

Intracellular Chloride Channels: Determinants of Function in the Endosomal Pathway

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Endosomes, and related subcellular compartments, contain various Cl⁻ channels in the CIC family. In this review, we describe the known roles of intracellular Cl⁻ channels and also explore some of the functional implications of transmembrane Cl⁻ flux in these organelles. Cl⁻ influx acts to control intraluminal pH, both by shunting the effects of the proton pump on membrane potential and, possibly, through direct effects of Cl⁻ on the proton pump. Changes in intraluminal pH likely help regulate membrane trafficking. We propose that changes in intraluminal Cl⁻ concentration ([Cl⁻]) could theoretically play a direct role in regulating membrane trafficking and organellar function through effects on chloride-sensitive proteins in the vesicular membrane, which could transduce information about intraluminal [Cl⁻] to the outside of the vesicle and thereby recruit various signaling molecules. We present a model in which regulation of cytosolic [Cl⁻] and vesicular Cl⁻ conductance could help control the amount or type of neurotransmitter stored in a particular population of synaptic vesicles.

Organellar Function Depends on Luminal Ionic Compositions

Cellular organelles have traditionally been defined by their morphological and functional characteristics. Endosomes and lysosomes, for example, are easily distinguished by their distinctive microscopic anatomy, their characteristic locations in the cell, and their respective functions in membrane trafficking and hydrolytic degradation. The current notion is that the distinct morphological and functional properties of each membranous subcellular compartment are determined by the particular protein and lipid composition of the organelle. For example, endosomes are characterized by certain small Rab guanosine triphosphatases (GTPases) not found in lysosomes, and lysosomes contain hydrolytic enzymes not resident in endosomes.

Organelles at different stages along the exocytic and endocytic pathways also have different luminal ionic compositions, most notably proton (H⁺) and chloride (Cl⁻) concentrations (1–3). In this review, we discuss the idea that the luminal ionic composition of an organelle plays a key role in its function. This notion is supported by the observation that perturbation of the ionic luminal organelle composition with drugs such as the sodium (Na⁺) ionophore monensin (4, 5), the weak base chloroquine (6), the proton pump inhibitor bafilomycin A1 (7–9), or the M2 proton channel of influenza virus (10) alters the morphology and impairs the function of the affected organelle. These reagents do not affect the endocytic pathway

in a homogeneous fashion. For example, monensin blocks the recycling of low density lipoprotein (LDL) receptor from endosomes to the cell surface and causes LDL receptors to accumulate in perinuclear endosomal compartments (4), whereas the influenza virus M2 proton channel disrupts the delivery of proteins to the apical plasma membrane when exogenously expressed in epithelial cells (10). These observations highlight the importance of the luminal ionic composition in the function of intracellular membranous organelles. In addition, the fact that these perturbations have different effects suggests that functionally different subpopulations of vesicles contain different complements of ion channels or that ion channels exist in different regulatory states in different types of vesicles.

Here, we focus on the role of Cl⁻ and Cl⁻-selective ion channels in regulating the pH and ionic composition of organellar compartments and develop the theme that Cl⁻ permeability and Cl⁻ concentration ([Cl⁻]) contribute to the unique identity of functionally different membrane compartments. Indeed, the ionic composition of different vesicular populations very likely plays a key role in determining the function of these vesicles and in vesicle-mediated mechanisms that sort membrane proteins to different locations in the cell. We propose that these functional effects are due to the influence of luminal ions on the conformation or activity (or both) of various enzymes and receptors in the vesicular membrane. We begin by discussing evidence that Cl⁻ channels play a role in membrane traffic through the regulation of luminal pH and then develop the idea that [Cl⁻] itself may be a regulator of vesicle function. We then use the synaptic vesicle to illustrate how Cl⁻ channels may provide novel mechanisms to regulate neurotransmitter packaging.

A Link Between Cl⁻ Channels and Organellar Function: Dent's Disease

Among the most important recent discoveries in the Cl⁻-channel field have been the elucidation of the roles of the CIC family of Cl⁻ channels in intracellular organelles and membrane trafficking (11). CIC channels, the largest known family of Cl⁻ channels, are found in organisms from bacteria to mammals. In mammals, the CIC family comprises nine genes. CIC-1, CIC-2, CIC-Ka, and CIC-Kb are expressed on the plasma membrane, whereas CIC-3 through -7 are intracellular Cl⁻ channels, although they may be trafficked to the plasma membrane under certain circumstances (11, 12).

In determining the role of CIC-5 in the pathogenesis of Dent's disease, Thomas Jentsch's lab was the first group to establish genetically the intracellular function of a CIC channel. CIC-5 is located on endosomes in renal proximal tubule cells. Dent's disease, which is characterized by a urinary loss of low molecular weight proteins, Ca²⁺, and phosphate, and by the development of kidney stones, is caused by a mutation in CIC-5. Knockout of CIC-5 in mice, which produces a disorder that resembles Dent's disease in humans (13), results in a disruption of endocytic traffic as measured by uptake of both fluorescent β -lactoglobulin and fluorescent dextran (13–17). Moreover, disruption of CIC-5 expression in a human colon carcinoma cell line (Caco-2 cells) by transfection with antisense oligonu-

