Expression of multiple Na$^{+}$/H$^{+}$ exchanger isoforms in rat parotid acinar and ductal cells

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Expression of multiple Na$^{+}$/H$^{+}$ exchanger isoforms in rat parotid acinar and ductal cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G470–G478, 1999.—Several members of the Na$^{+}$/H$^{+}$ exchanger gene family (NHE1, NHE2, NHE3, and NHE4) have unique functional properties. We examined the molecular and pharmacological properties of Na$^{+}$/H$^{+}$ exchange in rat parotid salivary gland cells. In acinar cells superfused with a physiological salt solution (145 mM Na$^{+}$), Na$^{+}$/H$^{+}$ exchanger activity was inhibited by low concentrations of the amiloride derivative ethylisopropyl amiloride (EIPA; IC$_{50}$ = 0.014 ± 0.005 µM), suggesting the expression of amiloride-sensitive isoforms of NHE1 and/or NHE2. Semi quantitative RT-PCR confirmed that NHE1 transcripts are most abundant in this cell type. In contrast, the intermediate sensitivity of ductal cells to EIPA indicated that inhibitor-sensitive and -resistant Na$^{+}$/H$^{+}$ exchanger isoforms are coexpressed. Ductal cells were about one order of magnitude more resistant to EIPA (IC$_{50}$ = 0.754 ± 0.104 µM) than cell lines expressing NHE1 or NHE2 (IC$_{50}$ = 0.076 ± 0.013 or 0.055 ± 0.015 µM, respectively). Conversely, ductal cells were nearly one order of magnitude more sensitive to EIPA than a cell line expressing the NHE3 isoform (IC$_{50}$ = 6.25 ± 1.89 µM). Semi quantitative RT-PCR demonstrated that both NHE1 and NHE3 transcripts are expressed in ducts. NHE1 was immunolocalized to the basolateral membrane of rat parotid acinar cells, whereas NHE3 was exclusively seen in the apical membrane of ductal cells. Immunoblotting, immunolocalization, and semiquantitative RT-PCR experiments failed to detect NHE2 expression in either cell type. Taken together, our results demonstrate that NHE1 is the dominant functional Na$^{+}$/H$^{+}$ exchanger in the plasma membrane of rat parotid acinar cells, whereas NHE1 and NHE3 act in concert to regulate the intracellular pH of ductal cells.

Four different isoforms of the Na$^{+}$/H$^{+}$ exchanger gene family (NHE1, NHE2, NHE3, and NHE4) have been described in epithelial tissues (31, 41). A non epithelial Na$^{+}$/H$^{+}$ exchanger, NHE5 (15), and a mitochondrial Na$^{+}$/H$^{+}$ exchanger, NHE6 (28), have also been identified. Virtually all mammalian cells utilize Na$^{+}$/H$^{+}$ exchangers to maintain the intracellular pH (for recent views, see Refs. 27, 30, 40, and 43). This function is especially important in salivary acinar cells, in which upregulation of Na$^{+}$/H$^{+}$ exchange buffers the acidification that results from HCO$_3^-$ secretion in response to Ca$^{2+}$-mobilizing agonists (16, 23). Upregulation of parotid gland cells has two distinct phases. Initially, cell shrinkage rapidly activates Na$^{+}$/H$^{+}$ exchange independent of the intracellular Ca$^{2+}$ concentration as well as protein kinase C (PKC) and calmodulin activity (22). Secondarily, a slow, Ca$^{2+}$-dependent upregulation (half-time >5 min) occurs that is separate from PKC and calmodulin activation (20, 21) as well as phosphorylation of NHE1 (35). This delayed activation of Na$^{+}$/H$^{+}$ exchange is apparently the result of both an increase in the maximum rate of uptake and a shift in the intracellular pH sensitivity of the exchanger (20).

