

Secretion and cell volume regulation by salivary acinar cells from mice lacking expression of the *Clcn3* Cl⁻ channel gene

Jorge Arreola*†‡, Ted Begenisich*‡, Keith Nehrke*†, Ha-Van Nguyen*†, Keerang Park*†, Linda Richardson†, Baoli Yang§, Brian C. Schutte§, Fred S. Lamb§ and James E. Melvin†

† Center for Oral Biology in the Aab Institute of Biomedical Sciences and ‡ Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY 14642, and § Department of Pediatrics, University of Iowa, Iowa City, IA 52242, USA

Salivary gland acinar cells shrink when Cl⁻ currents are activated following cell swelling induced by exposure to a hypotonic solution or in response to calcium-mobilizing agonists. The molecular identity of the Cl⁻ channel(s) in salivary cells involved in these processes is unknown, although ClC-3 has been implicated in several tissues as a cell-volume-sensitive Cl⁻ channel. We found that cells isolated from mice with targeted disruption of the *Clcn3* gene undergo regulatory volume decrease in a fashion similar to cells from wild-type littermates. Consistent with a normal regulatory volume decrease response, the magnitude and the kinetics of the swell-activated Cl⁻ currents in cells from ClC-3-deficient mice were equivalent to those from wild-type mice. It has also been suggested that ClC-3 is activated by Ca²⁺-calmodulin-dependent protein kinase II; however, the magnitude of the Ca²⁺-dependent Cl⁻ current was unchanged in the *Clcn3*^{-/-} animals. In addition, we observed that ClC-3 appeared to be highly expressed in the smooth muscle cells of glandular blood vessels, suggesting a potential role for this channel in saliva production by regulating blood flow, yet the volume and ionic compositions of *in vivo* stimulated saliva from wild-type and null mutant animals were comparable. Finally, in some cells ClC-3 is an intracellular channel that is thought to be involved in vesicular acidification and secretion. Nevertheless, the protein content of saliva was unchanged in *Clcn3*^{-/-} mice. Our results demonstrate that the ClC-3 Cl⁻ channel is not a major regulator of acinar cell volume, nor is it essential for determining the secretion rate and composition of saliva.

(Received 5 April 2002; accepted after revision 28 August 2002; first published online 27 September 2002)

Corresponding author J. E. Melvin: Center for Oral Biology, University of Rochester, Medical Center Box 611, 601 Elmwood Avenue, Rochester, New York 14642, USA. Email: james_melvin@urmc.rochester.edu

It has been proposed that the ClC-3 channel, one of nine members of the mammalian Cl⁻ channel (CLC) gene family, produces the swelling-activated Cl⁻ currents present in most, if not all mammalian cells (Duan *et al.* 1997). Expressed ClC-3 channels exhibit a weak outwardly rectifying steady-state current–voltage (*I*–*V*) relationship, inhibition by phorbol esters and time-dependent inactivation at very positive voltages (Kawasaki *et al.* 1994; Duan *et al.* 1997), much like the swell-activated Cl⁻ currents found in most native cells (Strange *et al.* 1996). In agreement with these expression studies, both antisense oligonucleotides and intracellular dialysis with an anti-ClC-3 antibody decrease the magnitude of the swell-activated Cl⁻ currents in bovine epithelial and canine pulmonary arterial smooth muscle cells (Wang *et al.* 2000; Duan *et al.* 2001). In contrast, others have shown that ClC-3 currents display outward rectification and are neither activated by osmotic swelling (Ogura *et al.* 2002) nor inhibited by phorbol esters (Li *et al.* 2000), properties that are comparable to those exhibited

by ClC-4 and ClC-5 (Friedrich *et al.* 1999; Li *et al.* 2000), which are intracellular CLCs with the greatest homology to ClC-3. Other investigators were unable to record Cl⁻ currents or enhanced Cl⁻ permeability after heterologous expression of ClC-3 (Steinmeyer *et al.* 1995; Stegen *et al.* 2000; Weylandt *et al.* 2001).

Although ClC-3 channels can apparently target the plasma membrane under some conditions (Huang *et al.* 2001; Weylandt *et al.* 2001), cell fractionation studies indicate that ClC-3 is primarily an intracellular channel located in the late endosomal compartment of liver cells, as well as synaptic vesicles in the brain, where in this latter tissue it may play a critical role in intravesicular acidification. Indeed, disruption of ClC-3 expression leads to postnatal degeneration of the hippocampus and the retina (Stobrawa *et al.* 2001). Dickerson *et al.* (2002) described generally similar age-dependent retinal and neural pathology in an independently generated *Clcn3*-null mutant mouse, and this group has also described other major findings

* These authors contributed equally to this study.

including astrogliosis, abnormal gait, kyphosis and altered vascular reactivity (Dickerson *et al.* 2001, 2002).

Stobrawa *et al.* (2001) also demonstrated that targeted disruption of the *Clcn3* gene has no effect on the swell-activated Cl^- current in pancreatic acinar cells and hepatocytes. In agreement with these observations, immunofluorescence studies show that ClC-3 is expressed primarily in the canalicular membranes of hepatocytes (Shimada *et al.* 2000). It is interesting to note that ClC-Ka and ClC-Kb, two kidney-specific members of the ClC gene family, target the plasma membrane only in the presence of Barttin, an apparent β -subunit (Estévez *et al.* 2001). Moreover, a splice variant of ClC-3 contains a domain that interacts with scaffolding proteins, which enhances targeting of ClC-3 to the plasma membrane (Ogura *et al.* 2002). These latter studies may explain the conflicting ClC-3 functional results; observed differences among various tissues and expression systems may reflect different expression levels of a chaperone protein or the ClC-3 splice variant, resulting in tissue- and cell-specific localization patterns for ClC-3. Moreover, Cl^- currents activated by Ca^{2+} -calmodulin-dependent protein kinase II appear when human ClC-3 is stably expressed in epithelial cells (Huang *et al.* 2001). With regard to this latter finding, activation of a Ca^{2+} -gated Cl^- channel is critical for initiating secretion by salivary acinar cells (Begenisich & Melvin, 1998). Thus, the function of ClC-3 may vary in different cell types depending on the targeting and the expression of regulatory proteins.

This raises the possibility that ClC-3 Cl^- channels in salivary gland acinar cells play a role in fluid secretion and/or volume regulation by directly contributing to or modulating Cl^- channel activity in the plasma membrane. Alternatively, ClC-3 may be localized to an intracellular compartment in acinar cells where it could regulate salivary protein secretion. To test these hypotheses, *Clcn3*-gene-targeted knockout mice were studied. Cell volume regulation, swell-activated Cl^- currents, and Ca^{2+} -dependent Cl^- currents were unaltered in *Clcn3*^{-/-} mice. ClC-3 also appeared to be highly expressed in the smooth muscle layer of blood vessels in the gland, indicating that ClC-3 may regulate salivation by modulating blood flow. However, in response to *in vivo* cholinergic stimulation, the protein content, ionic composition and the volume of saliva were unchanged. These results indicate that the ClC-3 Cl^- channel is not a major participant in cell volume regulation or secretion of saliva by acinar cells. Portions of this work have been published previously in abstract form (Melvin *et al.* 2000).

METHODS

Clcn3 mutant mice

Targeted disruption of the murine *Clcn3* gene was performed as described previously (Dickerson *et al.* 2002). Heterozygous 129/SvJ-C57BL/6 offspring were used to establish breeding

colonies at the University of Rochester vivarium. Animal breeding was performed as approved by the University of Rochester Committee on Animal Resources (IACUC). All animals were housed in micro-isolator cages and had access to laboratory chow and water *ad libitum*, with a 12 h:12 h light:dark cycle. A total of 459 pups were born in our University of Rochester facility. Of these, 115 (25.1%) were wild-type, 268 (58.4%) were heterozygous and 76 (16.6%) were homozygous for the null mutation. As described previously (Dickerson *et al.* 2002), many of the knockout mice die due to an unknown cause (30 out of 76 died by 6 weeks of age). Homozygous *Clcn3*^{-/-} mice exhibited reduced body weight. Salivary gland wet weights were comparable, with the exception of the submandibular gland, which was significantly smaller in *Clcn3*^{-/-} male mice.

