Clen2 encodes the hyperpolarization-activated chloride channel in the ducts of mouse salivary glands

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1The Center for Oral Biology and the Department of Pharmacology and Physiology, and 2Department of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, New York; 3Centro de Estudios Científicos, Valdivia; and 4Instituto de Anatomía, Histología y Patología, Universidad Austral de Chile, Valdivia, Chile

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Romanenko VG, Nakamoto T, Catalán MA, Gonzalez-Begne M, Schwartz GJ, Jaramillo Y, Sepúlveda FV, Figueroa CD, Melvin JE. Clcn2 encodes the hyperpolarization-activated chloride channel in the ducts of mouse salivary glands. Am J Physiol Gastrointest Liver Physiol 295: G1058–G1067, 2008. First published September 18, 2008; doi:10.1152/ajpgi.90384.2008.—Transepithelial Cl− transport in salivary gland ducts is a major component of the ion reabsorption process, the final stage of saliva production. It was previously demonstrated that a Cl− current with the biophysical properties of CIC-2 channels dominates the Cl− conductance of unstimulated granular duct cells in the mouse submandibular gland. This inward-rectifying Cl− current is activated by hyperpolarization and elevated intracellular Cl− concentration. Here we show that CIC-2 immunolocalized to the basolateral region of acinar and duct cells in mouse salivary glands, whereas its expression was most robust in granular and striated duct cells. Consistent with this observation, nearly 10-fold larger CIC-2-like currents were observed in granular duct cells than the acinar cells obtained from submandibular glands. The loss of inward-rectifying Cl− currents in cells from Clcn2−/− mice confirmed the molecular identity of the channel responsible for these currents as CIC-2. Nevertheless, both in vivo and ex vivo fluid secretion assays failed to identify significant changes in the ion composition, osmolality, or salivary flow rate of Clcn2−/− mice. Additionally, neither a compensatory increase in Cflr Cl− channel protein expression nor in Cflr-like Cl− currents were detected in Clcn2 null mice, nor did it appear that CIC-2 was important for blood-organ barrier function. We conclude that CIC-2 is the inward-rectifying Cl− channel in duct cells, but its expression is not apparently required for the ion reabsorption or the barrier function of salivary duct epithelium.

inward-rectifying chloride current; barrier function; NaCl absorption

THE CIC FAMILY of Cl− channels and transporters (also known as CLCN) is comprised of nine members in mammals and can be grouped according to their mode of Cl− transport and cellular location (24). The ubiquitously expressed CIC-2 protein belongs to the group of Cl− channels that function mostly in the cytoplasmic membrane (24, 41, 46). Other members of this gene family act as intracellular Cl− channels or H+/Cl− exchangers (31). Analysis of genetic diseases in humans and of the phenotype of Clcn2 null mice suggests important physiological roles for CIC-2 in several different tissues. Human mutations in the gene encoding CIC-2 (CLCN2) have been linked to epilepsy (3, 18, 20), whereas Clcn2−/− mice develop severe degradation of the retina and the testis (6, 35). It has been hypothesized that disruption of CIC-2 expression impairs transepithelial Cl− transport of the blood-organ barrier, and this change in the environment of photoreceptors and spermatozoa leads to cell death and ultimately to blindness and infertility (6). Moreover, impairment of the blood-brain barrier has been observed in Clcn2−/− mice, although these mice do not appear to develop epilepsy (5). It has also been proposed that stimulation of CIC-2 channels can induce recovery of the epithelial barrier function of ischemia-injured ileum and colon (32, 33). The functional significance of CIC-2 for epithelial transport and barrier functions in most organs remains to be evaluated.

Three major pairs of glands (parotid, submandibular, and sublingual) produce saliva in response to autonomic stimulation (9, 30). The cells within the secretory endpieces are distinct in each gland type (in rodents: parotid-serous cells, submandibular-seromucous cells, and sublingual-mucous with serous demilune cells). Nevertheless, all salivary glands make saliva in two stages, both of which are Cl− dependent. Initially, transepithelial Cl− movement across acinar cells generates a plasma-like isotonic fluid, the so-called primary saliva. Cl− channels are the major anion efflux pathway in the apical membrane of acinar cells, whereas the basolateral Na+/coupled Cl− cotransporter Nkcc1 is the primary Cl− uptake mechanism (17). In an earlier study of mouse parotid acinar cells we characterized the properties of CIC-2-like currents (35), one of several types of Cl− currents in this cell type (2, 44). Functional analysis of gene-targeted Clcn2 null mice demonstrated that the CIC-2 channel does not appear to participate in the secretion of saliva (35). This result is consistent with the currently accepted secretory model whereby Ca2+-activated Cl− activated Cl− channels have a central role in this process (9, 29, 30).

