ENHANCED FORMATION OF A HCO₃⁻ TRANSPORT METABOLON IN EXOCRINE CELLS OF Nhe1⁻/⁻ MICE
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Cl⁻ influx across the basolateral membrane is a limiting step in fluid production in exocrine cells and often involves functionally linked Cl⁻/HCO₃⁻ (Ae) and Na⁺/H⁺ (Nhe) exchange mechanisms. The dependence of this major Cl⁻ uptake pathway on Na⁺/H⁺ exchanger expression was examined in the parotid acinar cells of Nhe1⁻/⁻ and Nhe2⁻/⁻ mice, both of which exhibit impaired fluid secretion. No change in Cl⁻/HCO₃⁻ exchanger activity was detected in Nhe2-deficient mice. Conversely, Cl⁻/HCO₃⁻ exchanger activity increased nearly 4-fold in Nhe1-deficient mice, despite only minimal or any change in mRNA and protein levels of the anion exchanger Ae2. Acetazolamide completely blocked the increase in Cl⁻/HCO₃⁻ exchanger activity in Nhe1 null mice suggesting that increased anion exchange required carbonic anhydrase activity. Indeed, the parotid glands of Nhe1⁻/⁻ mice expressed higher levels of carbonic anhydrase 2 (Car2) polypeptide. Moreover, the enhanced Cl⁻/HCO₃⁻ exchange activity was accompanied by an increased abundance of Car2–Ae2 complexes in the parotid plasma membranes of Nhe1⁻/⁻ mice. Anion exchanger activity was also significantly reduced in Car2-deficient mice, consistent with an important role of a putative Car2–Ae2 HCO₃⁻ transport metabolon in parotid exocrine cell function. Increased abundance of this HCO₃⁻ transport metabolon is likely one of multiple compensatory changes in the exocrine parotid gland of Nhe1⁻/⁻ mice that together attenuate the severity of in vivo electrolyte and acid-base balance perturbations.

Fluid secretion by exocrine glands is a complex process that requires the coordinated activity of multiple ion transport and regulatory proteins to drive transepithelial Cl⁻ movement and apical HCO₃⁻ efflux (1-4). Cl⁻ uptake across the basolateral membrane of exocrine cells is generally mediated by two distinct mechanisms, the functionally linked Cl⁻/HCO₃⁻ and Na⁺/H⁺ exchangers (which support NaCl uptake in exchange for intracellular HCO₃⁻ and protons) and a Na⁺/K⁺/2Cl⁻ cotransporter. In concert, these two Cl⁻ uptake pathways concentrate Cl⁻ 4-5 fold above its electrochemical equilibrium while intracellular HCO₃⁻ is generated by carbonic anhydrase. Stimulation generates a transepithelial potential difference due to apical Cl⁻ and HCO₃⁻ efflux via anion channels and basolateral K⁺ loss through K⁺ channels. Na⁺ crosses the tight junction in response to the resulting lumen-negative, transepithelial potential difference, and water follows the consequent osmotic gradient to produce a NaCl-rich, plasma-like secretion.

The Cl⁻/HCO₃⁻ exchanger isoform Ae2 (also known as Slc4a2) and the Na⁺/H⁺ exchanger Nhe1 (Slc9a1) co-localize to the basolateral membrane in salivary exocrine cells (5-8). Consistent with a functional linkage between these two exchangers, targeted disruption of the Nhe1 gene decreases fluid secretion (6). During stimulation, Nhe1 maintains an alkaline intracellular pH critical for secretion (6,9,10). The elevated intracellular pH favors Cl⁻ entry across the basolateral membrane via Cl⁻/HCO₃⁻ exchange (11). Nhe2 (Slc9a2), on the other hand, is present in the apical membranes of salivary exocrine cells (6). Nhe2 does not play a significant role in global intracellular pH regulation (10,12) but nevertheless appears to regulate saliva secretion (6), possibly by modulating activity of the pH-sensitive apical anion channels expressed in this cell type (13). Hyposalivation from the parotid glands of Nhe1 null mice (6) is associated with increased expression of Nkcc1 (Slc12a2) transcript, suggesting that the Na⁺/K⁺/2Cl⁻ cotransporter partially compensates for the loss of Nhe1-regulated fluid secretion. Direct evidence demonstrating the functional importance of
the Na+/K+/2Cl− cotransporter was provided in mice lacking Nkcc1 gene expression (12). These animals exhibit defective Cl− uptake and, in consequence, a dramatic 60-70% reduction of fluid output by parotid glands in vivo. The residual fluid production in Nkcc1+/− mice is mediated by the paired Cl−/HCO3− exchangers (12). It was found that Nkcc1+/− mice express increased levels of Ae2 transcript and Cl−/HCO3− exchanger activity to partially compensate for the loss of Na+/K+/2Cl− cotransporter activity (12). Moreover, inhibition of carbonic anhydrase, Cl−/HCO3− exchange or Na+/H+ exchange activity severely limits secretion (see (14,15)), indicating that these HCO3− (H+) metabolism and transport mechanisms are important for fluid production.