The kinetic properties of Na$^{+}$/H$^{+}$ exchanger activity suggest that at least two different NHE isoforms are functionally expressed in rat parotid glands. In the present study, molecular and pharmacological techniques were used to address the possibility that multiple NHE isoforms are involved in salivary gland function. Our results demonstrate that NHE1 is the dominant NHE isoform in acinar cells. In contrast, NHE3 is a major contributor to pH regulation in ductal cells, although this cell type also expresses high levels of NHE1. NHE1 is located in the basolateral membranes of rat parotid acinar and ductal cells, whereas NHE3 was only found in the apical membranes of ductal cells. Some aspects of this work have been previously presented in abstract form (32).

METHODS

Isolation of rat parotid cells. Parotid glands were removed from male 150- to 250-g Wistar rats, and single acinar cells were dispersed by trypsin-collagenase digestion as described previously (3). Salivary gland cells were plated onto poly-l-lysine-coated glass coverslips, and individual acinar cells or small acini were isolated by using glass micropipettes. Ductal cells were isolated as small fragments. The lack of contamination was confirmed by PCR using acinar and ductal cell-specific primers (17).

To express NHE3, cDNA (RKNHE2–1; see Ref. 31) was ampliﬁed by using a rat NHE3-speciﬁc sense primer corresponding to nucleotides (nt) 16–37, 5′-acaagacttccggtatgcgtgctctgtcgg-3′, and an antisense primer corresponding to nt 4566–4586, 5′-actctagaagaaggggtactactgagaaatgat-3′.
TGA-3'. The lowercase italic letters indicate the addition of Hind III and Xba I restriction sites to 5' and 3' ends of the PCR products, respectively, to facilitate subcloning of the amplified NHE3 cDNA into a mammalian expression vector. The amplified 4640-bp cDNA product was digested with Hind III and Xba I, separated by electrophoresis on a 3% Tris-borate-EDTA (TBE)-agarose gel, and eluted using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). The isolated NHE3 cDNA was inserted into linearized pCMV (cytomegalovirus) vector plasmid (Tropix, Bedford, MA). This pCMV-NHE3 mammalian expression construct was stably transfected into the NHE-deficient PS120 cell line by calcium phosphate precipitation (10). NHE3 stable transfectants (PS3) were selected into the NHE-deficient PS120 cell line by calcium phosphate precipitation (10). NHE3 stable transfectants (PS3) were selected by the antibiotic Geneticin (G418; GIBCO BRL) and by an H+ killing method (11, 40). The NHE3 cell line (PS3), as well as cell lines expressing recombinant rat NHE1 (PS1C), NHE2 (PS2-5), and NHE4 (PS4-4), was used to determine the sensitivity of the different rat NHE isoforms to various Na+/H+ exchange inhibitors.

Semiquantitative RT-PCR amplification of rat parotid acini and ducts. Salivary glands are complex tissues primarily composed of acinar cells, although other cell types contribute as much as 20% to the composition of the gland. Therefore, to eliminate nonacinar cells from the RNA preparation, single parotid acinar cells were enzymatically dispersed and isolated using a glass micropipette for subsequent single-cell RT-PCR. Because ductal cells are difficult to positively identify at the single-cell level in parotid glands, several dozen cells were typically isolated as short intact stretches of duct. Total RNA was prepared from 5–10 single acinar cells or 2–4 ductal segments using TRIzol reagent (GIBCO BRL). RNA was reverse-transcribed to cDNA using oligo(dT) and random hexamer primers according to the manufacturer’s instructions (1st-STRAND cDNA synthesis kit; Clontech, Palo Alto, CA).

The cDNA was amplified using PCR primer sets that recognize specific NHE isoforms. PCR amplification was performed in a DNA thermal cycler (PTC-200 Peltier thermal cycler; MJ Research, Watertown, MA) using AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). In initial studies, PCR amplification products were separated on 2% TBE-agarose gel, and the identity of the products was verified by restriction endonucleases and DNA sequencing (36).