Secondarily, salivary gland ducts reabsorb much of the Na+ and Cl− secreted by acinar cells, and because ducts are relatively impermeable to water, the final saliva is markedly hypotonic (9). NaCl reabsorption is most robust in the submandibular gland. The duct system in all salivary glands is composed of intercalated, striated, and excretory ducts, with granular duct cells being especially prominent in the submandibular glands of rodents. Although the ion transport machin-
ery is not well understood in salivary ducts, Cl− channels are thought to be required for efficient NaCl reabsorption (9, 30). Indeed, several distinct Cl− currents have been identified in salivary duct cells that might support transepithelial Cl− transport. Among them is a cAMP-activated current generated by the Cfr Cl− channel located in the apical membrane of salivary gland duct cells (21, 45). Functional studies suggest that the Cfr channel very likely contributes to NaCl reabsorption across the apical membrane (21, 44, 45). In contrast, the basolateral Cl− efflux pathway is unknown but may involve Cl− channels. Other Cl− currents expressed in duct cells include inward-rectifying, Ca2+-activated, and volume-regulated currents, as well as currents similar to those associated with CIC-0 (22, 29, 44, 45). The molecular identities of Cl− channels involved in generating these latter Cl− currents remain unknown. Of particular interest are the inward-rectifying, CIC-2-like currents observed in rat (44) and mouse submandibular duct cells (27). Currents with similar properties to CIC-2 and localized to the basolateral membranes have been postulated to support NaCl reabsorption in the intestinal tract (7, 8, 36). The inward-rectifying current in salivary gland duct cells is activated by hyperpolarization and increased intracellular Cl− concentration ([Cl−]i) (15, 27), in agreement with the requirements of a basolateral Cl− efflux pathway in a duct cell model (29). Nevertheless, neither the physiological importance of these CIC-2-like currents nor the molecular identity of the associated Cl− channel has been demonstrated in salivary gland ducts.

Here we build on our previous results in the mouse salivary gland (35) using immunolocalization, electrophysiology, and ex vivo and in vivo model systems to address the molecular basis of NaCl reabsorption in salivary gland duct cells. Comparison of wild-type and Clcn2−/− mice demonstrated that Clcn2 encodes for the inward-rectifying Cl− currents in the basolateral membrane of duct cells. Nevertheless, the Cl− currents generated by CIC-2 do not appear to play a major role in NaCl reabsorption or the barrier function of salivary gland ducts. Moreover, compensation for the loss of CIC-2 by increased Cfr expression did not occur in Clcn2 null mice.

Materials and Methods

General procedures. Clcn2 and Cfr null mice were generated as previously described (35). Clcn2−/− and Clcn2+/− littersmates 2–6 mo old were used. All experimental procedures were approved by the Animal Resources Committee of the University of Rochester. Mice were anesthetized with chloral hydrate (400 mg/kg ip), and depth of anesthesia was assessed by testing for their responsiveness to toe pinch; their respiratory rate, depth, and character; and their response to whisker manipulation. At the end of each procedure, the animal was rendered unconscious by exposure to carbon dioxide and the diaphragm and aorta were severed to ensure death. Sucrose was purchased from J. T. Baker (Philipsburg, NJ) and all other reagents were from Sigma-Aldrich (St. Louis, MO) except where indicated. Data are presented as means ± SE. Differences between means were determined by Student’s t-test with \( P < 0.05 \) considered significant. A repeated-measures analysis was carried out using SAS 9.1 for the data shown in Fig. 3A. To increase robustness of the study findings, inference for the linear model was carried out using the estimating equations approach, which does not require the normal distribution assumption for the dependent variable. We used \( \chi^2 \) tests subsequent to the overall analysis for time, group, and time-by-group comparisons.

Electrophysiological recordings. Electrophysiological data were acquired via an Axopatch 200B amplifier and a Digidata 1320A digitizer (Axon Instruments, Foster City, CA). Pipettes from Corning 8161 patch glass (Warner Instruments, Hamden, CT) were pulled to 1 mV; therefore, no correction was made. In some experiments, 0.3 mM CdCl2 or 0.5 mM DIDS was added.