In mammalian cells carbonic anhydrases (Ca) appear to associate directly with Cl−/HCO3− exchangers, including members of both the SLC4A and SLC26A gene families, to form functional complexes termed transport metabolons (16-24). Such transport metabolons, through direct coupling of transmembrane bicarbonate transport with physically proximate (and possibly bound) carbonic anhydrase activity, may maximize transmembrane bicarbonate fluxes associated with the regulation of intracellular and extracellular or lumenal pH (25) (but see (26)). CaII can bind to acidic amino acid clusters in SLC4 anion exchanger C-terminal peptides or their fusion proteins (18,19,27) or to the STAS domain of SLC26A6 (24). Anion exchanger activity can be inhibited by mutations that interfere with CaII–anion exchanger interaction, either by over-expression of a catalytically inactive CaII mutant (22,27) or by phosphorylation of the anion exchanger SLC26A6 near its CaII binding site in the STAS domain (24). Collectively, these observations suggest that bound CaII elevates rates of HCO3− production and consumption within a micro-domain near the intracellular anion binding and translocation site(s) of Cl−/HCO3− exchangers. Conversely, peptides with sequences that include the proposed CaII binding site of the anion exchanger Ae1 strongly activate CaII (28). Thus, close proximity of CaII with anion exchanger polypeptides may be required for maximal bicarbonate transport activity.

To gain further insight into the functional relationship between the Cl−/HCO3− and Na+/H+ exchangers of the basolateral membrane of exocrine acinar cells, we examined the expression and activity of HCO3− metabolism and transport proteins in parotid acini of mice deficient in Nhe1 and in Nhe2. Our results demonstrate that Cl−/HCO3− exchanger activity was significantly enhanced in the parotid exocrine cells of Nhe1-deficient mice, but not in mice lacking Nhe2. Moreover, the interaction of murine carbonic anhydrase II (Car2) with Ae2 was enhanced in Nhe1-deficient mice, suggesting an in vivo correlation between increases in Cl−/HCO3− exchanger activity and formation of a functional Car2–Ae2 bicarbonate transport metabolon.

**EXPERIMENTAL PROCEDURES**

**Materials and Null Mutant Animals—** BCECF-AM was purchased from Molecular Probes (Eugene, OR). Collagenase P and Liberase RI were from Boehringer-Mannheim GmbH (Penzberg, Germany) and Roche Applied Science (Indianapolis, IN), respectively. EZ-Link Sulfo-NHS-SS-Biotin was from Pierce (Rockford, IL) and M-280 streptavidin beads were from Dynal Biotech ASA (Oslo, Norway). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Targeted disruptions of the murine Nhe1, Nhe2, and Ae2 genes were performed as previously described (10,29,30). Founder heterozygous mice were used to establish breeding colonies in the University of Rochester vivarium. A null mutation in the Car2 gene was induced by exposure to N-Ethyl-N-nitrosourea (31). PCR genotyping of the Car2 mutation followed a strategy developed in Dr. M. Soleimani's Laboratory, based on information from the Jackson Laboratories (Bar Harbor, Maine):

**Wildtype Car2 primers:** CGC TAT TTT TTG AAG ATT GGA CCT GCC ACA C and TGG AAG CAA TTA TTT ACT CCG GGT AAA CTG Wildtype primers: CGC TAT TTT TTG AAG ATT GGA CCT GCC ACA T and TGG AAG CAA TTA TTT ACT CCG GGT AAA CTG

**Car2−/− primers:** CGC TAT TTT TTG AAG ATT GGA CCT GCC ACA T and TGG AAG CAA TTA TTT ACT CCG GGT AAA CTG

All animals were housed in micro-isolator cages with free access to laboratory chow and water ad libitum with a 12-hour light/dark cycle. Experiments were performed on sex- and age- matched animals between 2 and 5 months old. All experimental protocols were approved by the Animal Resources Committee of the University of Rochester.

**Acinar Cell Preparation and in vitro Intracellular pH Measurements—** Parotid gland acinar cells were prepared as previously described (10). Briefly, CO2-narcotized mice were killed by exsanguination prior to isolation of acinar cells by collagenase digestion. Acinar cells were BCECF-loaded by 20-30 min
incubation at room temperature with BCECF-AM (2 
µM). Acinar cells spontaneously adhere to the base of a superfusion chamber mounted on a Nikon Diaphot 200 microscope interfaced with an Axon Imaging Workbench (Foster City, CA) or TILL Photonics Imaging System (Pleasanton, CA). Cells were excited alternately at 490 and 440 nm and the emitted fluorescence measured at 530 nm. Superfusion solutions contained (in mM): 110 NaCl, 25 NaHCO3, 5.4 KCl, 0.4 KH2PO4, 0.33 Na2HPO4, 0.8 MgSO4, 1.2 CaCl2, 10 glucose, 20 Hepes, pH 7.4 with NaOH, and was gassed with 5% CO2-95% O2 for at least 30 minutes before pH adjustment to 7.4 with NaOH. Chloride salts were replaced with equimolar gluconate in Cl--free solutions, and additional calcium was added to compensate for chelation.

Intracellular pH was estimated by in situ calibration of the fluorescence ratio F490/F440 performed using the nigericin-high K+ method of (32). The high K+ solution contained (in mM): 120 KCl, 20 NaCl, 0.8 MgCl2, 20 Hepes and 0.005 nigericin. pH was adjusted across the range from 5.6 to 8.0 with Tris-base. Data presented in the figures showing pHi changes vs. time are average results from a single representative experiment where multiple acinar aggregates were analyzed. Values quoted are the means ± S.E.M. for the number of acinar aggregates examined. All experiments were performed with 3 or more separate gland preparations, as summarized in the bar graphs.

