Expression of sodium bicarbonate cotransporter and its role in pH\textsubscript{i} regulation in guinea pig salivary glands

Jingchao Li\textsuperscript{1}, Na-Youn Koo\textsuperscript{1}, Ik-Hyun Cho\textsuperscript{1}, Tae-Hwan Kwon\textsuperscript{2}
Se-Young Choi\textsuperscript{1}, Sung J. Lee\textsuperscript{1}, Seog B. Oh\textsuperscript{1}, Joong-Soo Kim\textsuperscript{1}, and Kyungpyo Park\textsuperscript{1}

\textsuperscript{1}Department of Physiology, College of Dentistry, Seoul National University and Dental Research Institute, Seoul 110-749 and
\textsuperscript{2}Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu 700-422, Korea

Running head: Functional expression of NBC in guinea pig salivary glands

Correspondence to: Kyungpyo Park
Department of Physiology,
College of Dentistry,
Seoul National University,
Yeongeondong 28, Chongno Ku,
Seoul 110-749, South Korea

Tel: 822-740-8658; Fax: 822-762-5107; Email: kppark@snu.ac.kr
ABSTRACT

Patterns of salivary $\text{HCO}_3^-$ secretion vary and depend on species and gland type. However, the identities of the transporters involved in $\text{HCO}_3^-$ transport and the underlying mechanism of intracellular pH ($\text{pH}_i$) regulation in the salivary glands still remain unclear. In this experiment, we examined the expression of the $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC) and its role in $\text{pH}_i$ regulation in guinea pig salivary glands, which can serve as an experimental model to study $\text{HCO}_3^-$ transport in human salivary glands. RT-PCR, immunohistochemistry, and $\text{pH}_i$ measurements from the 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) loaded cells were performed. Amiloride-sensitive $\text{Na}^+/\text{H}^+$ exchange (NHE) played a putative role in $\text{pH}_i$ regulation in salivary acinar cells, and also appears to be involved in regulation in salivary ducts. In addition to NHE, NBC also played a role in $\text{pH}_i$ regulation in both acini and ducts. In the parotid gland, NBC1 was functionally expressed in the basolateral membrane (BLM) of acinar cells and the luminal membrane (LM) of ducts. In the submandibular gland, NBC1 was expressed only in the BLM of ducts. The NBC1 expressed in these two types of salivary glands take up $\text{HCO}_3^-$ and are involved in $\text{pH}_i$ regulation. Although NBC3 immunoreactivity was also detected in submandibular gland acinar cells and in the ducts of both glands, it is unlikely that NBC3 plays any role in $\text{pH}_i$ regulation. We conclude that NBC1 is functionally expressed and plays a role in $\text{pH}_i$ regulation in guinea pig salivary glands, but that its localization and role are different depending on the type of salivary glands.

Keywords: intracellular pH ($\text{pH}_i$), $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC), $\text{Na}^+/\text{H}^+$ exchange (NHE), guinea pig, salivary glands.
INTRODUCTION

Regulation of the intracellular pH (pH_i) in epithelial cells is critical for fluid and electrolyte absorption and secretion. Therefore, epithelial cells are equipped with diverse pH_i regulatory mechanisms. However, there are some differences in the pH_i regulatory mechanisms among species and gland types. In the salivary acinar cells, the initial response to a fluid secretion stimulus is an acidification of the cytosol resulting from an HCO_3^- efflux into the lumen (10, 13, 19, 37), and the secreted HCO_3^- plays a role in buffering the acid produced by oral bacteria and in providing an optimal pH for salivary amylase activity (6).

It is known that intracellular HCO_3^- is generated by carbonic anhydrase, which converts the CO_2 that has freely diffused into the cells from the blood into H^+ and HCO_3^- (21). The H^+ generated during this HCO_3^- production is then extruded outside the cells by an Na^+/H^+ exchange (NHE) (19, 22, 35). However, relatively little is known about the transport mechanisms involved in HCO_3^- uptake or reabsorption in either the acinar cells or the ducts. The rapid changes in HCO_3^- concentration over a wide range in human parotid glands (2) suggests that additional or alternate pathways for HCO_3^- transport may exist in the acini and/or ducts.

After the cloning of cDNA encoding an electrogenic Na^+/HCO_3^- cotransporter (NBC) in the kidney (29), several other subtypes from the NBC family, including pNBC1 (1), NBCn1 (4), and NBC3 (27), have been functionally characterized. The recent immunohistochemical studies on the electrogenic Na^+/HCO_3^- cotransporter NBC1 and the electroneutral NBC3 in salivary glands (5, 23, 31) suggest that these NBCs may
also contribute to the supply of intracellular $\text{HCO}_3^\text{−}$. Guinea pigs have been used as an experimental model for studying $\text{HCO}_3^\text{−}$ transport in the human pancreas (8). We also found that the $\text{HCO}_3^\text{−}$ concentrations of the resting and stimulated whole saliva of guinea pig are very similar to those of humans (2), which suggests that guinea pig salivary glands can also serve as an experimental model for studying $\text{HCO}_3^\text{−}$ transport in human salivary secretion. Therefore we examined the expression patterns and functions of NBC1 and NBC3 in guinea pig parotid and submandibular glands (SMG).