To test for Ca2+-activated K+ channels the external solution contained (in mM) 150 Na-glutamate, 5 K-glutamate, 2 CaCl2, 2 MgCl2, 10 HEPES, pH 7.2; the pipette solution contained 135 K-glutamate, 5 MgCl2, 10 EGTA, 10 HEPES, pH 7.2. The level of free [Ca2+]i up to 1 \( \mu \)M in the pipette solution was obtained with CaCl2 following WEBMAXC calculation (http://www.stanford.edu/~cpatton/webmaxC.htm). We evaluated whether Ca2+-activated Cl− channels were present in granular duct cells using an external solution that contained 139 TEA-Cl, 0.5 CaCl2, 100 mM-nitritom, 20 HEPS, pH 7.3; the pipette solution contained (in mM) 140 TEA-Cl, 0.5 CaCl2, 20 HEPES, pH 7.3; the intracellular solution contained 80 NMDG-glutamate, 50 NMDG-EGTA, 30 CaCl2 and 20 HEPS, pH 7.3. The level of free Ca2+ in the latter solution was estimated to be 250 nM. cAMP-activated Cl− currents were recorded with an external solution that contained 150 NaCl, 1 CaCl2, 1 MgCl2, 10 HEPS, 20 sucrose, and 0.3 mM CdCl2, pH 7.4; the pipette solution contained 120 CsCl, 10 TEACl, 1 MgCl2, 0.5 EGTA, 1 ATP, 20 HEPS, pH 7.4. Current was activated by superfusion with 10 \( \mu \)M forskolin and 100 \( \mu \)M IBMX.

Immunohistochemistry. Mice were anesthetized by intraperitoneal chloral hydrate injection (400 mg/kg) prior to arterial perfusion (Orion Sage Syringe Pump, Thermo Fisher Scientific, Waltham, MA) via the left ventricle, initially with PBS, followed by Bouin’s fixative (75 ml of saturated picric acid, 25 ml of 40% formaldehyde, and 5 ml of glacial acetic acid) at room temperature. The salivary glands were removed and incubated 48 h in the same fixative solution and then stored in 70% ethanol at room temperature prior to being embedded in paraffin.

Sections of 5-\( \mu \)m thickness were dewaxed and hydrated before immunostaining. Pseudoperoxidase activity was eliminated by treatment with absolute methanol and 10% hydrogen peroxide. Before immunostaining, all tissues were microwaved with 50 mM Tris·HCl pH 10.0 for 5 min. The microwave’s temperature-sensitive probe was used to clamp the temperature of the antigen retrieval buffer at 90°C using a maximum power of 1,150 W. Twenty minutes after treatment, sections were washed with three changes of 50 mM Tris·HCl pH 7.8 and then incubated overnight with various dilutions of an anti-peptide antibody directed to residues 888-906 of rat ClC-2 (1:100 to 1:500). This antibody recognizes the COOH-terminus epitope RSRHGLPRE antibody directed to residues 888-906 of rat ClC-2 (1:100 to 1:500). Antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Sections were washed with three changes of 50 mM Tris·HCl pH 7.8 containing 1% immunoglobulin-free bovine serum albumin. Bound immunoglobulins were detected with the LSAB kit (Dako, Carpinteria, CA) and peroxidase was visualized by incubation with 3,3’-diaminobenzidine (Dako) for 5 min. When immunostaining was complete, the sections were rinsed with distilled water and contrasted with Harris hematoxylin for 30 s. Finally, the sections were dehydrated in ethanol, cleared with xylene, and mounted by using Canada balsam.

Electrophysiological recordings. Electrophysiological data were acquired via an Axopatch 200B amplifier and a Digidata 1320A digitizer (Axon Instruments, Foster City, CA). Pipettes from Corning 8161 patch glass (Warner Instruments, Hamden, CT) were pulled to 1 mV; therefore, no correction was made. In some experiments, 0.3 mM CdCl2 or 0.5 mM DIDS was added. The level of free [Ca2+]i up to 1 \( \mu \)M in the pipette solution was obtained with CaCl2 following WEBMAXC calculation (http://www.stanford.edu/~cpatton/webmaxC.htm). We evaluated whether Ca2+-activated Cl− channels were present in granular duct cells using an external solution that contained 139 TEA-Cl, 0.5 CaCl2, 100 mM-nitritom, 20 HEPS, pH 7.3; the pipette solution contained (in mM) 140 TEA-Cl, 0.5 CaCl2, 20 HEPES, pH 7.3; the intracellular solution contained 80 NMDG-glutamate, 50 NMDG-EGTA, 30 CaCl2 and 20 HEPS, pH 7.3. The level of free Ca2+ in the latter solution was estimated to be 250 nM. cAMP-activated Cl− currents were recorded with an external solution that contained 150 NaCl, 1 CaCl2, 1 MgCl2, 10 HEPS, 20 sucrose, and 0.3 mM CdCl2, pH 7.4; the pipette solution contained 120 CsCl, 10 TEACl, 1 MgCl2, 0.5 EGTA, 1 ATP, 20 HEPS, pH 7.4. Current was activated by superfusion with 10 \( \mu \)M forskolin and 100 \( \mu \)M IBMX.
and the current was effectively inhibited with 5 μM CFTR inhibitor CFTR(Imh)-172.