Measurement of Mouse Ae2 Activity Expressed in Xenopus Oocytes— Oocytes were prepared by collagenase digestion from ovarian fragments harvested from adult female X. laevis following surgical protocols approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center. Individual oocytes were defolliculated by hand. Ae2a cRNA was transcribed from linearized template using T7 MEGAScript kit (Ambion, Woodlands, TX). Oocytes were microinjected with 50 nL water containing 10 ng Ae2a cRNA or no RNA, and incubated 3 days at 19°C in ND-96, pH 7.4 containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 Hepes, supplemented with 2.5 mM sodium pyruvate and with gentamicin (100 µg/100 ml). Oocytes were then injected with 50 nL 36Cl-- (≈12,000 cpm), allowed to reseal for 10 min in Cl--free ND-96, and 36Cl efflux was measured as previously described (11,33) into ND-96 (lacking pyruvate and gentamicin), first in the absence and then presence of 100 µM ACTZ (acetazolamide), and finally in the additional presence of 200 µM DIDS.

Biotinylation of Cell Surface Proteins— Biotinylation of cell surface proteins was performed according to the manufacturer's instructions (Pierce). Briefly, parotid exocrine cells were isolated as described above from Nhe1+/+ or Nhe1−/− mice. Mice used to detect anion exchanger Ae2 protein by western analysis were injected subcutaneously with heparin (13.6 units/gm body weight) and perfused via the left ventricle with PBS prior to gland excision to remove erythrocytes, and then, eliminate or minimize the presence of immuno-crossreactive erythroid anion exchanger Ae1 (34). Dispersed parotid cells were washed twice with ice-cold PBS (pH 8.0). After the addition of 10 mM Sulfo NHS-SS-Biotin stock solution (80µl per ml of PBS), cells were incubated for 30 minutes at 20°C or (not shown, n = 4) on ice. The reaction was stopped by addition of ice-cold 50 mM Tris (pH 8.0) for 10 minutes, and then the cells were washed twice with ice-cold PBS.

Preparation of Crude and Affinity-enriched Plasma Membrane Proteins— Enriched plasma membranes were isolated essentially as previously described (35,36). Biotinylated cells were collected by centrifugation at 1,000g for 45 sec and the pellet resuspended in 5 ml of ice-cold homogenizing buffer (JT Baker; Philipsburg, NJ; containing: 250 mM sucrose, 10 mM triethanolamine, 1 µg/ml leupeptin and 0.125 M phenylmethylsulfonylfluoride). Cells were homogenized with a glass-teflon tissue grinder (20 passes; Wheaton Science Products; Millville, NJ). Unbroken cells and nuclei were pelleted at 4,000g for 10 minutes at 4°C. The supernatant was saved and the pellet resuspended and centrifuged in the same volume of homogenization buffer as before. The collected supernatants were pooled and centrifuged at 22,000g for 20 min at 4°C. The pellet was suspended in the same buffer and centrifuged at 46,000g (Beckman SS28 rotor) for 30 minutes at 4°C. The resultant crude pellet was resuspended in 1 ml of hypotonic buffer (containing 100 mM NH4HCO3, pH 7.5; 5 mM MgCl2) and then incubated with 200 µl of Dynabeads M-280 streptavidin at 4°C overnight. Beads were collected with a magnetic plate and washed with hypotonic buffer to obtain the membrane associated fractions. The streptavidin beads carrying the enriched plasma membrane fractions were suspended in 100 mM DTT for 2 hr
according to the manufacturer’s instructions (Dynal Biotech ASA) and then centrifuged at 10,600g for 3 minutes. The supernatants were collected and used for electrophoresis and western blot analysis.

Electrophoresis and Western Blot Analysis— Protein (30 µg) was heated at 95°C for 5 minutes prior to separation in a 7.5% or 10% SDS-PAGE Tris-glycine mini-gel (Bio-Rad; Hercules, CA). Protein was transferred onto polyvinylidene (PVDF) membranes (Invitrogen, Carlsbad, CA) overnight at 4°C using a transfer buffer containing 10 mM CAPS, pH 11 in 10% methanol. Membranes were blocked overnight at 4°C with 5% non-fat dry milk in 25 mM Tris pH 7.5, 150 mM NaCl and then incubated with primary antibody for Ae2 aa 1224-1237 (5), CaII or CalV (Santa Cruz Biotechnology, Inc., CA; 1:2,000; 1:1,000; or 1:200 dilutions, respectively) in 2.5% non-fat dry milk solution at 4°C overnight. After washing with TBS containing 0.05% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated goat-anti rabbit IgG secondary antibody (Pierce) at a dilution of 1:2,500 in TBST/2.5% non-fat dry milk for 1 h at room temperature for the anti-Ae2 antibody; or with 1:20,000 donkey anti-sheep/goat IgG conjugated to horseradish peroxidase (Biodesign International, ME) for anti-CaII and anti-CalV antibodies. Labelled proteins were visualized using enhanced chemiluminescence (ECL detection kit, GE-Amersham Biosciences; Piscataway, NJ). In control experiments, the CalV and Ae2 antibodies were pre-absorbed (incubation at 4°C overnight) with 2µg/ml or 24µg/ml of the immunizing peptide, respectively (not shown). Values quoted are the means ± S.E.M. for the number of gland preparations examined.