To verify that the cDNA preparations were free of genomic DNA, a β-actin sense primer corresponding to nt 2451–2480, CCTCAGACTTCCGACCAAGAGATGCCACTG, and an anti-sense primer corresponding to nt 2749–2720, CGGATGTCCTCCTAGACTTCGAGCAAGAGATGGCCACTG, were designed to amplify products with similar sizes and melting temperatures from unique 3′-untranslated regions. The PCR product was digested with Hind III and Xba I restriction enzymes and separated by electrophoresis on a 3% Tris-borate-EDTA agarose gel, and the fluorescence images of PCR products were resolved by 4% polyacrylamide gel. PCR products were resolved by 4% polyacrylamide gel-8 M urea, and the fluorescence images of PCR products were generated by infrared IRD41 fluorophore and detected using a LI-COR digital imaging system.

The normalized DNA concentration for each isoform-specific plasmid was normalized by first estimating the amount of DNA present in a given plasmid DNA preparation by 10.220.33.1 on October 29, 2017 http://ajpgi.physiology.org/ Downloaded from

The cDNA PCR products for NHE1, NHE2, NHE3 and NHE4 were resolved on a 3% TBE-agarose gel and stained with ethidium bromide. The intensity of PCR products was measured using a densitometer (IS1000 Digital Imaging System; Alpha Innotech, San Leandro, CA) and was plotted to generate standard curves and estimate the amount of NHE-specific cDNA in the acinar and ductal cell preparations. PCR reaction conditions were optimized using different numbers of amplification cycles as previously described (25, 45).

Enhanced sensitivity for resolving amplification products was obtained in the second detection method by labeling the PCR primers with infrared IRD41 fluorophore and determining the amount of PCR product on a LI-COR 4000 automated sequencer (LI-COR, Lincoln, NB). The IRD41-labeled primers were the same as those described above.

The IRD41-labeled PCR1 primers were used to normalize the DNA concentrations for each NHE isoform-specific plasmid DNA. PCR products were resolved by 4% polyacrylamide gel-8 M urea, and the fluorescence images of PCR products were generated on the LI-COR automated sequencer. The intensity of PCR products was quantified using the IS1000 digital imaging system.

Preparation of polyclonal antibody to the COOH-terminal region of rat NHE1. The rat NHE1-specific sense primer was used to amplify the COOH-terminal region corresponding to amino acids Pro-602 to Glu-740. The rat NHE1-specific sense primer corresponded to nt 1804–1831, 5′-GAACCGGAGCTGAATGAAGC-GAAGATTCAAAGGGTCTAAA-3′, and the antisense primer corresponded to nt 2703–2732, 5′-CAACCTTTATCCGGCTATCCAGTCTATTA-3′. The size of the PCR amplification product was 154 bp. The amplified DNA was ligated into the pCR2.1 vector. The normalized DNA concentration for each isoform-specific plasmid was then amplified by its respective set of NHE isoform-specific primers to create standard curves for semi-quantitative PCR.

PCR products were quantified by one of two techniques. In the first method, the PCR products were resolved by 3% TBE-agarose gel and stained with ethidium bromide. The intensity of PCR products was measured using a densitometer (IS1000 Digital Imaging System; Alpha Innotech, San Leandro, CA) and was plotted to generate standard curves and estimate the amount of NHE-specific cDNA in the acinar and ductal cell preparations. PCR reaction conditions were optimized using different numbers of amplification cycles as previously described (25, 45).

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sequence was verified by protein sequencing (model 473A; Applied Biosystems, Norwalk, CT). The NHE1 fusion protein was then used to generate a rabbit polyclonal antibody as previously described (24). The NHE1-specific antibody was purified using a BNHE1 antigen-linked affinity column.