In vivo stimulated fluid secretion. Mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg body wt) prior to saliva collection as described (17). Secretion was stimulated by intraperitoneal injection of the cholinergic receptor agonist pilocarpine-HCl (10 mg/kg body wt). Gland-specific saliva was collected by isolating the ducts from the submandibular and parotid glands and inserting their distal ends into individual calibrated glass capillary tubes (Sigma-Aldrich). Body temperature was maintained at 37°C via a regulated blanket (Harvard Apparatus, Holliston, MA), and the trachea was incised to ensure a patent airway. The progression of saliva within the capillary tube was recorded at 5-min intervals. At the end of the saliva collection period, the glands were dissected, blotted dry, and weighed. Secreted saliva was expressed as microliters of saliva per minute.

Ex vivo perfused submandibular glands. The mouse submandibular gland was perfused essentially as previously described (37). Briefly, mice were anesthetized with chloral hydrate (400 mg/kg intra-peritoneal) and all branches of the common carotid artery were ligated with 4-0 silk. An aliquot of 100 μl of tissue was minced. Cells were homogenized with a glass-Teflon tissue grinder (Wheaton Science Products; Millville, NJ) in ice-cold buffer containing 2.5% sucrose, 10 mM HEPES, 1 CaCl2, and 1 MgCl2 at 37°C and gassed with 95% O2-5% CO2. The gland was perfused at a flow rate of ~0.8 ml/min via a peristaltic pump (Ismatec IPC, Glattbrugg, Switzerland). Saliva was collected from the perfused gland by inserting the main duct into a calibrated glass capillary tube. Salivation was stimulated by perfusion with the cholinergic receptor agonist carbachol (CCh, 0.1 μM) or with 0.1 μM CCh + the β-adrenergic receptor agonist isoproterenol (IPR, 5 μM). The saliva volume was recorded every minute and the flow rate expressed as microliters of saliva per minute. At the end of saliva collection, the glands were blotted dry and weighed.

To evaluate the permeability of the paracellular pathway, ex vivo submandibular glands were perfused with the above solution containing 9.7 mg/ml iohexol (Omnipaque, GE Healthcare, Amersham Division, Princeton, NJ) for 30 min to allow extracellular equilibration of iohexol. The ex vivo glands were then stimulated by the addition of 0.3 μM CCh + 5 μM IPR in the continued presence of iohexol. The concentration of iohexol in saliva was determined by HPLC analysis and expressed as micrograms per milliliter of saliva (39).

Western blot analysis. Clcn2 and Cfr wild-type and null mice were rendered unconscious by exposure to CO2 prior to exsanguination and submandibular gland removal. Cfr glands were dispersed by collagenase digestion as before (17) whereas Clcn2 glands were finely minced. Cells were homogenized with a glass-Teflon tissue grinder (Wheaton Science Products; Millville, NJ) in ice-cold buffer containing 250 mM sucrose, 10 mM triethanolamine, leupeptin (1 μg/ml), phenylmethylsulfonyl fluoride (0.1 μg/ml), and 0.5% Triton X-100. An aliquot of 100 μl of cell lysate was stored at −80°C, and the remainder was pelleted at 4,000 g for 10 min at 4°C to remove unbroken cells and nuclei. The supernatant was centrifuged at 22,000 g for 20 min at 4°C, and the pellet was resuspended in the same buffer and then centrifuged at 46,000 g (Beckman SW28 rotor) for 30 min at 4°C. The resultant crude plasma membrane pellet was stored at −80°C for electrophoresis analysis.

Clcn2 and Cfr cell lysates and plasma membrane fractions from knockout mice (60 μg/lane) were heated at 55°C for 20 min prior to separation in a 10% SDS-PAGE Tris-glycine mini-gel (Bio-Rad, Hercules, CA). Protein was transferred overnight onto polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA) by using a transfer buffer containing 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11, in 10% methanol at 4°C. Membrane was blocked overnight at 4°C with 5% nonfat dry milk in 25 mM Tris·HCl pH 7.5, 150 mM NaCl (TBS), and then incubated with anti-Cfr antibody (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1,000 in 2.5% nonfat dry milk solution at 4°C overnight. After being washed with TBS containing 0.05% Tween-20 (TBS-T), the membrane was incubated with horseradish peroxidase-conjugated goat-anti rabbit IgG secondary antibody (Pierce, Rockford, IL) at a dilution of 1:2,500 in TBS-T/2.5% nonfat dry milk for 1 h at room temperature. Labeled proteins were visualized by enhanced chemiluminescence (ECL detection kit, GE-Amersham Biosciences, Piscataway, NJ).