Co-immunoprecipitation of Ae2 and Car2–Proteins— Extracts of affinity-enriched membrane protein fractions (100 µg) from Nhe1+/+ or Nhe1−/− mice were incubated overnight at 4°C with 10µg of goat polyclonal anti-CaII antibody. ImmunoPure Immobilized Protein G-agarose beads (100 µl; Pierce) previously equilibrated with PBS (pH 7.4) were added to the immunoprecipitates and rocked overnight at 4°C. Following centrifugation at 10,000g, the beads and attached immune complexes were washed 5X with ice-cold PBS pH 7.4. Beads carrying the immunoprecipitated complexes were resuspended in 50 µl 2X SDS-PAGE sample buffer and boiled 5 minutes prior to SDS-PAGE separation on a 7.5% Tris-Glycine gel. The proteins were transferred onto a PVDF membrane and blocked as described above. The blocked membrane was incubated with Ae2 primary antibody (1:2,000 dilution) overnight at 4°C and then incubated with antibody goat anti-rabbit IgG horseradish peroxidase for 1hr at room temperature. The labeled proteins were identified by the ECL detection method.

Northern Blot Analysis— Total RNA was isolated from parotid glands using Trizol reagent (Gibco BRL), followed by poly-(A) selection on an oligo (dT) cellulose column (Life Technologies, Inc.). Each sample contained glands pooled from three mice. Northern blots were prepared and hybridized as previously described (37) using a cDNA probe for Ae2 (mouse nt 1300–1776, accession number J04036). A cDNA for mouse ribosomal messenger RNA L32 (mouse nt 3078-3244, accession number K02060) was used to normalize expression between preparations (not shown). Quantitative analysis of the northern blots was performed using a BioRad Molecular Imager (Hercules, CA).

RESULTS

CI/HCO3- Exchanger Activity is Enhanced in Nhe1-Deficient Mice. Knocking out the expression of an ion transport pathway is frequently associated with up-regulation of other functionally related transport proteins (29,38). Thus, a Na+/H+ exchanger null mutation might be expected to give rise to compensatory changes to enhance the movement of CI- and HCO3- by increasing CI-/HCO3- exchanger activity. The Na+/H+ exchanger Nhe1 regulates fluid secretion by maintaining an intracellular alkaline pH, which in turn facilitates CI- uptake via the action of the basolateral CI-/HCO3- exchanger in salivary gland acinar cells (4,11,37,39).

To examine the effects of Nhe1 gene disruption on CI-/HCO3- exchanger activity we monitored the intracellular pH of parotid exocrine cells loaded with the pH-sensitive dye BCECF. An intracellular alkalinization occurs following the removal of bath CI-, as extracellular HCO3- exchanges for intracellular CI- in cells expressing anion exchange. Such an alkalinization was observed in exocrine acinar cells from both wildtype (left panel) and Nhe1-deficient (right panel) mice (Fig. 1A). CI-/HCO3- exchange was inhibited greater than 85% by the anion exchange inhibitor DIDS (filled circles) in both wildtype and Nhe1-deficient cells (Fig. 1A). Figure 1B summarizes results from experiments like those...
shown in 1A as the DIDS-sensitive component of the rate of alkalization (wildtype = 0.08±0.01 pH units/min, n = 19; Nhe1−/− = 0.30±0.06 pH units/min n = 17; p<0.001). The rate of DIDS-sensitive anion exchanger activity in Nhe1−/− mice was nearly 4-fold faster than the activity in parotid cells from wildtype animals.

Nhe2 is expressed in the apical membranes of mouse parotid acinar cells (6). The observation of reduced in vivo fluid secretion in mice deficient in either Nhe1 or Nhe2 suggested the involvement of both Na+/H+ exchangers in this process (6). However, Figure 1B shows that the rate of DIDS-sensitive Cl-/HCO3− exchanger activity in cells from Nhe2−/− mice (0.05±0.01 pH unit/min, n = 25) did not differ from that of wildtype littermates (0.04±0.01 pH unit/min, n = 12). Therefore, unlike the dramatic 4-fold enhancement of activity in Nhe1 null mice, disruption of the Nhe2 gene produced no upregulation of DIDS-sensitive Cl-/HCO3− exchanger activity.