In our experiments, where animals 8–20 weeks old were used, the body weight of *Clcn3*^{+/+} mice for males was 30.8 ± 2.0 g ($n = 11$) and 21.1 ± 1.6 g ($n = 6$) for females, whereas the body weight for male *Clcn3*^{-/-} mice was significantly less at 22.1 ± 1.8 g ($n = 7$; $P < 0.008$) and 17.8 ± 0.9 g ($n = 5$; $P < 0.12$) for females. The salivary gland weights were, for parotid glands: in *Clcn3*^{+/+} mice 76.3 ± 8.0 mg ($n = 9$) for males and 67.0 ± 13.7 mg ($n = 5$) for females and in *Clcn3*^{-/-} mice 67.7 ± 5.3 mg ($n = 5$) for males and 63.5 ± 6.2 mg ($n = 4$) for females; for submandibular glands: in *Clcn3*^{+/+} mice 168.1 ± 26.3 mg ($n = 5$) for males and 67.8 ± 8.1 mg ($n = 3$) for females; and in *Clcn3*^{-/-} mice 104.4 ± 24.5 mg ($n = 3$) for males and 63.5 ± 2.8 mg ($n = 4$) for females; and for sublingual glands: in *Clcn3*^{+/+} mice 20.1 ± 4.1 mg ($n = 5$) for males and 18.0 ± 1.2 mg ($n = 3$) for females; and in *Clcn3*^{-/-} mice 16.0 ± 3.5 mg ($n = 3$) for males and 23.4 ± 2.0 mg ($n = 4$) for females. The mouse submandibular gland is generally two- to threefold larger in males due to the expression of testosterone-sensitive granular convoluted duct (GCD) cells. However, morphological examination of four wild-type and four null mutant mice demonstrated that the decreased size of the submandibular gland in male *Clcn3*^{-/-} mice could not be explained by the loss of GCD cells (data not shown). The number and size of these cells appeared normal, although detailed morphometric analyses were not performed.

Acinar cell preparation

Parotid acini from adult *Clcn3*^{+/+} and *Clcn3*^{-/-} gender- and age-matched littermates were isolated as described previously (Evans *et al.* 2000) using a protocol approved by the IACUC. Briefly, mice were killed by exsanguination following exposure to CO_2 gas, and the glands were removed and finely minced in digestion medium (Eagle's modified essential medium, EMEM; Biofluids, Rockville, MD, USA) containing collagenase P (0.3 mg 7.5 ml^{-1} per animal; Boehringer Mannheim, Germany) and 1% bovine serum albumin (BSA). The minced glands were incubated at 37°C in a shaker for 80 min and dispersed by gentle pipetting every 20 min. At the end of the digestion period, the acinar preparation was resuspended in EMEM and incubated at 22°C in 5% CO_2 -95% O_2 .

Single cells were isolated from parotid glands for patch-clamp experiments by digesting for 10 min with 0.02% trypsin, followed by 60 min digestion with 0.04 mg ml^{-1} collagenase P (Arreola *et al.* 1995). Cells were resuspended in Eagle's basal medium (Life Technologies) containing 2 mM glutamine with penicillin and streptomycin, attached to poly-L-lysine-coated coverslips, and incubated at 37°C in 5% CO_2 /95% air.

Electrophysiological analysis

The whole-cell configuration of the patch-clamp technique was used to record currents from single parotid acinar cells. Glass

pipettes were fabricated to have 3–4 M Ω resistance when filled with the internal solution. Recordings were performed at room temperature (22°C) using either an Axopatch 1-D or 200B amplifier (Axon Instruments, Foster City, CA, USA). Measured junction potentials for the solutions used were less than 3 mV and so no correction was applied.

Currents through swell-activated Cl⁻ channels were recorded in cells dialysed with a pipette solution containing (mM): tetraethylammonium chloride 140, EGTA 10, MgATP 5, Hepes 20, pH 7.3. Cells were bathed in a solution containing (mM): NMDG-Cl 140, CaCl₂ 0.5, Hepes 10, D-mannitol 50, pH 7.4. Hypotonic and hypertonic solutions were made by omitting D-mannitol and by increasing the D-mannitol concentration to 100 mM, respectively. Swell-activated Cl⁻ channels were activated by exposing cells to the hypotonic solution ~5 min after breakthrough. Data acquisition was performed using a 12 bit analog/digital converter controlled by a personal computer. Currents were filtered at 1 kHz using a four-pole low-pass Bessel filter and then sampled at 5 kHz. Voltage-clamp steps of 0.75 s duration were applied in 20 mV increments from -100 to +100 mV from a -50 mV holding potential.

Currents through Ca²⁺-dependent Cl⁻ channels were measured with an external solution containing (mM): KCl 5, NaCl 120, CaCl₂ 2, MgCl₂ 0.8, glucose 10, Hepes 10, pH 7.4. In order to minimize swell-activated currents, the tonicity of this solution was increased with 30 or 70 mM mannitol. The internal solution used for these recordings contained (mM): HCl 45.8, glutamic acid 80, NMDG 133, CaCl₂ 7.1, EGTA 10, Hepes 20, pH 7.2 (with NMDG). The free Ca²⁺ concentration of this solution was computed to be 400 nM according to the Sliders version 2.00 computer program (Bers *et al.* 1994). Voltage-clamp steps of 3 s duration from -30 to +90 mV were applied from a -50 mV holding potential.

Immunohistochemistry

Parotid glands from *Cln3*^{-/-} (negative control) and wild-type animals were removed and incubated overnight in 4% paraformaldehyde at 4°C. Paraformaldehyde-treated tissue was paraffin-embedded and sectioned at 5 μ m. Deparaffinized sections were incubated overnight at 4°C in PBS, 0.8% BSA, 0.1% gelatin, 0.1% Triton X-100, and 5% normal goat serum containing a 1:250 dilution of an anti-CIC-3 antibody (Alomone Laboratories, Israel), and then treated with 1:1000 Alexa-594 fluor-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA) diluted in the above buffer for 1 h at room temperature. Images were recorded and analysed using a Nikon E800 upright microscope equipped with a fluorescent source and a Spot2 CCD camera.

Western blot analysis

Parotid gland homogenates were prepared from *Cln3*^{-/-} and wild-type animals to identify native CIC-3 protein. Tissues were prepared as described previously by Turner *et al.* (1986). Glands were homogenized twice by 10 s strokes at power level 5 with a polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) in 5 ml (g tissue)⁻¹ of homogenization solution containing 10 mM Hepes adjusted to pH 7.4 with Tris, 10% sucrose, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), with one tablet of COMPLETE protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) per 50 ml. Homogenates were centrifuged at 2500 g for 15 min at 4°C and the supernatants saved. The pellets were resuspended in 5 ml of homogenization buffer per gram of starting tissue, then homogenized and recentrifuged as

above. The supernatants were combined and crude membrane proteins were precipitated by centrifugation at 22 000 g for 20 min at 4°C. The supernatants from this step were discarded and the pellets were resuspended in PBS containing 1 mM EDTA, 1 mM PMSF, and COMPLETE protease inhibitor (1 tablet per 50 ml) then passed once through a 25-gauge needle and once through a 30-gauge needle. Aliquots were quickly frozen in liquid N₂ and stored at -85°C until use. Approximately 100 mg of crude membrane protein was separated by two-phase tricine (T) polyacrylamide gel electrophoresis (10%T/6%C resolving layer, 4%T/3%C stacking layer), and transferred onto nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA) in buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid adjusted to pH 11 and 10% methanol. The blot was blocked overnight at room temperature in PBS blocking buffer containing 1% Tween-20, 4% BSA, and 1% normal goat serum. After blocking, the blot was incubated for 2 h at room temperature in blocking buffer containing a 1:250 dilution of a rabbit anti-CIC3 polyclonal antibody (Alomone Laboratories), washed three times with PBS-T (PBS containing 1% Tween-20), incubated for 1 h at room temperature in blocking buffer containing a 1:5000 dilution of HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and again washed three times with PBS-T. Immune complexes were detected on film using enhanced chemiluminescence (Amersham Pharmacia Biotech).