Specificity of the antibody was verified by Western analysis of the truncated NHE1 expressed protein (BNHE1) used to raise the antibody and membrane preparations from PS120 cells expressing the different NHE isoforms. Figure 1A, lane 1, shows the size of the BNHE1 protein stained with Coomassie blue. Incubation with preimmune serum failed to detect the protein (lane 2), whereas the affinity-purified antibody detected the 21-kDa fusion protein (lane 3). Furthermore, Fig. 1B shows that the anti-BNHE1 antibody did not cross-react with NHE2, NHE3, and NHE4 expressed in PS120 cells (lanes 2, 3, and 4, respectively) or with proteins isolated from the NHE-deficient cell line PS120 (lane 5). However, anti-BNHE1 detected a 95- to 105-kDa protein in the PS1C cell line (Fig. 1B, lane 1), which displays stable expression of NHE1.

Membrane preparation. Crude membranes were prepared from rat parotid glands (19) and from tissue culture cells (1) as previously described. Aliquots were quickly frozen in liquid N2 and stored at −85°C until use. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Western blot analysis. The purified antigen (BNHE1), membranes from rat parotid glands, and membranes from NHE-expressing cell lines (70 µg/lane) were resolved by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) as previously described (33). Membranes were then incubated with primary antibody (anti-BNHE1 and anti-NHE2) or preimmune serum overnight at 4°C, followed by detection with a horseradish peroxidase-linked goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratory, West Grove, PA) and enhanced chemiluminescence (Amer sham, Arlington Heights, IL).

Immunocytochemistry. Glands were immediately frozen in 2-methylbutane on dry ice, and sections were cut at 10 µm on a cryostat (HistoSTAT microtome, Scientific Instruments). The sections were treated as previously described (17) with polyclonal anti-NHE1 antibody (anti-BNHE1), preimmune serum, antisera for NHE2 (2M5; see Ref. 7), or NHE3 (no. 1314; see Ref. 1). The NHE2 and NHE3 antisera were presorbed for 1 h at room temperature with parotid gland homogenate prepared from NHE2 and NHE3 knockout mice, respectively. Sections were then treated with FITC-labeled secondary antibody (goat anti-rabbit; Jackson Immunoresearch Laboratory) at room temperature for 1 h. Samples were viewed and analyzed on a Zeiss Axioplan microscope using a ×40 neofluor objective (Carl Zeiss, Oberkochen, Germany).

Intracellular pH determinations. Intracellular pH was monitored using the pH-sensitive fluorescent dye seminaphthoradifluor-1 (SNARF-1; Molecular Probes, Eugene, OR) as previously described (4). NHE-expressing cell lines were grown on glass coverslips for 24–72 h before experiments. Immediately before the start of an experiment, rat parotid acinar and ductal cells were attached to a glass coverslip mounted in a superfusion chamber on the microscope stage of an Ultima confocal laser cytometer (Meridian Instruments, Okemos, MI).

SNARF-1-loaded cells were acidified by using a NH4Cl prepulse technique essentially as previously described (29). Briefly, cells were exposed to 60 mM NH4Cl in a physiological salt solution (NaCl was replaced by NH4Cl) for 10 min, which was then switched to an Na+/free salt solution (Na+ was replaced by N-methyl-d-glucamine). Approximately 5 min later, the extracellular Na+ was restored to a physiological concentration to initiate the Na+/H+ exchanger-mediated intracellular pH recovery (44). The physiological salt solution contained (in mM) 145 NaCl, 5.4 KCl, 0.4 KH2PO4, 0.33 NaH2PO4, 10 glucose, 20 HEPES, 1.2 CaCl2, and 0.8 MgSO4 (pH 7.4). The intracellular pH was estimated by the high potassium-nigericin technique as previously described (44). The estimated intracellular pH after acid loading was ~6.0. After the readdition of extracellular Na+, the intracellular pH rose to ~7.5. The raw data are presented.