Anion Exchanger Ae2 Transcript and Protein Levels in Nhe1-deficient Mice. The results shown in Figure 1 demonstrate that Cl-/HCO3− exchanger activity was dramatically enhanced in parotid acini from Nhe1-deficient mice. The DIDS-sensitive anion exchanger Ae2 is expressed in the basolateral membranes of salivary acinar cells (5,7,8) and its expression is upregulated in Nkce1-deficient mice (12), consistent with Ae2 being the dominant anion exchanger in this cell type. To determine whether the increased Cl−/HCO3− exchanger activity was related to increased expression of Ae2 in Nhe1−/− mice, northern blot and immunoblot analyses were performed. A small increase (20±1%, n = 4) in the level of Ae2 mRNA was detected in poly(A)-selected RNA prepared from parotid glands of Nhe1 null mice (Fig. 2A; normalized to L32 expression, not shown). However, mRNA expression does not necessarily relate directly with protein levels. The correlation between Ae2 protein expression and transport activity was evaluated with immunoblots of plasma membrane preparations from Nhe1 knockout mice (40). Figure 2B shows that the anti-Ae2 antibody detected a ~170 kDa protein in homogenates of the parotid gland (left panel) and the stomach (right panel). Preadsorption of the anti-Ae2 antibody with peptide antigen (24µg/ml) eliminated reactivity (not shown). Antibody specificity is further confirmed by the absence of Ae2 protein in homogenates from Ae2−/− stomach (30). The failure of Ae2−/− mice to survive and reach the post-weaning period (30), during which much of the postnatal development of salivary glands occurs in mice, precluded our use of this animal model to test Ae2 function in the adult parotid gland. In parallel with mRNA level (Fig. 2A), Ae2 protein expression in the salivary glands isolated from Nhe1-deficient mice increased only 11.5% (n = 3, Fig. 2B, left panel). Therefore, the nearly 4-fold enhanced anion exchanger activity observed in parotid acinar cells from Nhe1-deficient mice is not explained by increased abundance of Ae2 gene products.

Carbonic Anhydrase-dependent Cl−/HCO3− Exchange in Parotid Exocrine Cells. The lack of an obvious association between Ae2 gene expression and transporter function suggested that the enhanced Cl−/HCO3− exchanger activity in acinar cells from Nhe1-deficient mice was likely related to another mechanism. One possibility is that the enhanced anion exchanger activity reflects increased carbonic anhydrase activity. To test this possibility in the exocrine salivary gland, we examined the sensitivity of Cl−/HCO3− exchanger activity to the carbonic anhydrase inhibitor ACTZ.

Figure 3A shows that ACTZ indeed inhibits Cl−/HCO3− exchange activity in the mouse parotid gland. The summary (Fig. 3B) of experiments like those shown in Figure 3A illustrates that Cl−/HCO3− exchanger activity in parotid exocrine cells isolated from wildtype mice (open circles) was reduced ~40% by 100 µM ACTZ (filled circles). Remarkably, the component of increased anion exchanger activity observed in parotid cells from Nhe1-deficient mice was entirely dependent on carbonic anhydrase activity such that nearly 90% of the anion exchanger activity was sensitive to ACTZ (summarized in Fig. 3B). This inhibitory effect of ACTZ did not reflect direct inhibition of Ae2-mediated anion exchange. Fig. 4 shows that ACTZ inhibited Ae2-mediated DIDS-sensitive Cl−/Cl− homoexchange by only 16±2% (p<0.05). Although Cl−/Cl− exchange by Ae2 is not completely insensitive to this concentration of ACTZ as is Ae1 (22,41), this modest degree of inhibition cannot account for the observed 90% inhibition of Cl−/HCO3− exchange by ACTZ. Taken together, these results suggest that a considerable portion of the anion exchanger activity in parotid acinar cells is dependent on carbonic anhydrase activity.

Increased Expression of Carbonic Anhydrase 2 (Car2) in Parotid Glands of Nhe1-deficient Mice. The fact that the enhanced Cl−/HCO3− exchange activity in acinar cells from Nhe1-deficient mice was
both ACTZ-sensitive and was not associated with increased Ae2 expression suggested that the increased Cl-/HCO₃⁻ exchange activity in these mice may reflect increased formation of a HCO₃⁻ transport metabolon (20,42). To explore further this possibility in the exocrine salivary gland, we examined whether the enhanced Cl-/HCO₃⁻ exchanger activity in Nhe1-deficient mice might correlate with increased expression of carbonic anhydrases Car2 or Car4. The cytosolic Car2 protein appears to associate with the cytoplasmic domain of overexpressed membrane transport proteins including Cl-/HCO₃⁻ and Na⁺/H⁺ exchangers (18,19,42,43), whereas the transmembrane Car4 protein interacts with exofacial domains of these transporters (23,27). Figure 5A shows that Car2 is expressed in parotid gland homogenates (Hom), and that a considerable amount of this Car2 is associated with the plasma membrane protein fraction (PM). The expression of Car2 in the parotid glands of Nhe1-deficient mice increased in both the plasma membrane fraction (PM; 62±14%, n = 3) and gland homogenates (Hom; 25±8%, n = 4). Although Car4 was also present in parotid gland homogenates, Car4 abundance did not differ in glands from wildtype and from Nhe1-deficient mice (Fig. 5B). Pre-adsorption of the CaIV antibody with peptide antigen eliminated reactivity, confirming the immunospecificity of the CaIV antibody (not shown). Car2-dependent Cl⁻/HCO₃⁻ Exchange in Parotid Cells. CaII has been shown in heterologous expression systems to interact with anion exchanger proteins or their fusion protein products to form a HCO₃⁻ transport metabolon (17-20,22-24,44,45), but similar physical interaction has not yet been demonstrated in vivo. Here we show that a CaII-specific antibody co-immunoprecipitates Ae2 polypeptide (Fig. 6A), suggesting a robust in vivo interaction between these two proteins in salivary glands. Importantly, the abundance of recovered Car2–Ae2 complexes in the parotid cells of Nhe1⁻/⁻ mice was 57±18% higher (n = 5) than in wildtype cells (Fig. 6A, left panel).

