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Chloride in Vesicular Trafficking and Function

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Abstract

Luminal acidification is of pivotal importance for the physiology of the secretory and endocytic pathways and its diverse trafficking events. Acidification by the proton-pumping V-ATPase requires charge compensation by counterion currents that are commonly attributed to chloride. The molecular identification of intracellular chloride transporters and the improvement of methodologies for measuring intraorganellar pH and chloride have facilitated the investigation of the physiology of vesicular chloride transport. New data question the requirement of chloride for pH regulation of various organelles and furthermore ascribe functions to chloride that are beyond merely electrically shunting the proton pump. This review surveys the currently established and proposed intracellular chloride transporters and gives an overview of membrane-trafficking steps that are affected by the perturbation of chloride transport. Finally, potential mechanisms of membrane-trafficking modulation by chloride are discussed and put into the context of organellar ion homeostasis in general.

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CHLORIDE CONCENTRATION AND pH OF INTRACELLULAR ORGANELLES

Vacuolar-type ATPase (V-ATPase): also known as H^+-ATPase; a multisubunit enzyme complex that utilizes the energy of ATP hydrolysis to translocate protons across membranes

CFTR: cystic fibrosis transmembrane conductance regulator

Chloride (Cl^-) is the most abundant anion in the extra- and intracellular spaces of animal cells; the cytosolic Cl^- concentration ($[Cl^-]$) ranges from approximately 5 to 40 mM. Cl^- transport through the plasma membrane is involved in numerous physiological processes as diverse as systemic electrolyte homeostasis, modulation of cellular excitability, and cellular volume regulation. Subcellular Cl^- transport has been thought mostly to merely provide an electrical shunt for the acidification of cellular organelles. Acidification is mediated primarily by the electrogenic proton pumping of the vacuolar (V-type) ATPase (V-ATPase) (1). Without parallel counterion transport, the resulting inside-positive transmembrane potential would rapidly shut down the activity of the V-ATPase before a significant pH gradient was generated. Along the secretory pathway, the luminal pH drops from the near-cytosolic value of 7.2 within the endoplasmic reticulum (ER) to 6.7–6.4 along the Golgi complex to approximately 5.2–5.4 in secretory granules (**Figure 1**). Compartments of the endocytic pathway are also progressively acidified, yielding a pH of approximately 6.0 in early endosomes and a final pH of less than 5.0 in lysosomes. Consistent with Cl^- being the counterion for H^+ entry, the concentration of luminal Cl^- ($[Cl^-]_{lumen}$) also increases along the endocytic pathway (**Figure 1**). Immediately after endocytosis, $[Cl^-]_{lumen}$ drops from the extracellular value of ~140 mM to ~20 mM (2). This drop is thought to be due to Cl^- being expelled by negative surface charges in the forming vesicles (2). Subsequently $[Cl^-]_{lumen}$ rises again to reach ~40 mM in early and recycling endosomes, ~50 mM in the Golgi complex, and ~60 mM in late endosomes (2, 3). Exact measurements of lysosomal $[Cl^-]$ have not been reported due to the lack of suitable Cl^- sensors for the high $[Cl^-]$ at low pH. However, estimated from measurements with cells treated with low Cl^- and from model calculations (4), lysosomal $[Cl^-]_{lumen}$ is greater than 80 mM.

Recent data strongly point to a crucial role of Cl^- transport in organellar physiology that goes beyond merely providing the electrical shunt for proton pumping by the V-ATPase (4, 5). Although various proteins are known to be regulated by Cl^-, the mechanism by which (mainly luminal) Cl^- affects membrane traffic and organellar function has remained elusive. Ion depletion studies using in vitro experiments globally demonstrated a role for Cl^- in vesicular acidification, but more detailed insights could be obtained only after relevant Cl^- transport proteins were identified. Over the past two decades, the role of various Cl^- transport proteins in vesicular physiology—in particular CLC transporters and the cystic fibrosis transmembrane conductance regulator (CFTR)—has been studied by using a battery of in vitro and cell-based systems, as well as animal models and human hereditary diseases. Further candidates for intracellular Cl^- transport proteins that may have a role in vesicular ion transport have emerged. In this review, first we give an overview of the growing number of confirmed or candidate vesicular Cl^- transport proteins because much of our current insight into the role of vesicular Cl^- comes from studies in which specific transporters were eliminated or modified. Then we discuss the various membrane-trafficking steps involving Cl^-, with an emphasis on the Cl^- transporters thought to be involved. Finally, we discuss potential mechanisms by which Cl^- may affect membrane traffic and organellar function and how these processes integrate into a broader picture of vesicular ion homeostasis.

INTRACELLULAR CHLORIDE TRANSPORT PROTEINS

A reasonable number of Cl^- channels and transporters have been identified on the molecular level, but many Cl^- conductances identified by biophysical methods still lack a known molecular correlate (reviewed in References 6 and 7). All Cl^- transport proteins are synthesized at the endoplasmic reticulum (ER), from where they travel to their respective subcellular destination,

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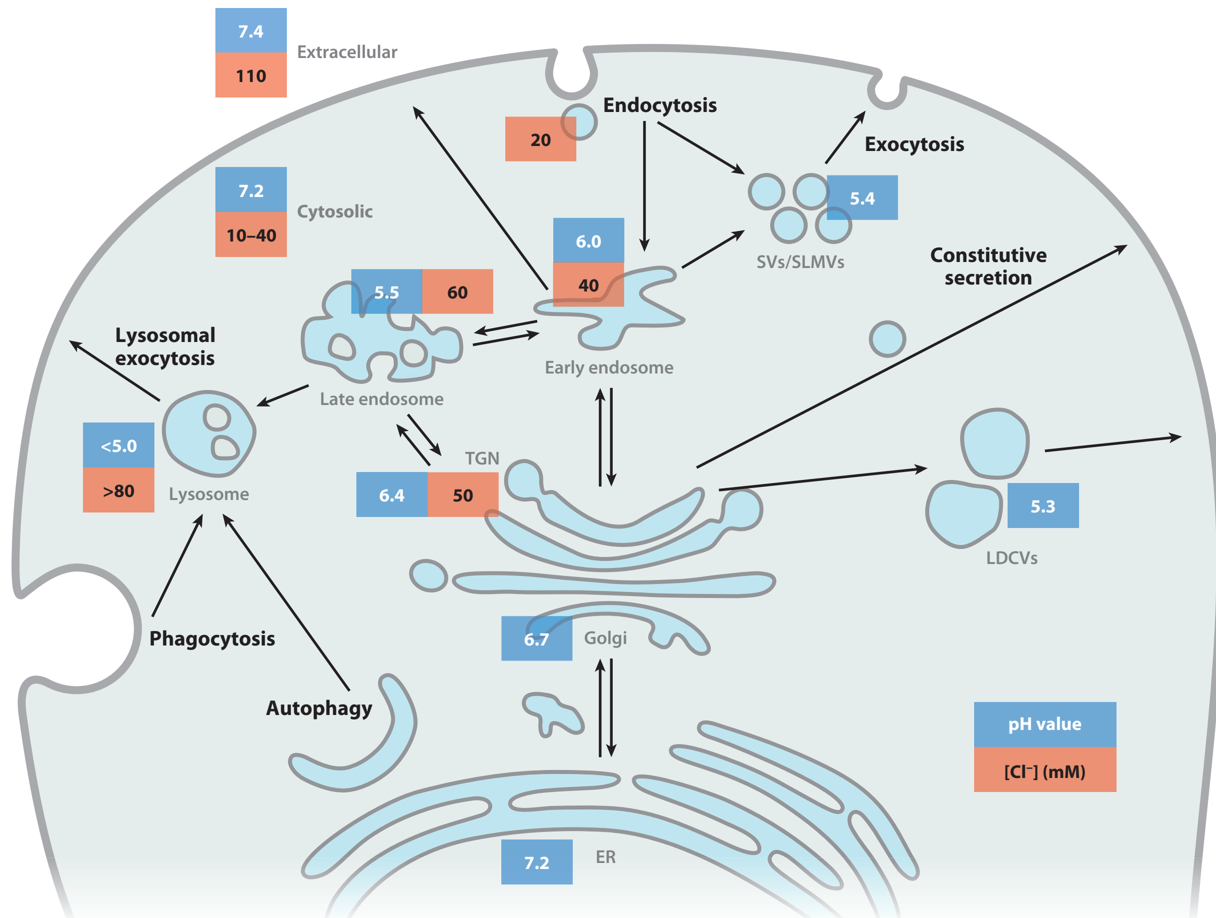