RESULTS

Differences in EIPA sensitivity of Na+/H+ exchanger activity in parotid acinar and ductal cells. The different NHE isoforms display unique sensitivities to various Na+/H+ exchanger inhibitors including the 5-amino acid derivative of amiloride (EIPA). NHE1 and NHE2 are sensitive, whereas NHE3 and NHE4 are resistant, to inhibition by EIPA in heterologous expression systems (9, 29, 42). Inhibitor sensitivities were thus used to characterize Na+/H+ exchanger activity in native parotid cells.

The EIPA sensitivity was determined in rat parotid acinar and ductal cells by monitoring the intracellular...
pH recovery after an acid load generated by an NH₄Cl prepulse. Figure 2 shows typical examples of the intracellular pH recovery from an acid load for parotid acinar and ductal cells. EIPA (200 nM) blocks the initial rate of the intracellular pH recovery by 90% in acinar cells (Fig. 2A). In contrast, EIPA inhibits the pH recovery by 15% in ductal cells (Fig. 2B). Washout of EIPA restored Na⁺/H⁺ exchanger activity to control rates (data not shown). Figure 3 summarizes the effects of the EIPA concentration on the intracellular pH recovery in rat parotid acinar and ductal cells. The EIPA sensitivity of ductal cells (IC₅₀ = 0.754 ± 0.104 µM) was 50-fold less sensitive than that of acinar cells (IC₅₀ = 0.014 ± 0.005 µM).

EIPA sensitivity of recombinant rat NHE1, NHE2, and NHE3 expressed in PS120 cells. The conditions for monitoring the EIPA sensitivity of parotid Na⁺/H⁺ exchanger activity in the present studies are different from those used earlier to characterize recombinant rat NHE1, NHE2, and NHE3 (29, 42). Previously, the EIPA sensitivity of ²²Na uptake was determined in low external Na⁺ (<1 mM). In contrast, we monitored the EIPA sensitivity of the NHE-dependent pH recovery in a physiological Na⁺ concentration (145 mM). Na⁺ is known to compete with amiloride (2); thus the potency of EIPA for the different rat NHE isoforms was unknown under our experimental conditions. Figure 4 shows that, when conditions identical to those used with acinar and ductal cells were utilized, recombinant rat NHE1 was very sensitive to 1 µM EIPA (Fig. 4A), whereas NHE3 was very resistant (Fig. 4B).

Figure 5 summarizes the effects of the EIPA concentration on the intracellular pH recovery in PS120 cells expressing rat recombinant NHE1, NHE2, and NHE3. The EIPA sensitivity of cells expressing NHE3 (IC₅₀ = 6.25 ± 1.89 µM) was about two orders of magnitude less sensitive compared with that of cells expressing NHE1 or NHE2. In contrast, the EIPA sensitivity was not significantly different between rat NHE1 and NHE2 (IC₅₀ = 0.076 ± 0.013 and 0.055 ± 0.015 µM, respectively).

NHE4 activity is very resistant to EIPA (9). Therefore, the relative resistance of ductal cells to EIPA suggests that NHE4 expression may potentially contribute to intracellular pH regulation in these cells (see Fig. 2). However, under our experimental conditions, Na⁺/H⁺ exchanger activity was not detected in the PS4-4 cell line expressing the NHE4 isoform (data not shown), even when hypertonic conditions were used to activate the exchanger as previously reported (6). Chambrey et al. (8, 9) have also reported difficulty in detecting NHE4 activity in an expression system as well as in native
cells. Thus the failure of the NHE4 cell line to express detectable Na\(^+\)/H\(^+\) exchange activity most likely indicates that NHE4 may be quiescent in acinar and ductal cells under the conditions of our experiments.