RESULTS

CIC-2 protein expression in salivary ducts. Immunohistochemistry was performed to evaluate the cellular and subcellular localization of CIC-2 protein in mouse submandibular and parotid glands. Submandibular salivary gland tissue from wild-type mice revealed CIC-2 staining in acinar cells and in all types of duct cells (Fig. 1A). Robust staining was observed in granular duct and striated duct cells, whereas staining in the acini and in excretory duct cells was less prominent. Tissue obtained from the Clcn2−/− mice revealed no significant immunostaining in duct cells and only weak background staining was detected in the acinar cells (Fig. 1A, inset).

At a higher magnification the subcellular staining pattern could be clearly observed in the granular and striated duct cells (Fig. 1, B and C, respectively) of the submandibular gland. Most of the staining in both types of duct cells was localized to near the basal and lateral membranes (arrows), whereas the apical membranes had no detectable staining (dental lumens are marked by asterisks).

Similar intense staining was observed in the striated duct cells of the parotid gland (Fig. 1D; parotid glands do not have granular ducts), whereas weak staining was detected in the acini and in excretory duct (not shown) cells. No specific immunostaining was detected in the parotid glands of Clcn2−/− mice (Fig. 1D, inset). Figure 1E shows a higher magnification image of a striated duct where immunostaining was localized to near the basal and lateral membranes (arrows).

Functional CIC-2 channels in granular duct and acinar cells. Preparations enriched with either single granular duct or acinar cells were prepared from submandibular glands (34, 37). Consistent with previous reports, the submandibular gland preparations from both wild-type and Clcn2−/− male mice (Fig. 2A) contained relatively big cells, termed granular duct cells, marked by the presence of large granules that are easy to visually identify and differentiate from acinar cells (15, 43). Positive identification of individual striated and excretory duct cells was not possible. Preparations from female submandibular glands did not contain granular duct cells that we could positively identify (these cells and their granules are much smaller in females), and thus preparations from male glands were used for patch-clamp experiments. The morphological identification of the granular duct cells was further confirmed electrophysiologically by the absence of Ca2+-activated K+ and Cl− channels, which are hallmark currents of acinar cells (29). In whole-cell experiments, dialysis of granular duct cells with either 250 mM or 1 μM free Ca2+ did not activate either K+ or Cl− currents (n = 3 and 4, respectively, not shown).

Expression of CIC-2-like current was observed in all submandibular granular duct cells tested from wild-type mice (n = 8; average amplitude at −120 mV was −76 ± 15 pA/pF). Figure 2B shows representative traces and plots of their current-voltage relation (I-V) of the Cl− current recorded at high intracellular [Cl−]. This current had an electrophysiological footprint, i.e., time dependence and strong inward rectification.
of the steady-state current, consistent with the previously observed endogenous Cl\(^{-}\) current in granular duct cells and with heterologously expressed ClC-2 channels (11, 15). As expected for ClC-2, the current was sensitive to Cd\(^{2+}\) but insensitive to 0.5 mM DIDS (95 ± 7% of the control current, \(n = 3, P = 0.893\)). Superfusion with 0.3 mM CdCl\(_2\) resulted in 80 ± 8% inhibition of the Cl\(^{-}\) current measured at the end of a 2-s voltage pulse to −120 mV (from −76 ± 15 to −21 ± 12 pA/pF, \(n = 7, P = 0.005\)). The current resistant to CdCl\(_2\) did not display time or voltage dependence and was similar in magnitude to the current seen in Clcn2\(^{-/-}\) mice (see below this section). Cd\(^{2+}\) block was reversible, suggesting that patched salivary gland cells remain intact under these conditions (not shown).

Submandibular acinar cells also expressed a ClC-2-like current, albeit of a significantly smaller amplitude than in granular duct cells (−9 ± 2 pA/pF at −120 mV, \(n = 6\), not shown). As expected, these currents were insensitive to 0.5 mM DIDS (99 ± 9% of the control current, \(n = 3\)) but were strongly inhibited by 0.3 mM Cd\(^{2+}\) to −3 ± 1 pA/pF (\(n = 5\)). Comparison of the amplitude of the currents in acinar and duct cells suggests that duct cells express nearly 10-fold more ClC-2-like, Cd\(^{2+}\)-sensitive current (−55 and −6 pA/pF for duct and acinar cells, respectively, at −120 mV) under identical experimental conditions.

To verify the molecular identity of the inward-rectifying Cl\(^{-}\) current we analyzed the currents in cells obtained from Clcn2\(^{-/-}\) mice. Figure 2C shows a representative current trace and I-V plot from a submandibular granular duct cell from a knockout mouse. The observed Cl\(^{-}\) current was time- and voltage-independent, and the current density amplitude was similar to that found in wild-type cells after inhibition of the ClC-2-like current with Cd\(^{2+}\). The relatively small current in the Clcn2 null cells was insensitive to Cd\(^{2+}\); the current densities before and after addition of 0.3 mM CdCl\(_2\) were −24 ± 6 pA/pF (\(n = 11\)) and −25 ± 9 pA/pF (\(n = 7\)) at −120 mV, respectively.