RNA analysis

Total RNA was prepared from enzymatically dispersed parotid acinar cells from adult *Cln3*^{+/+} and *Cln3*^{-/-} littermates using Trizol reagent (Life Technologies) and analysed by RT-PCR as described previously (Park *et al.* 1999). Four overlapping RT-PCR primers were designed to amplify the CIC-3 message from isolated parotid acinar cells including two sense primers (U1 and U2) complementary to codons 34–40 and 384–391, respectively, and two nonsense primers corresponding to codons 209–214 and 590–597 (L1 and L2, respectively). The PCR products were inserted into the pCR2.1 TA cloning vector (Invitrogen) and sequenced using the T7 Sequenase 7-deaza-dGTP DNA sequencing kit (Amersham Life Science).

Cell volume determinations

Cell volume was estimated by confocal microscopy, as described previously (Schreiber *et al.* 1999). The dispersed acini were loaded with the fluoroprobe calcein by incubation for 15 min at room temperature in 2 μ M calcein-AM (Molecular Probes). Acini were allowed to adhere to the base of a superfusion chamber that was mounted on an Olympus PMT2 fluorescence microscope interfaced with an Ultima confocal microscope (Genomic Solutions, Ann Arbor, MI, USA). Intracellular dye was excited with the 488 nm band of an argon laser and emitted fluorescence was measured at 530 nm. Changes in cell volume were monitored by measuring the fluorescence intensity of intracellular calcein within a user-defined volume. In combination with an Olympus DplanApo \times 40 objective, a 225 μ m confocal pinhole produces an approximately 4- μ m-thick optical section in the z-direction. Using Ultima software, an x-y area of the two-dimensional image was circumscribed within individual acini. Cell volume is expressed in arbitrary units as 1/normalized calcein fluorescence (1/calcein Fn).

Stimulated flow rates and saliva composition

Adult age- and gender-matched littermates (*Cln3*^{+/+}, *n* = 17; and *Cln3*^{-/-}, *n* = 12) were anaesthetized with 300 mg chloral hydrate (kg body weight)⁻¹ (i.p.) and then stimulated with 10 mg

pilocarpine-HCl (kg body weight)⁻¹ (I.P.). Whole saliva was collected, primarily representing a combination of parotid and submandibular gland secretions, with a very minor component from sublingual, minor salivary, nasal and tracheal glands. Saliva was collected from the lower cheek pouch by a suction device at intervals of 5, 10 and 15 min, and volumes are expressed in microlitres per 5 minutes. Time zero was designated as the point in time when saliva was first noted, typically about 1 min after pilocarpine injection (mice have little or no resting saliva flow). The protein concentration of saliva was estimated using the

Bradford method (Pierce Chemical, Rockford, IL, USA). Collected saliva samples were analysed for total Na⁺ and K⁺ content by atomic absorption using a Perkin-Elmer 3030 spectrophotometer. Sample osmolality was measured using a Wescor 5500 vapour pressure osmometer, and Cl⁻ activity was estimated using an Orion EA 940 expandable ion analyser.

Statistical analyses

Experiments were repeated using preparations from at least three separate animals in each group. Data were analysed by a two-tailed Student's *t* test, and differences between test and control values at *P* < 0.05 were considered to be statistically significant.

RESULTS

Loss of ClC-3 expression in mouse parotid cells

The ClC-3 Cl⁻ channel is highly expressed in many major organ systems, including brain, lung, spleen, adrenal glands and kidney (Kawasaki *et al.* 1994). To verify the loss of wild-type ClC-3 Cl⁻ channel expression in salivary glands, Western blot analysis was performed. Targeted deletion of the *Clcn3* gene results in the excision of exons 6 and 7, which contain the coding sequence for transmembrane domains 2–4, regions that are critical for channel function (Dickerson *et al.* 2002). Deletion of this fragment of *Clcn3* in the *Clcn3*^{-/-} mouse resulted in the absence of a protein band of the appropriate apparent molecular mass for ClC-3 (Fig. 1, upper panel). An additional weaker band that migrated at a higher apparent molecular mass than ClC3 also disappeared in the knockout mice, and may represent post-translational modifications to the protein. Bands of a lesser apparent molecular mass than ClC-3 did not disappear in the knockout animals, but vanished when the primary antibody was pre-absorbed with a threefold excess by weight of antigen (ClC-3 amino acids 592–661 + GST fusion protein provided by Alomone Laboratories), demonstrating that the remaining protein bands in knockout mice are the result of antibody cross-reactivity (see below).

Immunohistochemistry was performed to localize ClC-3 in mouse parotid salivary glands. The upper left panel of Fig. 2 shows heavy staining of the muscular walls of blood vessels (arrows) and of duct cells, with less intense staining of acinar cells and the stroma in the parotid glands of wild-type mice. The lower left (immunohistochemistry) and lower right (Nomarski image of the section shown in the lower left panel) panels of Fig. 2 show a higher magnification view of a blood vessel, with arrows indicating the thick muscular wall of this vessel. The upper right panel of Fig. 2 illustrates that there was none of the usual intense staining associated with the outer blood vessel wall in *Clcn3*^{-/-} mice, suggesting that ClC-3 is highly expressed in the smooth muscle layer of blood vessels, as suggested by a previous study (Lamb *et al.* 1999). In contrast, considerable non-specific labelling remained associated with duct and acinar cells, consistent with this antibody recognizing additional proteins besides ClC-3

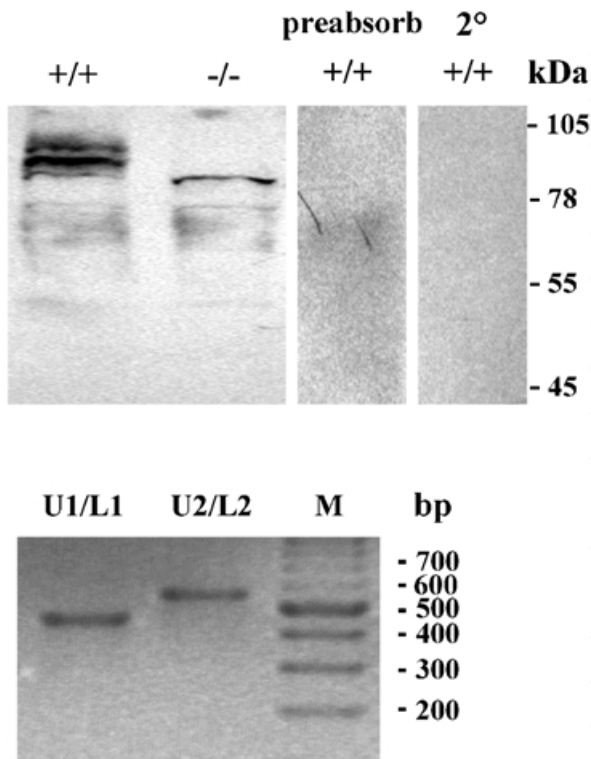


Figure 1. ClC-3 protein and mRNA expression in mouse parotid acinar cells

Protein was isolated from the parotid glands of ClC-3 wild-type and null mutant mice. Upper panel, an antibody to ClC-3 recognized several bands of the appropriate molecular mass for ClC-3 in protein samples from a wild-type mouse parotid gland (lane 1). In a protein sample from parotid glands of *Clcn3*^{-/-} mice, a band of the correct molecular mass for ClC-3 protein was not observed (lane 2), although several other bands remained immunoreactive. These most likely represent cross-reactivity of the antibody with non-specific proteins. When probed with an anti-ClC-3 antibody that had been pre-absorbed with a threefold excess of the peptide antigen to which the antibody had been raised (lane 3) or with secondary antibody alone (lane 4), none of the immunoreactive bands in the wild-type protein sample were recognized. Molecular weight markers are shown to the right of the blots. Lower panel, four overlapping RT-PCR primers were designed to amplify the ClC-3 message from isolated parotid acinar cells including two sense primers (U1 and U2) complementary to codons 34–40 and 384–391, respectively, and two nonsense primers corresponding to codons 209–214 and 590–597 (L1 and L2, respectively). The PCR product sizes using primer pairs U1/L1 and U2/L2 are 542 base pairs (bp) and 638 bp long, respectively. DNA sequencing confirmed 100% identity of the PCR products as ClC-3. Lane M is a 100 bp DNA ladder size marker.