Figure 1

Typical pH values and Cl⁻ concentrations in organelles of the secretory and endocytic pathways. In the endocytic pathway, the luminal pH drops from the extracellular value of 7.4 to less than 5.0 in the lysosome. After an initial drop to ~20 mM, the luminal Cl⁻ concentration increases concomitantly and reaches greater than 80 mM in lysosomes. Additionally, compartments of the secretory pathway are progressively acidified. Whereas the pH of ~7.2 in the endoplasmic reticulum (ER) is similar to that of the cytosol, the *trans*-Golgi network (TGN) displays a pH value of ~6.4. Specialized secretory vesicles, such as large dense-core vesicles (LDCVs), smaller synaptic vesicles (SVs), and synaptic-like microvesicles (SLMVs), are acidified to a pH of ~5.4. The Cl⁻ concentration in these compartments has not been determined.

sometimes by trafficking through the plasma membrane. In some cases, there are conflicting data about the involvement of a particular, confirmed Cl⁻ transporter in a given membrane-trafficking step. In contrast, in other cases there are not unambiguously clear data that certain proteins are Cl⁻ channels or transporters; instead, such proteins may have totally unrelated functions (6, 8). In this section, we give an overview of the (potential) intracellular Cl⁻ transporters (**Table 1**). Their roles in specific membrane-trafficking steps are discussed in the subsequent section.

ClC-3 Through ClC-7: Cl⁻/H⁺ Exchangers in the Endosomal Pathway

The by far most convincing set of data showing a role in vesicular Cl⁻ transport exists for the CLC proteins ClC-3 through ClC-7 (for recent comprehensive reviews, see References 9 and

Table 1 Established and proposed intracellular Cl^- transport proteins[a,b]

Protein	Ion transport	Localization	Cell biology	Associated disease(s)
ClC-3	Outwardly rectifying currents, probably $2Cl^-/1H^+$ exchange	Endosomes (ubiquitous); SVs and SLMVs	Endosomal and SV acidification, exocytosis of LDCVs in endocrine cells	Degeneration of the hippocampus and retina (KO mice)
ClC-4	Outwardly rectifying $2Cl^-/1H^+$ exchange	Endosomes (ubiquitous)	Endosomal acidification?	None found yet
ClC-5	Outwardly rectifying $2Cl^-/1H^+$ exchange	Apical EEs (kidney and intestine)	Endosomal acidification, Cl^- accumulation, renal endocytosis	Dent's disease in humans; similar phenotype in KO mice
ClC-6	Outwardly rectifying $2Cl^-/1H^+$ exchange	LEs (neurons)	LE/lysosome traffic?	Lysosomal storage, late-onset NCL (KO mice)
ClC-7/Ostm1	Outwardly rectifying $2Cl^-/1H^+$ exchange	LEs/lysosomes (ubiquitous), ruffled border (osteoclasts)	Lysosomal Cl^- accumulation, protein degradation	Osteopetrosis (humans and KO mice), lysosomal storage (NCL) with neuronal and retinal degeneration
CFTR	cAMP-activated Cl^- channel, linear *I-V* relation	PM of epithelia, apical EEs (renal PT cells)	Salt and water transport by epithelia	Cystic fibrosis (humans and animal models)
CLICs	Nonselective anion currents?	Cytosol, nucleus, PM, and intracellular membranes (ubiquitous); CLIC6 enriched in water-secreting tissue	Cell cycle (?), phagosomal acidification (?) (CLIC1), cell-hollowing tubulogenesis (CLIC4)	Mild bleeding disorder (CLIC1 KO mice), defective angiogenesis (CLIC4 KO mice), hair cell degeneration leading to hearing and vestibular dysfunction (*jitterbug* mice with mutated CLIC5)
AQP6	Anion and cation currents, slightly outwardly rectifying currents; activated by low pH	Apical EEs and PM in renal epithelia	Unknown	None found yet
TMEM16C–G (ANO3–7)	Probably ion channels; paralogous to plasma membrane TMEM16A and -B, which are CaCCs	ER?	Induces scramblase activity (TMEM16F)	Muscular dystrophy (TMEM16E) Scott syndrome (TMEM16F)
Bestrophin-1	CaCCs?	ER (epithelial cells)	ER Ca^{2+} movement	Best's macular dystrophy
GPHR	High conductance; nonrectifying, weakly anion selective	Golgi complex	Golgi complex and TGN acidification	None found yet

[a]See text for references.

[b]Abbreviations: AQP6, aquaporin 6; CaCCs, calcium-activated chloride channels; CFTR, cystic fibrosis transmembrane conductance regulator; CLIC, Cl^- intracellular channel; EEs, early endosomes; ER, endoplasmic reticulum; GPHR, Golgi pH regulator; *I-V*, current-voltage; KO, knockout; LDCVs, large dense-core vesicles; LEs, late endosomes; NCL, neuronal ceroid lipofuscinosis; PM, plasma membrane; PT, proximal tubule; SLMVs, synaptic-like microvesicles; SVs, synaptic vesicles; TGN, *trans*-Golgi network.

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10). The CLC gene family, first identified by expression cloning of ClC-0 from *Torpedo* (11), is present in organisms from bacteria to humans. In mammals, this family comprises nine members that can be grouped into three branches according to their sequence homology (9, 10). Members of the first branch—ClC-1, -2, -Ka, and -Kb—are Cl^- channels of the plasma membrane. By contrast, the members of the second branch (ClC-3, -4, and -5) and third branch (ClC-6 and -7) localize predominantly on distinct, yet overlapping compartments apparently covering the complete endosomal pathway (12–18) and on related organelles such as synaptic vesicles (SVs) and synaptic-like microvesicles (SLMVs) (13, 19). Some of these, like ClC-5, to some extent also reach the plasma membrane, where their ion transport activity can be conveniently studied (20–22). Many of their sorting motifs have been identified (18, 23, 24). In contrast to the CLC founding member ClC-0 and the mammalian CLCs of the first branch, but like the bacterial EcClC-1 (25), the intracellular CLCs are Cl^-/H^+ exchangers with an $\sim 2Cl^-:1H^+$ stoichiometry (4, 22, 26–29).

SVs: synaptic vesicles

SLMVs: synaptic-like microvesicles

Cl^- transport by CLC proteins has been extensively studied (reviewed in References 10 and 30), and crystal structures of bacterial (31, 32) and, later, eukaryotic (33) CLCs have bolstered structure/function studies of CLCs. CLC proteins form rhombus-like dimers of twofold symmetry (31). Each monomer displays a complex transmembrane topology with an independent ion translocation pathway (31, 34). Although CLC proteins usually function as homodimers, heterodimerization between members of the first homology branch and between members of the second homology branch occurs upon heterologous expression (16, 34, 35) and for ClC-4 and -5 in vivo (36). Four of the mammalian CLCs are known to possess auxiliary β subunits (37–39).

The intracellular CLCs mediate outwardly rectifying currents that have been characterized by exploiting heterologous expression systems and site-directed mutagenesis (21, 27, 29, 40). A conserved glutamate residue in the ion translocation pathway, referred to as a gating glutamate because of its role in the gating of plasma membrane CLC Cl^- channels (32, 41, 42), is critical for the rectification of the intracellular CLCs that likely occurs by voltage-dependent gating (29, 43). For those CLCs that work as $2Cl^-/H^+$ exchangers, such as ClC-4 through ClC-7, the same gating glutamate is required for the coupling of Cl^- to H^+ transport (4, 22, 26–29). ClC-3, which is highly homologous to ClC-4 and ClC-5, probably also functions as a $2Cl^-/H^+$ transporter (9, 10). The physiological importance of this exchange mechanism was revealed by mice in which ClC-5 and ClC-7 were converted to pure Cl^- conductors (4, 5). The equivalent mutation in the single yeast CLC that resides in a late Golgi complex/prevacuolar compartment revealed that proton coupling is crucial for this CLC's cellular function (44).