We have demonstrated that $\text{pH}_i$ was differently regulated depending on the type of salivary glands, even in the same species, namely the guinea pig. NHE was the exclusive $\text{pH}_i$ regulator in the SMG acinar cells. However, in addition to NHE, an EIPA-insensitive, $\text{Na}^\text{+}$- and $\text{HCO}_3^\text{−}$-dependent NBC1 substantially contributed to $\text{pH}_i$ regulation in the parotid acinar cells, and in the parotid and SMG duct cells in guinea pigs.
MATERIALS AND METHODS

Materials and solutions. Collagenase (CLSPA type) was purchased from Worthington Biomedical (Lakewood, NJ, USA), and the 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was obtained from Molecular Probes (Eugene, OR, USA). All other reagents, including the 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS), were obtained from Sigma (St. Louis, MO, USA). The physiological salt solution (PSS) consisted of 135 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgSO₄, 0.33 mM NaH₂PO₄, 0.4 mM KH₂PO₄, 10 mM glucose, and 20 mM HEPES (pH 7.4 with NaOH). The HCO₃⁻-free solution (HEPES buffered solution, HBS) contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES (pH 7.4 with NaOH), and 10 mM glucose. In the HCO₃⁻-containing solution (BBS), the 25 mM NaCl was replaced with an equal concentration of NaHCO₃. The BBS was gassed with 5% CO₂/95% O₂. The Na⁺-free solutions were prepared by replacing the Na⁺ with N-methyl-D-glucamine (NMDG). The NH₄⁺-containing solutions were prepared by replacing the 20 mM Na⁺ with 20 mM NH₄⁺. The Cl⁻-free solutions were prepared by replacing the Cl⁻ with an equimolar amount of gluconic acid. Additional Ca²⁺ was added to compensate for chelation by gluconate. NBC1 and NBC3 antibodies were purchased from Chemicon (Temacula, CA, USA).

Saliva collection and determination of HCO₃⁻ concentrations. All animals were used according to the protocol of the Animal Care and Use Committee of the Seoul National University. Male guinea pigs weighing 400-500 g were anesthetized intraperitoneally using sodium pentobarbital (50 mg/kg). The resting whole saliva of
the guinea pigs was collected over two five-minute intervals by aspiration. The collection of stimulated saliva was done by subcutaneous injection of pilocarpine (80 mg/kg). Resting and stimulated human whole saliva was obtained from five healthy volunteers. In the human subjects, stimulated saliva was collected using 3% citric acid to stimulation the tongue. At the end of the saliva collection, the concentration of HCO$_3^-$ was measured using an ion analyzer (Stat Profile pHox Plus, Nova Biomedical, MA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) of NBCs. Total RNAs from guinea-pig parotid glands were extracted by procedure of Qiagen RNeasy® Mini or Micro Kit (Qiagen Inc. Valencia, CA, USA), and subjected to RT-PCR using an oligo-dT reverse transcriptase primer and M-MLV reverse transcriptase (Invitrogen, CA, USA). Degenerate oligonucleotide primers derived from conserved NBC sequences of rat (GenBank accession no. AF124441), mouse (GenBank accession no. AF141934) and human (GenBank accession no. AF011390) were used to amplify cDNA clones of two different NBC isoforms (kNBC1 and pNBC1). Guinea-pig β-actin (GenBank accession no. AF508792) was amplified to assess the cDNA yield. Primers were designed to amplify specifically the following transcripts: guinea-pig β-actin (435 bp); TGCGTGACATCAAGGAGAAG (sense), GCTGGAAGGTGGAGAGTGAG (antisense). The degenerate primers for NBC1s were 5’ GAG AAA GGA TCC ATC ATG C, 5’AGC ATG ACA GCC TGC TGT A, 5’ TGT GCC CAC AAG GTT CTT GTT C, and 5’ TTC TCT CCA CCT GAG TAC ATA TT. The degenerative primers for NBC3 (GenBank accession no. AF047033) were 5’ AGG AAC ACA AAT TGA AGA AAG GAG and 5’ ACT CCC ATA TAA AGG AAA ACA CCA. Cycling parameters were as follows: 38 cycles of 94℃ for 55 seconds, 57℃ for 55 seconds; and 72℃ for 2 minutes. An expected PCR product of
approximately 437 bp, 182 bp, and 316 bp were generated and DNA sequencing was analyzed using dye terminator methods with BaseStation™ (MJ research Inc. MA, USA). These PCR fragment showed a 95% homology to the human NBC1 (GenBank accession no. AF011390) and NBC3 (GenBank accession no. AF047033). Subsequently, we designed specific primers for guinea-pig NBC1, 5’ GTG CAA GTA GGA TGT TCA A and 5’ ATC TCA TGG TAC GAC TTG TCT T. The expected PCR products are approximately 320 bp.

Acinar cell and intralobular duct preparation. The guinea pig parotid gland and SMG were dispersed into individual ducts (intralobular) and acini by a modification of the methods described previously (10). Briefly, the guinea pigs were anaesthetized by inhalation of diethyl-ether and killed by cervical dislocation. The parotid gland or SMG were quickly removed and finely minced in PSS supplemented with 0.1% sodium pyruvate, 0.02% trypsin inhibitor, and 0.1% bovine serum albumin (BSA). The tissues were then digested in the same solution containing collagenase (100 units/ml) at 37°C for 60 min with continuous agitation. During incubation, the cells were periodically dispersed by trituration at 20, 40, and 60 min.