(see also Fig. 1). Since it was not possible to localize the expression of CIC-3 to acinar cells with this antibody by immunohistochemical techniques, a fine glass pipette was used to isolate dispersed parotid acini, and RT-PCR was then performed to determine whether the CIC-3 message is present in these cells. The lower panel of Fig. 1 shows that amplification of RNA from isolated parotid acini results in PCR products of the expected size. Furthermore, DNA sequencing confirmed 100% identity of the PCR products to mouse CIC-3.

Regulatory volume decrease (RVD) and swell-activated Cl^- currents in parotid acinar cells from *Clcn3*^{+/+} and *Clcn3*^{-/-} mice

The Cl^- and K^+ channels in salivary gland acinar cells are activated by secretagogues or when swollen by a hypotonic challenge. The molecular identity of the Cl^- channel(s)

activated under these conditions is unknown. Regardless of the channel(s) involved, in both instances the resulting KCl efflux is accompanied by osmotic water loss and cell shrinkage. The observed shrinkage during a hypotonic shock is termed regulatory volume decrease (RVD). Cell volume measurements demonstrated that acinar cells isolated from mice lacking CIC-3 undergo RVD in a similar fashion to cells from wild-type littermates (Fig. 3, upper panel). Initial rate calculations verified that there was no statistical difference between the RVD for acinar cells isolated from wild-type and knockout mice (lower panel of Fig. 3).

The above RVD experiments suggest that the CIC-3 channel is not the major swell-activated Cl^- channel stimulated during RVD in salivary gland acinar cells. To verify that swell-activated Cl^- currents are still present, the

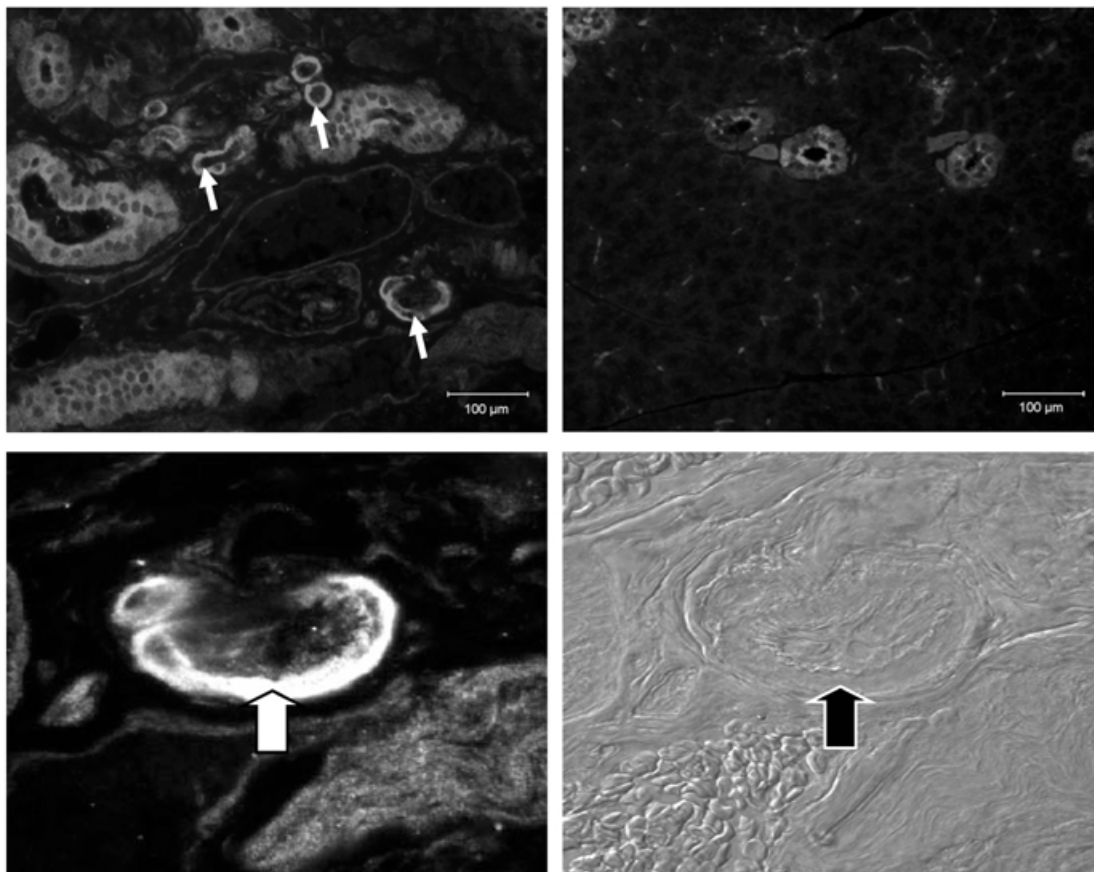


Figure 2. Immunohistochemical labelling using a CIC-3 antibody in the parotid glands of *Clcn3*^{+/+} and *Clcn3*^{-/-} mice

The parotid glands were removed from *Clcn3*^{+/+} and *Clcn3*^{-/-} mice and fixed in paraformaldehyde overnight prior to imbedding in paraffin and sectioning at 5 μm . The glands were exposed to anti-CIC-3 antibody (1:250) overnight. Reactivity was then detected by the use of an Alexa-594 fluorescent secondary antibody. Images were taken at several magnifications from $\times 200$ to $\times 600$, as indicated below. Upper left panel, section from wild-type parotid gland. Arrows indicate intense staining of blood vessels (magnification, $\times 200$). Upper right panel, section of parotid gland from null mutant mouse. Intense staining of blood vessels is absent, but non-specific staining of duct and acinar cells remains (magnification, $\times 200$). Lower left panel, section from wild-type parotid gland. Arrows indicate intense staining of blood vessels (magnification, $\times 600$). Lower right panel, Nomarski image of the section in the lower left panel (magnification, $\times 600$).

patch-clamp technique was used to examine Cl^- currents in parotid acinar cells from $\text{Clcn3}^{-/-}$ mice. Whole-cell recordings revealed that exposure to a hypotonic solution activated large outwardly rectifying Cl^- currents in acinar cells from $\text{Clcn3}^{+/+}$ mice as well as those derived from $\text{Clcn3}^{-/-}$ mice (Fig. 4, upper and middle rows, respectively). The left traces were obtained in isotonic conditions, while the centre traces represent the current after exposure to a hypotonic solution for 2–4 min. The hypotonic solution increased the current relative to that seen in a hypertonic solution 8- to 10-fold in both wild-type and knockout mice. The swell-activated current density was not statistically different between groups ($\text{Clcn3}^{+/+} = 29.2 \pm 8.2 \text{ pA pF}^{-1}$, $n = 6$; $\text{Clcn3}^{-/-} = 32.2 \pm 3.9 \text{ pA pF}^{-1}$, $n = 7$). The sensitivity of this current to cell swelling was confirmed when the hypotonic-activated currents disappeared following exposure to a hypertonic solution (right traces). The lower row of Fig. 4 shows the I - V relationship for the

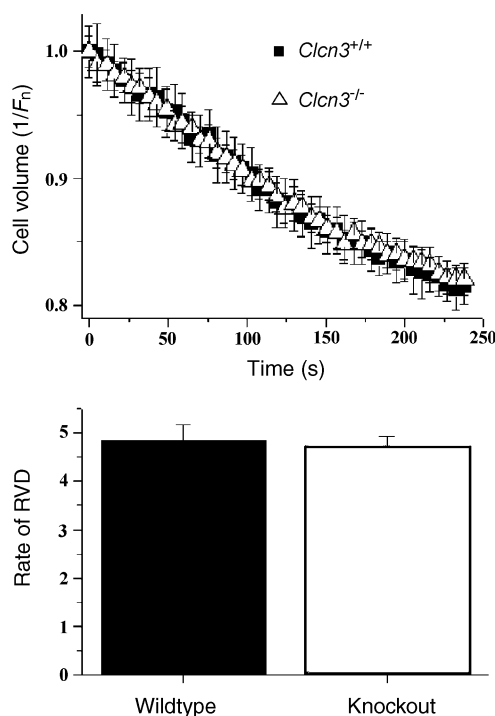


Figure 3. Targeted disruption of the *Clcn3* gene fails to inhibit the RVD response in parotid acinar cells