Human diseases and genetic mouse models have shed light on the physiological importance and function of these vesicular anion transporters. ClC-3 appears to be expressed in all mammalian tissues, where it localizes to (late) endosomes (9). The same subcellular localization occurs upon heterologous expression. In neurons and neuroendocrine tissue, ClC-3 localizes additionally to SVs and to SLMVs. ClC-3B, a splice variant of ClC-3 with unknown function, contains a C-terminal PDZ-binding motif (45), which brings ClC-3B to the Golgi complex through binding to the Golgi complex–localized protein GOPC (46). The most striking phenotype upon ClC-3 disruption in mice is the severe degeneration of the hippocampus and the retina (13, 47, 48). The molecular mechanisms leading to this phenotype, however, remain to be elucidated.

ClC-4 also displays broad tissue distribution, but its subcellular localization has not been unambiguously clarified (9). Upon heterologous coexpression, ClC-4 colocalizes with and can be coimmunoprecipitated with its close relatives ClC-3 and -5 (16). Like ClC-3 and ClC-5, ClC-4 is found on compartments of the endocytic pathway (16, 36, 49). Reports of different subcellular distributions, such as to the ER (50) or the plasma membrane (51), may be explained by the expression system or by the lack of knockout-controlled antibodies. The physiological function of ClC-4 is unknown, and mice lacking ClC-4 display no obvious phenotype (52, 53).

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ClC-5 expression is strongest in epithelial cells of the kidney and intestine (20, 54, 55), where it colocalizes with the V-ATPase to apical early endosomes (12, 56). Owing to its role in renal proximal tubular endocytosis (57), dysfunction of ClC-5 in humans and mice leads to the symptoms of Dent's disease, such as proteinuria, hyperphosphaturia, hypercalciuria, and kidney stones (recently reviewed in References 9, 10, and 58).

Proteinuria: loss of proteins into the urine

Osteopetrosis: a rare hereditary disease with increased bone mass and severe secondary effects; also known as marble bone disease

Neuronal ceroid lipofuscinosis (NCL): a family of neurorodegenerative lysosomal storage diseases

ClC-6 is expressed predominantly in neurons, where it localizes to late endosomes (15). Both central and peripheral neurons of ClC-6 knockout mice accumulate lysosomal storage material, which may underlie the observed decrease in pain sensitivity (15).

The ubiquitously expressed ClC-7 localizes to late endosomes/lysosomes (14). In osteoclasts, it is expressed prominently in the ruffled border, a lysosome-derived acid-secreting plasma membrane domain involved in bone resorption (14). ClC-7 requires a β subunit, the type I transmembrane protein Ostm1, for protein stability and ion transport activity (29, 38). Mutation or loss of either protein leads to osteopetrosis in mice and humans (14, 59) and to a neuronal ceroid lipofuscinosis (NCL)-like lysosomal storage disease accompanied by degeneration of neurons and the retina (38, 60).

CFTR: A cAMP-Activated Plasma Membrane Cl^- Channel with Possible Vesicular Roles

The gene encoding CFTR, a cAMP-activated Cl^- channel, was identified by positional cloning as the gene underlying cystic fibrosis (61). Cystic fibrosis, the most common lethal autosomal recessive disease in Caucasians, is a multisystemic disease caused by defective salt and water transport across epithelia that results in an alteration of fluid films covering epithelia and variable obstructions of, e.g., pancreatic ducts and the intestine (62). Changes in the composition of the mucus covering airway epithelia facilitate chronic infections of the lung, the most common cause of death.

CFTR belongs to the ATP-binding cassette (ABC) transporter family, with a tandem repeat of six putative transmembrane helices and a nucleotide-binding fold separated by a large regulatory domain (61). Various studies have unambiguously demonstrated that CFTR functions as a Cl^- channel (recently reviewed in Reference 63).

In agreement with its role in epithelial salt secretion, CFTR localizes to the apical surface of various epithelia, such as epithelia in airways, intestines, secretory glands, bile, and pancreatic ducts (64). In addition, several studies have reported CFTR to localize and function on organelles of the biosynthetic and endocytic pathways (65). In particular, an early-endosomal localization has been reported in the kidney, where CFTR is prominently expressed as well (reviewed in Reference 66).

CLICs: Highly Questionable Candidates for Intracellular Cl^- Channels

The CLIC (denoting Cl^- intracellular channel) gene family consists of six members in vertebrates (for recent reviews, see References 67 and 68). The founding member was isolated as a putative Cl^- channel from bovine kidney microsome fractions (69). The protein with an apparent molecular weight of 64 kDa (hence termed p64 and now also known as CLIC5B) was subsequently cloned and discovered to mediate Cl^- flux after reconstitution into vesicles (70). CLIC1 through CLIC4 and CLIC5A, a splicing variant of p64, lack the large N terminus of p64/CLIC5, leaving a conserved stretch of just ~240 amino acids. Apart from p64/CLIC5B, only CLIC6 (also known as parchorin) possesses a large N-terminal domain in addition to the C-terminal CLIC domain.

The sequence of CLIC proteins, however, lacks the hydrophobic stretches typical for ion channels and integral membrane proteins. CLICs appear to be dimorphic proteins that exist and

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function as soluble proteins, but they may also insert into lipid bilayers, a feature reminiscent of annexins and bacterial porins. Crystal structures for most CLICs are now available in their soluble form, whereas the mechanism of membrane insertion has not been clarified in sufficient detail (67, 68). The biophysical properties of anion transport associated with CLICs are still nebulous. Various biophysical studies, using either transfected cells or purified proteins reconstituted into artificial planar bilayers, reported different anion conductances that varied from 6 to 120 pS for CLIC1. In most studies, CLICs displayed poor anion selectivity or did not differentiate between anions and cations (reviewed in Reference 67). Reconstitution experiments are prone to artifacts, and heterologous overexpression can activate endogenous currents. So far no mutagenesis experiments that would alter the observed transport properties have been reported, leaving considerable doubt about their ion transport activity. Additionally, despite their ability to insert into membranes and their localization to various intracellular compartments, most, if not all, of the diverse cell physiological functions assigned to CLICs seem to be fulfilled by their soluble forms, and there is no convincing evidence that CLICs exert a physiological function as Cl^- channels. Instead, CLICs may serve different functions such as the mediation of cytoskeleton–plasma membrane interaction or transcriptional activation (67). Thus, the terminology CLIC (for Cl^- intracellular channel, as discussed above) may be grossly misleading.

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Further Proposed Cl^- Transporters of Various Intracellular Compartments

Biophysical studies of intracellular organelles, such as the nuclear envelope, the ER, and the Golgi complex, have reported multiple Cl^- conductances with distinct properties (e.g., References 71–75). These currents cannot yet be unambiguously assigned to transport proteins. Candidate proteins may emerge with the identification of new Cl^- transporter families or with the discovery that specific members of gene families generally performing other functions also mediate Cl^- transport.

An example of the latter case is AQP6, a member of the aquaporin water and glycerol channel family. AQP6 localizes predominantly to apical endosomes in renal epithelia and in particular to acid-secreting intercalated cells. AQP6 expression in *Xenopus* oocytes yielded slightly outwardly rectifying currents with moderate anion selectivity (76), but single-channel analysis found that AQP6 is equally permeable to Cl^- and Na^+ (77). Using a GFP-AQP6 fusion protein with drastically increased plasma membrane expression revealed high nitrate permeability (78). Interestingly, AQP6 is activated at pH values of less than 5.5, whereas ClC-5, which displays a similar subcellular localization, is inhibited by low pH (21). The physiological role of AQP6 is unknown.

Members of the TMEM16 [alternatively termed anoctamin (ANO)] gene family, TMEM16A/ANO1 and TMEM16B/ANO2, constitute long-sought Ca^{2+}-activated Cl^- channels (79–81). The ten TMEM16 family members (TMEM16A–H, -J, and -K or ANO1–10), which share a common predicted topology with cytoplasmic N and C termini and eight transmembrane domains, are involved in various physiological processes (recently reviewed in Reference 82). Although Cl^- channel activity has been shown only for TMEM16A and -B, the other members may also represent Cl^- channels. However, as exemplified by the CLC family that comprises both Cl^- channels and Cl^-/H^+ exchangers (9, 10), it cannot be excluded that some TMEM16 proteins exert other functions. For instance, it has been reported that TMEM16F/ANO6 induces scramblase activity that is absent in Scott syndrome patients carrying TMEM16F mutations (83). It is, however, presently unclear whether TMEM16F mediates lipid translocation. Upon heterologous expression, TMEM16C–G (ANO3–7) localized to intracellular membranes and the ER (84). However, in the absence of reliable antibodies, it is unclear whether they localize to the ER endogenously and, if so, what physiological role they would exert.

