 50mer oligonucleotide probes were synthesized to identify unique sequences within the transcribed mRNA of each isoform. The rPMCA-1 probe identifies nucleotides 334 to 383 of the transcribed rPMCA-1 gene, while the rPMCA-2 probe identifies nucleotides 562 to 611 of the rPMCA-2 gene [19].

The hPMCA-1 and hPMCA-4 cDNA probes as well as the rPMCA-1 and rPMCA-2 oligonucleotide probes were labeled using the Vector Laboratories (Burlingame, CA) photoactivatable biotin labeling technique.

Paraffin embedded tissue sections of rat and human SMG for *in situ* hybridization were deparaffinized and rehydrated through a series of ethanols to water. Sections were digested in pepsin-HCl for 2 minutes to improve access to target sequences. Tissue sections were processed for *in situ* hybridization as we have previously described [7]. The sites of binding of the biotinylated human cDNA probes and rat oligonucleotide probes to specific tissue mRNA sequences were visualized by incubating sections with a streptavidin-peroxidase solution. After incubation and washing, the sections were exposed to a diaminobenzidene-H_2O_2 solution for approximately 5 minutes until a brown colored reaction product developed over the sites of probe binding. Sections were then counter-stained with Mayer's hematoxylin and dehydrated before mounting for observation.

Results

Figure 1 shows staining of a rabbit SMG tissue section for plasma membrane Ca-pump. Specific staining is clearly seen over the interlobular and intralobular ducts. No staining is seen in the mucus or serous acini or in the intercalated ducts of the gland. No staining was seen in negative control sections of the same tissue stained by the same procedure but without anti-Ca-pump antibody. Figure 2, at a higher magnification of the same area seen in Figure 1, demonstrates the strongest staining for Ca-pump over the basal membranes of the ductal epithelial cells.

Western blot analysis of rabbit SMG homogenates with anti-Ca-pump antibody 5F10 (Figure 3), demonstrates a major band at 135 kDa. This band is of a molecular weight consistent with plasma membrane Ca-

pumps described in other tissues and species [3, 4, 5, 6]. Other lighter staining bands may result from endogenous or experimentally produced tissue proteolysis.

Figure 4 represents a tissue section from rat SMG that, like rabbit SMG, shows highly specific staining for Ca-pump protein in the interlobular and intralobular duct cells. Figure 5, at a higher magnification from another area on the same slide, shows basal staining of some duct cells and also shows highly variable staining between cells within the same duct. To help distinguish the cells that are staining from those which are not, some sections were double-stained for plasma membrane Ca-pump, and secretory granules using PAS. Figure 6 is a micrograph of Ca-pump positive rat SMG intralobular duct cells demonstrating a lack of Ca-pump staining in cells containing PAS positive secretory granules (arrow).

Figure 7 represents a Western blot of rat SMG tissue homogenates stained with anti-Ca-pump antibody 5F10. Like the Western blot for the rabbit SMG, this figure shows a major staining band at 135 kDa corresponding to the molecular weight of intact Ca-pump. A second band near 76 kDa is believed to be a product of proteolysis and corresponds to the molecular weight of a major fragment of the pump seen after trypsin digestion.

Figures 8 and 9 represent rat SMG tissue sections processed for *in situ* hybridization in order to compare the localization of mRNA for two Ca-pump isoforms derived from two separate genes, rPMCA-1 and rPMCA-2. Figure 8 shows hybridization of SMG interlobular duct cell cytoplasm to a 50mer oligonucleotide probe specific for rPMCA-1 mRNA. Figure 9 demonstrates hybridization of rat interlobular duct cell cytoplasm to a 50mer oligonucleotide probe specific for rPMCA-2 mRNA.

Figure 10 represents a section of human SMG from autopsy stained with antibody 5F10. Like rabbit and rat SMGs, this section shows highly specific staining for Ca-pump protein in the interlobular and intralobular duet cells. Figure 11 presents an enlargement from the same area which demonstrates a more defuse staining of the membranes and cytoplasm of the duet cells. The delay in fixation of autopsy tissues may cause migration of some Ca-pump antigen into the cytoplasm.