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cleotides produces altered receptor-mediated endocytosis (15). Thus, CIC-5 appears to play a critical role in endocytic traffic in the renal proximal tubule, and defects in traffic of endocytically sorted membrane proteins secondary to the loss of CIC-5 function could explain the mechanism underlying Dent's disease (15, 16). Although the precise trafficking defect in renal proximal tubule is still not understood, an attractive though speculative possibility is that the recycling of membrane proteins from endosomes is affected. Indeed, inhibition of CIC5 expression by antisense oligonucleotides reduces the recycling rates of an endocytic receptor, the transferrin receptor (15). This evidence supports a role of Cl⁻ channels in modulating sorting decisions made by membrane proteins at the endosome level. Defective recycling could also explain the results of Christensen *et al.* (18) in which the overall content of rab GTPases in early and late endosomes is normal in CIC-5^{-/-} kidney, yet the apical receptors megalin and cubilin are down-regulated, thus suggesting that impaired recycling could reduce the half-life of membrane proteins by targeting to lysosomes.

Cl⁻ Channels Regulate Organellar Transmembrane Potential and pH

The membrane-trafficking defect caused by disease-causing mutations in CIC-5 is apparently related, at least in part, to the inability of the endosomal vesicles bearing these mutant channels to acidify properly. Organellar acidification is driven by v-type adenosine triphosphatase (v-ATPase) proton pumps in the vesicular membrane, but the rate and degree of acidification are regulated by Cl⁻ channels, as well as by other pumps, transporters, and channels (Fig. 1) (19–22). Because the proton pump is electrogenic, it generates a membrane potential, which constitutes a major factor in determining the degree of vesicular acidification. This positive membrane potential provides a force that opposes the ability of the proton pump to move protons into the vesicle. However, pathways for an-

ion influx or cation efflux will dissipate the transmembrane potential generated by the proton pump and thereby facilitate acidification of the vesicle.

Endosomal acidification depends on the presence of cytoplasmic Cl⁻ (23–28). Furthermore, DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid), a Cl⁻ channel blocker, inhibits acidification of clathrin-coated vesicles (29). Membrane vesicles derived from mice deficient in certain CIC channels acidify more slowly than do vesicles from wild-type animals (16, 30). Thus, in the endosomal pathway, Cl⁻ channels, especially those in the CIC family, clearly play a key role in this shunting process.

Luminal pH Varies Along the Endosomal Pathway

The ability of Cl⁻ to modulate the membrane potential of intracellular vesicles provides a mechanism by which vesicular pH may be controlled. As shown in Fig. 2, intracellular membrane compartments differ in their luminal pH (1–3). Along the endosomal pathway, the pH of a newly formed endosome is similar to that of the extracellular solution it encloses (pH 7.4), but as the early endosome matures to a late endosome its luminal proton concentration increases by about two orders of magnitude until it reaches a pH near 5.3.

The different pH's of various compartments along the endocytic pathway is important in determining the subcellular destination of endocytosed receptors and their ligands (1). Receptors that are rapidly recycled between endosomes and the plasma membrane dissociate from their ligands at a less acidic pH than do receptors that are not trafficked to the plasma membrane. For example, LDL receptors that are involved in cholesterol uptake are rapidly recycled to the plasma membrane and dissociate from LDL particles in early endosomes at a pH of less than 6.8, whereas mannose-6-phosphate receptors that target enzymes to lysosomes and recycle to the Golgi dissociate from their ligands at the more acidic pH of late endosomes.

If luminal Cl⁻ plays a role in determining the different pH's along the endocytic pathway, then it is reasonable to predict that luminal [Cl⁻] should increase along the endocytic pathway.

Chloride Concentration in the Endosomal Pathway

The kinetics of changes in endosomal proton and Cl⁻ concentration have recently been elegantly demonstrated by Verkman and colleagues (31, 32), who used novel Cl⁻- and pH-sensitive dyes to follow changes in luminal ionic composition with time after endocytosis. [Cl⁻] was measured with a green Cl⁻-sensitive fluorophore, BAC [10,10'-bis(3-carboxypropyl)-9,9'-biacridinium dinitrate] coupled to a red-labeled receptor ligand (transferrin or α -macroglobulin). Transferrin is targeted to early and recycling endosomes, whose luminal pH is in the range of pH 6.0 to 6.8, whereas α -macroglobulin (which, unlike the transferrin receptor, is not recycled to the plasma