The results of Figure 6 strongly suggest that the increased expression of Car2, and its increased interaction with Ae2, may be responsible for the 4-fold increase in the anion exchanger activity in parotid acinar cells of Nhe1⁻/⁻ mice. To further test this hypothesis, we monitored the Cl⁻/HCO₃⁻ exchanger activity in acinar clusters from Car2⁻/⁻ mice and their wildtype littermates. The right panel of Figure 6A confirms the absence of CaII protein in Car2⁻/⁻ glands and so validates the specificity of the anti-CaII antibody used in the immunoprecipitation assays. Figure 6B shows that parotid cells from Car2⁻/⁻ mice (filled circles) display levels of Cl⁻/HCO₃⁻ exchanger activity ~40% lower than parotid cells from wildtype animals (open circles), a reduction comparable in magnitude to the effect of ACTZ on exchange activity in wildtype mice (see Figure 3B). These data are consistent with an important role for Car2 in regulation of Cl⁻/HCO₃⁻ exchanger activity in mouse parotid acinar cells.

**DISCUSSION**

The purpose of the present study was to investigate in the exocrine parotid salivary gland of the mouse the relationship between the expression of Cl⁻/HCO₃⁻ exchange activity and the Na⁺/H⁺ exchangers Nhe1 and Nhe2. The basolateral Na⁺/H⁺ exchanger Nhe1 is the primary regulator of intracellular pH in mouse salivary acinar cells (10,12). According to the current fluid secretion model, Nhe1 is functionally coupled to the Cl⁻/HCO₃⁻ exchanger Ae2. Together, they exchange extracellular NaCl for intracellular protons and bicarbonate. On the other hand, Nhe2 is located in the apical membrane of exocrine cells (6) where it is less likely to be functionally linked to basolateral Cl⁻/HCO₃⁻ exchanger activity in parotid exocrine cells. However, this increase in activity in Nhe1⁻/⁻ mice was not associated with increased expression of the Cl⁻/HCO₃⁻ exchanger Ae2.

One possible explanation for this enhanced Cl⁻/HCO₃⁻ exchanger activity in the Nhe1 null mouse is increased expression of another anion exchanger. There are two gene families that express anion exchanger proteins, Scl4a and Scl26a (25,44). Of these, however, only Ae2 (Scl4a2) has to date been shown to be expressed in salivary gland acinar cells (5,7,8), suggesting that up-regulation of anion exchanger activity in the present study likely involves Ae2. In support of such a mechanism, it was previously shown that disruption of the Na⁺/K⁺/2Cl⁻ cotransporter gene Nkcc1 increased Ae2 expression and Cl⁻/HCO₃⁻ exchanger activity in parotid acinar cells (12). Therefore, it was somewhat surprising that the enhanced Cl⁻/HCO₃⁻ exchanger activity in Nhe1 knockout mice did not correspond to increased Ae2 expression. The lack of correlation between
increased anion exchanger activity and Ae2 abundance suggested that another mechanism is involved.

What then is the mechanism for the dramatic 4-fold enhancement of Cl-/HCO₃⁻ exchanger activity in parotid acini from Nhe1-deficient mice? Considerable evidence in heterologous expression systems suggests that Cl-/HCO₃⁻ exchangers bind CaII to form a HCO₃⁻ transport metabolon (45). Such an association of the catalytic enzyme CaII has been postulated by some to generate substrate near the transport site to maximize the transmembrane movement of HCO₃⁻ mediated by the anion exchanger Ae2. The reliance of maximal anion exchanger activity on CaII is apparently not the physical association, per se, but is dependent on carbonic anhydrase activity to generate HCO₃⁻ near the active substrate transport site of the exchanger (18-20,22,44). Our data are consistent with these latter observations. For example, the enhanced Cl-/HCO₃⁻ exchanger activity in Nhe1-deficient mice was entirely sensitive to the carbonic anhydrase inhibitor acetazolamide, as was much of the anion exchange in wildtype animals. Moreover, we found that mice lacking Car2 (Car2) also displayed a deficit in Cl⁻/HCO₃⁻ exchanger activity comparable to the ACTZ-sensitive proportion of anion exchanger activity in wildtype mice.

Therefore, our results support the hypothesis that the increased Cl⁻/HCO₃⁻ exchanger activity in Nhe1-deficient mice primarily reflected an increased formation of Car2-Ae2 complexes. However, we cannot rule out the possibility that another mechanism may also contribute to up-regulation. Ae2 activity in parotid acinar cells is [Ca²⁺]-sensitive such that a muscarinic-induced increase in [Ca²⁺] increases anion exchanger activity ~90% (5). Consistent with this latter observation, activation of mouse Ae2 expressed in Xenopus oocytes by hypertonic shrinkage or by ammonium is prevented by chelation of oocyte [Ca²⁺], (33). Cytosolic pH also influences intracellular Ca²⁺ metabolism, such that disruption of the gene encoding Nhe1, the primary regulator of pH in salivary acinar cells (9,37), might be expected to alter [Ca²⁺]. Specifically, intracellular acidification increases [Ca²⁺], in alveolar epithelium (46), but decreases [Ca²⁺], in cochlear hair cells (47) and rat parotid acini (48). In contrast, [Ca²⁺], mobilization in pancreatic acini appears to be independent of both Nhe1 activity and pH (49).