Figures 12, 13, and 14 represent human SMG tissue sections processed for *in situ* hybridization in order to compare the localization of mRNA for two Ca-pump isoforms derived from two separate genes, hPMCA-1 and hPMCA-4. Figure 12 shows hybridization of human SMG interlobular duet cell cytoplasm to a cDNA probe specific for hPMCA-1 mRNA. Figure 13 is a higher magnification micrograph of the same area demonstrating the specificity of the hybridization to the cytoplasm,
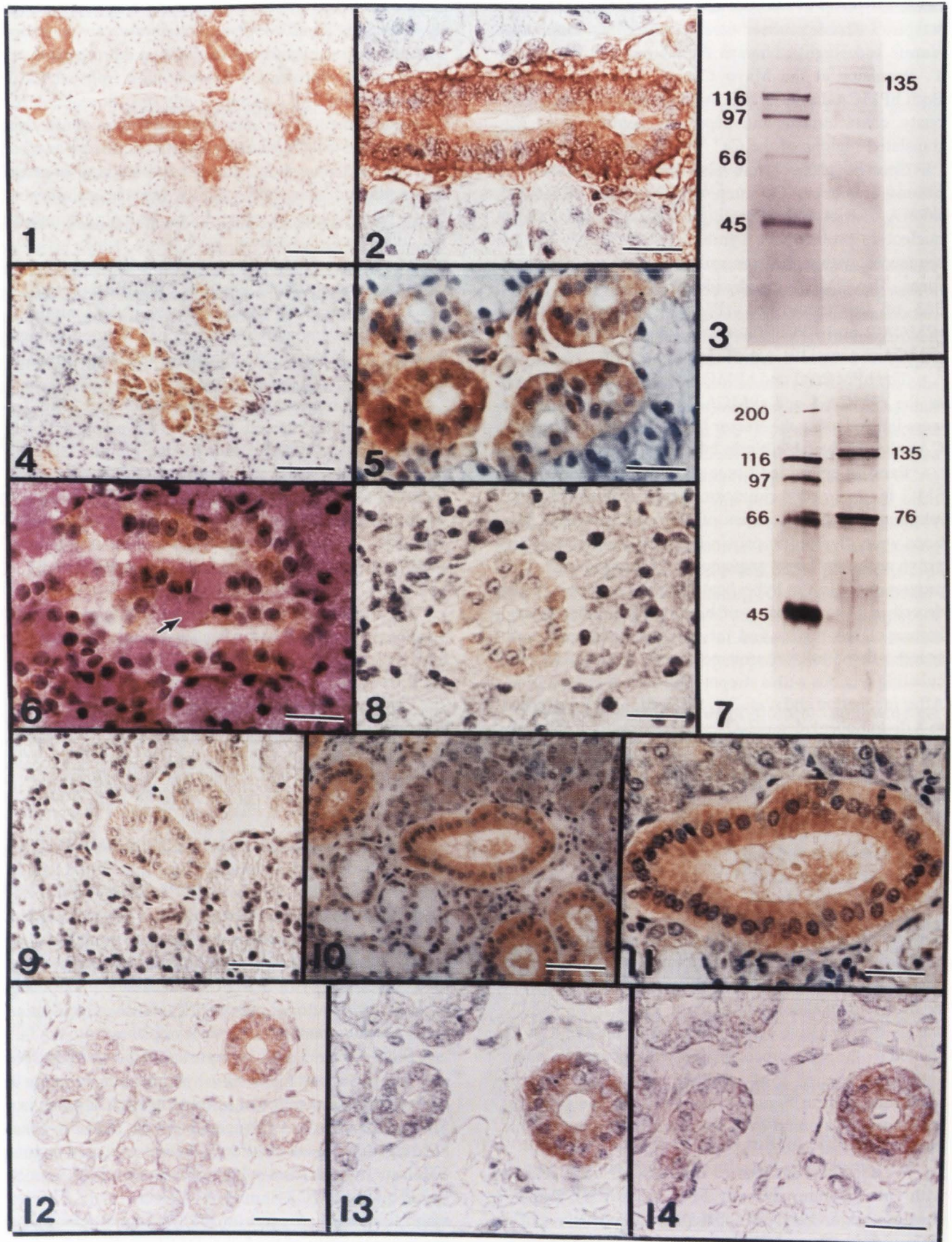
Legends for Figures 1-14 (on the facing page 820).