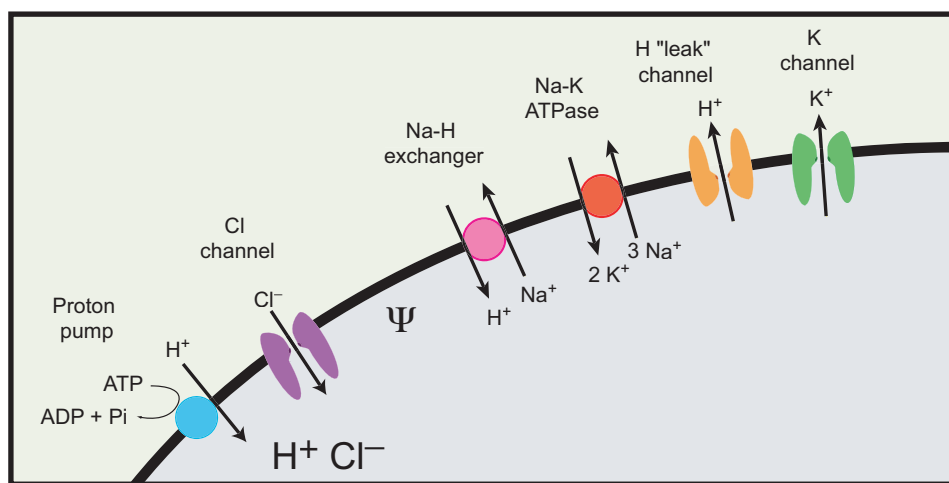


Fig. 1. Multiple vesicular channels and transporters affect the transmembrane proton electrochemical gradient ($\Delta\mu_{H^+}$). The electrical ($\Delta\Psi$) and chemical (ΔpH) components of $\Delta\mu_{H^+}$ are generated by proton accumulation in the vesicle lumen driven by the vacuolar ATPase (depicted as a blue circle). Different permeabilities to ions determined by organelle-specific channels and transporters define the ionic composition of the organelle lumen. For example, the Na⁺-K⁺ ATPase, present only in the early stages of the endocytic pathway, increases $\Delta\Psi$, whereas the activity of organelle-specific Cl⁻ or K⁺ channels decreases $\Delta\Psi$. The vesicular [Cl⁻] also regulates pH because activity of the v-ATPase is modulated by luminal Cl⁻ anions. On the other hand, pH is regulated by proton "leak" pathways and a family of electroneutral Na⁺-H⁺ exchangers that possess different subcellular localizations.