The role of ClC-3 in the regulatory volume decrease (RVD) response was examined in parotid acinar cells loaded with the fluoroprobe calcein, as described in Methods. Upper panel, parotid acini isolated from $\text{Clcn3}^{+/+}$ (■) and $\text{Clcn3}^{-/-}$ (△) mice were superfused in an isosmotic solution and then hyposmotic cell swelling was induced by switching the perfusate to a hypotonic medium (30% dilution with water). The cell volumes shown are normalized to the maximum volume achieved following exposure to a hypotonic solution. Changes in cell volume are represented as $1/\text{normalized calcein fluorescence}$ ($1/\text{calcein Fn}$). Lower panel, the initial rate of change expressed as $1/\text{calcein Fn} \times \text{min}^{-1} \times 10^{-2}$ ($\text{Clcn3}^{+/+}$, $n = 8$; $\text{Clcn3}^{-/-}$, $n = 11$; no statistical differences between groups).

currents depicted in the upper ($\text{Clcn3}^{+/+}$) and middle ($\text{Clcn3}^{-/-}$) rows. The I - V relationship for cells isolated from wild-type and from null mutant mice have the characteristic outward rectification of the swell-activated Cl^- currents seen in nearly all mammalian cells. Together, these data indicate that disruption of the *Clcn3* channel gene had neither an effect on RVD nor on the swell-activated Cl^- currents in mouse parotid acinar cells.

Ca²⁺-activated Cl^- currents in parotid acinar cells from $\text{Clcn3}^{+/+}$ and $\text{Clcn3}^{-/-}$ mice

Ca²⁺-dependent Cl^- channels are thought to play a central role in the secretion of saliva (Arreola *et al.* 1996). Because

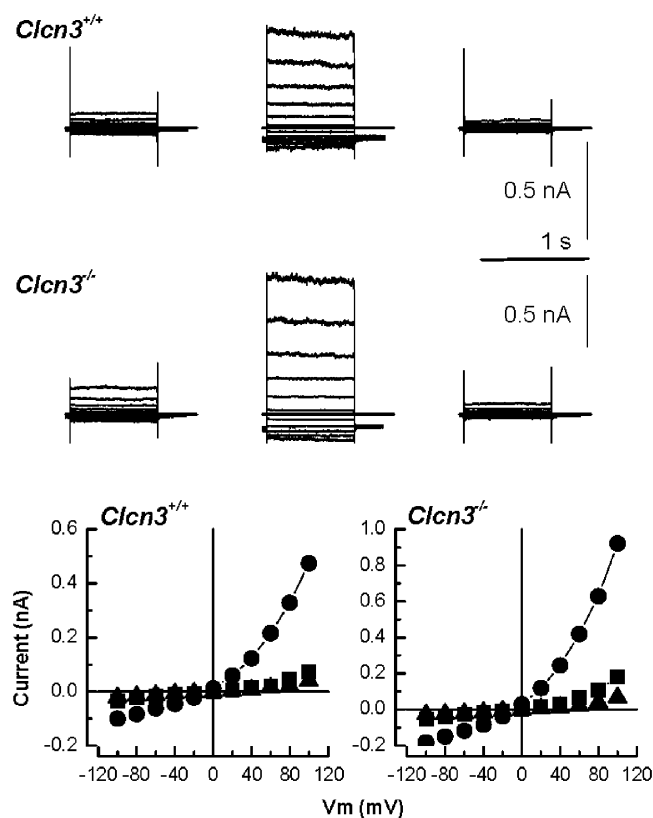


Figure 4. Swell-activated Cl^- currents in parotid acinar cells from $\text{Clcn3}^{+/+}$ and $\text{Clcn3}^{-/-}$ mice

Volume-sensitive Cl^- currents recorded from acinar cells isolated from $\text{Clcn3}^{+/+}$ (upper row) and $\text{Clcn3}^{-/-}$ (middle row) mice. Control traces (left column) were recorded after 5 min of dialysis under isotonic conditions; hypotonic-activated currents (centre column) were recorded after 2–3 min in the hypotonic solution; inhibition of Cl^- currents (right column) by cell shrinkage was assessed after 5 min exposure to a hypertonic solution. The lower row shows the current–voltage relationship for the data depicted in the upper and middle rows under isotonic (■), hypotonic (●) and hypertonic (▲) conditions obtained from acinar cells isolated from $\text{Clcn3}^{+/+}$ and $\text{Clcn3}^{-/-}$ mice. There was no statistical difference between the swell-activated current (current present in the hypotonic solution minus the current after switching to the hypertonic solution) from wild-type and null mutant mice ($\text{Clcn3}^{+/+} = 29.2 \pm 8.2 \text{ pA pF}^{-1}$, $n = 6$; $\text{Clcn3}^{-/-} = 32.2 \pm 3.9 \text{ pA pF}^{-1}$, $n = 7$).

an increase in the intracellular Ca^{2+} concentration occurs during a hypotonic shock (Mignen *et al.* 1999; Weskamp *et al.* 2000), these same Ca^{2+} -dependent Cl^- channels might contribute to the RVD response. Interestingly, the CIC-3 Cl^- channel cloned from the human T84 cell line is apparently activated by CaM kinase II, a Ca^{2+} - and calmodulin-dependent kinase (Huang *et al.* 2001). Even if CIC-3 is not a CaM kinase II-dependent channel in salivary gland acinar cells, deletion of the *Clcn3*^{-/-} gene could result in compensatory changes in other Cl^- channels, including those activated by intracellular Ca^{2+} . Thus, we compared the level of expression of Ca^{2+} -activated Cl^- current in wild-type and *Clcn3*^{-/-} mice.

The upper panel of Fig. 5 shows an example of anion currents recorded from a wild-type mouse. Illustrated are currents at several membrane potentials with a fixed intracellular Ca^{2+} concentration of about 400 nM (see Methods). The time course of current activation and deactivation are characteristic of Ca^{2+} -activated Cl^- channels in this cell type (Arreola *et al.* 1996). As an estimate of the level of expression of this channel, we determined the magnitude of the current at the end of the 3 s pulse to +90 mV corrected for cell size (cell capacitance). The average current from wild-type cells was $140 \pm 20 \text{ pA pF}^{-1}$ (S.E.M., $n = 10$). The lower panel of Fig. 5

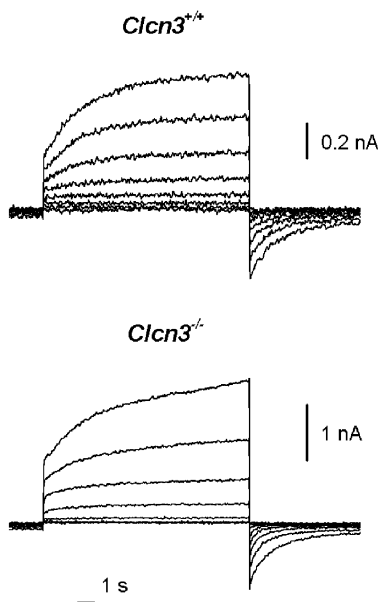


Figure 5. Ca^{2+} -dependent Cl^- currents in parotid acinar cells from *Clcn3*^{+/+} and *Clcn3*^{-/-} mice

Upper panel, currents from a parotid acinar cell from a *Clcn3*^{+/+} mouse in response to 3 s voltages pulses from -30 to $+90$ mV in 20 mV increments from a holding potential of -50 mV. Lower panel, currents at the same potentials from a parotid acinar cell from a *Clcn3*^{-/-} mouse. There was no statistical difference between the Ca^{2+} -activated current from wild-type and null mutant mice (*Clcn3*^{+/+}, $140 \pm 20 \text{ pA pF}^{-1}$; $n = 10$; *Clcn3*^{-/-}, $135 \pm 13 \text{ pA pF}^{-1}$; $n = 5$).

shows that disruption of the *Clcn3* gene did not eliminate expression of Ca^{2+} -activated Cl^- channels. Indeed, the average Ca^{2+} -activated current at $+90$ mV in acinar cells from *Clcn3*^{-/-} mice was not statistically different ($135 \pm 13 \text{ pA pF}^{-1}$, $n = 5$). These results indicate that CIC-3 is not the Ca^{2+} -activated Cl^- channel in mouse parotid acinar cells. Furthermore, the RVD observed in parotid acinar cells from *Clcn3*^{-/-} mice is not likely to be due to enhanced expression of Ca^{2+} -dependent Cl^- currents.