Semiquantitative RT-PCR in isolated acinar and ductal cells. The Na\(^+\)/H\(^+\) exchange inhibitor EIPA was unable to distinguish between rat recombinant NHE1 and NHE2 when expressed in NHE-deficient PS120 cells. As an alternative approach, we analyzed the transcript levels for NHE1, NHE2, NHE3, and NHE4 by semiquantitative RT-PCR methods to determine the expression levels of the different NHE isoforms in acinar and ductal cells. Figure 6 shows an example of control amplifications of the NHE1-containing plasmid run in duplicate at different concentrations to generate a standard curve. Standard curves were then used to empirically estimate the concentrations of the different NHE isoforms derived from acinar and ductal cells.

Figure 7 shows the results from a representative experiment (n = 4 for each isoform-specific set of primers). NHE1 was expressed most abundantly in

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**Fig. 4.** EIPA sensitivity of NHE activity in PS120 cells expressing either rat NHE1 (PS1C) or rat NHE3 (PS3). SNARF-1-loaded PS1C (A) and PS3 (B) cells were acidified using NH\(_4\)\(^+\)/NH\(_3\) prepulse technique (see Methods). Recovery from an acid load was initiated by readdition of a physiological concentration of Na\(^+\) at time indicated by arrow. Rate of recovery from an acid load was determined in presence and absence of 1 µM EIPA. Data are from representative examples with 10 or more cells per condition.

**Fig. 5.** Dependence of NHE inhibition on EIPA concentration in PS120 cells expressing either rat NHE1, NHE2, or NHE3. Concentration dependence of EIPA inhibition was tested using concentrations ranging from 2 nM to 30 µM EIPA for PS1C (▲) and PS2-5 (●) cells and from 1 to 800 µM EIPA for PS3 cells (■). Dotted line is a sigmoidal fit to NHE3 data (IC\(_{50}\) = 6.25 ± 1.89 µM), dashed line is fit to NHE1 data (IC\(_{50}\) = 0.076 ± 0.013 µM), and solid line is fit to NHE2 data (IC\(_{50}\) = 0.055 ± 0.015 µM). Number of cells tested for each EIPA concentration was 35 or more per condition. In all cases, SE was <5% of mean.

**Fig. 6.** Standard curve for semiquantitative PCR of pCR2.1-NHE1. The amount of NHE-specific cDNA from acinar and ductal cell preparations was empirically calculated using the corresponding standard curve. A: standard curve generated by plotting no. of molecules added vs. arbitrary fluorescence units (×10\(^2\)) for amplification of pCR2.1-NHE1 construct using NHE1-specific primers. This procedure was repeated for each NHE isoform (see Methods). B: amplification products from different concentrations of pCR2.1-NHE1 construct were run in duplicate. Fluorescence intensity was measured by densitometry. Size of pCR2.1-NHE1 amplification product was 190 bp. Lane M is a 100-bp ladder molecular weight size standard; estimated amounts of DNA were 6, 60, 600, or 6,000 molecules.
acinar (Fig. 7A, lane 1) and ductal cells (Fig. 7B, lane 1). NHE4 transcripts were also expressed in both cell types (Fig. 7A and B, lane 4). In contrast, NHE3 transcripts were detected at levels less than that observed for either NHE1 or NHE4 in ductal cells (Fig. 7B, lane 3), but NHE3 mRNA was not present in acinar cells (Fig. 7A, lane 3). NHE2 transcripts were below the detection limit of this assay in both acinar and ductal cells (Fig. 7, A and B, lane 2).

In additional experiments similar to those shown in Fig. 7, the number of PCR cycles was increased beyond the linear range of the assay (up to 60 cycles). Amplification products were observed for all four epithelial NHE isoforms in both acinar and ductal cells (data not shown). DNA sequencing confirmed their identity as NHE1, NHE2, NHE3, and NHE4. The cDNA prepared from individual acinar cells and duct fragments were free of genomic DNA (see METHODS). Although not quantitative, these data demonstrate that NHE2 mRNAs are present in both cell types, but at very low levels, likely orders of magnitude less than those of NHE1 and NHE4.

