Immunolocalization of electroneutral Na\(^+\)-HCO\(_3^-\) cotransporters in human and rat salivary glands

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Received 31 October 2001; accepted in final form 22 February 2002

Gresz, V., T.-H. Kwon, H. Vorum, T. Zelles, I. Kurtz, M. C. Steward, C. Aalkjaer, and S. Nielsen. Immunolocalization of electroneutral Na\(^+\)-HCO\(_3^-\) cotransporters in human and rat salivary glands. Am J Physiol Gastrointest Liver Physiol 283: G473–G480, 2002. First published March 6, 2002; 10.1152/ajpgi.00421.2001.—Patterns of salivary HCO\(_3^-\) secretion vary widely among species and among individual glands. In particular, virtually nothing is known about the molecular identity of the HCO\(_3^-\) transporters involved in human salivary secretion. We have therefore examined the distribution of several known members of the Na\(^+\)-HCO\(_3^-\) cotransporter (NBC) family in the parotid and submandibular glands. By use of a combination of RT-PCR and immunoblotting analyses, the electroneutral cotransporters NBC3 and NBC1 mRNA and protein expression were detected in both human and rat tissues. Immunohistochemistry demonstrated that NBC3 was present at the apical membranes of acinar and duct cells in both human and rat parotid and submandibular glands. NBC1 was strongly expressed at the basolateral membrane of striated duct cells but not in the acinar cells in the human salivary glands, whereas little or no NBC1 labeling was observed in the rat salivary glands. The presence of NBC1 at the basolateral membrane of human striated duct cells suggests that it may contribute to ductal HCO\(_3^-\) secretion. In contrast, the expression of NBC3 at the apical membranes of acinar and duct cells in both human and rat salivary glands indicates a possible role of this isoform in HCO\(_3^-\) salvage under resting conditions.

BICARBONATE TRANSPORTERS PLAY VITAL ROLES in the epithelia of the kidney and gastrointestinal tract (stomach, intestine, pancreas, salivary glands, etc.). A primary function is in the regulation of intracellular pH, which is important for normal cell function and is determined by the balance between the activities of various acid extruders and acid loaders. Many of the same transporters also play important roles in the net secretion or absorption of H\(^+\) and HCO\(_3^-\) to maintain systemic acid-base balance.

Several members of the Na\(^+\)-HCO\(_3^-\) cotransporter (NBC) family have recently been identified and functionally characterized, including NBC1, NBCn1, and NBC3 (25, 30). Regulation of these NBCs may play a role in the maintenance of intracellular pH and in regulating acid-base balance. Electrogenic NBCs can function as either acid extruders or acid loaders according to their stoichiometry as well as the electrochemical gradients of the transported ions (25). In general, those with a 1:3 stoichiometry, as observed in kidney proximal tubule (10, 34), mediate HCO\(_3^-\) efflux (acid loading), whereas those showing a 1:2 stoichiometry, as observed in many other tissues (2, 9, 12), mediate HCO\(_3^-\) uptake (acid extrusion).

Since the molecular identity of the first electrogenic NBC (NBC1) was established in the kidney (26, 27), several other NBC1 variants and NBC1-like proteins have been identified (1, 3, 6, 11, 24). In addition, a number of homologous, but less closely related, electroneutral NBCs has recently been cloned. These include NBC3 from human skeletal muscle (23) and NBC1 from rat aortic smooth muscle (5) and are considered by some to be splice variants of the retinal NBC2 isoform (30). In rat kidney, we have demonstrated that NBC3 is present at the collecting duct intercalated cells (13) and NBC1 is mainly present at the medullary thick ascending limb cells (33), and these are generally expected to mediate HCO\(_3^-\) uptake into cells (acid extrusion).

Saliva secretion in salivary glands is believed to occur in two stages: the production of an isotonic, plasmalike primary fluid by the acinar cells, and the subsequent net reabsorption of electrolytes, but not water, in the ductal system to produce a hypotonic saliva (8). The concentration of HCO\(_3^-\) in the final saliva and its dependence on flow rate vary greatly among species and also among individual glands (32, 35). In the rat, in which the salivary HCO\(_3^-\) concentra-
tion is low and falls with increasing flow rate, HCO₃⁻ secretion may occur largely as a result of secretion in the ducts in exchange for reabsorbed Cl⁻. In the parotid glands of humans and other primates, on the other hand, the concentration of HCO₃⁻ is much higher and increases with flow rate. In this case, it is possible that the acini produce an HCO₃⁻-rich primary fluid and that HCO₃⁻ is reabsorbed in the ductal system (4, 8, 35).