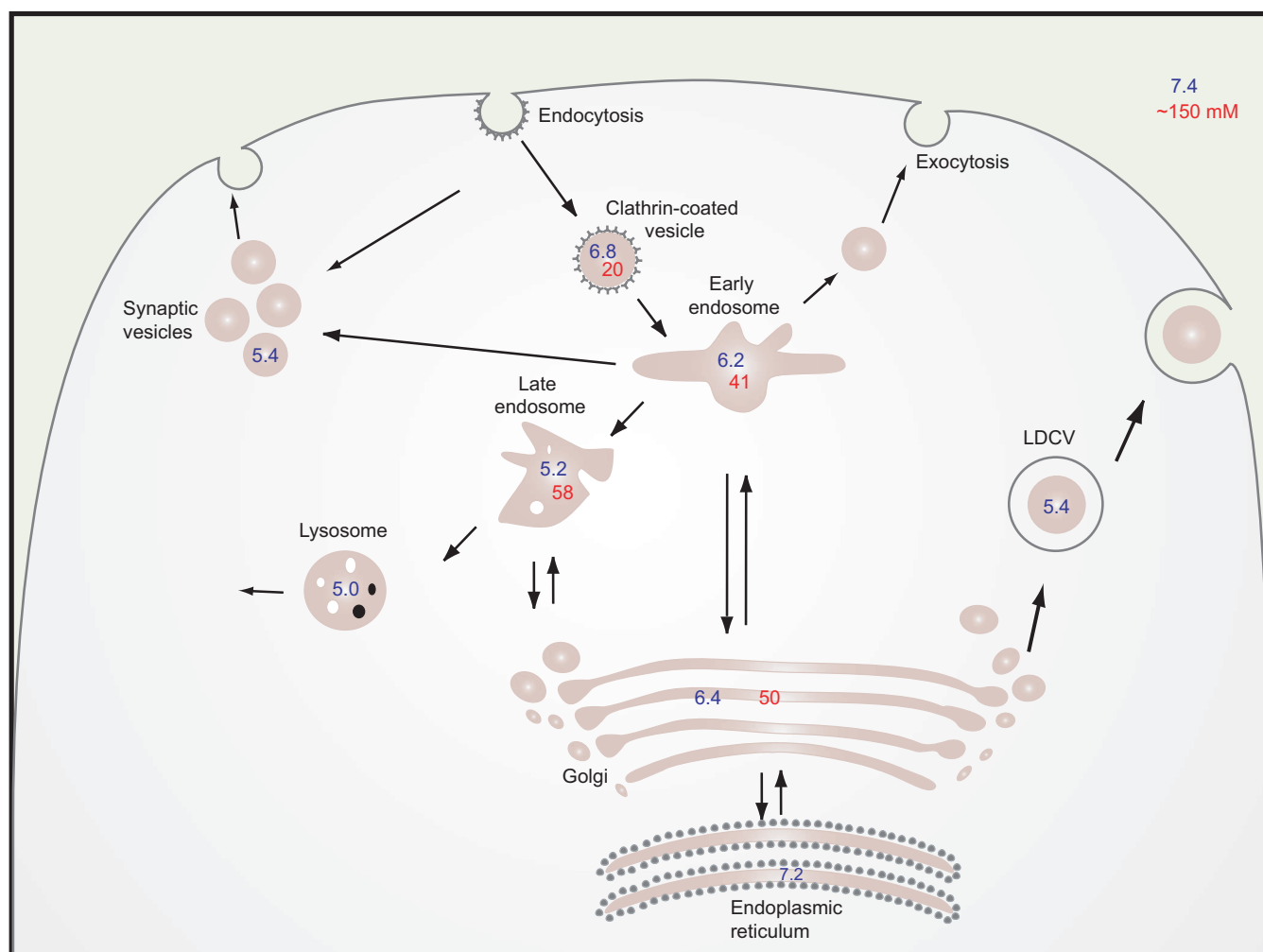


Fig. 2. Distinctive Cl^- concentrations and pH values in subcompartments of the exocytic and endocytic pathways. A prototypical mammalian secretory cell is depicted. Values in purple located in the organelle lumen correspond to experimentally determined pH values, whereas the figures in red represent the mM $[\text{Cl}^-]$ in the organelle lumen. Each compartment has a distinctive pH and $[\text{Cl}^-]$. The intravesicular $[\text{Cl}^-]$ in synaptic vesicles, large dense core vesicles (LDCV), and lysosomes has not yet been determined. An animation illustrating changes in $[\text{Cl}^-]$ and pH in subcompartments of various endosomal pathways can be accessed from <http://stke.sciencemag.org/cgi/content/full/sigtrans;2004/233/re8/DC1>.

membrane) ends up in late, more acidic (pH 5) endosomes. The rate of Cl^- accumulation in endosomes parallels that of endosomal acidification. Thirty seconds after internalization, endosomes have an internal $[\text{Cl}^-]$ of 20 mM. This drop in $[\text{Cl}^-]$ relative to that in the external solution (~ 150 mM) is thought to be due to the negative interior Donnan potential of the forming endosome. $[\text{Cl}^-]$ in the endosome increases to 41 mM over a period of about 10 min. In α -macroglobulin-labeled endosomes, the $[\text{Cl}^-]$ increases further to 58 mM by about 45 min after internalization. In different cell types, the final endosomal $[\text{Cl}^-]$ varies: Late endosomes in Chinese hamster ovary (CHO) cells exhibit a higher $[\text{Cl}^-]$ than do those in J774 cells (32). Because J774 cells are phagocytic, whereas CHO cells are fibroblastic, these differences in endosomal $[\text{Cl}^-]$ may be important in terms of the specialized functions of the endosomal pathway. Cl^- uptake into the endosome is clearly coupled to the proton pump, because Cl^- accumulation is blocked by the proton-pump inhibitor bafilomycin A1. In the presence of bafilomycin A1, Cl^- uptake can

be restored by the K^+ ionophore valinomycin, indicating that K^+ conductance in the native endosome is low and that K^+ ions are unlikely to provide appreciable counterion flux. Because the molar coupling of Cl^- to H^+ is 1:1, this suggests that Cl^- is the major counterion for protons. Endosomal acidification is inhibited by replacing Cl^- with gluconate and by the Cl^- channel blocker NPPB [5-nitro-2-(3-phenylpropylamino) benzoate]. These data provide a compelling picture of endosomal maturation being characterized by an increase in both H^+ - and Cl^- -ion concentration in the lumen of the vesicle.

Other Transporters and Channels Contribute to Luminal Ionic Composition

Although Cl^- ions play an important role in regulating vesicular membrane potential and pH, the contribution of Cl^- relative to that of other ions varies in different subcellular compartments. For example, the vesicular transmembrane potential can also be modified

by pumps such as the Na⁺/K⁺ ATPase (33). The Na⁺/K⁺ ATPase pumps ~3 Na⁺ ions into the vesicle for each 2 K⁺ ions pumped out, which will make the intraluminal potential more positive. This in turn will retard acidification. The Na⁺/K⁺ ATPase is restricted to early endosomes, and this localization may be a factor keeping the early endosomal pH less acidic.

Another major factor affecting vesicular acidification is the presence of leak pathways for protons, through channels, transporters, and the lipid bilayer itself. Proton leak pathways will oppose the development of a pH gradient by decreasing the effectiveness of the proton pump to accumulate protons inside the vesicle. Proton leak pathways that have been identified include the Na⁺/H⁺ exchanger and proton channels. In the secretory pathway, it has been suggested that the pH gradient that exists between the endoplasmic reticulum (pH 7.4) and mature secretory granules (pH 5.5) can be explained by increasing proton-pump density and decreasing proton leak in the progression toward mature secretory granules (34).

Mechanisms Linking Intravesicular pH With Membrane Traffic

The precise mechanisms by which intravesicular pH regulates membrane traffic in the cell remain unknown. However, a working hypothesis (illustrated in Fig. 3) is that changes in intravesicular pH alter the function or conformation of a receptor in the vesicle that recruits signaling molecules from the cytosol (35, 36). For example,

binding of the small GTPase Arf to microsomal vesicles can be modulated by pH (37). The binding of Arf to the membrane depends on ATP to power the proton pump and on extravesicular Cl⁻ to dissipate the pH gradient. Altering the luminal pH by equilibrating vesicles in media of differing pH in the presence of the K⁺/H⁺ exchanger nigericin has revealed an inverse relation between Arf binding and intraluminal pH. Likewise, the association of Arf-6 and its nucleotide exchange factor ARNO (ARF nucleotide-binding site opener) with endosomes is blocked by inhibitors of v-ATPase and by drugs that dissipate the proton gradient (38), indicating that this association depends on intravesicular pH.