***In vivo* stimulated salivary fluid, electrolyte and protein secretion in CIC-3 Cl^- channel-deficient mice**

The above data reveal that disruption of the *Clcn3* gene alters neither Cl^- current expression (swell-activated or Ca^{2+} -dependent) nor the RVD response found in mouse parotid acinar cells. Thus, it is improbable that CIC-3 is involved directly in fluid secretion or cell volume regulation in parotid acinar cells. However, because CIC-3 may play an important role in vesicular transmitter uptake and/or secretion in the hippocampus (Stobrawa *et al.* 2001), it is important to test for any possible role in fluid or protein secretion. Specifically, CIC-3 appears to be highly expressed in the muscular walls of blood vessels (Fig. 2), suggesting that this channel, by controlling the flow of blood through salivary glands, could indirectly regulate the saliva flow rate. To test this hypothesis we collected *in vivo* stimulated whole saliva from *Clcn3* wild-type and null mutant mice and determined the salivary flow rate ($\mu\text{l 5 min}^{-1}$) over a 15 min time period. Figure 6 shows that targeted disruption of *Clcn3*^{-/-} (\square) did not significantly reduce the flow rate of pilocarpine-stimulated saliva secreted during the 15 min collection period compared with *Clcn3*^{+/+} animals (\blacksquare). As reported previously, there

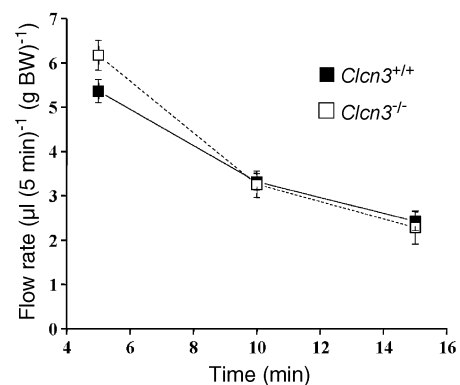


Figure 6. Targeted disruption of the *Clcn3* gene does not inhibit pilocarpine-induced *in vivo* salivation

The role of CIC-3 in the production of saliva was examined *in vivo* by stimulating with 10 mg pilocarpine-HCl ($\text{kg body weight (BW)}^{-1}$). Whole saliva was collected from the lower cheek pouch by suctioning at intervals of 5, 10 and 15 min and amounts are expressed as $\mu\text{l 5 min g BW}^{-1}$. Time zero was designated as the point when saliva was first noted following pilocarpine injection. *Clcn3*^{+/+} (\blacksquare), $n = 17$; *Clcn3*^{-/-} (\square), $n = 12$.

Table 1. Targeted disruption of the *Clcn3* gene. Effects on the osmolality, [Na⁺], [K⁺], [Cl⁻] and protein content of stimulated whole saliva

	<i>Clcn3</i> ^{+/+}	<i>Clcn3</i> ^{-/-}
Osmolality (mmol kg ⁻¹)	187.4 ± 8.0	171.0 ± 4.8
[Na ⁺] (mM)	94.8 ± 11.8	89.9 ± 3.9
[K ⁺] (mM)	37.7 ± 2.7	39.6 ± 2.4
[Cl ⁻] (mM)	69.8 ± 7.1	54.8 ± 9.4
Protein content (mg ml ⁻¹)	74.5 ± 12.4	89.3 ± 17.2

Anaesthetized adult *Clcn3*^{+/+} and *Clcn3*^{-/-} mice were stimulated with 10 mg pilocarpine-HCl (kg body weight)⁻¹. Whole saliva was collected and samples were analysed for total Na⁺ and K⁺ content by atomic absorption using a Perkin-Elmer 3030 spectrophotometer, for osmolality using a Wescor 5500 vapour pressure osmometer and for Cl⁻ activity with an Orion EA 940 expandable ion analyser. Protein concentration was estimated using the Bradford method (*n*=6 for each group; mean ± S.E.M.; no statistical difference between groups).

was a high initial flow rate seen at the commencement of secretion, which declined to a slower rate thereafter (Evans *et al.* 2000).

Salivation occurs in two stages, the initial step being the secretion of fluid with a plasma-like ionic composition from acinar cells. Subsequently, duct cells reabsorb much of the secreted NaCl to produce a hypotonic, NaCl-poor saliva. The transport pathways involved in NaCl reabsorption are not well understood, but may involve Cl⁻ channels (Komwatana *et al.* 1994; Dinudom *et al.* 1995). To examine the role of ClC-3 channels in NaCl uptake, we determined the osmolality, the total Na⁺ and K⁺ content, and the Cl⁻ activity of whole saliva. Table 1 shows that the ionic composition and the osmolality of saliva collected from ClC-3-deficient mice were not statistically different from that secreted by wild-type littermates, suggesting that *Clcn3*^{-/-} channels do not play a significant part in NaCl reabsorption in this tissue.

In addition to fluid and electrolytes, saliva is rich in secretory proteins. The movement of proteins into secretion granules and the stimulated discharge of these proteins are modulated by the pH of the various intracellular compartments in this pathway (Kleene *et al.* 1999; Williams & Webb, 2000; Wu *et al.* 2000). ClC-3 has been localized to endosomal compartments in the liver and synaptic vesicles of neurones, where it has been suggested to play a role in the pH regulation of these organelles (Stobrawa *et al.* 2001). Therefore, to examine whether ClC-3 regulates secretory protein discharge, we determined the protein concentration of the pilocarpine-stimulated saliva collected from *Clcn3*^{+/+} and *Clcn3*^{-/-} mice. Table 1 shows that the protein content of the saliva was comparable between *Clcn3* wild-type and null mutant mice. Taken together, our results demonstrate that disruption of the *Clcn3* gene fails to alter the *in vivo* flow rate or composition of saliva.

DISCUSSION

The ClC-3 Cl⁻ channel is highly expressed in many different organ systems, including salivary glands. This channel has been hypothesized to be the swell-activated Cl⁻ channel present in the heart and most other mammalian tissues (Duan *et al.* 1997). However, knockout of this gene fails to inhibit similar swell-activated Cl⁻ currents in mouse hepatocytes or pancreatic acinar cells (Stobrawa *et al.* 2001). Instead, mice lacking ClC-3 undergo postnatal degeneration of the retina and hippocampus (Stobrawa *et al.* 2001; Dickerson *et al.* 2002) and exhibit kyphosis, altered vascular reactivity and unexplained postnatal death (Dickerson *et al.* 2001, 2002). It appears that ClC-3 is an intracellular channel protein that may play a role in pH regulation of intracellular organelles (Stobrawa *et al.* 2001). In general agreement with these studies (Stobrawa *et al.* 2001; Dickerson *et al.* 2002), we found that disruption of ClC-3 expression had no effect on the swell-activated Cl⁻ currents in parotid acinar cells (neither the typical swell-activated currents nor the Ca²⁺-dependent Cl⁻ currents) or the ability of these cells to volume regulate in response to a hypotonic challenge. Majid *et al.* (2001) reported that volume-sensitive channel activation is much faster in submandibular salivary acinar cells, which express ClC-3 channels, than in lacrimal acinar cells, which do not. Majid *et al.* saw little or no current induced by swelling during the first 5 min of a hypotonic challenge in lacrimal acinar cells, whereas this current was maximal by this time in salivary cells. If ClC-3 is the fast-activating current in salivary acinar cells, as hypothesized by Majid *et al.*, then little or no swelling-induced current should have been observed shortly after cell swelling in the ClC-3 knockout mice. However, there did not appear to be a difference in the rates of channel activation in our experiments during the first 4 min of a hypotonic challenge, although no detailed kinetic analysis was done. Swelling-induced currents in parotid acinar cells from wild-type and *Clcn3* null mutant mice were generally recorded 2–4 min after switching to a hypotonic solution, and were of similar magnitude. Moreover, because the kinetics and current magnitudes were comparable, these results also show that ClC-3 is not a regulator of the volume-sensitive channel, an alternative hypothesis proposed by Majid *et al.* to explain the different activation kinetics. Clearly, the molecular identity of the swell-activated Cl⁻ current in parotid acinar cells as well as some other cell types (Stobrawa *et al.* 2001; Dickerson *et al.* 2002) is not ClC-3, nor is it likely to be another known member of the CLC gene family based on channel kinetics and intracellular localization patterns.