Figure 1. Low magnification micrograph showing specific binding of Ca-pump antibody 5F10 to rabbit SMG intralobular and interlobular ducts. Bar = 100 μm.

Figure 2. High magnification micrograph of the same area as Figure 1 showing heavy staining of the basal membrane of rabbit SMG intralobular duct cells. Bar = 31 μm.

Figure 3. Western blot analysis of rabbit SMG protein homogenate with Ca-pump antibody 5F10, showing a major band at 135 kDa.

Figure 4. Low magnification micrograph showing specific binding of Ca-pump antibody 5F10 to rat SMG intralobular and interlobular ducts. Bar = 80 μm.

Figure 5. High magnification micrograph from the same slide as figure 4 showing variable basal membrane staining of cells of the rat SMG intralobular and interlobular ducts. Bar = 25 μm.

Figure 6. Rat SMG duct cells double stained for plasma membrane Ca-pump and PAS. Cells containing PAS-positive secretory granules do not stain for Ca-pump. Bar = 25 μm.

Figure 7. Western blot analysis of rat SMG proteins with Ca-pump antibody 5F10, showing major bands at 135 kDa and 76 kDa.

Figure 8. *In situ* hybridization of rat SMG to a 50mer oligonucleotide probe specific for rat PMCA1 Ca-pump gene mRNA transcript. Probe binding is seen in the cells of an intralobular duct. Bar = 25 μm.

Figure 9. High magnification micrograph of the same area as Figure 1 showing heavy staining of the basal membrane of rabbit SMG intralobular duct cells. Bar = 80 μm.

Figure 10. Low magnification micrograph showing specific binding of Ca-pump antibody 5F10 to human SMG intralobular and interlobular ducts. Bar = 80 μm.

Figure 11. High magnification micrograph of the same area as Figure 10 showing staining of the human SMG intralobular duct cells. Bar = 31 μm.

Figure 12. *In situ* hybridization of human SMG to a cDNA probe specific for human PMCA1 Ca-pump gene mRNA transcript. Probe binding is seen in the cells of an intralobular duct. Bar = 80 μm.

Figure 13. Higher magnification micrograph showing *in situ* hybridization of human SMG to a cDNA probe specific for human PMCA1 Ca-pump gene mRNA transcript. Variable probe binding is seen in the cells of an intralobular duct. Bar = 25 μm.

Figure 14. Serial section of the same tissue as Figure 13, showing *in situ* hybridization of rat SMG to a cDNA probe specific for human PMCA4 Ca-pump gene mRNA transcript. Bar = 25 μm.

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and not the nuclei of human interlobular duct cells, and no binding to the adjoining connective tissue or surrounding acinar cells. Figure 14 represents a serial section from the same human SMG, but hybridized to an hPMCA-4 cDNA specific probe, and it shows specific labeling of the cytoplasm of the interlobular duct cells.

Discussion

The structure of the intralobular ducts in rat, rabbit and human SMGs, appear quite distinct histologically [10]. In rats, the majority of the intralobular ducts appear to be of a granular type epithelium, at different

stages of secretory activity. Some cells devoid of granules were found among the numerous secretory granule containing cells. In humans, the intralobular duct appears by light microscopy to be devoid of most secretory granules, and may be characterized as a classical striated epithelium having highly invaginated basal membranes and interspersed, radially-arranged mitochondria. Rabbit intralobular ducts contain both granular and agranular regions, with the granular regions located between the intercalated ducts and agranular regions of striated epithelium [10].