Isolation of interlobular ducts and microperfusion via ductal lumen. Microdissection of ducts was performed under the stereomicroscope by a previously described method, originally developed for isolating interlobular ducts from guinea pig pancreas (8). After the parotid gland and SMG were removed, they were injected with digestion buffer and coarsely chopped. Digestion buffer consisted of Dulbecco’s modified Eagle’s medium (DMEM) containing 80 unit/ml collagenase, 400 unit/ml hyaluronidase, and 2 mg/ml BSA. The chopped tissues were put into the digestion
buffer, gassed with 5% CO₂-95% O₂, shaken in a 37 °C water bath for 35 min, and then changed with fresh digestion buffer for another 30 min. After digestion, the tissues were washed three times with DMEM and resuspended in a storage buffer consisting of DMEM with 3% (w/v) BSA. Then, the interlobular ducts were microdissected under the stereomicroscope. Dissected interlobular ducts were placed in storage buffer on ice until the pHᵢ was measured. We constructed cannula using a yellow tip to make its diameter small enough to cannulate the interlobular excretory duct under the stereomicroscope. Microdissected interlobular duct was first cannulated by a modified yellow tip, and then its tip was connected again with the PE cannula (I.D 0.28 mm, O.D 0.61 mm) for the microperfusion study. A DAD-12 (ALA Scientific Instruments, Inc., Westbury, NY, USA) system with a controlled pressure of N₂ gas (30-80 psi) was used for microperfusion at a flow rate of 4 μl/10 min.

**pHᵢ measurements.** The isolated acini or intralobular ducts were incubated with 2 μM BCECF/AM for 30 min at room temperature and washed once with PSS containing 0.1% BSA. They were kept on ice until use. For the microperfusion study, 5 μM BCECF/AM was loaded via the ductal lumen. The fluorescence of the isolated acini or ducts was measured by photon counting using a Photon Technology International system (South Brunswick, NJ, USA). The BCECF fluorescence was recorded at excitation wavelengths of 440 and 490 nm, with an emission wavelength of 530 nm. The 490/440 fluorescence ratios were calibrated using the high potassium nigericin procedure described elsewhere (34). The values reported are the mean ± SEM of the number of acinar aggregates or ducts examined. Acidification of the cells was induced by exposure to 20 mM NH₄Cl for 1 min. We then observed the pHᵢ recovery for the
two different types of salivary glands. During the pH measurements, bath solution was superfused at 3 ml/min flow rate.

**Immunohistochemistry of NBCs.** Male guinea pigs were anaesthetized via inhalation of diethyl-ether. The parotid gland and SMG were removed and postfixed in cold fixative (3% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4) for 3 h. The tissues were rinsed and dehydrated in ethanol followed by xylene and embedded in paraffin and cut at 2 μm thickness. For immunohistochemistry, sections were dewaxed, rehydrated and incubated for 30 minutes with 3% H₂O₂ in 100% methanol to remove endogenous peroxidase activity. For antigen retrieval, sections were put in TEG buffer (1mM Tris solution supplemented with 0.5 mM EGTA, pH 9.0) and heated using microwave for 10 min. Nonspecific binding of immunoglobulin was prevented by incubating sections in 50 mM NH₄Cl in 0.01 M PBS (pH 7.4) for 30 min, followed by blocking with 0.01M PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin for 1 h at room temperature, then sections were incubated overnight at 4 °C with primary antibodies diluted in 0.01 M PBS containing 0.1% BSA and 0.3% Triton X-100. After washing three times in washing buffer (0.01 M PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin), the sections were incubated with a biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA) at a dilution of 1:200, for 1 h at room temperature followed by incubation with avidin and biotinylated horseradish peroxidase complex (Vector Labs, Burlingame, CA, USA) at 1:100 for 1 h at room temperature. Then, sections were visualized with 3,3’-diaminobenzidine (Sigma, St. Louis, MO, USA). Rat kidney was used as a positive control. After counterstaining with hematoxylin, sections were dehydrated and coverslipped. The slides were examined and photographed with a light microscope. The
polyclonal antibodies to the subunits of NBC1/pNBC and NBC3 were obtained from Chemicon (Temecula, CA, USA). The immunogens of the guinea pig anti-$\text{Na}^+\text{-HCO}_3^-$ cotransporter1 (NBC1/pNBC) and rabbit anti-$\text{Na}^+\text{-HCO}_3^-$ cotransporter3 (NBC3) were amino acids 928-1035 of the C-terminus of human NBC1 and a 19 amino acid peptide sequence with the cytoplasmic C-terminus of human NBC3, respectively.
RESULTS

$HCO_3^-$ concentrations in resting or stimulated whole saliva of guinea pigs and humans. The saliva collected from guinea pigs and humans was analyzed for $HCO_3^-$ concentration. We used whole saliva which is a mixture of salivas from 3 sets of major glands and many minor glands. The resting and stimulated whole saliva of the guinea pigs contained 4.0 ± 1.0 mM (n=5) and 16.2 ± 0.8 mM (n=5) $HCO_3^-$, respectively, while in humans, the resting and stimulated saliva contained 2.4 ± 0.3 mM (n=5) and 16.4 ± 3.1 mM (n=5) $HCO_3^-$, respectively. The results indicate that the $HCO_3^-$ concentrations of guinea pig saliva, in either a resting or stimulated state, are very similar to those of humans.