The role of NBCs in these processes is largely unknown. There is functional evidence of NBC activity in the acinar cells of bovine parotid (16), sheep parotid (22, 31), and mouse submandibular gland (17). Furthermore, immunohistochemical studies have indicated that NBC1 is present at the basolateral membrane of the acinar cells in rat parotid (28), human parotid (19), and mouse submandibular gland (17). Basolaterally located NBCs such as these could, together with acid extruders such as Na⁺/H⁺ exchanger 1 (NHE1), contribute to the supply of intracellular HCO₃⁻ ions for secretion across the apical membrane. They may, alternatively, simply play a housekeeping role in the regulation of intracellular pH.

Recent functional and immunohistochemical data suggest that electroneutral NBC isoforms are expressed at the apical membranes of both acinar and ductal cells in the mouse submandibular gland (17). These apically located NBCs could, together with NHE2 and NHE3, contribute either to ductal HCO₃⁻ reabsorption during secretion or to HCO₃⁻ "salvage" processes in the resting state. In view of the general lack of information about HCO₃⁻ secretory mechanisms in human salivary glands, our main aim in the present work has been to identify and localize some of the NBCs expressed in human and, for comparison, rat parotid and submandibular glands by the use of molecular and immunohistochemical techniques. Our studies have focused specifically on the electroneutral NBC isoforms NBC3, which was originally cloned from human skeletal muscle (23), and its rat ortholog, NBCn1, which has three splice variants (NBCn1B, NBCn1C, NBCn1D) and was originally identified in rat aorta (5).

MATERIALS AND METHODS

Tissue samples. Human parotid and submandibular salivary tissue was taken from adult patients undergoing surgery for various primary maxillofacial interventions. Patients gave informed consent, and the procedures were approved by the Semmelweis University of Budapest Regional Committee of Science and Research Ethics. Human kidney material was obtained as described in previous studies (18). The tissues used did not contain atypical cells when assessed microscopically. Rat tissue was derived from male Wistar rats weighing 250–300 g (M & B, Ry, Denmark) that were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water.

RT-PCR. For RT-PCR, samples were frozen in liquid nitrogen immediately after surgery and stored at −70°C until use. Total RNA was extracted from kidney and salivary gland tissue by the single-step guanidium thiocyanate method. Total RNA (1 μg) was reverse transcribed at 42°C for 50 min using oligo(dT)₁₅ primers and Expand RT (Boehringer Mannheim). RT products were stored at −20°C until required. Rat β-actin and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified to assess the cDNA yield, and both RT-negative and PCR-negative control reactions were performed.

Primers were designed to amplify specifically the following transcripts: rat β-actin (189 bp), sense 5'-TCC TAG CAT CAT GAA GAT GCT C-3', antisense 5'-AAA CCC AGC TCA GTA ACA G-3'; rat NBCn1B (473 bp), sense 5'-TCC GAT GCC AGT TCT ATA TGG-3', antisense 5'-CAG GGC TAT ATT TTA GGG TC-3'; rat NBCn1C (389 bp), sense 5'-ATA GGA AAA GGC CTG TCA GCC TC-3', antisense 5'-GAG AAG CCA AAA TCC CTG G-3'; rat NBCn1D (2.9 kb), sense 5'-CTG ACC TTC ACT TGC TGG AA-3', antisense 5'-TCA TGG AAA GTG CCT TCC AC-3'; human GAPDH (605 bp), sense 5'-AGA AGG TGA AGG TCG GAG TC-3', antisense 5'-CAT TAG AGG CAG GGA TGA TG-3'; human NBC3 (983 bp), sense 5'-GCC ACT CTT TGC GAA CAG GTC-3', antisense 5'-CAA ACC ACC AAA AAG CCG TCC-3'.