In our studies, striated ducts in all three species contain high levels of Ca-pump protein, primarily in the basal membranes. This suggests that plasma membrane Ca-pump may play a role in Ca efflux from the ductal lumen into the plasma. The presence of high levels of Ca-pump protein (relative to surrounding cells), in the three species tested, suggests that a mechanism for Ca efflux from the ductal lumen into the plasma may be a generalized feature of SMG duct cells from many species. Of additional interest is the observation that only the duct cells devoid of granules appear to bind to anti-Ca-pump antibody.

The secretions of the SMG acini are very similar to that of plasma, and are nearly isotonic [18]. Secretions of the acini contain high concentrations of ions including, sodium, potassium, chloride and calcium. It is well established that the striated ducts actively resorb sodium and other ions from the acinar cell secretion, with the ultimate secretion reaching the oral cavity being hypotonic [17]. The finding of plasma membrane Ca-pump exclusively within the basal membranes of intralobular and interlobular duct cells is consistent with a role for the Ca-pump in duct cell electrolyte transport.

Interpretation of earlier studies of the Ca-ATPase in the plasma membranes of salivary glands may have been confounded by the presence of an ecto-ATPase which is also located on the plasma membrane, but which does not play a role in Ca transport [15]. Our study uses a specific monoclonal antibody directed against the Ca-Mg-ATPase Ca-pump, which does not cross-react with proteins of the same molecular weight as the SMG ecto-ATPase (100,000 kDa). Ca-pump protein is a low abundance protein representing approximately 0.1% of membrane protein [8]. Because of the small amount of protein presumably present in our samples, we have overloaded some gels to assist in detection. This creates background staining, but which is easily distinguishable from the Ca-pump protein bands.

Our study also shows specific mRNA for the plasma membrane Ca-pump isoforms rPMCA-1 and rPMCA-2 in the SMG of rats, and hPMCA-1 and hPMCA-4 in the SMG of humans. The finding of Ca-pump mRNA derived from three different Ca-pump genes from two dis-tinct species in the same cells of the striated ducts suggests that Ca regulation is an important and highly conserved mechanism in this tissue. As mentioned above, the PMCA1 gene is believed to code for proteins with a role in maintaining cell Ca at approximately 10^{-7} M, in the face of 1000 fold higher extracellular Ca concentration [14]. Proteins derived from this gene may be found in many cell types including cells with no role in Ca transport. The PMCA2 and PMCA4 genes are believed to code for proteins with specific roles in the vectorial movement of Ca [14]. Antibody 5F10 recognizes all three isoforms of the Ca-pump (J.T. Penniston, personal communication). No Ca-pump mRNA or protein was found in human, rabbit, or rat salivary gland elements other than intralobular and interlobular ducts.

The present studies suggest that plasma membrane Ca-pumps may help maintain the low intracellular Ca concentration of the interlobular and intralobular duct cells of SMGs in the face of high Ca concentrations in the surrounding plasma, extracellular fluid, and primary salivary secretions. These studies also suggest, that plasma membrane Ca-pumps in the SMG striated duct cells of rabbits, rats, and man are in a position to play a role in the regulation of the final Ca concentration of saliva delivered to the oral environment.

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Discussion with Reviewers

A. Kittel: How do the authors explain those many bands on the Western blot with higher molecular weight than 135 kDa?

Authors: Ca-pump protein represents less than 1% of membrane protein. Because of the small amounts present in many tissues, it is sometimes necessary to overload the gel as in Figure 3. This creates non-specific binding to other proteins in the tissue to some extent. We believe the dominance of the 135 kDa band is still clear.

J.R. Martinez: The authors used monoclonal antibodies to localize Ca^{++} pumps in submandibular cells of three species and *in situ* hybridization to study the distribution of the mRNA's isoforms. The findings are of interest, but puzzling, as they show no mRNA or pump protein in the serous or mucous acini although abundant physiological evidence suggests that acinar cells must have a plasma membrane Ca^{++} pump which operates to maintain a low (i.e., nanomolar) cytoplasmic level (see, for example, Tepikin and Petersen, 1992). The authors should explain this discrepancy.