Although present mainly in the plasma membrane, bestrophin-1 may also reside in the ER, where it may facilitate Ca^{2+} movement (85). There is rather compelling evidence that bestrophins can act as Ca^{2+}-activated Cl^- channels (86), but they may also have other functions, such as modulating Ca^{2+} channels (87, 88). It remains unclear whether Cl^- currents through bestrophin-1 facilitate ER Ca^{2+} release. Intriguingly, bestrophin-1 has recently been reported to mediate glial release of neurotransmitters such as GABA (89).

trans-Golgi network (TGN): the major site of cargo sorting in the secretory pathway

DIDS: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid

Expression cloning identified GPHR (Golgi pH regulator) as responsible for impaired Golgi complex acidification in a mutant cell line (90). GPHR, which is predicted to be a multimembrane-spanning protein, is localized predominantly to the Golgi complex. Reconstitution of purified GPHR into planar lipid bilayers yielded nonrectifying, weakly selective anion currents (90). However, no mutagenesis altering the associated conductance was shown to ascertain that GPHR is an anion channel.

SVs express the ClC-3 Cl^- transporter, but the vesicular glutamate transporter VGLUT1 was also reported to conduct Cl^- (91), and the GABA transporter VGAT cotransports Cl^- with GABA in lipid bilayer reconstitution (92). It remains to be confirmed whether these neurotransmitter transporters transport Cl^- in a native setting.

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MEMBRANE-TRAFFICKING PATHWAYS INVOLVING CHLORIDE TRANSPORT

Cl^- is considered to provide the main electrical shunt for the acidification of most organelles of the secretory and endocytic pathways but may also affect vesicle transport and function by other means. In this section, we focus on particular membrane-trafficking steps that are impinged by the lack or dysfunction of Cl^- transport proteins.

Cl^- Transporters in the Secretory Pathway: A Role in Golgi Complex Sorting and Large Dense-Core Vesicle Exocytosis?

In the Golgi complex and the *trans*-Golgi network (TGN), cargo of the biosynthetic pathway undergoes extensive posttranslational modifications, such as proteolytic cleavage and glycosylation, and is sorted for transport to its destination. These processes depend on an adequate luminal acidification (93, 94). As Cl^- currents provide at least part of the electrical shunt required for proton pumping by the V-ATPase (95), impairment of the respective Cl^- conductance is expected to increase the luminal pH. CFTR was proposed to mediate such a counterion transport when the Golgi complex and TGN of cells with mutated CFTR were reported to be more alkaline (96). However, subsequent studies found Golgi complex pH to be normal in the absence of CFTR (reviewed in Reference 97). More recently, researchers found that dysfunction of the putative Golgi-resident Cl^- channel GPHR diminishes the acidification of the Golgi complex and TGN, leading to Golgi complex fragmentation and delayed transport and impaired glycosylation of proteins (90). However, it remains to be seen whether counterion transport by GPHR is directly responsible for this effect.

Whereas the role of Cl^- for constitutive secretion beyond the TGN remains obscure (98), regulated secretion of storage granules likely needs a Cl^- conductance for further acidification and exocytic release (99). Priming of insulin granules for exocytosis depends on acidification by the V-ATPase and may be blocked by DIDS, an unspecific inhibitor of anion transporters and other proteins (100). ClC-3 was proposed to provide this Cl^- conductance (100–102). Further indirect evidence of this notion is the reduction in acidification of insulin granules by Ins(3,4,5,6)P_4 (103), a second messenger that may inhibit ClC-3 (104). Steady-state pH of ClC-3 knockout secretory

granules was reported to be more than one pH unit more alkaline than in the wild type, but that value was clearly outside the measuring range (101), and we could not detect differences in granular acidification in our laboratory (T. Maritzen, O. Scheel & T.J. Jentsch, unpublished results). Additionally, the localization of ClC-3 to large dense-core vesicles (LDCVs) is questionable (9, 105). In knockout-controlled experiments, ClC-3 was not found on LDCVs, but rather on endosomes and SLMVs, both in adrenal chromaffin cells and in pancreatic β cells (19). Nonetheless, exocytosis of LDCVs in ClC-3-deficient chromaffin cells was reduced (19), and glucose-induced insulin release was reportedly diminished in mice globally disrupted for ClC-3 (101, 102). However, glucose tolerance tests were normal in *Clcn3*$^{-/-}$ mice (48). Moreover, these mice are systemically sick and, for instance, also display changed leptin concentrations. No changes in insulin and leptin were found in mice lacking ClC-3 only in β cells (19). The mechanism by which lack of ClC-3 impairs LDCV exocytosis remains obscure and may be an indirect consequence of a trafficking defect in the endosomal system to which ClC-3 localizes.

LDCVs: large dense-core vesicles

Adrenal chromaffin cells: catecholamine-releasing neuroendocrine cells of the adrenal gland medulla

β cells: the insulin-releasing cells of the pancreatic islets

PT: proximal tubule

Megalin: an apical recycling receptor responsible for the endocytosis of many proteins and other substances

Impaired Endocytosis upon Dysfunction of Intracellular Cl$^-$ Transporters

The Cl$^-$/H$^+$ exchangers ClC-4 and -5 localize to early and recycling endosomes. Mutations in ClC-5 underlie Dent's disease (106). A constant symptom of that X-linked disorder is low-molecular-weight proteinuria that points to reduced apical endocytosis by renal proximal tubule (PT) cells (107). The localization of ClC-5 to apical endosomes in PT cells (12) suggested that ClC-5 may be directly involved in early endocytosis. Two studies on independent ClC-5 knockout mouse models confirmed this role (57, 108). Well-controlled studies of endocytosis in vivo were performed in heterozygous female mice that have chimeric PTs with cells expressing or lacking ClC-5 due to random X-chromosomal inactivation (57). ClC-5-deficient PT cells showed a drastic, cell-autonomous reduction of both fluid-phase endocytosis and receptor-mediated endocytosis of intravenously injected, fluorescently labeled endocytic cargo (57). In addition, the decreased levels of the endocytic receptors megalin and cubilin indicated that their recycling to the cell surface is affected by lack of ClC-5 (57, 109), suggesting that receptor-mediated endocytosis is even more severely affected than fluid-phase endocytosis. ClC-5 is thus required for endosomal acidification (110, 111) and/or Cl$^-$ accumulation (5). However, the particular transport step within the endocytic pathway in which ClC-5 exerts its crucial function is yet to be elucidated. Although no gross morphological changes in the endocytic pathway were observed in *Clcn5*$^-$ PTs (57), the lack of ClC-5 also affects lysosome biogenesis through its role in endocytosis (112). Hyperphosphaturia and kidney stones upon ClC-5 dysfunction may be explained as secondary effects of defective endocytosis (see sidebar on indirect consequences of impaired PT endocytosis).

ClC-4 may colocalize and interact with ClC-5 in renal PT cells (36), implicating similar roles for ClC-4 and ClC-5. ClC-4-deficient mouse fibroblasts were reported to display elevated endosomal pH and impaired endocytic uptake of transferrin (113). However, the severe phenotype of Dent's disease in mice and humans shows that loss of ClC-5 is not compensated by ClC-4. Moreover, *Clcn4*$^{-/-}$ mice do not present either detectable proteinuria or defective PT endocytosis, and the additional disruption of ClC-4 in ClC-5-deficient PT cells does not further compromise their endocytosis (53).

CFTR may colocalize with ClC-5 in early endosomes in distal segments of PTs (114). CFTR-deficient mice display mild proteinuria, and receptor-mediated endocytosis by PT cells is moderately reduced (114, 115). Impaired endosomal recycling was reported earlier for cells with defective CFTR (116). In contrast to ClC-5 knockout mice, CFTR-deficient mice have unaltered levels of megalin (114), which refutes a general recycling defect. Instead, the endocytic defect seems to be due to a mechanistically obscure destabilization of the megalin-cubilin interaction that entails a urinary loss of cubilin (114).