ClC-3 is one of nine members of the CLC mammalian Cl⁻ channel gene family. Like ClC-3, the hyperpolarization-activated ClC-2 Cl⁻ channel is widely expressed and may be regulated by cell volume (Jordt & Jentsch, 1997),

although ClC-2 has very different channel kinetics from swell-activated currents (Strange *et al.* 1996). A null mutation of this gene results in the lack of inward rectifier Cl⁻ current in parotid acinar cells, but has no effect on swell-activated currents or the ability of these cells to volume regulate (Nehrke *et al.* 2002). Targeting of some members of the CLC gene family to the plasma membrane appears to involve interactions with other proteins. Plasma membrane targeting of two kidney specific isoforms, ClC-Ka and ClC-Kb, apparently requires expression of a β -subunit termed Barttin (Estévez *et al.* 2001). Mutations in the human *CLCNKB* or Barttin genes cause Bartter's syndrome, a disease associated with severe renal wasting (Simon *et al.* 1997; Estévez *et al.* 2001). Thus, other chaperone proteins may determine the function of some CLC channels by regulating the ultimate destination of these proteins to either intracellular structures or the plasma membrane. Indeed, an alternatively spliced variant of ClC-3 expresses a PDZ binding that apparently interacts with a scaffolding protein to enhance expression at the plasma membrane (Ogura *et al.* 2002).

So if the ClC-3 Cl⁻ channel does not play a major role in cell volume regulation, what then is its function in salivary glands? Expression in the smooth muscle cells of blood vessels in the parotid gland suggests that ClC-3 could be important for indirectly regulating saliva production. Indeed, altered vascular reactivity has been noted in the aorta of ClC-3-deficient mice (Dickerson *et al.* 2001). However, we found that *in vivo* stimulation with the cholinergic agonist pilocarpine failed to affect salivary gland flow rates. Moreover, our results revealed that a *Clcn3* gene null mutation did not alter the osmolality or the ionic composition of the saliva produced, indicating that the ClC-3 Cl⁻ channel is unlikely to be involved in NaCl reabsorption. Similarly, disruption of *Clcn3* does not grossly alter renal function (L. W. Dickerson & F. S. Lamb, unpublished observations).

Our results show clearly that ClC-3 is neither the swell-activated Cl⁻ channel nor the Ca²⁺-dependent Cl⁻ channel in salivary acinar cells. Alternatively, ClC-3 might play an important role in the regulation of endocytic or secretory vesicle sorting, a pH-dependent process in some cells (Castle *et al.* 1987). Stobrawa *et al.* (2001) found that ClC-3 targets synaptic vesicles, where it may control the pH of this organelle and thereby have an effect on neurotransmitter uptake/release by these vesicles. Vesicle fusion events leading to protein movement often require an intraorganelle acidification via the action of the H⁺-ATPase. It appears that Cl⁻ channels may be involved in pH regulation of some intracellular organelles by acting as an electrogenic shunt (Stobrawa *et al.* 2001). Nevertheless, the protein content of *in vivo* stimulated saliva from *Clcn3*^{+/+} and *Clcn3*^{-/-} mice was comparable, indicating that ClC-3 channel activity is not essential for secretory protein

discharge by salivary acinar cells. However, secretory granule fusion with the plasma membrane in this cell type may not be dependent upon acidification of the granules, where the intragranular pH is apparently neutral (Castle *et al.* 1987).

In summary, our results indicate that the ClC-3 Cl⁻ channel is not a major regulator of the functions typically associated with salivary gland cells. Cell volume regulation, swell-activated Cl⁻ currents, and Ca²⁺-dependent Cl⁻ currents in salivary acinar cells were unaltered in *Clcn3*^{-/-} mice, demonstrating clearly that the ClC-3 channel is not involved in the RVD response. Moreover, the *in vivo* volume, total protein content and ionic composition of saliva secreted in response to stimulation were unchanged, suggesting that ClC-3 does not play a major role in these processes. Further study is required to determine the function of this channel in salivary acinar cells.

REFERENCES

- ARREOLA, J., MELVIN, J. E. & BEGENISICH, T. (1995). Activation of calcium-dependent chloride channels in rat parotid acinar cells. *Journal of General Physiology* **108**, 35–47.
- ARREOLA, J., MELVIN, J. E. & BEGENISICH, T. (1996). Volume-activated chloride channels in rat parotid acinar cells. *Journal of Physiology* **484**, 677–687.
- BEGENISICH, T. & MELVIN, J. E. (1998). Regulation of chloride channels in secretory epithelia. *Journal of Membrane Biology* **163**, 77–85.
- BERS, D. M., PATTON, C. W. & NUCCITELLI, R. (1994). A practical guide to the preparation of Ca²⁺ buffers. *Methods in Cell Biology* **40**, 3–29.
- CASTLE, J. D., CAMERON, R. S., ARVAN, P., VON ZASTROW, M. & RUDNICK, G. (1987). Similarities and differences among neuroendocrine, exocrine, and endocytic vesicles. *Annals of the New York Academy of Sciences* **493**, 448–460.
- DICKERSON, L. W., BARNA, T. J., SCHUTTE, B. C., CAO, X. R., YANG, B., & LAMB, F. S. (2001). Decreased reactivity to norepinephrine (NE) in aortae from male ClC3 (-/-) mice. *FASEB Journal* **15**, A114.
- DICKERSON, L. W., SCHUTTE, B. C., YANG, B., BONTIUS, D. J., BARNA, T. J., BAILEY, M. C., NEHRKE, K., WILLIAMSON, R. A. & LAMB, F. S. (2002). Altered GABAergic function accompanies hippocampal degeneration in mice lacking ClC-3 voltage-gated chloride channels. *Brain Research* **956** (in the Press).
- DINUOM, A., KOMWATANA, P., YOUNG, J. A. & COOK, D. I. (1995). A forskolin-activated Cl⁻ current in mouse mandibular duct cells. *American Journal of Physiology* **268**, G806–812.
- DUAN, D., WINTER, C., COWLEY, S., HUME, J. R. & HOROWITZ, B. (1997). Molecular identification of a volume-regulated chloride channel. *Nature* **390**, 417–421.
- DUAN, D., ZHONG, J., HERMOSO, M., SATTERWHITE, C. M., ROSSOW, C. F., HATTON, W. J., YAMBOLIEV, I. I., HOROWITZ, B. & HUME, J. R. (2001). Functional inhibition of native volume-sensitive outwardly rectifying anion channels in muscle cells and *Xenopus* oocytes by anti-ClC-3 antibody. *Journal of Physiology* **531**, 437–444.
- ESTÉVEZ, R., BOETTGER, T., STEIN, V., BIRKENHAGER, R., OTTO, E., HILDEBRANDT, F. & JENTSCH, T. J. (2001). Barttin is a Cl⁻ channel β -subunit crucial for renal Cl⁻ reabsorption and inner ear K⁺ secretion. *Nature* **414**, 558–561.