ClC-2-like Cl\(^{-}\) currents were also absent in all acinar cells obtained from Clcn2\(^{-/-}\) mice (\(n = 7\)). The mean current density measured at −120 mV was −3 ± 1 pA/pF. In these experiments the average seal resistance was 3.3 ± 0.5 GΩ and the average cell capacitance was 15 ± 2 pF. Therefore, the measured current density in Clcn2\(^{-/-}\) mice is equivalent to the expected background current from patch-clamp measurements with multigigaohm seals.

In vivo stimulated fluid secretion from submandibular glands. The above results demonstrate that several types of salivary duct cells express ClC-2 protein (Fig. 1), and the ClC-2-like currents were confirmed to be due to ClC-2 channel expression in the granular duct cells of submandibular glands using Clcn2 knockout mice (compare Fig. 2, B vs. C). Consequently, because ClC-2-like currents in salivary gland duct cells were abolished in Clcn2\(^{-/-}\) mice, we could evaluate the physiological importance of these Cl\(^{-}\) channels for ductal NaCl reabsorption.

We demonstrated in our previous study that a null mutation of the Clcn2 gene did not impair the secretion of whole saliva (35). It is important to note that whole saliva is primarily composed of secretions from the three pairs of major glands, the morphology and functional properties of which are unique. Approximately 90% of whole saliva in mice is generated by the two largest of these paired glands, the parotid and submandibular. This earlier study did not evaluate whether the function of individual salivary glands were altered in Clcn2 null mice. Consequently, to determine whether Clcn2 gene ablation affects secretion of the submandibular and/or parotid glands, and to exclude possible compensation of one of the glands for the loss of secretion from the other gland, we collected in vivo ductal saliva from individual mouse submandibular and parotid glands. Figure 3 shows that the volume of fluid secreted by the...
submandibular (Fig. 3A) and parotid (Fig. 3B) glands of Clcn2 knockout and wild-type animals were not statistically different at individual time points (Student’s t-test). To increase robustness of the study findings, inference for the repeated-measures analysis was also carried out for the submandibular duct data by the estimating equations approach, which does not require the normal distribution assumption for the dependent variable. Although the overall analysis of main effects and interaction failed to reveal significant differences, inspection of the curves led us to believe that differences between the wild-type and knockout mice, although not present at the earlier time points, may be present at the later end of the observation period. This was true, where tests at 10 and 15 min find the glands not significantly different, whereas at 20 min and later the differences were significant. These P values do not reflect a correction for multiple comparisons, although when using a Bonferroni adjustment comparisons at 25 and 30 min remain significantly different (P ≤ 0.01).

Thus the results in left panels of Fig. 3 demonstrate that CIC-2 channels do not play a major role in the fluid secretion mechanism of either submandibular or parotid acinar cells. Furthermore, we found no effect of Clcn2 ablation on the ion composition or the osmolality of secreted saliva (right panels of Fig. 3), suggesting that the CIC-2 channel does not appear to be required for NaCl reabsorption by the salivary ducts in either gland. However, we have shown previously that in vivo saliva production in response to systemic administration of a broad-range cholinergic secretagogue (pilocarpine) may in some cases not faithfully reflect perturbations of the saliva production machinery (37). To circumvent this concern, we used an ex vivo perfused submandibular gland preparation in the following section.

Ex vivo stimulated fluid secretion from perfused submandibular glands. To directly test the function of the CIC-2 channel for NaCl reabsorption, an ex vivo perfused submandibular gland preparation was used that not only eliminates circulating factors and central neural inputs that complicate the interpretation of in vivo experiments but also permits control of the ion composition and agonist concentration of the perfusate. The ex vivo preparation was found to be a sensitive and robust model system that produces results consistent with data obtained by other functional methods (37).

Figure 4 illustrates the saliva secretion rate and ion composition in response to perfusion of the ex vivo submandibular gland with agonists under two different conditions. Figure 4A shows results from experiments with the muscarinic agonist CCh, whereas Fig. 4B is from experiments using carbachol with IPR to stimulate β-adrenergic receptors and raise the intracellular cAMP content of duct cells (12, 16) and thus further enhance Cl− reabsorption through the activation of Cfr (25, 38). As expected, the addition of IPR enhanced Cl− uptake from saliva by ~50% in wild-type glands, and to a comparable magnitude in Clcn2 null glands. None of the saliva parameters that we monitored (flow rate, ion concentration, or osmolality) were significantly affected by ablation of the CIC-2 channel with either type of agonist stimulation. Thus CIC-2 does not appear to be required for fluid secretion or Cl− reabsorption by salivary ducts.