Western analysis of NHE proteins. Pharmacological characterization and semiquantitative RT-PCR analysis suggest that NHE1 is the most abundant Na⁺/H⁺ exchanger isoform in rat parotid gland. To verify that the NHE1 protein is expressed in the membranes of this tissue, a rabbit polyclonal antibody was prepared against a nonconserved 139-amino acid region of the COOH-terminal domain of rat NHE1, and the specificity was verified (see METHODS; Fig. 1). Western blot analysis of rat parotid membranes detected a 90- to 100-kDa protein with the BNHE1 polyclonal antibody (Fig. 8A, lane 1), whereas preimmune serum failed to label protein (lane 2). The size of the NHE1 protein shown is similar to that previously described in rabbit kidney (5). Based on the primary sequence, the NHE1 protein is predicted to be 91 kDa.

Na⁺/H⁺ exchanger activity in acinar cells was very sensitive to inhibition by EIPA, suggesting that these cells functionally express NHE1 and/or NHE2. However, RT-PCR failed to detect significant levels of NHE2 mRNA. In agreement with the semiquantitative RT-PCR experiments, the NHE2-specific antibody 2M5 did not detect a 90-kDa protein in a rat parotid gland membrane preparation (Fig. 8B, lane 2). In contrast, the NHE2-specific antibody detected an ~90-kDa protein in rat submandibular glands (Fig. 8B, lane 1 at arrow). The size of the NHE2 protein in rat submandibular gland is similar to that previously described in rat intestine and kidney (7) and is consistent with the predicted molecular mass (90 kDa). However, nonspecific bands are also labeled with the NHE2 antibody. To verify that the 90-kDa band labeled in the rat submandibular protein preparation is NHE2, Western blots of proteins isolated from parotid glands of mice lacking NHE2 expression were probed (38). Comparable to rat submandibular glands, parotid proteins isolated from
wild-type mice contained a 90-kDa protein labeled by the NHE2 antibody (Fig. 8B, lane 3). However, the band representing NHE2 was absent in NHE2 knockout mice (Fig. 8B, lane 4), whereas the staining intensity of proteins labeled nonspecifically remained constant.

Immunolocalization of NHE proteins. NHE1 has been localized to the basolateral membrane of epithelial cells (5), and this includes rat submandibular and parotid glands (12, 35). The NHE1-specific polyclonal antibody, anti-BNHE1, was used to confirm the site of this protein in the plasma membrane of rat parotid glands. Figure 9A shows that NHE1 is localized to the basolateral membrane of both acinar cells (less intense meshwork of staining) and ductal cells (intense labeling). NHE1 expression is considerably more abundant in ductal cells (arrows). Figure 9B is a Nomarski image of the same field as in Fig. 9A. In contrast, the polyclonal NHE3-specific antibody no. 1314 (1) recognized the apical membrane of ductal cells (Fig. 9, C and D, arrows) but failed to label acinar cells.

Our results suggest the relative absence of NHE2 mRNA or protein in rat parotid acinar and ductal cells (see Figs. 7 and 8). Consistent with these observations, immunolocalization studies in which an NHE2-specific antibody was used failed to detect NHE2 protein in rat parotid glands (Fig. 10, A and B). However, as previously reported (12), the apical membranes of rat submandibular ductal cells were labeled with the NHE2-specific antibody (Fig. 10, C and D, arrows).

DISCUSSION

$Na^+/{H}^+$ exchange plays an important role in regulating the intracellular pH of salivary gland cells. When acinar cells are stimulated to secrete, $HCO_3^-$ efflux...
produces a drop in the intracellular pH that is rapidly buffered by the activation of Na\(^+\)/H\(^+\) exchangers (16, 23, 44). A recent report suggests that NHE1 is most likely the Na\(^+\)/H\(^+\) exchanger isoform involved in this process (35). However, earlier studies indicate that upregulation of Na\(^+\)/H\(^+\) exchange in this gland is different compared with recombinant NHE1. NHE1 is regulated by protein kinase A (PKA) and PKC pathways (14, 18, 37) as well as calmodulin (26). In contrast, upregulation of Na\(^+\)/H\(^+\) exchanger activity in parotid cells is not associated with PKA or PKC pathways (20, 21) and is not blocked by calmodulin antagonists (22).