Hot-start PCR was performed in 25-μl final reaction volume (0.8 μM of each primer, 1.5 mM MgCl₂, 0.4 mM dNTP, and 0.75 U Tag DNA polymerase (Boehringer Mannheim)) under the following reaction conditions: denaturation for 4 min at 96°C, addition of Taq at annealing temperature (50°C for β-actin and GAPDH, 58°C for hNBC3 and rNBCn1B, 60°C for rNBCn1C) and then 35 cycles (20 for GAPDH) of 30 s at 92°C (denaturation), 30 s at annealing temperature, and 30 s at 72°C (extension), followed by a final extension period of 10 min at 72°C. For rNBCn1D, the 35 cycles were 1 min at 94°C, 150 s at 60°C and 150 s at 72°C (17). The amplified RT-PCR products were visualized by ethidium bromide-stained agarose gel electrophoresis. All of the short PCR products were isolated and sequenced to verify their identity. Approximately 600–700 bases from each end of rNBCn1D were sequenced, and those fragments were found to be 100% identical to the corresponding sequences. Antibodies. Antibodies were as follows. First, electroneutral NBC3: a synthetic peptide corresponding to amino acids 1197–1214 of the COOH terminus of human NBC3 (GenBank accession no. AF070475) (33). Note that, because the COOH terminus of all NBCn1 (GenBank accession no. AF047033) (23) was used to generate a polyclonal antibody (13). This region of NBC3 is nearly identical in human, rat, and rabbit, and the antibody shows cross-reactivity among species (13). Second, electroneutral NBCn1: anti-NBCn1 was raised in rabbits against a synthetic peptide corresponding to amino acids 1204–1218 of the COOH terminus of NBCn1 (GenBank accession no. AF070475) (33). Note that, because the COOH terminus of all three rat NBCn1 splice variants are identical (5), the antibody does not distinguish among NBCn1B, NBCn1C, and NBCn1D.

Membrane fractionation and immunoblotting. Tissues were homogenized in an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany) for 15 s on ice in dissection buffer containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5.5 μM leupeptin (pH 7.2). The homogenate was centrifuged in an Eppendorf 5403 centrifuge (Eppendorf, Germany) at 4,000 g for 15 min at 4°C to remove whole cells and nuclei. The supernatant was then centrifuged at 200,000 g for 1 h to produce a pellet enriched for membranes. From the resultant pellets, gel samples were made containing 2% SDS Laemmli sample buffer. Prepared samples were loaded and run under reducing conditions on 6 or 10% homogenous Tris-glycine minigels (Novex, San Diego, CA). After transfer by electroelution to nitrocellulose membranes, blots were blocked for 1 h with 5% milk in PBS-Tris [80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween 20 (pH 7.5)]. They
were then incubated overnight at 4°C with anti-NBC3 (diluted 1:1,000) or anti-NBCn1 (diluted 1:100). The labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (P217, DAKO, Glostrup, Denmark) with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunolabeling controls were performed using antibodies preabsorbed with immunizing peptide.

**Immunohistochemistry.** Human salivary gland tissues destined for immunohistochemistry were cut into small pieces and immediately immersed in cold fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4) for at least 2–4 h before washing in buffer. Human kidney tissues were processed as described previously (18). Male Wistar rats were anesthetized with halothane inhalation, and salivary glands were fixed by transcardiac perfusion, or kidneys were fixed by retrograde aortic perfusion, with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer. The tissues were removed and postfixed in the same fixative solution for 1 h. The tissue blocks were rinsed and dehydrated in ethanol followed by xylene and embedded in paraffin. For immunoperoxidase and immunofluorescence microscopy, the paraffin-embedded tissues were cut at 2-μm thickness on a rotary microtome (Leica, Germany). The staining was carried out using indirect immunoperoxidase or indirect immunofluorescence. The tissues were dewaxed and rehydrated.

For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.5% H2O2 in absolute methanol for 30 min at room temperature. For antigen retrieval, sections were put in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA (3.6-dioxaoctamethylenedinitrilo-tetraacetic acid) and were heated using a microwave oven for 10 min. Nonspecific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH4Cl in 0.01 M PBS (pH 7.4) for 30 min, followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.1% BSA and 0.3% Triton X-100. The sections were then rinsed three times in PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 10 min. The labeling was visualized with horseradish peroxidase-conjugated goat antirabbit secondary antibodies (P448, DAKO; diluted 1:200 in PBS containing 0.1% BSA and 0.3% Triton X-100) followed by incubation with 3,3-diaminobenzidine for 10 min. The sections were washed three times for 10 min in PBS and counterstained for 2 min in Mayers hematoxylin, rinsed for 20 min under running tap water, dehydrated, and mounted with Eukitt mounting medium (O. Kindler). The microscopy was carried out using a Leica DMRE microscope.