Cfr channels in submandibular cells. An alternate possibility for the apparent lack of effect of the Clcn2 null mutation on Cl− reabsorption is that another Cl− channel(s) may have been upregulated to compensate for the loss of CIC-2 in duct cells. Duct cells express Cfr, a cAMP-activated Cl− channel involved in NaCl absorption and fluid secretion. Cfr has been localized to the apical membrane of salivary gland duct cells. It is assumed that Cfr acts as the apical Cl− uptake pathway for transudctal Cl− absorption (13, 45). Thus, if compensatory
mechanisms are activated in the Clcn2 null gland, it might be predicted that both the apical (Cftr) and an alternative basolateral Cl− flux pathway are upregulated to compensate for loss of the basolateral CIC-2 Cl− channel.

To test this possibility we compared the level of Cftr expression in the submandibular salivary glands of wild-type and Clcn2 null mice. Figure 5A shows that there was no apparent change in the levels of Cftr protein expression in whole-cell lysates (WC) or in the plasma membranes (PM) of Clcn2 null glands. Note also that there was no detectable protein signal in Cftr null mice (far right lanes), verifying the specificity of the antibody. Incubation of duct cells with a Cftr activation cocktail containing forskolin and IBMX to increase the intracellular cAMP level readily upregulated Cl− current with characteristic properties of Cftr, i.e., time- and voltage-independence and sensitivity to the Cftr-specific inhibitor CFTR(Inh)-172 (Fig. 5B). Moreover, there was no significant change in the kinetic properties of the cAMP-activated Cl− current in Clcn2 null mice (Fig. 5C). The magnitudes of the current at +100 mV in the granular duct cells of the submandibular glands of Clcn2 null mice were comparable to those found in wild-type cells (Clcn2 wild-type, 63.4 ± 12.6 pA/pF; n = 5; Clcn2 null, 83.8 ± 26.9, n = 10, P > 0.6). Together, these results suggest that an increase in Cftr expression did not appear to compensate for the loss CIC-2 expression in duct cells. Cftr-like currents were not detected in acinar cells from wild-type or Clcn2 null mice (not shown).

Tight junction permeability in submandibular glands. Finally, it has been hypothesized that CIC-2 regulates the trans-epithelial transport and barrier properties of different organs (6, 35). Here we tested whether paracellular permeability was altered in Clcn2 null mice using iohexol as an index of tight junction permeability in the ex vivo submandibular gland. Iohexol is a nonionic contrast agent with a molecular weight of 821 Da used as an extracellular indicator to monitor glomerular filtration rate (1, 42) and for detecting epithelial barrier defects in the intestines (1). Iohexol inclusion in the perfusate did not alter the stimulated flow rate or the ion composition of the collected saliva (not shown). Moreover, there was no change in the iohexol concentration in the saliva collected from the submandibular glands of wild-type and Clcn2 null mice (Clcn2 wild-type, n = 7, 122.8 ± 40.7 µg iohexol/ml of saliva; Clcn2 null, n = 10, 115.8 ± 39.9 µg iohexol/ml of saliva, P > 0.9). These results indicate that the paracellular permeability was apparently not altered in Clcn2 null mice.

**DISCUSSION**

Transepithelial Cl− movement drives both fluid secretion and NaCl reabsorption. The role of the CIC-2 Cl− channel in these two separate functions of epithelia remains controversial. The direction of Cl− movement determines whether an epithelial cell is secretory or absorptive, which is dictated by the basolateral and apical locations of the various Cl− transport pathways in the plasma membrane. Thus, to understand the function of the CIC-2 Cl− channel in epithelia, its subcellular localization has to be determined. In secretory epithelia, the basolateral Na+/K+/2Cl− cotransporter is the primary Cl− uptake mechanism (17, 19), whereas an apical Cl− channel acts as the efflux pathway. Therefore, if CIC-2 Cl− channels
are located in the apical membrane of secretory cells, then activation of the inward-rectifying Cl\(^{-}\) current generated by this channel would lead to transepithelial Cl\(^{-}\)/H\(^{+}\) movement and fluid secretion. In agreement with this secretion model, ClC-2 has been localized to the apical membrane of human intestinal cells (10, 28), mouse lung epithelia (4), and the parietal cells of rabbit stomach (40). However, our results using Clcn2 null mice demonstrate that the ClC-2 channel was diffusely expressed in acinar cells and did not appear to specifically target to the apical membrane. Consistent with this observation, functional studies with the Clcn2 knockout mice showed that only subtle changes in salivary gland fluid secretion was observed in either the parotid or submandibular gland (Figs. 3 and 4). Thus the ClC-2 Cl\(^{-}\)/H\(^{+}\) channel does not appear to be targeted to the apical membrane of secretory cells in salivary glands, and disruption of the Clcn2 gene indicated that it is not likely to be involved in fluid secretion.