The present study therefore investigated further the properties of the Na\(^+\)/H\(^+\) exchangers regulating intracellular pH in the rat parotid gland by using a combination of molecular, pharmacological, and immunochemical techniques. It has been previously suggested that the activities of NHE1 and NHE2 can be distinguished on the basis of their sensitivities to the Na\(^+\)/H\(^+\) exchange inhibitor EIPA (40, 43). In basolateral membrane vesicles prepared from rat parotid glands, Manganel and Turner (19) found that amiloride inhibited Na\(^+\)/H\(^+\) exchange with an IC\(_{50}\) of \(\sim 1.6\) \(\mu\)M. This was the first observation suggesting that Na\(^+\)/H\(^+\) exchanger activity in the rat parotid gland is due to NHE1 and/or NHE2 expression. In the present study, the sensitivity of rat parotid acinar cells to EIPA (Fig. 3) was comparable to the sensitivity of expressed recombinant rat NHE1 and NHE2 proteins (Fig. 5). These results are consistent with previous reports for rat NHE1 and NHE2 (8, 9, 29, 42).

Neither proteins (Figs. 8 and 10) nor transcripts (Fig. 7) for NHE2 and NHE3 were detected in acinar cells. NHE1 and NHE4 transcripts were observed in single parotid acinar cells. In contrast to NHE1, NHE4 activity is very resistant to EIPA (IC\(_{50}\) > 10 \(\mu\)M; see Ref. 8). Although NHE4 transcripts were relatively abundant in parotid acinar cells, the total block of Na\(^+\)/H\(^+\) exchanger activity by low concentrations of EIPA indicates that NHE4 is not actively involved in intracellular pH regulation in these cells. The explanation for this observation may be that NHE4 is quiescent under most physiological conditions (6, 8, 9). Although NHE4 appears to be inactive, we cannot rule out the possibility that this NHE isoform has an unknown mode of regulation that uniquely activates this exchanger in salivary cells.

In ductal cells, transcripts and protein for NHE3 were seen in addition to those for NHE1 and NHE4. These results are consistent with an earlier report suggesting that NHE1 is most likely the primary Na\(^+\)/H\(^+\) exchanger in parotid acinar cells (35), but the results differ in that NHE3 and NHE4 are also expressed in rat parotid salivary glands. One potential reason for this apparent inconsistency might be the loss of ductal cells during the isolation procedure (35). Alternatively, the efficiency of the PCR primers used in the two studies may be different. Indeed, we failed to get a PCR product when we attempted to amplify our cDNA preparations with the NHE3 and NHE4 primers previously described (35).

Although we could not distinguish between NHE1 and NHE2 with EIPA, PCR-based and immunocytochemical methods demonstrate that NHE1 is by far the dominant exchanger for regulating the intracellular pH in rat parotid acinar cells. In contrast to acinar cells, ductal cells were much more resistant to EIPA, indicating functional expression of multiple NHE isoforms. Ductal cells were less sensitive to EIPA than the cell lines expressing recombinant rat NHE1, but they were more sensitive to EIPA than NHE3. In agreement with these observations, NHE1 and NHE3 transcripts (Fig. 7) and proteins (Figs. 8 and 9) are expressed in ductal cells. Therefore, both NHE1 and NHE3 contribute significantly to intracellular pH regulation in ductal cells. The apical location of NHE3 suggests a potential role for this Na\(^+\)/H\(^+\) exchanger isoform in Na\(^+\) reabsorption by ductal cells, as has been recently demonstrated (39) in other Na\(^+\) conserving tissues such as mouse kidney and intestine.

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