Authors: The lack of Ca-pump epitopes and mRNA in the serous or mucus acini, however, is not interpreted as a discrepancy by us. In our immunohistochemical procedures, we are looking at the relative amount of Ca-pump mRNA and protein present in different cell types. While we acknowledge the fact that most cell types probably contain a plasma membrane Ca-pump, the amount expressed in different cell types, (i.e., Ca^{++}-transporting epithelia versus cells that do not transport Ca^{++}), may be many-fold different, which is precisely the point of our findings. Our study shows that the amount of Ca-pump in the inter- and intralobular duct cells is many-fold greater than in the other cell types. While Ca-pump mRNA and protein may be present in the serous and mucus acini, the amount present is below the sensitivity of our detection methods.

J.R. Martinez: Salivary duct cells behave in general like relatively tight absorptive epithelia and the presence of Ca^{++} in these cells does not necessarily mean that Ca^{++} is a mediator in monovalent ion transport, but merely that duct cells have an energy-requiring Ca^{++}

extrusion mechanism (as many secretory and other cells do). Although the Ca^{++} concentration of primary (acinar) secretion has not been directly measured, the Ca^{++} concentration of final saliva is actually low (the free Ca^{++} is probably lower than plasma), there is little reason to support ductal Ca^{++} secretion as a mechanism controlling salivary Ca^{++}. If Ca^{++} in the primary (acinar) secretion is bound to proteins and glycoproteins, it could become concentrated in the ducts when flow rates are low, but primarily as a result of water reabsorption. Only a portion (70-80%) of salivary Ca^{++} is probably in the ionized form and it is not known if it changes appreciably in the duct system.

Authors: We do not support the concept of a ductal Ca^{++} secretory mechanism regulating salivary Ca^{++} concentration. As Figure 2 clearly shows by the basal orientation of the immunohistochemical reaction, we suggest that our data supports a Ca^{++} resorptive mechanism regulating salivary Ca^{++} concentration. The regulated, energy requiring Ca^{++} extrusion mechanism would move Ca^{++} out of the duct and back into the plasma, as is the case with the kidney distal tubule, and the intestinal epithelium in which this has been more thoroughly studied. The bulk of salivary Ca^{++} must come from acinar secretion, but our data suggests that the plasma membrane Ca-pump may not play a major role in this mechanism, only in the "fine tuning" of the ductal Ca^{++} concentration via the reabsorption by the duct cells.

H. Lesot: Was the goal of the paper to compare the differential expression of proteins belonging to the same family among species and, if this is the case, what led to the choice of these three species?

Authors: The specific goal of our paper is stated in the final paragraph of the **Introduction**. The choice of multiple species was done in order to suggest that our findings were part of a generalized mechanism found in multiple species. More importantly, however, certain limitations of our techniques would not allow us to limit our studies exclusively to human tissues. For example, the human findings are clearly the most relevant, but rapid and adequate fixation of tissues for this study was limited, and therefore, subcellular localization of Ca-pump to either the basal or luminal membrane was not determinable. Appropriately rapid fixation of the rabbit and rat tissue was possible, but subcellular localization of Ca-pump within the cells of the rabbit was clearly more well defined than in the rat so both were included, (see Figure 2 versus Figure 5). In addition, the sequence of the Ca-pump genes in the rabbit have not yet been published, and probes are only available for the rat and human isoforms. By looking at all three species, we believe we have made an adequate case for

Ca-pump proteins in the basal membrane and Ca-pump mRNA isoforms in the cytoplasm as a general feature of SMG duct cells, within the limitations of the techniques.

H. Lesot: Does the specificity of the 5F10 monoclonal antibody recognizes all five forms of the protein and, if not, which ones are recognized? An immunoblot or a reference to published [work] might be introduced in this paper to replace the "personal communication" mentioned in the **Discussion**.

Authors: The specificity of the 5F10 antibody has been well characterized in our previous study (Borke *et al.*, 1989b). At this time, the work demonstrating that 5F10 cross reacts with all the isoforms has not yet been published. That study was submitted by Dr. John Penniston's group from the Mayo Clinic in Rochester, Minnesota, but has not yet been accepted for publication. It is, therefore, necessary to cite this as a "personal communication".

Additional Reference

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