In contrast to reports in different cell types (4, 40), CIC-2 was localized to the basolateral membrane of surface colonic cells and villus duodenal enterocytes (36), results that were confirmed by using Clcn2 null mice (36). Basolateral localization in these cells suggests that CIC-2 channel may be involved in NaCl absorption by the gastrointestinal tract. Taking into consideration the limits of immunolocalization methods, CIC-2 appears to be targeted to the basolateral membranes in the duct cells of mouse submandibular and parotid glands. This raised the possibility that CIC-2 regulates NaCl absorption in salivary glands, acting as the basolateral efflux pathway. Examination of the biophysical and pharmacological footprint of the Cl\(^{-}\) current in the submandibular granular duct cells of wild-type mice revealed that it was comparable to that described for the CIC-2 channel (24, 41). Moreover, this current was absent in granular duct cells from Clcn2\(^{-/-}\) mice, which agreed with the disappearance of CIC-2 immunostaining in the Clcn2 null mice. It should be noted that the magnitude of the CIC-2-like current in granular duct cells was more nine times larger than that found in acinar cells. This observation is consistent with the much more intense immunostaining of the granular duct cells and with CIC-2 playing an important functional role in duct cells.

In the currently accepted saliva production model, the duct system modifies the concentration of electrolytes of the isotonic plasmalike primary saliva secreted by acinar cells. Salivary duct epithelium reabsorbs most of the Na\(^{+}\)/H\(^{+}\) and Cl\(^{-}\)/H\(^{+}\) secreted by acinar cells, and because the duct is relatively impermeable to water the final saliva is hypotonic. The transepithelial flux of Cl\(^{-}\) is supported by distinct transport pathways in the apical and basolateral membranes of the duct cells. The main route for Cl\(^{-}\) absorption across the lumen appears to be through Cfr, an apical cAMP-activated Cl\(^{-}\) channel. Indeed, we found that increasing the intracellular cAMP content both activated Cfr-like currents in the duct cells (Fig. 5, A and B) and enhanced Cl\(^{-}\) uptake (compare Fig. 4, A to B). In contrast, the mechanism by which Cl\(^{-}\) efflux is mediated across the
Fig. 5. Functional expression of Cftr channel protein and cAMP-activated current in submandibular granular duct cells. A: Western blot analysis demonstrating the expression level of Cftr channel protein in the submandibular glands of Clcn2 wild-type (+/+), Clcn2 null (−/−), and Cftr null (−/−) mice. Lanes were loaded with either whole-cell lysate (WC) or plasma membrane fraction (PM). The approximate molecular weight of Cftr is 168 kDa. B: a representative cAMP-activated, Cftr-like current recorded in the presence of 0.3 mM Cd2+ to inhibit the inward-rectifying current. Insets: raw currents recorded in response to 2-s voltage steps from −100 to +100 mV in 20 mV increments from 0 mV holding potential. The currents were recorded before (top left) and after addition of a Cftr activation cocktail (+Forsk+IBMX), and after superfusion with CFTR inhibitor CFTR(Inh)-172 in the continued presence of the Cftr activation cocktail (right). Main panel: current-voltage relation constructed from the raw currents. C: same as in B but the cells were obtained from a Clcn2+/− animal. Calibration bars for current amplitude and time: 0.5 nA and 0.5 s, respectively.

The approximate molecular weight of Cftr is 168 kDa. The functional expression of Cftr channel protein and cAMP-activated current in submandibular granular duct cells is shown in Fig. 5. A Western blot analysis demonstrates the expression level of Cftr channel protein in the submandibular glands of Clcn2 wild-type (+/+), Clcn2 null (−/−), and Cftr null (−/−) mice. Lanes were loaded with either whole-cell lysate (WC) or plasma membrane fraction (PM). The approximate molecular weight of Cftr is 168 kDa. A representative cAMP-activated, Cftr-like current recorded in the presence of 0.3 mM Cd2+ to inhibit the inward-rectifying current. Insets: raw currents recorded in response to 2-s voltage steps from −100 to +100 mV in 20 mV increments from 0 mV holding potential. The currents were recorded before (top left) and after addition of a Cftr activation cocktail (+Forsk+IBMX), and after superfusion with CFTR inhibitor CFTR(Inh)-172 in the continued presence of the Cftr activation cocktail (right). Main panel: current-voltage relation constructed from the raw currents. C: same as in B but the cells were obtained from a Clcn2+/− animal. Calibration bars for current amplitude and time: 0.5 nA and 0.5 s, respectively.

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For example, an additional basolateral Cl⁻ transport pathway and/or a paracellular Cl⁻ permeability pathway may have been upregulated to compensate for the loss of CIC-2 expression. Future studies are required to address the lack of supporting evidence of such pathways in salivary gland ducts.

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