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HYPERPHOSPHATURIA AND KIDNEY STONES: INDIRECT CONSEQUENCES OF IMPAIRED ENDOCYTOSIS IN PROXIMAL TUBULES

ClC-5 and OCRL, both proteins whose dysfunction can underlie Dent's disease, function in the early steps of the endocytic pathway (57, 166), which is compatible with the proteinuria seen in this disease. According to an experimentally supported hypothesis (57), the primary defect in endocytosis, specifically that of calciotropic hormones, also accounts for the hyperphosphaturia and the more variable symptoms of hypercalciuria and kidney stones. After passing the glomerular filter, parathyroid hormone (PTH) is normally endocytosed from the primary urine by proximal tubule (PT) cells. Defective endocytosis entails elevated PTH levels in the lumen of later PT segments, enhancing the stimulation of apical PTH receptors. This stimulation triggers the internalization and degradation of the cell surface–resident phosphate transporter NaPi-2a (57), thereby causing phosphaturia. Like PTH, vitamin D_3 is endocytosed by PT cells from the primary urine. Both reduced uptake of 25(OH)-vitamin D_3 and the increased PTH signaling upon defective endocytosis by PT cells would increase the activity of the hydroxylase that produces active vitamin D_3 [1,25-$(OH)_2$-$VitD_3$]. However, the increased hydroxylase activity is counteracted by the diminished availability of the inactive precursor. Thus, this hypothesis states that the balance between two opposing mechanisms (reduced substrate availability and increased activation rate) governs whether 1,25-$(OH)_2$-$VitD_3$ levels are elevated. Elevated levels would increase intestinal Ca^{2+} absorption and favor hypercalciuria and kidney stones.

Cl^- Transport Proteins in Late-Endosomal/Lysosomal Transport and Function

Various Cl^- transport proteins have been implicated in late-endosomal/lysosomal trafficking and in the function of lysosomes and lysosome-related organelles (LROs). Comparing hepatocytes cultured from wild-type and ClC-3-deficient mice revealed that ClC-3 participates in normal acidification and in increasing $[Cl^-]_{lumen}$ in early and late endosomes (117). A role of ClC-3 in late-endosomal traffic is suggested by the observation that its overexpression in mammalian cells entails a drastic enlargement of vesicular structures positive for late-endosomal/lysosomal markers (118). Endocytic cargo destined for lysosomes accumulated in these compartments. It remains, however, unclear whether the neuronal and retinal degeneration of ClC-3-deficient mice (13, 47, 48) is due to disturbed endosomal trafficking or to a role of ClC-3 on SVs. One of the three independent ClC-3 mouse models (47) reportedly displayed features of the lysosomal storage disease NCL, but closer examinations of another ClC-3-deficient mouse strain could not confirm this finding (13, 60). By contrast, neuronal accumulation of lysosomal storage material was observed in mice lacking ClC-6, a late-endosomal CLC expressed almost exclusively in neurons (15), demonstrating a role of ClC-6 in late-endosomal/lysosomal function.

Lysosome-related organelles (LROs): cell-specific intracellular organelles similar to lysosomes in terms of biogenesis, protein content, and acidic pH

The only CLC found on lysosomes is the ubiquitously expressed ClC-7 (14, 28, 60). Its importance for lysosomal function is obvious from the lysosomal pathology of mice lacking either ClC-7 [*Clcn7*$^{-/-}$ mice (14)] or its obligate β subunit, Ostm1 (spontaneous *grey-lethal* mutant mice). Neurons and PT cells of these mice accumulate lysosomal storage material (38, 60). Whereas these deposits are located within the initial axon segment of *Clcn6*$^{-/-}$ neurons (15), they are scattered throughout the somata of ClC-7- or Ostm1-deficient neurons (38, 60). The lysosomal storage of ClC-7/Ostm1-deficient mice displays features of NCL, such as an accumulation of subunit c of the mitochondrial ATP synthase and progressive CNS neurodegeneration that is accompanied by microglial activation and astrogliosis (38, 60, 119). Forebrain-specific disruption of ClC-7 in mainly excitatory neurons demonstrated that neuronal accumulation of storage material is cell autonomous and that neurodegeneration can be extremely severe (17). In neurons and renal PT cells of mice lacking ClC-7/Ostm1, the morphologic integrity of the

late-endosomal/lysosomal system is disturbed. In brains of *Clcn7*$^{-/-}$ mice, ClC-3 and ClC-6 are shifted partially into lysosomal fractions, as revealed by subcellular fractionation (15). Whereas staining of the late-endosomal/lysosomal marker protein lamp-1 (lysosome-associated membrane protein-1) appears more diffuse in ClC-7/Ostm1-deficient neurons (17, 60), lamp-1-positive structures are drastically enlarged in PT cells (17). Both receptor-mediated endocytosis and fluid-phase endocytosis are unaffected in ClC-7-deficient PT cells, and endocytic cargo is delivered to the enlarged late endosomes/lysosomes (17). Quantitative in vivo pulse-chase experiments using mice with a chimeric deletion of ClC-7 in PTs (generated by crossing floxed ClC-7 mice with mice expressing cre-recombinase under the control of the ApoE promoter) revealed retarded degradation of endocytosed protein (17). Intriguingly, the additional disruption of ClC-5, which reduces endocytic uptake, does not abolish the enlargement of late endosomes/lysosomes in ClC-7-deficient PT cells (17). This finding suggests that the morphological alteration of the endosomal/lysosomal pathway is not due to an accumulation of undegraded, endocytosed cargo but may rather result from disturbed trafficking or budding/fusion events.

lamp-1: lysosome-associated membrane protein-1

Pheomelanin: a red-yellow class of melanin pigments

ROS: reactive oxygen species

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Brains and kidneys of ClC-7 knockout mice display increased levels of LC3-II, indicative of an accumulation of autophagic structures (17). It is unclear whether this phenotype is due to a defect in lysosomal clearance (consistent with the slowed lysosomal degradation) of autophagic material or to an increase in autophagy (possibly as a cellular answer to lysosomal storage accumulation). ClC-7/Ostm1 seems also involved in the function of LROs such as melanosomes. In a genetic background with normally brown fur, the coat color of *Clcn7*$^{-/-}$ (14) and spontaneous Ostm1-deficient *grey-lethal* mice is gray. The exact role of ClC-7/Ostm1 in pigmentation, however, is unknown. The transporter may be required for the generation or exocytosis of pheomelanin. A reduction in exocytosis seems supported by the observation that the acid-secreting ruffled border of osteoclasts, which is built up by massive lysosomal exocytosis, is severely underdeveloped in *Clcn7*$^{-/-}$ mice (14).

CFTR may also play a role in lysosomal and phagolysosomal degradation in some cells. Macrophages internalize invading microorganisms, such as bacteria, in phagosomes. Nascent phagosomes fuse with lysosomes to form phagolysosomes that maintain an acidic pH for bacterial clearance by enzymatic breakdown. Lack or dysfunction of CFTR impairs the bactericidal activity specifically of alveolar—but not that of, e.g., peritoneal—macrophages via diminished acidification of lysosomes and phagolysosomes, despite normal phagosome-lysosome fusion (120, 121). Ceramide levels are increased in the lungs of CFTR-deficient mice, which may result from impaired ceramide degradation due to elevated lysosomal pH in airway epithelial cells (122). However, several subsequent studies using superior pH measurement techniques (123) failed to confirm a role of CFTR in lysosomal acidification in either alveolar macrophages or respiratory epithelial cells (124–127). A different explanation for the inflammatory phenotype of airway epithelia in cystic fibrosis was recently provided by a study that showed that defective CFTR inhibits autophagy, thereby entailing a sequestration of anti-inflammatory proteins into aggresomes (128). Defective CFTR may impair this process indirectly through an increase in intracellular reactive oxygen species (ROS) (128), but the link between CFTR and increased ROS remains obscure. CLIC1 was also recently implicated in phagosome acidification, but a direct demonstration that this is due to a chloride conductance is lacking (129).

MECHANISTIC FUNCTION OF CHLORIDE TRANSPORT AND CONCENTRATION

For a long time, Cl^- transport over intracellular membranes was thought to serve purely as an electrical shunt for the movement of physiologically more relevant cations. The main role of

Cl^- channels in the secretory and endocytic pathways was thought to be allowing and modulating organellar acidification, which may affect secondary active transport processes, trafficking, and enzymatic activities. However, several findings have altered this view. Cl^- transport seems dispensable for the acidification of some organelles, and recent data strongly suggest that Cl^- transport exerts functions beyond simply providing the counterion for acidification.