RT-PCR analysis for NBC1 and NBC3 expression in guinea pig salivary glands. RT-PCR was performed to determine if the electrogenic NBC1 and the electroneutral NBC3 mRNA are expressed in the parotid and submandibular glands (SMG) of guinea pigs. NBC1 transcripts with a predicted molecular size of 437 bp (N-terminal) and of 182 bp (middle portion) were observed from the whole parotid tissue (Fig. 1A). We confirmed that our RT-PCR product was NBC1 by comparing its partial sequence with the sequence of a human NBC1 (1) and guinea pig NBC1 gene (11), which is our accompanying paper. It was found that the NBC1 in our experiments is the pancreatic type (pNBC1), which shows a 95% sequence homology with the human pNBC1 (GenBank accession no. AF011390). Subsequently, using the specific pNBC1 primer for guinea pig (expected product size is 320 bp), we then characterized NBC1 mRNA expression in acini and ducts from two different glands, the parotid gland and the SMG (Fig. 1B). The upper panel of Fig. 1B shows that NBC1 transcripts are
expressed in both the acini and ducts of the parotid glands, but only in the ducts of SMG. The middle panel of Fig. 1B shows NBC3 transcripts with a predicted molecular size of 316 bp. In parotid glands, the mRNA of NBC3 was detected only in ducts, but NBC3 mRNA was expressed both in acinar and ducts in SMG.

**Immunohistochemical localization of NBC1 and NBC3.** Immunohistochemistry was performed to examine the cellular and subcellular localization of the NBCs in guinea pig salivary glands. Rat kidney (32) was used as a positive control, which revealed NBC1 expression (indicated by arrows) at the basolateral membrane (BLM) of the proximal tubules (Fig. 2A). Fig. 2B, C and D shows staining of NBC1 in guinea pig parotid glands. Strong labelings are observed in the BLM (Fig. 2B) of acinar cells. In the intralobular (Fig. 2C) and interlobular ducts (Fig. 2D), strong stainings of NBC1 were shown in the luminal membrane (LM). In SMG, NBC1 staining was evident in the BLM of the intralobular (Fig. 2E) and interlobular (Fig. 2F) ducts, but not in the acini. Fig. 3 shows NBC3 expression (indicated by arrows). Staining in intercalated cells of the cortical collecting duct in rat kidney were used as positive control (12) (Fig. 3A). In the SMG, NBC3 staining was strong in the LM of acinar cells (Fig. 3 B-D), LM of the intralobular (Fig. 3B and C) and interlobular ducts (Fig. 3D). In parotid glands, NBC3 staining was observed in the BLM of intra (Fig. 3E) and interlobular (Fig. 3F) ducts, but not in acini.

**Intracellular pH (pH\textsubscript{i}) recovery in parotid and SMG acinar cells.** Throughout the experiments, we used an NH\textsubscript{4}Cl prepulse technique to acidify the cells. When cells are exposed to NH\textsubscript{4}Cl for 1 min (indicated by dark bars), they are transiently alkalinized, then become acidified later. Using this technique, we measured the pH\textsubscript{i} recovery rate from the cell acidosis and investigated the involved transporters using
specific blockers. Fig. 4 shows the pH_i recovery patterns of acinar cells from two different types of guinea pig salivary glands, the SMG (A and B), and the parotid glands (C and D). Fig. 4A and 4C show the pH_i recovery in HBS while Fig. 4B and 4D show the pH_i recovery in BBS. The pH_i of the acinar cells in both salivary glands did not recover after the NH_4Cl pulse in the Na^+-free solution (indicated by white bar), suggesting that the pH_i recovery is Na^+-dependent. We first examined the effect of an amiloride derivative, EIPA, which is a NHE inhibitor, on pH_i recovery. As shown in Fig. 4A and 4C, 1 μM EIPA completely inhibited the pH_i recoveries in both types of acinar cells in HBS. The experiment was repeated in a different bath solution, BBS (B and D). Although the pH_i recovery was also completely inhibited by 1 μM EIPA in the SMG acinar cells (Fig. 4B), 1 μM EIPA only partly inhibited the pH_i recovery in the parotid acinar cells (Fig. 4D).

**Functional expression of NBC1 in parotid acinar cells.** Fig. 5A summarizes the results of the experiments in Fig. 4. The pH_i recovery rate of the SMG acinar cells was not much different between the two bath solutions, HBS and BBS. Furthermore, a complete inhibition of pH_i recovery in SMG acinar cells in the presence of 1 μM EIPA in both HBS and BBS suggests that NHE1 is the exclusive pH_i regulator in this tissue. In parotid gland acinar cells, the rate of pH_i recovery in BBS (0.687±0.091 pH unit /min, n=5) was much faster (P<0.01) than in HBS (0.288±0.028 pH unit /min, n=12). In the presence of HCO_3^−, EIPA did not completely inhibit the pH_i recovery (0.151±0.034 pH unit /min, n=11), suggesting that in parotid gland acinar cells, an EIPA-insensitive, HCO_3^−- and Na^+-dependent mechanism, in addition to NHE, is involved in pH_i recovery. Thus, we further examined whether the residual component of pH_i recovery is inhibited by DIDS, a specific NBC1 blocker. Fig. 5B shows a
representative figure of four experiments in BBS. The EIPA-insensitive pHi recovery component in the parotid acinar cells was completely inhibited by 1 mM DIDS.