For immunofluorescence microscopy, the labeling was visualized with incubation with FITC-conjugated goat antirabbit secondary antibodies (Alexa 488, Molecular Probes Europe, Leiden, The Netherlands). The sections were then rinsed in PBS three times for 10 min and mounted with glycerol supplied with antifading reagent (N-propyl galate).

**RESULTS**

**RT-PCR analyses.** As shown in Fig. 1, RNA samples from rat kidney, parotid, and submandibular glands were assessed by RT-PCR for transcripts of NBCn1B (473 bp), NBCn1C (389 bp), and NBCn1D (2.9 kb), using specific primers designed to the available nucleotide sequences. Samples of human kidney, parotid, and submandibular glands were also examined for NBC3 (983 bp; Fig. 1). Transcripts of the housekeeping genes for β-actin (189 bp) and GAPDH (605 bp) were used as references for the rat and human tissue samples, respectively. As shown in Fig. 1, RT-PCR yielded
products that ran as single bands of the expected size as detected by ethidium bromide-stained agarose gel electrophoresis. Sequencing of all the products showed 100% identity with the published nucleotide sequences.

The RT-PCR data indicate that, as in the mouse submandibular gland (17), mRNAs for all three NBCn1 splice variants (NBCn1B-D) were detected in the rat salivary glands. We also show a novel finding that NBC3 mRNA is expressed in the human parotid and submandibular glands. Rat and human kidney gave the expected results as positive controls.

Immunoblotting analyses. Immunoblotting was performed with antibodies to NBC3 and NBCn1, which cross-react between rat and human, by use of membrane fractions prepared from human and rat salivary glands as well as from human and rat kidney as positive controls (Fig. 2). Both isoforms were detected in the salivary glands of both species. The NBC3 antibody revealed an ~200-kDa band in both human and rat salivary glands as well as in kidney (Fig. 2A). The NBCn1 antibody, which does not distinguish between the B, C, and D splice variants, labeled an ~180-kDa band in both human salivary glands, and a weak signal was also obtained in the rat submandibular gland (Fig. 2B). There was only a very faint band, however, in the rat parotid gland. Immunolabeling controls using peptide-preabsorbed antibodies showed absence of labeling in each case (data not shown).

Immunohistochemical localization of NBCs in human and rat salivary glands. Immunohistochemistry was performed to examine the cellular and subcellular localization of the electroneutral isoforms NBC3 (Fig. 3) and NBCn1 (Fig. 4) in human and rat parotid and submandibular glands and in kidney as a positive control.

NBC3. As shown in Fig. 3, sections of rat (Fig. 3A) and human (Fig. 3B) kidney used as a positive control revealed NBC3 labeling in collecting duct intercalated cells, consistent with previous studies (13). In the rat parotid gland (Fig. 3C), there was strong apical labeling of acinar cells (arrow) and intralobular ducts (arrowhead), in particular, striated ducts. In the rat submandibular gland (Fig. 3D), there was also apical labeling in the intralobular ducts (arrowheads), whereas staining in the acini was less prominent.

In the human parotid gland (Fig. 3E) and submandibular gland (Fig. 3F), very strong apical labeling of the NBC3 was observed in the striated ducts. This is also seen at higher magnification in Fig. 3G. Apical labeling was also evident in the intercalated ducts (arrowhead, ID in Fig. 3G) and main excretory ducts of both glands (arrowheads in Fig. 3, I and J). The purely serous acinar cell population of the human parotid gland was also labeled at the apical membrane (arrow, SA in Fig. 3G), whereas labeling in the mixed submandibular gland was confined to the apical membrane of the serous acini and serous demilunes (Fig. 3, F and H) and absent from the mucous acini of the human submandibular gland (MA in Fig. 3, F and H).

NBCn1. As shown in Fig. 4, NBCn1 labeling in rat (Fig. 4A) and human (Fig. 4, B and C) kidney was associated with the basolateral membranes of the medullary thick ascending limbs and intercalated cells of the collecting ducts. This is consistent with previous observations in the rat kidney (33). In both human parotid (Fig. 4, F and G) and submandibular (Fig. 4, H and I) glands, very strong NBCn1 labeling was seen in the striated ducts. This was confined to the extensively folded basolateral plasma membrane domains (arrows in Fig. 4, F–I) whereas there was no labeling observed in the acini (Fig. 4, F–I). The immunolabeling controls were negative (insets in Fig. 4, F and H).