Resorption lacuna: the sealed space between an osteoclast and bone that is acidified and equipped with enzymes for the degradation of bone material

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Many, But Not All, Organelles Require Cl^- as a Counterion for Their Acidification

The pivotal importance of the generation and maintenance of pH gradients across the membrane of intracellular vesicles is undisputed. Posttranslational modification of secreted proteins, regulation of receptor-ligand interactions, cargo sorting, recruitment of transport machinery components, neurotransmitter loading, and degradation by lysosomal enzymes are among the numerous processes in the secretory and endocytic pathways that depend on an acidic luminal pH (reviewed in, e.g., References 93–95). To prevent the buildup of an inside-positive electrical potential across the membrane, which would hamper further entry of protons, a cation efflux and/or an anion influx must accompany H^+ influx. Early studies on isolated Golgi complex vesicles and endosomes showed that external Cl^- is required for efficient acidification (e.g., References 130–132). This notion was corroborated by studies that monitored $[Cl^-]_{lumen}$ in the endocytic pathway (2, 3). After an initial drop to approximately 20 mM after endocytic compartments pinch off from the plasma membrane, $[Cl^-]_{lumen}$ increases as these compartments are gradually acidified. On the basis of their endosomal localization, ClC-3, -4, and -5 are good candidates to supply the necessary Cl^- conductance. Consistent with this idea, an endosome-enriched preparation from livers of *Clcn3*$^{-/-}$ mice reportedly displayed a slightly less acidic pH compared with that of wild type (47). Acidification and increase in $[Cl^-]_{lumen}$ of early and late endosomes are diminished in ClC-3-deficient cultured hepatocytes (117). ClC-3 may also be involved in the acidification of SVs (13, 133). The requirement of ClC-5 for the acidification of renal endosomes was demonstrated convincingly with isolated endosomes (5, 110) and by monitoring endosomal pH and $[Cl^-]_{lumen}$ in cultured PT cells (111) from *Clcn5*$^-$ mice.

In contrast to the situation in endosomes, the need for Cl^- transport proteins for the establishment of the acidic pH of lysosomes is a matter of ongoing debate (123, 134). For specialized cell types, in particular alveolar macrophages and respiratory epithelial cells, lack or inhibition of CFTR has been reported to entail elevated lysosomal pH (120–122). Subsequent studies, however, showed no influence of CFTR on lysosomal pH (124–127). The studies refuting a role for CFTR appear more reliable, as they exploited technically superior, ratiometric methods to measure lysosomal pH (123). There is also some controversy about the role of ClC-7, which localizes to late endosomes/lysosomes in most, if not all, cells and to the acid-secreting ruffled border facing the resorption lacuna of bone-resorbing osteoclasts. The acidification of this lysosome-like extracellular space is impaired in osteopetrotic ClC-7 knockout mice (14). This impairment can be attributed in part to an underdevelopment of the ruffled border (14). A severe underdevelopment is also seen when ClC-7 is converted into a pure Cl^- conductance (4). Although staining with an acidotropic dye had shown the presence of acidified vesicles in ClC-7-deficient cells (14), it came as a surprise that lysosomes in cultured neurons and fibroblasts from mice lacking ClC-7 or its obligate β subunit, Ostm1, displayed no detectable elevation of their acidic steady-state pH (38, 60), a finding also made with neurons lacking the late-endosomal ClC-6 (15). Further studies confirmed the dispensability of ClC-7 for lysosomal steady-state pH in alveolar macrophages (127) and for in vitro acidification of lysosomes (4). In an apparently contradicting report (28), the reduced total lysotracker signal in HeLa cells upon partial ClC-7 knockdown with a single siRNA was interpreted as indicative of elevated lysosomal pH. Obviously, the ratiometric pH

determinations in the former studies are more trustworthy (123). Majumdar et al. (135) recently reported that lysosomal acidification in microglia is enhanced upon microglial activation with macrophage colony–stimulating factor in parallel with upregulation of lysosomal ClC-7/Ostm1. A direct causative role of Cl^- transport by ClC-7/Ostm1 remains to be tested, but a role of ClC-7 in lysosomal acidification in some specialized cell types cannot be excluded.

Not only the role of particular Cl^- transporters but also that of Cl^- itself as the required counterion for lysosomal acidification are disputable. Cl^- influx is actually dispensable for ATP-dependent lysosomal acidification after transient dissipation of the pH gradient in vitro and in cultured macrophages (4, 127). Although Cl^- influx can support lysosomal reacidification, efflux of monovalent cations seems to provide a major part of the required electrical shunt (127). The higher cation conductance of lysosomes compared with that of endosomes (132) may underlie the different requirements of Cl^- for the acidification of endosomes and lysosomes.

The steady-state pH in the Golgi complex is determined mainly by proton leakage rather than by a limiting countercharge conductance for acidification (136). The decreasing rate of proton leakage along the secretory pathway may underlie the drop in luminal pH from the ER to secretory granules (137). The nature of this proton conductance is still obscure, but this conductance may be a key regulator of steady-state pH in all intracellular acidic compartments (138). Nonetheless, experiments using in situ pH measurements suggested that both cation and Cl^- conductances can support Golgi complex acidification (139), to which Cl^- may even contribute to a larger extent than do cations (3).

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The Electrochemical Proton Gradient Drives Secondary Active Transport: The Case of Synaptic Vesicles

The secondary active filling of SVs with neurotransmitters is driven by the electrochemical gradient of protons generated by the V-ATPase. The chemical component, i.e., ΔpH, plays a more important role in loading with some neurotransmitters, such as monoamines, whereas the vesicular glutamate transporters (VGLUT1–3) depend primarily on the electrical voltage (V or $\Delta\Psi$) across the SV membrane, and uptake of glycine and GABA seems to rely equally on both parameters (recently reviewed in Reference 140). Dependence on ΔpH indicates an exchange of neurotransmitters for H^+, whereas dependence on V ($\Delta\Psi$) means that neurotransmitter uptake moves net negative charge into SVs. The electrical component of the electrochemical H^+ gradient can be selectively diminished by ionic conductances, resulting in larger ΔpH, whereas ΔpH may be reduced (and V increased) by electroneutral exchangers involving H^+. Indeed, K^+/H^+ exchange may stimulate glutamate uptake by increasing the portion of the electrical gradient over the SV membrane (141). Proton translocation into SVs requires the parallel influx of anions, usually Cl^-, for charge compensation (140). Due to its localization to SVs (13, 142, 143), ClC-3 is expected to provide the required Cl^- conductance. Indeed, in vitro acidification of SVs from *Clcn3*$^{-/-}$ mice was diminished (13, 133). Nonetheless, the remaining acidification was sufficient to allow for efficient uptake of dopamine. GABA loading seemed unaffected by the lack of ClC-3, as no alterations in miniature inhibitory postsynaptic currents (mIPSCs), which depend on the GABA content of individual SVs and on the density of postsynaptic receptors, were detected (13). The observed reduction in vesicular glutamate uptake was explained by reduced VGLUT1 levels that may be due to a selective loss of glutamatergic SVs (13). The observed slight increase in miniature excitatory postsynaptic current (mEPSC) amplitudes (13), which may hint at higher glutamate content of individual SVs, is difficult to interpret, as neurons begin to degenerate even at the young age of mice used in this study (13).

A subsequent study has questioned that ClC-3 provides a major electrical shunt for SV acidification (91). ClC-3 was reported to be present on fewer than 0.1% of SVs (91), a finding that our

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lab, however, could not reproduce by using equivalent methods (S. Weinert & T.J. Jentsch, unpublished data). Instead, Schenck et al. (91) attributed the electric shunt to VGLUT1 and reported a lack of Cl^--dependent acidification of SVs from VGLUT1 knockout mice (91), attributing the previously observed reduced acidification of ClC-3-deficient SVs (13) to the reduced VGLUT1 levels in these SVs. Although it is widely accepted that VGLUT1 can facilitate SV acidification by the import of negatively charged glutamate ions (which, however, needs allosteric activation by Cl^-), these authors also reported that VGLUT1 transports Cl^- and therefore facilitates SV acidification in the absence of glutamate (91). However, experiments by others (144) refuted Cl^- transport by VGLUT1 (and VGLUT2).