**pHi recovery and NBC1 expression in the intralobular ducts.** We next examined pHi regulation in the intralobular striated ducts from two different types of salivary glands, parotid and SMG. Fig. 6A and 6B show superimposed raw traces for pHi recoveries from NH₄Cl-induced acidosis at various EIPA concentrations in HBS. The degree of inhibition by EIPA was in proportion to its concentrations in both ducts. The 1 µM EIPA concentration that had completely inhibited pHi recovery in acinar cells (see Fig. 4A and 4C) partly inhibited the pHi recovery of the parotid and SMG ducts by 59±9% (n=4) and 73±11% (n=3), respectively, compared to their full recovery rates. Only the higher concentrations of EIPA, 100 and 10 µM, could almost completely inhibit pHi recovery by 97±8% (n=3) and 93±7% (n=4), respectively, in parotid and SMG ducts. The IC₅₀ was estimated as 0.33±0.12 µM (n=4) and 0.18±0.17 µM (n=3) in the parotid (dotted lines) and SMG (solid) ducts, respectively (Fig. 6C). By contrast, in BBS (Fig. 6D and 6E), the higher concentrations (100 and 10 µM) of EIPA inhibited pHi recovery by only 42±6% (n=4) and 49±9% (n=3) in the parotid and SMG ducts respectively, compared with the normal recovery. These residual pHi recoveries were further inhibited by 93±4% (n=3) and 91±8% (n=3) in the parotid and SMG ducts by 1 mM DIDS, suggesting that, in addition to NHEs, NBC1 is functionally expressed in the two intralobular duct types. Based on the immunohistochemistry data, intralobular NBC1 appears to be expressed in the LM of the parotid ducts and the BLM of the SMG ducts. However, the immunohistochemical data alone may not be sufficient to verify a functional expression of NBC1. Therefore, we went on further to perform a microperfusion study in the interlobular excretory
ducts, which enable separated perfusions, one via bath, and the other via the ductal lumen.

Subcellular localization and NBC1 expression in the interlobular ducts. Fig. 7 shows a series of microperfusion studies in the interlobular ducts of the parotid glands and SMG. When we studied the pH_i recovery in the LM of the ducts, the bath was superfused with Na^+ -free solution to inhibit the pH_i recovery that may occur in the BLM of ducts. The addition of Na^+ (140 mM) into the ductal lumen caused pH_i recovery from the NH_4^+Cl-induced acidification, and 1 μM EIPA completely inhibited the pH_i recovery in HBS in parotid interlobular ducts (Fig. 7A). However, the same concentration of EIPA did not block the pH_i recovery in BBS, and the residual pH_i recovery component was completely blocked by addition of 1 mM DIDS (Fig. 7B). This result suggests that NBC1 is functionally expressed in the LM of the interlobular duct. We also wanted to know whether Cl^-/HCO_3^- exchange is expressed in the LM. Fig. 7C shows one of three typical experiments. When we omitted Cl^- (indicated by the dark bar) from the ductal lumen, pH_i was dramatically increased, and the increased pH_i was restored to the prestimulus level by the readdition of Cl^-, suggesting that Cl^-/HCO_3^- exchange is also functionally expressed in the LM. We then added 1 mM DIDS into the ductal lumen to examine whether NBC1 functions in a resting state. The application of DIDS for 5 min slowly decreased the pH_i from 7.45 to 7.2, suggesting that the HCO_3^- in the ductal lumen is reabsorbed in the resting state. However, the pH_i recovery pattern in the LM of the SMG interlobular ducts was different. Fig. 7D shows superimposed traces of pH_i recovery under different EIPA concentrations in HBS. We found that 5 μM EIPA only partly (71±4%, n=3) inhibited pH_i recovery and that 50 μM EIPA inhibited pH_i recovery by 94±6% (n=3)
compared with normal recovery. This result indicates that the LM of the SMG interlobular ducts express EIPA-resistant NHE isoforms, probably NHE3. Furthermore, Fig. 7E shows the inhibitory effect of highest concentration of EIPA, 50μM, on the pH\textsubscript{i} recovery was not significantly different (p>0.1) between HBS (94±6%) and BBS (92±5%, n=3), suggesting that NBC1 is not functionally expressed in the LM of SMG.

We next examined the pH\textsubscript{i} recovery mechanisms in the BLM of interlobular ducts. The ductal lumen was perfused with Na\textsuperscript{+}-free solution to inhibit the pH\textsubscript{i} recovery that may occur in the LM of ducts. Fig. 8A and 8B show the pH\textsubscript{i} recovery patterns from the parotid glands. The addition of Na\textsuperscript{+} to the bath solutions neither induced pH\textsubscript{i} recovery in HBS (Fig. 8A) nor BBS (Fig. 8B), although the addition of Na\textsuperscript{+} into the ductal lumen rapidly restored pH\textsubscript{i}. This result strongly suggests that any Na\textsuperscript{+}-dependent pH\textsubscript{i} recovery mechanisms such as NHE1 does not exist in the BLM of parotid ducts. In contrast to the parotid glands, the addition of Na\textsuperscript{+} to the bath solutions rapidly restored pH\textsubscript{i} in the SMG ducts, and the pH\textsubscript{i} recovery rate was further increased by the addition of Na\textsuperscript{+} into the ductal lumen in HBS from the rate of 0.094±0.016 pH unit/min (n=3) to 0.502±0.023 pH unit/min (n=3) (Fig. 8C). Fig. 8D shows that 1 μM EIPA only partly inhibits pH\textsubscript{i} recovery by 38±12% (n=3) compared with the normal recovery in BBS and the residual component was further inhibited by 90±2% (n=3) by the addition of 1 mM DIDS, suggesting that NBC1 is functionally expressed in the BLM. All our main findings are summarized in Table 1.
DISCUSSION

*Guinea pig salivary glands as a HCO$_3^-$ transport model.* HCO$_3^-$ is the most important buffering system in saliva. Although its concentration is very low in unstimulated saliva, it can be increased up to 60 mM at high flow rates in humans (2). However it is still unclear what mechanisms mediate the HCO$_3^-$ secretion and absorption in salivary glands. The marked differences in salivary HCO$_3^-$ secretion among species and among glands within species suggests that the expression patterns of HCO$_3^-$ transporter may well differ both among species and among glands. For example, with increasing salivary flow rate, HCO$_3^-$ concentration rise in human parotid gland, but fall in rat parotid gland (33). Interestingly, we found the value of HCO$_3^-$ concentration of whole saliva in guinea pig was similar with those of human, suggesting that guinea pig salivary glands may be served as an appropriate experimental model for studying the HCO$_3^-$ transports in human.