In the rat salivary glands, faint marginal labeling of NBCn1 was detected in the granular ducts of the submandibular glands (Fig. 4E) but not in the acini. No labeling was seen in either the acini or the ducts of the rat parotid glands (Fig. 4D), although there was some weak staining of blood vessels (not shown).

DISCUSSION

Fluid secretion by salivary acinar cells is driven by secondary active transport of anions, principally Cl− ions in rodents. The cellular and molecular mechanisms responsible for acinar Cl− secretion and its subsequent reabsorption in the ductal system are relatively well established (8, 32). However, most salivary glands also secrete significant amounts of HCO3−. The concentration varies widely, reaching 100 mM or more in the parotid secretions of ruminants and some primates (35), but relatively little is known about the transport mechanisms involved in HCO3− secretion, in
either the acinar cells or the ducts. In many cases, it is not even known whether the secreted HCO$_3^-$ is of acinar or ductal origin.

Until recently, it has been widely assumed that intracellular HCO$_3^-$ is generated in salivary acinar and ductal cells mainly from CO$_2$. This would result from carbonic anhydrase activity working in conjunction with H$^+$ extrusion, principally by Na$^+$/H$^+$ exchange at the basolateral membrane. HCO$_3^-$ efflux across the apical membrane is then believed to occur via anion conductances in the acinar cells or via anion exchangers in the ductal cells. The discovery of the NBC family of Na$^+$-coupled HCO$_3^-$ transporters with a variety of stoichiometries has raised the possibility that NBCs may also contribute to these processes.

Given the marked differences in salivary HCO$_3^-$ secretion among species and among glands within species, patterns of expression of the different NBCs may

Fig. 3. Immunohistochemical analysis of NBC3 distribution in rat and human salivary glands and kidney. Immunoperoxidase and immunofluorescence labeling of paraffin sections. A and B: positive controls showing specific staining of collecting duct intercalated cells in rat (A) and human (B) kidney. B: apical and subapical labeling of type A intercalated cells (arrows) and basal labeling of type B intercalated cells (arrowhead) in the cortical collecting duct. C–H: apical labeling of serous acini (SA, arrows), demilunes (D), and ducts (arrowheads; ID, intercalated duct; GD, granular duct; SD, striated duct) in rat and human parotid and submandibular glands. Only the mucous acini (MA) of the human submandibular gland are not labeled. I and J: apical labeling of excretory duct cells (arrowheads). Immunolabeling controls were negative (insets in C–F). Magnifications: ×250 (E, F, J); ×650 (A–D, G, H); ×1,000 (I).
well differ both among species and among glands. Functional evidence for NBC activity was detected some time ago in the acinar cells of ruminant parotid glands (16, 22, 31), but the particular isoform has not been established. More recently, the electrogenic NBC1 isoform has been identified by molecular techniques in mouse (17), rat (28), and human (19) salivary glands. We have now used RT-PCR and immunolabeling techniques to examine the molecular identity and cellular localization of some of the electroneutral NBC isoforms in the parotid and submandibular glands of the human and rat. Table 1 summarizes our main findings: all of the known electroneutral NBC variants appear to be expressed to a greater or lesser extent in both human and rat salivary glands. There are striking differences, however, in their localization within the glands, and there are interesting differences among the species.

Although it has been suggested that the acinar cells of the human parotid might be the main source of
salivary HCO$_3^-$ (35), the presence of NBCn1 at the basolateral plasma membrane of the striated ducts raises the possibility that a significant amount of HCO$_3^-$ may enter the saliva there. Although this is generally assumed to be the major site of Cl$^-$ reabsorption, it is possible that some of the Cl$^-$ reabsorbed at the luminal membrane of the striated duct cells is exchanged for HCO$_3^-$, which has been brought into the cells across the basolateral membrane as a result of NBC activity. It is worth noting that the 1:1 stoichiometry of NBCn1 would ensure that the driving force for HCO$_3^-$ transport would remain inwardly directed, supporting HCO$_3^-$ secretion, regardless of any changes in basolateral membrane potential that might arise as a result of Cl$^-$ reabsorption. On the other hand, the basolateral Na$^+$/H$^+$ exchanger working in conjunction with carbonic anhydrase would also generate intracellular HCO$_3^-$. Thus an evaluation of the relative contributions of different basolateral transporters to HCO$_3^-$ secretion in the ducts must await further functional studies.