Arf GTPases play a central role in recruiting cytoplasmic protein complexes that bind to vesicular membrane proteins “coating” them. These coat complexes are translocated from the cytosol to membranes, promote membrane vesiculation, and carry out specific sorting functions. Because Arf membrane recruitment is regulated by luminal pH, one would predict that the subsequent recruitment of coat complexes should also be regulated by luminal pH. Indeed, recruitment of COPI, a coat protein complex, to endosomes is inhibited by the proton pump inhibitor bafilomycin A1. This provides evidence for a critical role of pH in coat recruitment. A hypothetical pH-sensitive protein (PSP) has been postulated to play a role in the association of Arf and coat proteins to the endosome (35, 38–40). Changes in luminal pH are thought to alter the conformation of the PSP and consequently its affinity for recruited cytosolic pro-

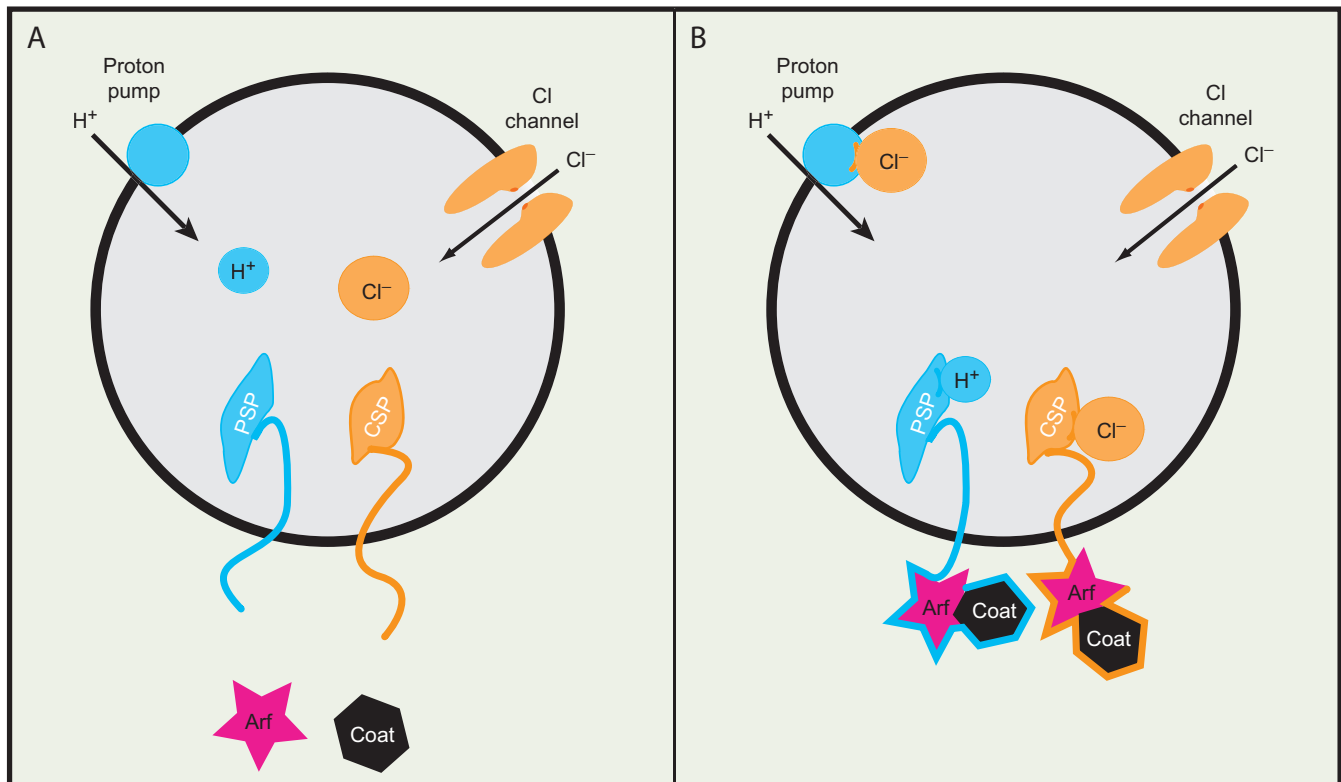


Fig. 3. Ion-sensitive transmembrane proteins transduce signals from the vesicle lumen to the cytoplasmic face of the organelle. **(A)** Hypothetical transmembrane receptors for luminal H⁺ and Cl⁻ are present in the organelle membrane. Their cytosolic tails convey information about the luminal ionic composition to the cytoplasmic side of the membrane, which can affect the recruitment of Arf GTPases and Arf-interacting proteins, such as coats. **(B)** Recruitment of coat components and Arf GTP exchange factors to organelles depends on the transmembrane $\Delta\mu\text{H}^+$. Chloride-sensitive proteins in vesicular membranes could transduce information about the intracellular [Cl⁻] to recruit signaling molecules.

teins. Because this hypothetical PSP has not yet been identified, the mechanistic links between intravesicular pH and membrane traffic remain speculative.

Importance of Cl⁻ Concentration

Although most attention has been focused on intravesicular pH as the parameter that controls membrane traffic, intravesicular [Cl⁻] could also affect vesicle trafficking directly independent of its effect on pH. It is well established that various proteins have Cl⁻-ion binding sites and that the conformation or activity of these proteins can be altered by Cl⁻ binding. Proteins that have Cl⁻-binding sites or whose function is altered by [Cl⁻] include hemoglobin (41), α -amylase (42), ANP (atrionatriuretic peptide) receptor (43, 44), various ion channels and transporters (45–48), angiotensin-converting enzyme I (49), AML1 (acute myeloid leukemia-1) transcription factors (50), heterotrimeric GTP-binding proteins (G proteins) (51), cathepsin-C (52), and certain kinases (53). We propose that in addition to PSPs, there may also be chloride-sensitive proteins (CSPs) in vesicular membranes that could transduce information about the intracellular [Cl⁻] to the vesicular membrane to attract signaling molecules.