In contrast to the earlier finding of reduced Cl^--dependent, ATP-driven SV acidification in ClC-3-deficient mice (13), normal Cl^--dependent, ATP-driven acidification was reported (133) for a crude SV preparation from a different ClC-3 knockout mouse strain (48). This preparation should contain mostly glutamatergic vesicles, but the authors did not check for the abundance of VGLUT1 in their preparation. Hence no direct comparison to the earlier study (13), and no conclusion about a role of VGLUT1, is possible. When the vesicle population was enriched for inhibitory SVs by depleting glutamatergic vesicles with antibodies against VGLUT1, the remaining SVs from *Clcn3*$^{-/-}$ mice acidified less efficiently than those from wild-type mice (133). This observation appears consistent with the previous study in which VGLUT1 levels were reduced within the total SV population (13). Riazinski et al. (133) also immunodepleted a rat SV preparation presumably enriched for inhibitory vesicles (by first immunodepleting for glutamatergic vesicles) with an antibody against ClC-3. The remaining vesicles acidified less efficiently than the starting preparation. But contrary to the claim that this constitutes a "rat model for the *Clcn3*$^{-/-}$ vesicles," this depleted vesicle population most likely differs not only in the amount of ClC-3 but, in many respects, from wild-type vesicles, and no conclusion as to a specific role of ClC-3 is possible. In support of their hypothesis that ClC-3-facilitated acidification of SV drives GABA uptake, the authors showed a reduction in amplitude and frequency of mIPSCs in hippocampal slices (133). This finding, however, contrasts with earlier findings in another *Clcn3*$^{-/-}$ mouse line that there are no such differences (13).

Given these rather confusing and contradictory data, much more work is needed to clarify the roles of ClC-3 and VGLUT1 in SV acidification. The situation is complicated by the heterogeneous nature of vesicles used in acidification assays. In view of the early-onset neurodegeneration in *Clcn3*$^{-/-}$ mice, it is desirable to use young mice, with the clear caveat that SV composition may change during postnatal development. Lastly, it remains to be elucidated whether the loss of neuronal cells in ClC-3 knockout mice results from the absence of ClC-3 from SVs or whether a defect in the endosomal system, to which ClC-3 also localizes, underlies this neurodegeneration.

The Need for Cl^-/H^+ Exchange Suggests Additional Roles for Cl^-

The functional characterization of ClC-4 and -5 as Cl^-/H^+ exchangers rather than as Cl^- channels (22, 26) suggested that their physiological function may not be limited to providing compensating currents for luminal acidification (49). The highly electrogenic exchange with a ratio of approximately $2Cl^-:1H^+$ (22, 26, 145) would still perfectly fulfill the requirement, but at higher energetic cost, because overall electroneutrality demands that one out of three protons transported by the ATP-consuming V-ATPase leave the lumen. One idea was that ClC-5 might directly mediate acidification of early endosomes by exchanging the high $[Cl^-]_{lumen}$ of endocytic vesicles for protons immediately after the vesicles pinch off from the plasma membrane (22). This H^+-ATPase-independent mechanism was recently proposed to effectively contribute to the acidification of early endosomes (146). However, theoretical considerations put this mechanism very much in doubt (for

calculations, see Reference 9): With the usual extracellular and cytosolic [Cl^-] and a typical proton buffer capacity, the initial $[Cl^-]_{lumen}$ will not suffice to accumulate enough protons in the lumen to obtain sufficiently acidic pH values. Moreover, the efflux of negative charge by the electrogenic exchange would require charge compensation, which the H^+-ATPase could not provide, as its activity would increase, rather than decrease, the inside-positive voltage created by the hypothetical Cl^- efflux through ClC-5. The requirement of Cl^- transport for H^+-ATPase-mediated early-endosome acidification (5, 110, 132) argues against a significant electrical shunt mediated by cations. The efflux of Cl^- and cations would furthermore lead to an osmotic shrinkage of the acidifying endosome. This scenario conflicts with the observed volume increase reported for early endosomes (2). Lastly, the observation that $[Cl^-]_{lumen}$ is initially low in newly formed endosomes and increases during acidification (2, 3, 111) also argues against the postulated acidification through Cl^-/H^+ exchange.

In an alternative role of the Cl^-/H^+ exchange mechanism—in analogy to vacuolar nitrate accumulation by AtClCa in *Arabidopsis* (147)—the endosomal/lysosomal CLC exchangers may accumulate Cl^- in the lumen of their respective organelle (49), in addition to electrically shunting proton pumping as shown for ClC-5 (110). This possibility was experimentally approached by generating two knock-in mouse models in which the gating glutamate of endosomal ClC-5 and lysosomal ClC-7 was mutated to alanine (4, 5). This mutation uncouples Cl^- from proton countertransport and converts these outwardly rectifying CLC exchangers into linear Cl^- conductances (4, 21, 22, 26, 29). Such an uncoupled ClC-5 transporter is expected to efficiently support endosomal acidification according to the classical model of charge compensation by Cl^- channels. Isolated renal endosomes from these *Clcn5*^{unc} (unc for uncoupled) mice indeed displayed normal V-ATPase-mediated acidification (5). Nonetheless, receptor-mediated endocytosis and fluid-phase endocytosis in PT cells were as severely impaired as those in mice lacking ClC-5, as revealed by their proteinuria and by in vivo endocytosis experiments with PTs chimeric for uncoupled and Cl^-/H^+-exchanging ClC-5 (5). Like ClC-5-deficient mice, *Clcn5*^{unc} mice had drastically reduced levels of the endocytic receptors megalin and cubilin in PTs and displayed hyperphosphaturia and hypercalciuria (5). Because endosomal acidification is normal, these phenotypes of *Clcn5*^{unc} mice can be ascribed specifically to the uncoupling of Cl^- transport from proton countertransport.

Mice expressing ClC-7^{unc} instead of the wild-type protein accumulate lysosomal storage material in neurons and display retinal and CNS degeneration and secondary microgliosis symptoms that are as pronounced as those in ClC-7-deficient mice (4). Like lysosomes from ClC-7-deficient fibroblasts, lysosomes derived from *Clcn7*$^{unc/unc}$ mice display an unaltered steady-state pH and normal V-ATPase-mediated acidification in vitro (4). However, the lysosomal pH reached after protonophore treatment, which is determined by the voltage generated by the lysosomal conductances and respective ion gradients, differs between fibroblasts obtained from wild type, *Clcn7*$^{-/-}$, and *Clcn7*$^{unc/unc}$ mice. The relatively acidic steady-state pH of protonophore-treated, ClC-7-deficient lysosomes suggests the presence of a lysosomal cation conductance that creates a lumen-negative potential in the presence of a lumen-to-cytoplasm cation gradient that could be accounted for only by Na^+ (4). This conclusion agrees with the observation that Cl^- is dispensable for lysosomal reacidification (4, 127) and with more direct experiments showing lysosomal cation conductance (127). Due to the additional presence of a Cl^- conductance, which presumably adds a lumen-positive potential owing to a lumen-to-cytoplasm gradient of Cl^-, protonophore-treated lysosomes from fibroblasts expressing wild-type ClC-7 or ClC-7^{unc} reach a higher pH (4). The observation that protonophore-treated lysosomes from wild-type mice reach a more alkaline pH than those from *Clcn7*$^{unc/unc}$ mice can be explained by the higher $[Cl^-]_{lumen}$ of wild-type lysosomes. Indeed, a ratiometric Cl^--sensitive dye chased into lysosomes of fibroblasts revealed significantly reduced steady-state $[Cl^-]_{lumen}$ in both *Clcn7*$^{-/-}$ lysosomes and *Clcn7*$^{unc/unc}$ lysosomes (4). If a reduction in lysosomal [Cl^-] were directly responsible for lysosomal storage in *Clcn7*$^{-/-}$

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or *Clcn7*$^{unc/unc}$ mice, one would expect that cells with low cytoplasmic [Cl^-] would show more pronounced pathology. CNS neurons, but not hepatocytes, show intracellular deposits (14), and indeed neurons display exceptionally low cytoplasmic [Cl^-] owing to Cl^- extrusion by the KCl cotransporter Kcc2 (148). However, it is not clear how the lysosomal pathology of ClC-7-deficient renal PT cells (17) fits into this picture.