- EVANS, R. L., PARK, K., TURNER, R. J., WATSON, G. E., NGUYEN, H. V., DENNETT, M. R., HAND, A. R., FLAGELLA, M., SHULL, G. E. & MELVIN, J. E. (2000). Severe impairment of salivation in $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC1)-deficient mice. *Journal of Biological Chemistry* **275**, 26720–26726.
- FRIEDRICH, T., BREIDERHOFF, T. & JENTSCH, T. J. (1999). Mutational analysis demonstrates that ClC-4 and ClC-5 directly mediate plasma membrane currents. *Journal of Biological Chemistry* **274**, 896–902.
- HUANG, P., LIU, J., DI, A., ROBINSON, N. C., MUSCH, M. W., KAEZEL, M. A. & NELSON, D. J. (2001). Regulation of human ClC-3 channels by multifunctional Ca^{2+} /calmodulin dependent protein kinase. *Journal of Biological Chemistry* **276**, 20093–20100.
- JORDT, S. E. & JENTSCH, T. J. (1997). Molecular dissection of gating in the ClC-2 chloride channel. *EMBO Journal* **16**, 1582–1592.
- KAWASAKI, M., UCHIDA, S., MONKAWA, T., MIYAWAKI, A., MIKOSHIBA, K., MARUMO, F. & SASAKI, S. (1994). Cloning and expression of a protein kinase C-regulated chloride channel abundantly expressed in rat brain neuronal cells. *Neuron* **12**, 597–604.
- KLEENE, R., KASTNER, B., ROSSER, R. & KERN, H. (1999). Complex formation among rat pancreatic secretory proteins under mild alkaline pH conditions. *Digestion* **60**, 305–313.
- KOMWATANA, P., DINUDOM, A., YOUNG, J. A. & COOK, D. I. (1994). Characterization of the Cl^- conductance in the granular duct cells of mouse mandibular glands. *Pflügers Archiv* **428**, 641–647.
- LAMB, F. S., CLAYTON, G. H., LIU, B. X., SMITH, R. L., BARNA, T. J. & SCHUTTE, B. C. (1999). Expression of CLCN voltage-gated chloride channel genes in human blood vessels. *Journal of Molecular and Cellular Cardiology* **31**, 657–666.
- LI, X., SHIMADA, K., SHOWALTER, L. A. & WEINMAN, S. A. (2000). Biophysical properties of ClC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. *Journal of Biological Chemistry* **275**, 35994–35998.
- MAJID, A., BROWN, P. D., BEST, L. & PARK, K. (2001). Expression of volume-sensitive Cl^- channels and ClC-3 in acinar cells isolated from the rat lacrimal gland and submandibular salivary gland. *Journal of Physiology* **534**, 409–421.
- MELVIN, J. E., NEHRKE, K., PARK, K., RICHARDSON, L., YANG, B., LAMB, F. & SCHUTTE, B. C. (2000). Normal RVD and salivation in parotid acini from ClC3 -deficient mice. *Journal of Dental Research* **79**, 345a.
- MIGNEN, O., LE GALL, C., HARVEY, B. J. & THOMAS, S. (1999). Volume regulation following hypotonic shock in isolated crypts of mouse distal colon. *Journal of Physiology* **515**, 501–510.
- NEHRKE, K., ARREOLA, J., NGUYEN, H.-V., PILATO, J., RICHARDSON, L., OKUNADE, G., BAGGS, R., SHULL, G. E. & MELVIN, J. E. (2002). Loss of hyperpolarization-activated Cl^- current in salivary acinar cells from Cln2 knockout mice. *Journal of Biological Chemistry* **277**, 23604–23611.
- OGURA, T., FURUKAWA, T., TOYOZAKI, T., YAMADA, K., ZHENG, Y., KATAYAMA, Y., NAKAYA, H. & INAGAKI, N. (2002). ClC-3B , a novel ClC-3 splicing variant that interacts with EB50 and facilitates expression of CFTR-regulated ORCC. *FASEB Journal* **16**, 863–865.
- PARK, K., OLSCHOWKA, J. A., RICHARDSON, L. A., BOOKSTEIN, C., CHANG, E. B. & MELVIN, J. E. (1999). Expression of multiple Na^+/H^+ exchanger isoforms in rat parotid acinar and ductal cells. *American Journal of Physiology* **276**, G470–478.
- SCHREIBER, R., NITSCHKE, R., GREGER, R. & KUNZELMANN, K. (1999). The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. *Journal of Biological Chemistry* **274**, 11811–11816.
- SHIMADA, K., LI, X., XU, G., NOWAK, D. E., SHOWALTER, L. A. & WEINMAN, S. A. (2000). Expression and canalicular localization of two isoforms of the ClC-3 chloride channel from rat hepatocytes. *American Journal of Physiology – Gastrointestinal Liver Physiology* **279**, G268–276.
- SIMON, D. B., BINDRA, R. S., MANSFIELD, T. A., NELSON-WILLIAMS, C., MENDONCA, E., STONE, R., SCHURMAN, S., NAYIR, A., ALPAY, H., BAKKALOGLU, A., RODRIGUEZ-SORIANO, J., MORALES, J. M., SANJAD, S. A., TAYLOR, C. M., PILZ, D., BREM, A., TRACHTMAN, H., GRISWOLD, W., RICHARD, G. A., JOHN, E. & LIFTON, R. P. (1997). Mutations in the chloride channel gene, CLCNKB , cause Bartter's syndrome type III. *Nature Genetics* **17**, 171–178.
- STEGEN, C., MATSKEVICH, I., WAGNER, C. A., PAULMICHL, M., LANG, F. & BROER, S. (2000). Swelling-induced taurine release without chloride channel activity in *Xenopus laevis* oocytes expressing anion channels and transporters. *Biochimica et Biophysica Acta* **1467**, 91–100.
- STEINMEYER, K., SCHWAPPACH, B., BENS, M., VANDEWALLE, A. & JENTSCH, T. J. (1995). Cloning and functional expression of rat CLC-5 , a chloride channel related to kidney disease. *Journal of Biological Chemistry* **270**, 31172–31177.
- STOBRAWA, S. M., BREIDERHOFF, T., TAKAMORI, S., ENGEL, D., SCHWEIZER, M., ZDEBIK, A. A., BOSL, M. R., RUETHER, K., JAHN, H., DRAGUHN, A., JAHN, R. & JENTSCH, T. J. (2001). Disruption of ClC-3 , a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* **29**, 185–196.
- STRANGE, K., EMMA, F. & JACKSON, P. S. (1996). Cellular and molecular physiology of volume-sensitive anion channels. *American Journal of Physiology* **270**, C711–730.
- TURNER, R. J., GEORGE, J. & BAUM, B. J. (1986). Evidence for a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport system in basolateral membrane vesicles from rabbit parotid. *Journal of Membrane Biology* **94**, 143–152.
- WANG, L., CHEN, L. & JACOB, T. J. (2000). The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. *Journal of Physiology* **524**, 63–75.
- WESKAMP, M., SEIDL, W. & GRISSMER, S. (2000). Characterization of the increase in $[\text{Ca}^{2+}]_i$ during hypotonic shock and the involvement of Ca^{2+} -activated K^+ channels in the regulatory volume decrease in human osteoblast-like cells. *Journal of Membrane Biology* **178**, 11–20.
- WEYLANDT, K. H., VALVERDE, M. A., NOBLES, M., RAGUZ, S., AMEY, J. S., DIAZ, M., NASTRUCCI, C., HIGGINS, C. F. & SARDINI, A. (2001). Human ClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. *Journal of Biological Chemistry* **276**, 17461–17467.
- WILLIAMS, R. M. & WEBB, W. W. (2000). Single granule pH cycling in antigen-induced mast cell secretion. *Journal of Cell Science* **113**, 3839–3850.
- WU, M. M., LLOPIS, J., ADAMS, S., MCCAFFERY, J. M., KULOMAA, M. S., MACHEN, T. E., MOORE, H. P. & TSIEN, R. Y. (2000). Organelle pH studies using targeted avidin and fluorescein-biotin. *Chemistry and Biology* **7**, 197–209.

Acknowledgements

We thank Jodi Pilato and Jill Thompson for technical assistance. This work was supported in part by National Institutes of Health Grants DE09692 and DE13539 (J.E.M.).