Chloride as a Regulator of the Proton Pump: A Possible CSP?

Although intracellular Cl⁻ channels clearly function as a pathway for shunting the membrane potential generated by the proton pump (19), there is also abundant evidence that Cl⁻ may have a direct regulatory effect on the proton pump. For example, when Cl⁻ ions on the cytoplasmic side of the vesicle are replaced with NO₃, the ability of the vesicle to acidify is abolished (37). This effect cannot be explained by the loss of a counterion shunt to dissipate membrane potential, because NO₃ exhibits a high conductance through ClC channels. Rather, Cl⁻-ion concentration itself is likely to be important for the function of the vesicle. Indeed, experiments with isolated vesicles have demonstrated clearly that the activity of the vesicular proton pump depends on the presence of Cl⁻ ions (48, 54).

Role of Cl⁻ Channels in Neurotransmitter Uptake Into Synaptic Vesicles

Synaptic vesicles are specialized endocytic vesicles that are defined by the type of neurotransmitter they contain. Although the complement of Cl⁻ channels present in synaptic vesicles has not been fully elucidated, ClC-3 is certainly one of the important synaptic vesicle Cl⁻ channels (30). Knockout of ClC-3 in mice produces postnatal degeneration of the hippocampus and retina. This is associated with a decreased rate of acidification of synaptic vesicles (30).

The accumulation of transmitter in the vesicle is performed by specific neurotransmitter transporters [including transporters for acetylcholine (ACh), glutamate, and γ -aminobutyric acid (GABA)]; however, the energy source that powers these transporters is linked to the proton electrochemical gradient ($\Delta\mu_{\text{H}^+}$) established by the proton pump (55). As we have discussed, the relative Cl⁻ conductance of a vesicle modulates the ratio of the electrical ($\Delta\Psi$) and the chemical (ΔpH) gradients. For instance, high Cl⁻ conductance favors a low $\Delta\Psi$ and a high ΔpH . Thus, the Cl⁻ conductance can control transmitter uptake into the vesicle, depending on which component of the electrochemical gradient is more important for a specific transporter. Changes

in Cl⁻-channel activity (which might be controlled by phosphorylation, for example) or changes in cytosolic [Cl⁻] thus have the potential to alter the quantity of transmitter packaged in a vesicle.

Glutamate Uptake Depends More on $\Delta\Psi$ Than on ΔpH

Synaptic vesicles that store glutamate provide a specific example of the roles that Cl⁻ channels may play in vesicle function. Glutamatergic vesicles incubated *in vitro* in the presence of ATP generate a proton electrochemical gradient. The relative magnitude of the ΔpH and $\Delta\Psi$ components is controlled by the cytoplasmic [Cl⁻] (56, 57). Low Cl⁻ concentrations (4 mM) create a vesicular environment in which $\Delta\Psi$ is the predominant component. In contrast, at high Cl⁻ concentrations (≥ 80 mM), $\Delta\Psi$ collapses and ΔpH predominates (58). *In vitro* at low glutamate concentrations (50 μM), glutamate uptake is maximal at low cytoplasmic [Cl⁻] (high $\Delta\Psi$ and low ΔpH) and is negligible at high [Cl⁻] (high ΔpH and low $\Delta\Psi$) (56, 57, 59). However, at high (millimolar) glutamate concentrations, the steady-state rate of glutamate uptake is less sensitive to [Cl⁻] than it is at low glutamate concentrations. The simplest interpretation of these data is that $\Delta\Psi$ increases the affinity of the transporter for glutamate and also provides the driving force for glutamate transport (60). These data obtained *in vitro* lead one to predict that the amount of glutamate stored in a synaptic vesicle would depend on the cytosolic glutamate and Cl⁻ concentrations.

Glutamate Concentration in a Vesicle May be Regulated by Cl⁻

Indeed, at the single-synapse level, the quantity of glutamate contained in individual synaptic vesicles, as inferred from the amplitudes of miniature postsynaptic potentials, is highly variable and can differ by one order of magnitude (61, 62). Ishikawa and co-workers have shown that when cytosolic glutamate concentration in the presynaptic terminal is low, some vesicles apparently do not contain glutamate (63). When cytosolic glutamate concentration is increased, these empty vesicles become filled. One possible explanation of this result is that certain vesicles remain unfilled when cytosolic glutamate is low because they have a small $\Delta\Psi$ as a consequence of a high Cl⁻ conductance. When cytosolic glutamate concentration is increased, the small $\Delta\Psi$ would have less impact on the rate of glutamate uptake (60).

Another piece of evidence that Cl⁻ conductance may provide a tonic inhibition on filling of glutamatergic vesicles is the observation that in ClC-3 knockout mice, the amplitudes of hippocampal miniature excitatory postsynaptic potentials, which reflect the amount of glutamate packaged in each vesicle, are modestly increased (30). Thus, a formal possibility exists that changes in Cl⁻-channel activity or in cytosolic [Cl⁻] could provide a tuning mechanism to control the neurotransmitter content of glutamatergic vesicles. Whether this hypothesis holds water awaits experimental verification.