*Functional expression of NHEs in guinea pig salivary glands.* We found that there was a significant difference in the threshold concentrations of EIPA required to inhibit NHE activity between two tissues, the acini and ducts from two salivary glands, the parotid and the SMG. Amiloride-sensitive NHEs, probably NHE1 and/or NHE2, played a putative role in pH$_i$ regulation in salivary acinar cells, because 1 µM of EIPA, a specific blocker for NHE1 or NHE2, completely inhibited pH$_i$ recovery in HBS. However, the inhibition threshold of EIPA in the duct cells was more than one order of magnitude higher than that in acinar cells. Based on the inhibitory efficiency of the known EIPA to different NHE isoforms (17), another type of NHE isoform, probably NHE3 located in the LM (24), appears to act together with NHE1 to regulate pH$_i$ in
ducts. We did not further examine the molecular identities of the NHE isoforms expressed in these ducts, since our main aim in the present study was to elucidate an HCO₃⁻ transport mechanism in relation with the expression of NBCs. Thus, after determining the maximal concentration of EIPA needed to inhibit NHEs, we studied the activity of NBCs in the presence of the maximal concentration of EIPA.

Functional expression of NBCs in guinea pig salivary glands. Table 1 summarized our main findings for NBC1 and NBC3 expression including all the RT-PCR, immunohistochemistry and functional studies. Our results provided evidence for a heterogeneous distribution of NBCs in guinea pig salivary glands. NBC1 showed distinct membrane domain localization; it is expressed in the BLM of parotid acinar cells and the LM of intra- and interlobular parotid ducts. Particularly, it is worth to note that NBC1 was functionally expressed in the LM of the parotid ducts. Roussa et al. (30) also reported NBC1 expression by immunohistochemistry in the LM of human parotid gland striated ducts. We not only verified its localization by immunohistochemistry, but also confirmed its function by pHᵢ measurements using microperfusion. Although immunolabeling of NBC3 was also detected in parotid and SMG ducts, we did not find any evidence of NBC3 involvement in pHᵢ regulation. Throughout the experiments, 1 mM of DIDS completely inhibited all of the residual EIPA-insensitive pHᵢ recovery components in BBS. The absence of pHᵢ recovery in the BLM of the parotid ducts suggests that NBC3, its location confirmed by immunohistochemistry, does not play any roles in pHᵢ regulation. However, in the SMG duct, we cannot rule out the possibility that high concentrations of EIPA (more than 50 μM) inhibit NBC3 activity. Whether high concentrations of EIPA inhibit NBC3 activity (27) or not (25) is still controversial.
The physiological role of NBCs in guinea pig salivary glands. We found that when guinea pig parotid acinar cells were acidified, the pH recovered to the prestimulus level. In this process, in addition to NHE1 and/or NHE2, NBC1 in the BLM also contributed to pH recovery by taking up HCO$_3^\text{-}$. The favorable electrical potential due to a membrane depolarization by Cl$^\text{-}$ and/or HCO$_3^\text{-}$ efflux (9, 10, 26) can further provide a driving force for HCO$_3^\text{-}$ influx via NBC1. The concentration of HCO$_3^\text{-}$ varies with the flow rate. Unstimulated saliva contains very little HCO$_3^\text{-}$, whereas stimulated saliva contains a much higher concentration (2). One possible explanation for the very low HCO$_3^\text{-}$ concentration in unstimulated saliva is due to the reabsorption of HCO$_3^\text{-}$ in ducts. However, there is no candidate HCO$_3^\text{-}$ transporters reported, especially in the parotid ducts that may mediate HCO$_3^\text{-}$ reabsorption. To our knowledge, this is the first report of a functional expression of NBC1 in the LM of parotid ducts in mammalian salivary glands. Our results strongly suggest that the luminal NBC1 plays a dominant role in HCO$_3^\text{-}$ reabsorption. Although NBC3 has been believed to play an HCO$_3^\text{-}$ salvage role in mouse SMG (16), we did not find any evidence of NBC3 expression in the LM of the parotid ducts. This discrepancy may be due to the fact that HCO$_3^\text{-}$ transporters involved in salvage of HCO$_3^\text{-}$ depend on the species and type of salivary glands. As in acinar cells, duct cells are also depolarized by agonists. The depolarization can be induced by Cl$^\text{-}$ or HCO$_3^\text{-}$ exit via the cystic fibrosis transmembrane regulator (CFTR) (7). This favorable electromotive force for the influx mode of NBC1 may further accelerate reabsorption of HCO$_3^\text{-}$ from the ductal lumen. This luminal NBC1 also appears to function in the resting state, since there was a slow decrease of ductal pH in the presence of 1 mM DIDS. It’s unlikely that other DIDS sensitive HCO$_3^\text{-}$ transporters, such as AE1-3 of SLC4 family (28)
and some electrogenic Cl⁻/HCO₃⁻ extrangers of the SLC26 family (20), involve in this process. Although the direction of Cl⁻ and HCO₃⁻ movement via Cl⁻/HCO₃⁻ exchanger depends on the local gradients, it is likely that the Cl⁻/HCO₃⁻ exchanger in our experiment generate net Cl⁻ uptake and HCO₃⁻ efflux, because the Cl⁻ concentration, 115 mM, of microperfusion solution via ductal lumen may be much higher than that of duct cells. Although any data for the intracellular Cl⁻ concentration in guinea-pig duct cells is not available, they have reported the Cl⁻ concentration of 38±8 mM and 57.7±4.1 mM in rat (36) and rabbit SMG ducts(14), respectively. If we assume that the Cl⁻ concentration of guinea-pig duct cells are not much different from those of other rodents such as rat or rabbit, the Cl⁻/HCO₃⁻ exchanger in our experiment should generate net Cl⁻ uptake and HCO₃⁻ efflux in resting states. To keep the HCO₃⁻ concentration of the saliva low would be advantageous to facilitate a sour taste in the resting state (18). The high HCO₃⁻ concentration in stimulated parotid saliva can be explained partly by the limited time for the duct system to reabsorb HCO₃⁻ at high flow rates, and partly by the activation of the luminal Cl⁻/HCO₃⁻ exchanger (15). HCO₃⁻ is also one of the main components in SMG saliva (3). The presence of NBC1 at the BLM of the SMG ducts suggests that NBC1 may mediate HCO₃⁻ uptake into the duct cells to compensate for the HCO₃⁻ secretion through the CFTR. In this tissue, a strong HCO₃⁻ reabsorption mechanism mediated by the NBC1 observed in parotid ducts may not be necessary, because the amount of HCO₃⁻ in primary saliva secreted from SMG acinar cells will be much less than that secreted by the parotid glands by the absence of basolateral NBC1.