Nevertheless, the magnitude of the increase in anion exchanger activity in the present study was considerably larger (more than 4× greater) than the activation stimulated by an increase in the intracellular [Ca²⁺] in salivary acinar cells (5), indicating that only a relatively small fraction of the enhanced Cl⁻/HCO₃⁻ exchanger activity observed in the present studies might be related to increased [Ca²⁺].

In summary, this is the first demonstration that Car2 and Ae2 interact in vivo. Moreover, the correlation between the increased Car2-Ae2 interaction and the increased Cl⁻/HCO₃⁻ exchange activity in Nhe1-/- mice suggests that Car2 and Ae2 form a functional bicarbonate transport metabolon in parotid acinar cells. Our results demonstrate that Nhe1 gene ablation leads to a compensatory up-regulation of this HCO₃⁻ metabolism and transport pathway, likely in order to limit the extent of electrolyte and acid-base perturbations in the exocrine parotid gland. This interaction of Car2 and Ae2 in Nhe1-deficient mice is likely to be important not only in transepithelial Cl⁻ movement but also for cellular pH regulation.
REFERENCES


FOOTNOTES
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The abbreviations used are: PMSF, phenylmethyl sulfonyl fluoride; Ca, carbonic anhydrase; Car2, murine carbonic anhydrase 2; Car4, murine carbonic anhydrase 4; Nhe, Na+/H+ exchanger; Ae, anion exchanger; Nkcc, Na+/K+2Cl- cotransporter; pHo, intracellular pH; [Ca2+]i, intracellular Ca2+ concentration; BCECF-AM, 2’-7’-bis(carboxyethyl)-5-carboxyfluorescein-AM; Sulfo-NHS-SS-Biotin, Sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate; CAPS, 3-[Cyclohexylamino]-1-propanesulfonic acid; ACTZ, acetazolamide; DIDS, 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid.

FIGURE LEGENDS

Figure 1. Increased Cl-/HCO3- exchanger activity in parotid exocrine cells from Nhe1-/- mice. Intracellular pH was measured in BCECF-loaded parotid acinar cells prepared from Nhe1+/+ and Nhe1-/- mice. Panel A: Cl-/HCO3- exchanger activity was monitored as the DIDS-sensitive alkalinization that occurs after changing to a Cl--free external solution (hatched bar). Cl-/HCO3- exchanger activity in parotid acini from Nhe1+/+ (Left) and from Nhe1-/- (Right) mice in the absence (open circles) or presence of 500 µM DIDS (filled circles). Panel B: summary of the initial rates of the DIDS-sensitive Cl-/HCO3- exchanger activity in parotid acini from Nhe1+/+ (0.08±0.01 pH units/min, n = 19), Nhe1-/- (0.30±0.06 pH units/min, n = 16), Nhe2+/+ (0.04±0.01 pH units/min, n = 12) and Nhe2-/- (0.05±0.01 pH units/min, n = 25) mice. *Initial rates in parotid acini from Nhe1+/+ mice were significantly greater than in Nhe1-/- mice, p < 0.001; there was no significant difference between Nhe2+/+ and Nhe2-/- mice. There was no significant difference in the initial resting intracellular pH between Nhe1+/+ and Nhe1-/- mice (7.27±0.05 and 7.23±0.06, respectively) and between Nhe2+/+ and Nhe2-/- mice (7.24±0.08 and 7.25±0.05, respectively).

Figure 2. Anion exchanger Ae2 mRNA and protein expression in mouse parotid glands. Parotid glands were isolated and the Ae2 mRNA and protein contents determined as described in “Experimental Procedures.” Panel A: Northern blot of poly(A)-selected RNA (2.5 µg/lane) from parotid glands of wildtype (+/+) and null (-/-) Nhe1 mice detected a 4.4 kb transcript when hybridized with a rat Ae2 cDNA probe. Normalization of Ae2 expression was performed by controlling mRNA concentrations with an L32 probe (data not shown). No significant difference was detected between Nhe1+/+ and Nhe1-/- mice, n = 4. Panel B:
An anti-Ae2 antibody (1:2,000) detected an ~170 kDa protein in homogenates (30 µg/lane) prepared from parotid glands of wildtype (+/+) and null (-/-) Nhe1 mice (Left). Expression of Ae2 protein in parotid glands from Nhe1<sup>+/+</sup> mice were not significantly different than in Nhe1<sup>+/+</sup> mice, n = 3. Antibody pre-adsorbed with an Ae2 peptide antigen (24 µg/ml) failed to react (not shown). Similarly, protein isolated from the stomachs of Ae2<sup>-/-</sup> mice did not react with the anti-Ae2 antibody (Right).