Cytosolic Cl⁻ Concentration Is Dynamic

The recent realization that cytosolic [Cl⁻] is highly dynamic bolsters the suggestion that Cl⁻ conductance may regulate transmitter content in synaptic vesicles. Cytosolic [Cl⁻] undergoes substantial changes both on a slow time scale during development, and on a rapid time scale in response to neurotransmitters. During early neuronal development, responses to the neurotransmitter GABA, which opens ligand-gated Cl⁻ channels, are depolarizing instead of hyperpolarizing as they are in the adult (64). The develop-

mental change from depolarizing to hyperpolarizing responses is due to a decrease in cytosolic $[Cl^-]$ and a change in the electrochemical driving force for Cl^- movement. Kuner and Augustine (65) have estimated the decrease in cytosolic $[Cl^-]$ from embryonic day 18 to postnatal day 14 to be over 100 mM (from 130 to 20 mM). This developmental decrease in cytosolic $[Cl^-]$ is mediated by the regulated expression of the K^+ - Cl^- cotransporter (64, 66).

Cytosolic $[Cl^-]$ also changes over a shorter time scale in response to GABA application to a neuron. Currents as small as 100 pA produce 10 to 20 mM changes in cytosolic $[Cl^-]$, and 500-pA currents produce changes as large as 100 mM (65). The fact that cytosolic $[Cl^-]$ can vary over the same range that modulates $\Delta\Psi$ and ΔpH in synaptic vesicles raises the possibility that Cl^- plays an important role in loading synaptic vesicles with transmitters. As an extreme example, we could imagine two different synapses, each of which contains glutamatergic vesicles that differ only in the presence or absence of Cl^- conductance, thus defining whether the synapse does or does not secrete glutamate. Moreover, even if synaptic vesicles in two distinct synapses possess similar Cl^- conductance, differences in the mechanisms controlling cytosolic $[Cl^-]$ at each synapse could, in principle, provide an alternative route to regulate $\Delta\Psi$ and thereby the storage of glutamate into synaptic vesicles. From a broader perspective, regulation of $\Delta\Psi$ could provide a nongenomic mechanism to switch the secretory phenotype of a nerve terminal.

Differential Regulation of Neurotransmitter Transporters By Cl^- Conductance

Different neurotransmitter transporters derive their energy from different components of $\Delta\mu_{H^+}$. For example, vesicular ACh transport depends predominantly on ΔpH (67), vesicular GABA transport depends on both $\Delta\Psi$ and ΔpH (68, 69), and vesicular glutamate uptake depends more on $\Delta\Psi$ than on ΔpH (56, 59). Because Cl^- conductance can modulate the ratio of $\Delta\Psi$ and ΔpH , it is conceptually possible that the Cl^- conductance could define which neurotransmitters are released from a given synapse. For example, imagine a synapse containing synaptic vesicles that have transporters for both GABA and glutamate. If the Cl^- conductance in these vesicles is high, the vesicles will accumulate GABA (because the GABA transporter derives its energy largely from ΔpH), but will accumulate little glutamate (because the glutamate transporter derives its energy largely from $\Delta\Psi$). If the Cl^- channel shuts down or the cytoplasmic $[Cl^-]$ falls substantially, the situation would be reversed and the synapse could be switched between GABAergic and glutamatergic. Although there are at present no documented examples of switches from GABAergic to glutamatergic transmission, a developmental switch from GABAergic to glycinergic transmission has recently been shown to occur at the level of single synaptic vesicles (70). Furthermore, some synapses contain the necessary biochemical machinery to allow GABA-to-glutamate switches. For example, the vesicular glutamate transporter vGLUT3 is expressed in certain GABAergic, cholinergic, and serotonergic neurons that are not traditionally thought to release glutamate (71, 72). In particular, in the CA1 region of the hippocampus, vGLUT3 colocalizes with the GABA biosynthetic enzyme GAD in presynaptic endings on pyramidal cells that have the morphological features of GABAergic synapses. This observation certainly raises the question of whether certain GABAergic synapses have the capability of releasing glutamate. If so, the fact that the filling of GABAergic vesicles and glutamatergic vesicles exhibit different dependences on ΔpH and $\Delta\Psi$ sug-

gests that cytosolic Cl^- and vesicular Cl^- -channel activity could play important roles in modulating the proportion of GABA and glutamate released. Measurements of the Cl^- content of vGlut3-containing vesicles could provide evidence to support or refute this model.

Summary

In this article, we develop the idea that intracellular Cl^- channels, especially CIC Cl^- channels, play an important role in regulating the pH of intracellular vesicles and that intravesicular pH and $[Cl^-]$ impinge on mechanisms of vesicular trafficking. We also discuss the possibility that Cl^- channels could play a role in regulating the neurotransmitter content of synaptic vesicles. It may be prudent, however, to emphasize which aspects of our reasoning are supported by strong experimental evidence and which are more speculative.