This study demonstrates that the mechanisms for pH regulation in the guinea pig parotid gland and SMG are different. NBC1, in addition to NHEs, is involved in
regulating the pH\textsubscript{i} in parotid acinar cells and in both ducts of the parotid gland and SMG. We also provide new evidence that NBC1 is expressed in the LM of parotid ducts and may play a role in HCO\textsubscript{3}\textsuperscript{-} reabsorption.

GRANTS

This work was supported by a Korea Research Foundation Grant, KRF-2002-041-E00215.
REFERENCES


FIGURE LEGENDS

Figure 1. RT-PCR analysis of NBC isoforms in the parotid gland (PG) and SMG of guinea pig. (A) NBC1 transcripts with a predicted molecular size of 437 bp (N-terminal) and 182 bp (middle portion) were generated using degenerative oligonucleotide primers derived from conserved NBC1 sequences of rat, mouse, and human. (B) NBC1 transcripts in acini and ducts (the upper panel). In this case, we used specific primers (N-terminal) for guinea pig NBC1. NBC1 transcripts were detected from both acini and ducts in the PG, but only from the ducts in the SMG. NBC3 transcripts in acini and ducts (the middle panel) with a predicted molecular size of 316 bp. The NBC3 mRNA expressed in parotid gland duct, SMG duct and acini.

Figure 2. Labeling of NBC1 (indicated by arrows). (A) Rat kidney used as positive control which revealed NBC1 labeling at the basolateral side of proximal tubules. (B) NBC1-antibody-stained BLM of parotid gland acinar cells; Strong labelings of NBC1 in the LM of (C) intralobular ducts and (D) interlobular excretory ducts. (E) Strong labelings of BLM were observed in the SMG intralobular duct cells, but there was no NBC1 staining in the SMG acinar cells. (F) Strong labeling of NBC1 in the BLM of the interlobular ducts of the SMG. Magnification: A-F, x400

Figure 3. Immunoperoxidase labeling of NBC3 (indicated by arrows) in rat kidney (A), submandibular gland (B-D) and parotid gland (E and F) from guinea pig. (A) NBC3 immunolabeling is seen at the intercalated cells of the cortical collecting duct in rat kidney as a positive control. (B-D) In the submandibular glands, NBC3 labeling is associated with apical plasma membrane of the acini (B-D), intralobular duct (C)
and interlobular duct (D). (E-F) In the parotid glands, NBC3 labeling is associated with basolateral plasma membrane of the intralobular duct (E) and interlobular duct (F), whereas no labeling is seen in the acini (E). Magnification: A-F, x630

Figure 4. pH$_i$ recovery patterns from the NH$_4$Cl (dark bars)-induced acidification in guinea pig salivary glands and the effects of EIPA. (A) Normal pH$_i$ recovery in SMG acinar cells and complete inhibition by 1 μM EIPA (hatched bar) in HBS. (B) A complete inhibition of pH$_i$ recovery in SMG acinar cells in the presence of 1 μM EIPA in BBS. (C) Normal pH$_i$ recovery and complete inhibition by 1 μM EIPA in parotid gland (PG) acinar cells in HBS. (D) A partial inhibition of pH$_i$ recovery in PG acinar cells by 1 μM EIPA in BBS. Each figure is a representative figure from five to twelve experiments.