Together, the phenotypes of the *Clcn5*^{unc} mice and *Clcn7*$^{unc/unc}$ mice show that Cl^-/H^+ exchange by CLCs, rather than Cl^- conductance alone, is of pivotal importance in the endosomal/lysosomal pathway and collectively suggest a physiological role for luminal Cl^- accumulation. A minimal mathematical model for V-ATPase-mediated vesicular acidification not only confirms the plausibility of a higher $[Cl^-]_{lumen}$ due to a $2Cl^-:1H^+$ coupling but also leads to the counter-intuitive prediction that the vesicle reaches an inside-negative voltage with a Cl^-/H^+ exchanger (4). Accordingly, by their own mode of transport, CLC exchangers would ensure a voltage at which these strongly outwardly rectifying transporters would not be virtually inactive due to the inside-positive potential. However, this prediction conflicts with some reports on inside-positive endosomes and lysosomes (e.g., References 131 and 149). By creating a more negative luminal potential, Cl^-/H^+ exchange, as opposed to a pure Cl^- conductance, is furthermore predicted to facilitate the establishment of a more acidic pH (4).

Intriguingly, and in contrast to *Clcn5*^{unc} mice and *Clcn5*$^{-}$ mice, *Clcn7*$^{unc/unc}$ mice and *Clcn7*$^{-/-}$ mice have overlapping, but not identical, phenotypes. *Clcn7*$^{unc/unc}$ mice have wild-type-like brown coat color in a genetic background in which mice lacking ClC-7/Ostm1 have gray fur, demonstrating that Cl^-/H^+ exchange is dispensable for the role of ClC-7 in hair pigmentation that depends on melanocyte function (4). *Clcn7*$^{unc/unc}$ mice also develop less severe osteopetrosis than do ClC-7-deficient mice (4). In analogy to ClC-5 on early endosomes, the conductance of the ClC-7^{unc} protein could in principle support the acidification of the resorption lacuna [which is impaired in *Clcn7*$^{-/-}$ mice (14)]. The diminished bone resorption in *Clcn7*$^{unc/unc}$ mice, however, may rather be due mainly to the underdevelopment of the ruffled border (4), which in turn may be explained by reduced lysosomal exocytosis, thereby indirectly ascribing the osteopetrotic phenotype to a lysosomal defect.

Cl^- May Affect Membrane Traffic by Regulating Organellar Ion Homeostasis and Osmolarity

The importance of luminal Cl^- accumulation, independent of a function in charge compensation for acidification, raises the question as to what role luminal Cl^- may play. In the endocytic pathway, very few roles have been assigned directly to luminal Cl^-. These roles include a moderate effect on iron release from transferrin (150, 151) and the regulation of the lysosomal enzyme cathepsin C (152). However, reduced activity of cathepsin C due to the reduced $[Cl^-]_{lumen}$ upon lack or uncoupling of ClC-7 (4) is unlikely to explain the retarded degradation (17) and storage material accumulation (38, 60) in ClC-7/Ostm1-deficient lysosomes, for the [Cl^-] of 50 mM for full activation of cathepsin C is exceeded in *Clcn7*$^{-/-}$ lysosomes and *Clcn7*$^{unc/unc}$ lysosomes (4). The Cl^- gradient across the lysosomal membrane may also be used to drive export of catabolites in a Cl^- cotransport process. Alternatively, the presence of the late-endosomal marker lysobisphosphatidic acid on enlarged structures in ClC-7-deficient PT cells and the fact that the enlargement of late endosomes/lysosomes is not diminished by a strong reduction of degradative cargo (17) suggest that the observed impairment of lysosomal protein degradation is secondary to a defect in membrane traffic within the late-endosomal/lysosomal pathway. Similarly, $[Cl^-]_{lumen}$ may influence trafficking of renal early and/or recycling endosomes where ClC-5 plays the dual function of acidification and Cl^- accumulation (5).

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The V-ATPase may also function as a luminal pH–sensing and –signaling molecule that recruits membrane-trafficking machinery components in response to luminal pH (153). So far, there is no evidence for an analogous luminal Cl^-–sensing signal transducer. Instead, Cl^- may have an indirect impact on membrane traffic through its effect on other ions, both by its luminal concentration and by the Cl^- transmembrane gradient. For example, $[Cl^-]_{lumen}$ may affect Ca^{2+} efflux, which plays various roles in membrane traffic within the endosomal/lysosomal system (154), by modulating endosomal Ca^{2+} channels (155). Ca^{2+} release through second messenger–activated two-pore channels or mucolipins (156) may require the Cl^- gradient for counterion transport. The requirement of Cl^- transport for Ca^{2+} release from other intracellular stores such as the ER is well accepted, although the need for specialized Cl^- channels is disputed (157). Mucolipin-1 may play various roles, including one in lysosomal exocytosis, in the endosomal/lysosomal pathway (158). Additionally, transport of further ions may require Cl^- transport. For example, Cl^- efflux through ClC-3 has been proposed to support phagocytosis and the respiratory burst in neutrophils by balancing charge translocation during NADPH oxidase activity (159). This possibility would be consistent with the voltage dependence of intracellular CLCs because NADPH oxidase transports negative charge into the vesicular lumen, thereby activating these CLCs. However, most data and theoretical considerations argue that charge compensation in this process is mediated largely by voltage-gated proton channels (160).

$[Cl^-]_{lumen}$ and the transmembrane voltage, both parameters that are affected by Cl^-/H^+ exchange in the endosomal/lysosomal system (4), influence organellar ion homeostasis in general. Changing compositions of Cl^-, Na^+, K^+, and Ca^{2+} likely have different effects on various steps of vesicular transport (161). Cl^- accumulation (and possibly a cation influx due to an inside-negative potential) changes the osmolarity of the respective compartment, which should lead to a volume increase. Indeed, the relative volume of endosomes increases by a factor of 2.5 within the first 15 min after cellular uptake (2). The enlargement of endosomal compartments upon ClC-3 overexpression (118) may also result from an increase in H^+ gradient–driven Cl^- accumulation because this enlargement is prevented either by dissipation of the pH gradient by bafilomycin or by mutating the gating glutamate (analogous to the uncoupling mutations of *Clcn5*^{unc} mice and *Clcn7*$^{unc/unc}$ mice) (118). Vesicular volume increase is involved in the exocytosis of secretory granules in mast cells (162) and in pancreatic acinar cells (163). Moreover, osmotic gradients may increase the volume and alter the shape of a given subcellular compartment, thereby influencing events such as vesicle budding (164) and endocytosis (165). The presence of both a cation conductance and $2Cl^-/H^+$ exchange on lysosomes (4, 127) may minimize the osmotic effect during lysosomal acidification.

Finally, vesicular $[Cl^-]$ must be viewed in the context of other ions, conductances, electroneutral transporters, and pumps, as well as in the context of the dynamic budding, fusion, and trafficking that occurs in the endocytic and secretory pathways. For instance, a higher-than-cytoplasmic Na^+ concentration in lysosomes, which is required in mathematical models for cation-dependent lysosomal acidification (4), might in principle arise from the activity of cation/H^+ exchangers as well as from the endocytic delivery of extracellular Na^+ (4). Ultimately, a thorough understanding of vesicular ion homeostasis and of ion-dependent vesicular trafficking must integrate all these different processes in a complex model.

SUMMARY POINTS

1. Depending on the compartment, Cl^- and/or cations provide the countercharge for V-ATPase-dependent acidification.

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2. Disturbance of vesicular Cl^- transport leads to pathologies such as impaired endocytosis and lysosomal storage, neurodegeneration, and osteopetrosis.
3. Such pathologies cannot be attributed merely to impaired vesicular acidification.
4. The need for Cl^-/H^+ exchange in the endosomal/lysosomal pathway suggests a crucial role for luminal Cl^- accumulation.
5. The role of Cl^- must be considered in the context of general organellar ion homeostasis.

FUTURE ISSUES

1. What is the role of luminal Cl^-?
2. What is more important: the luminal concentration of Cl^- or its gradient across the vesicular membrane?
3. Is there a transmembrane sensor for luminal Cl^-?
4. Are the roles of bestrophins, CLICs, and GPHR related to Cl^- transport?
5. What is the role of intracellular TMEM16 proteins?
6. What is the role of ClC-3 on synaptic vesicles?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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2. Shows increases in endosomal Cl^- and volume in parallel with acidification.

4. Mouse models and mathematical modeling reveal the important role of luminal Cl^- accumulation by CLC exchangers.

5. Together with Reference 4, demonstrates that Cl^- conductance cannot substitute for vesicular Cl^-/H^+ exchange.

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