There is strong experimental evidence that functionally different types of intracellular membrane vesicles have different intraluminal pH and $[Cl^-]$ and that the accumulation of Cl^- in vesicles correlates with pH. The ability of vesicles to acidify depends on the extravesicular $[Cl^-]$ and can be reduced by Cl^- channel blockers. Furthermore, disruption of CIC Cl^- -channel function by knockout of CIC-3 or CIC-5 results in decreased vesicular acidification. Together, these data constitute a rather consistent picture of CIC Cl^- channels playing a key role in regulation of vesicular pH. These data do not exclude a role for other types of Cl^- channels or other counterions in regulating pH. Indeed, it seems reasonable to think that CIC Cl^- channels may be important only in certain subsets of vesicles. The mechanism by which Cl^- channels increase pH can be explained by Cl^- ions acting as counterions to dissipate the potential gradient produced by the proton pump.

The link between intravesicular pH and $[Cl^-]$ and membrane traffic is more tentative, although the data are strongly suggestive. The most compelling data come from the CIC-3 and CIC-5 knockouts, where membrane traffic is disturbed. Although the implication that membrane traffic is disrupted because intravesicular pH and $[Cl^-]$ are abnormal is logical and also fits with the effects of agents that disrupt vesicular pH and membrane trafficking, the mechanisms remain unknown. The existence of pH-sensitive or Cl^- -sensitive transducing proteins is speculative, and further research is required to establish the precise roles of Cl^- channels in vesicular traffic.

Our proposal for the role of Cl^- channels in regulation of the neurotransmitter content of synaptic vesicles is somewhat fanciful, yet it is consistent with a number of experimental observations. This proposal provides experimentally testable ideas to explain some puzzling observations, such as why some GABAergic neurons contain glutamate transporters and why glutamate content in synaptic vesicles is so variable.

Puzzles That Remain

Although the importance of intracellular Cl^- channels seems clear, there are many fundamental problems that remain to be elucidated. Foremost is the major challenge of identifying the complement of different kinds of Cl^- channels that define different subpopulations of vesicles and in understanding how these channels may be turned on and turned off. The challenge is daunting, considering that another Cl^- channel, CFTR (cystic fibrosis transmembrane conductance regulator), is also located in intracellular membranes, but its intracellular role has remained elusive despite substantial effort (25, 73–77). Some reports have suggested that CFTR regulates intravesicular pH, whereas other reports have denied this function for CFTR.

There are two puzzling features of CIC channels that suggest that their regulation and function in intracellular membranes are incompletely understood. When CIC-4 and CIC-5 channels are overexpressed so that they traffic to the plasma membrane, the channels carry Cl⁻ ions very poorly in the direction of the extracellular space (the current “outwardly rectifies”) (11). Because the extracellular space is topologically equivalent to the lumen of the vesicle, this suggests that these channels are ill-suited to transport Cl⁻ into the vesicle. There are several possible alternatives. One possibility is that the channels behave differently in intracellular membranes than when they are expressed on the plasma membrane. It is known that the properties of CIC channels, including rectification, change when they are expressed as heteromeric channels (78). One possibility is that CLC channels in intracellular membranes exist as heteromeric channels that do not outwardly rectify. In addition, channel function might be modulated by association with other proteins or by phosphorylation. Localization of kinases or modulatory subunits in different subcellular compartments could provide differential regulation of Cl⁻ conductance.

Another puzzling feature concerns the effects of pH on channel function. Both CIC-4 and CIC-5 are inhibited by low pH: The currents are ~50% inhibited at pH 5.5 relative to pH 7.4 (79). This suggests that the function of these CIC channels might decrease as the acidity of the vesicular compartment falls. This could be a mechanism to prevent vesicles from becoming too acidic, because the proton pump is thermodynamically capable of acidifying the vesicle to below pH 4 (80). However, this inhibitory effect of pH raises questions about the relative contribution of CIC channels to electroneutrality in different kinds of endosomes. The inhibition of CIC channels by acid suggests the possibility that other species of Cl⁻ channels—or possibly other kinds of ion channels—might be important in providing the counterion shunt for the proton pump in the later stages of the endosomal pathway to promote acidification.

Finally, although Dent’s disease has provided our first mechanistic insights into the intracellular roles of CIC channels, it is not clear that the Dent’s disease phenotype is fully explained by changes in endosomal pH. Changes in endosomal pH in CIC-5 knockout mice have not yet been demonstrated. Furthermore, there appear to be multiple sorting problems in Dent’s disease, some of which are unlikely to be related to endosomal pH. Christensen *et al.* (18) have demonstrated decreased endocytosis, increased retention of receptors in endosomes, and decreased trafficking from endosomes to lysosomes in Dent’s disease. However, the decreased endocytosis is unlikely to be explained by decreased endosomal pH because the initial steps of endocytosis are pH-independent. If this conclusion is substantiated, it suggests that CIC-5 may have other functions independent of vesicular pH regulation.

Prospects for the field of intracellular Cl⁻ channels are exhilarating. Powerful new tools are now available to test some of the ideas discussed here. For example, proteomic approaches will certainly be applied to investigating the ion-channel complements of different subpopulations of membrane vesicles. Coupled with targeted ion-sensitive dyes that can be used to track changes in ion concentrations in different subcellular compartments, modified channel expression by small interfering RNA (siRNA), and genetically modified animals, new and exciting insights are certain to come. Finally, although our excitement about intracellular Cl⁻ channels has been stimulated by the discoveries of the roles of CIC channels, it seems very likely that new families of Cl⁻ channels will emerge to complicate the picture even further.

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