Figure 5. (A) A summarized result of pH$_i$ recoveries of acinar cells (n= 5 to 12) and the effects of DIDS. The pH$_i$ recovery rates of the SMG acinar cells were not significantly different (P>0.1) between HBS (-) and BBS (+). By contrast, in the parotid acinar cells, the pH$_i$ recovery rate in BBS was much faster (indicated by **, P<0.01) than those in HBS. 1 μM EIPA only partly blocked the pH$_i$ recovery of the parotid acinar cells in BBS (indicated by *). (B) A complete inhibition of pH$_i$ recoveries in PG acinar cells in the presence of 1 μM EIPA and 1 mM DIDS (grey bar) in BBS. The EIPA-insensitive pH$_i$ recovery component (0.218±0.044 pH unit/min, n=4) in parotid acinar cells was completely blocked by 1 mM DIDS.

Figure 6. pH$_i$ regulation in the intralobular ducts from two different types of salivary glands: the parotid gland (PG) and the SMG. Superimposed raw traces for pH$_i$
recoveries from an NH₄Cl-induced acid load in the presence of various concentrations of EIPA in (A) PG ducts and in (B) SMG ducts. (C) The average IC₅₀ values of EIPA in HBS from the two ducts. (D) pHᵢ recovery in PG ducts (0.721±0.047 pH unit/min, n=4) only partly inhibited by 100 μM EIPA (0.416±0.045 pH unit/min, n=4) in BBS, and the residual pHᵢ recovery component was further inhibited by 1 mM DIDS (0.050±0.026 pH unit/min, n=3). (E) pHᵢ recovery pattern in SMG ducts using the same protocol as for Fig. 6D. In this experiment, 10 μM EIPA was used. The normal pHᵢ recovery (0.729±0.027 pH unit/min, n=4) was partly inhibited by 10 μM EIPA (0.369±0.064 pH unit/min, n=3), and the residual pHᵢ recovery component was further inhibited by 1 mM DIDS (0.066±0.055 pH unit/min, n=3). Fig. 6D and 6E shows a representative curve each from three experiments.

Figure 7. pHᵢ recovery patterns in the LM of the parotid (A, B and C) and SMG(D and E) interlobular ducts by microperfusion technique via the ductal lumen. (A) Normal pHᵢ recovery (0.097±0.003 pH unit/min, n=3) was completely inhibited by 1 μM EIPA in parotid ducts in HBS. (B) The pHᵢ recovery pattern in the presence of 1 μM EIPA in BBS (0.101±0.011 pH unit/min, n=3). EIPA only partly inhibited pHᵢ recovery, and the residual pHᵢ recovery component was further completely inhibited by 1 mM DIDS. (C) A rapid increase of pHᵢ induced by Cl⁻ removal from the ductal lumen. The decreased pHᵢ was recovered to the prestimulus levels by the readdition of Cl⁻. The application of 1 mM DIDS slowly decreased pHᵢ from 7.45 to 7.2 over 5 min. (D) Superimposed raw traces of pHᵢ recoveries in various concentrations of EIPA (0, 5 and 50 μM) in HBS. The degree of inhibition of the pHᵢ recovery by EIPA was in proportion to its concentrations. The highest concentration of EIPA, 50 μM, was
needed to almost completely inhibit pH$_i$ recovery in the LM. (E) pH$_i$ recovery pattern in the presence of the highest concentration of EIPA, 50μM, in BBS (0.037±0.022 pH unit/min, n=3). There was no significant difference (p>0.1) between BBS and HBS (0.025±0.009 pH unit/min, n=3). The figures in 7A-C and 7E are representative ones from three or four experiments.

Figure 8. pH$_i$ recovery patterns in the BLM of the parotid (A and B) and SMG interlobular ducts (C and D) by microperfusion technique. It is noticeable that addition of Na$^+$ into the bath solutions have neither induced pH$_i$ recovery in HBS (A) nor BBS (B) until the addition of Na$^+$ into the ductal lumen rapidly restored pH$_i$. (C) In the SMG interlobular ducts, the addition of Na$^+$ into the bath solution induced pH$_i$ recovery (0.094±0.016 pH unit/min, n=3), which further increased by the addition of Na$^+$ into the ductal lumen (0.502±0.023 pH unit/min, n=3). A concentration of 1 μM EIPA almost completely inhibited this pH$_i$ recovery in the BLM (0.021±0.004 pH unit/min, n=3). (D) 1 μM EIPA only partly inhibited pH$_i$ recovery (0.078±0.005 pH unit/min, n=3) in BBS, and the residual pH$_i$ recovery component was almost completely inhibited by 1 mM DIDS (0.013±0.003 pH unit/min, n=3) in the BLM. All figures in 7A-D are representative ones selected from three or four experiments.

Table 1. Summary of NBC1 and NBC3 expression patterns in guinea-pig salivary glands.

+ : Positive response , - : negative response
Fig. 1
Fig. 2
Fig. 4

A. SMG acinar cells in HBS

B. SMG acinar cells in BBS

C. P3 acinar cells in HBS

D. P3 acinar cells in BBS

PG: parotid gland
SMG: submaxillary gland
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Table 1. Summary of NBC1 and NBC3 expression patterns in guinea-pig salivary glands

<table>
<thead>
<tr>
<th></th>
<th>Parotid gland</th>
<th></th>
<th>Submandibular gland</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acinar cells</td>
<td>duct</td>
<td></td>
<td>Acinar cells</td>
</tr>
<tr>
<td></td>
<td>intralobular</td>
<td>intralobular</td>
<td></td>
<td>intralobular</td>
</tr>
<tr>
<td></td>
<td>LM</td>
<td>BLM</td>
<td>LM</td>
<td>BLM</td>
</tr>
<tr>
<td>NBC1 RT-PCR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>immunostaining</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pH regulation</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(functional study)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBC3 RT-PCR</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>immunostaining</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH regulation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(functional study)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>