From a comparative viewpoint, it is interesting to note that NBCn1 was only weakly expressed in the striated ducts of the rat parotid gland and, apart from some expression in blood vessels, not at all in the secretory tissue of the rat parotid gland. These glands, particularly the rat parotid, secrete significantly less HCO$_3^-$ than the human glands (35), so it is tempting to suggest that there is a correlation between NBCn1 expression in the striated ducts and salivary HCO$_3^-$ concentrations.

In contrast to the NBCn1 isoform, the other electroneutral cotransporter, NBC3, appears to be uniformly expressed at the apical membranes of both the acinar and ductal cells in all four glands examined in this study. This finding is probably consistent with recent observations in the mouse submandibular gland (17). In that study, however, the apical labeling obtained with the NBC3 antibody was attributed to the NBCn1 splice variants, which share many sequence similarities with NBC3 at the COOH terminus. Our results, however, suggest that NBC3 and NBCn1 are immunologically distinct, and we would therefore predict that the luminal membrane isoform in the mouse is actually NBC3. Although NBCn1B fragments were successfully amplified from mouse duct cell preparations by RT-PCR (17), this isoform may actually be expressed at the basolateral membrane, as appears to be the case for NBCn1 in the human glands.

On the basis of data from mouse, rat, and human, the presence of NBC3 at the apical membrane of acinar and ductal cells appears to be a general feature of salivary glands. The presence of this and other Na$^+$-dependent HCO$_3^-$-absorbing mechanisms in the apical membranes of HCO$_3^-$-secreting tissues such as the pancreas (14, 36) and the salivary glands (15, 17, 20, 21) is perhaps surprising. However, these mechanisms may play an important role in reabsorbing HCO$_3^-$ from the luminal fluid when fluid secretion ceases. The need for so-called “HCO$_3^-$ salvage” is particularly understandable in the pancreas, where a lowering of luminal pH will help to reduce the proteolytic activity of pancreatic enzymes and thus reduce the risk of autodigestion. It is less clear why such mechanisms would be advantageous in the salivary glands, although they would also help to reduce the risk of calcareous stone formation.

Clearly, electroneutral NBC3 and NBCn1 have different cellular distribution patterns in the human and rat salivary glands. We have shown previously that the NBC3 and NBCn1 antibodies also show markedly different labeling patterns in the kidney (13, 33). Thus, although the amino acid sequence of human NBC3 is 89–92% identical to that of the rat NBCn1 clones and they may well be splicing variants of the corresponding gene in the two species (5, 30), it seems likely that their physiological roles are quite different.

The presence of different isoforms, or splice variants, of the same transporter in the apical and basolateral domains of epithelial cells has been extensively observed in the kidney (7, 29). In some cases, one may be involved in transepithelial electrolyte transport, whereas the other has a housekeeping role in maintaining normal cytosolic ion concentrations. In the salivary glands, we would suggest that NBC3 and NBCn1 are both involved in transepithelial transport but under quite different physiological conditions. The basolateral NBCn1 may participate in ductal HCO$_3^-$ secretion and contribute to the relatively high HCO$_3^-$ concentrations observed in human saliva, whereas the apically localized NBC3 may have a role in HCO$_3^-$ salvage during periods of low salivary flow.

We thank Dr. I. Bögi for access to human tissue, and I. M. Paulsen and M. Pedersen for expert technical assistance.

The Water and Salt Research Center at the University of Aarhus was established by support from Danmarks Grundforskningsfond. Support for this study was provided by the Karen Elise Jensen Foundation, the Novo Nordic Foundation, the Danish Medical Research Council, the University of Aarhus Research Foundation, the University of Aarhus, the Wellcome Trust, and the Commission of the European Union (K.A. 3.1.2 and K.A. 3.1.3. Programmes).

Table 1. Summary of immunohistochemical data

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NBC3, Na$^+$ – HCO$_3^-$ cotransporter cloned from human skeletal muscle; NBCn1, a splice variant of the rat ortholog of NBC3; AP, apical; BL, basolateral; ser, serous acini only.
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