**Figure 3.** ACTZ-sensitive Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity in mouse parotid glands. Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity was monitored as the alkalization that occurs after changing to a Cl-− free external solution. **Panel A:** Cl/HCO<sub>3</sub>-<sub>−</sub> exchange activity in parotid acini from Nhe1<sup>+/+</sup> mice in the absence (open circles) or presence of 100 µM ACTZ (filled circles). **Panel B:** summary of the initial rates of the Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity in parotid acini from Nhe1<sup>+/+</sup> mice in the absence (0.070±0.003 pH units/min, n = 18) or presence (0.040±0.002 pH units/min, n = 25) of 100 µM ACTZ and Nhe1<sup>-/-</sup> mice in the absence (0.270±0.006 pH units/min, n = 25) or presence (0.030±0.003 pH units/min, n = 25) of ACTZ. *Initial rates were significantly less in the presence of ACTZ, p < 0.01. There was no significant difference in the initial resting intracellular pH between Nhe1<sup>+/+</sup> and Nhe1<sup>-/-</sup> mice (7.22±0.06 and 7.22±0.04, respectively).

**Figure 4.** Mouse Ae2 is insensitive to ACTZ. **Panel A:** Traces of 36Cl<sup>-</sup> efflux from a representative oocyte expressing mouse Ae2 (filled circles) and a representative oocyte previously injected with water rather than with cRNA (open circles), both exposed sequentially to ND-96, ND-96 plus 100 µM ACTZ, and ND-96 containing 100 µM ACTZ plus 200 µM DIDS. **Panel B:** Summarized data for 16 oocytes subjected to the protocol shown in panel A. *ACTZ only modestly inhibited Ae2-mediated Cl/Cl<sup>-</sup> exchange, p < 0.05, n = 16.

**Figure 5.** Increased carbonic anhydrase II expression in parotid glands from Nhe1<sup>-/-</sup> mice. Specific antibodies to CaII and CaIV (1:1,000 and 1:200 dilutions, respectively) were used to examine carbonic anhydrase expression in parotid homogenates (Hom) and plasma membranes (PM) prepared from wildtype (+/+) and null (-/-) Nhe1 mice. **Panel A:** expression of an ~29 kDa Car2 band was detected in parotid glands from Nhe1<sup>-/-</sup> and Nhe1<sup>+/+</sup> mice. Expression of Car2 was significantly increased in parotid homogenates and plasma membranes (30 µg of protein/lane) prepared from Nhe1<sup>-/-</sup> mice [25±8% (n = 4) and 62±14% (n = 3), respectively; p<0.01]. **Panel B:** the CaIV antibody detected an ~36 kDa band in parotid plasma membrane fractions (30 µg of protein/lane) prepared from Nhe1<sup>-/-</sup> and Nhe1<sup>+/+</sup> mice. No significant changes were detected. Pre-adsorption of this antibody with 2 µg of immunizing CaIV peptide eliminated the signal (not shown, n = 3).

**Figure 6.** Increased Car2-Ae2 complex formation and decreased Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity in Car2-deficient mice. Immunoprecipitation of a Car2-Ae2 complex was performed as described in “Experimental Procedures.” Specific antibodies to Ae2 or CaII (1:1,000 and 1:200 dilutions, respectively) were used to examine protein expression in parotid plasma membranes prepared from wildtype (+/+) and null (-/-) Nhe1 and Car2 littermates. Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity was monitored as the alkalization that occurs after changing to a Cl-− free external solution in BCECF-loaded acinar cells. **Panel A:** Significantly more Ae2 was pulled down by the CaII antibody in acinar cell lysates from Nhe1<sup>-/-</sup> than from wildtype mice (Left, n = 4). Expression of a ~30 kDa protein was detected with an anti-CaII antibody in parotid glands from Car2<sup>+/+</sup> but not Car2<sup>-/-</sup> mice (Right, n = 4). **Panel B:** Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity in parotid acini from Car2<sup>+/+</sup> (open circles) or Car2<sup>-/-</sup> (filled circles) mice. Summary of the initial rates of the Cl/HCO<sub>3</sub>-<sub>−</sub> exchanger activity in parotid acini from Car2<sup>+/+</sup> mice (0.066±0.007 pH units/min, n = 12) and Car2<sup>-/-</sup> mice (0.032±0.016 pH units/min, n = 15). *Initial rates were significantly less in Car2<sup>-/-</sup> mice, p < 0.01. There was no significant difference in the initial resting intracellular pH between Car2<sup>+/+</sup> and Car2<sup>-/-</sup> mice (7.26±0.04 and 7.28±0.05, respectively).
Figure 1. Increased Cl⁻/HCO₃⁻ exchanger activity in parotid exocrine cells from Nhe1⁻⁻ mice.
Figure 2. Anion exchanger Ae2 mRNA and protein expression in parotid glands from Nhe1−/− mice.
Figure 3. Enhanced acetazolamide-sensitive Cl⁻/HCO₃⁻ exchanger activity in parotid glands from Nhe1⁻/- mice.
Figure 4. ACTZ-insensitive Cl⁻/HCO₃⁻ exchanger activity in Xenopus oocytes expressing mouse Ae2.
Figure 5. Increased carbonic anhydrase II expression in parotid glands from Nhel<sup>−/−</sup> mice.
Figure 6. Increased CaII-Ae2 complex formation and decreased Cl⁻/HCO₃⁻ exchanger activity in CaII-deficient mice.
Enhanced formation of a HCO3- transport metablon in exocrine cells of Nhe1-/- mice
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