Sensors and regulators of intracellular pH

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Abstract | Protons dictate the charge and structure of macromolecules and are used as energy currency by eukaryotic cells. The unique function of individual organelles therefore depends on the establishment and stringent maintenance of a distinct pH. This, in turn, requires a means to sense the prevailing pH and to respond to deviations from the norm with effective mechanisms to transport, produce or consume proton equivalents. A dynamic, finely tuned balance between proton-extruding and proton-importing processes underlies pH homeostasis not only in the cytosol, but in other cellular compartments as well.

Proton-motive force (ψ_{H+}). The driving force for proton (or equivalent) movement, consisting of the proton concentration gradient and the transmembrane electrical potential.

pH buffering capacity
A measure of the ability of a solution to withstand changes in pH. It is defined as $\beta = dn/dpH$, where n is the number of acid or base equivalents that need to be added to alter pH.

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Eukaryotic cells are highly compartmentalized, segregating specific functions within membrane-bound organelles. Compartmentalization probably evolved from the need to provide distinct environmental conditions for the optimal operation of individual metabolic pathways, and also to store energy as electrochemical gradients across the membrane dielectric. Protons (and proton equivalents) have a crucial role in this context. Virtually all proteins depend on pH to maintain their structure and function, and protonation–deprotonation events dictate the charge of biological surfaces and are an integral part of many metabolic reactions[1]. Moreover, the proton-motive force (ψ_{H+}) is key to the generation and conversion of cellular energy. It is therefore not surprising that intracellular pH is stringently regulated, nor that it varies greatly among the different organelles (FIG. 1). The objective of this Review is to provide an up-to-date overview of the sensors, determinants and regulators of the pH of individual subcellular compartments. For simplicity, our discussion will concentrate on the mechanisms that are present in most non-polarized mammalian cells, addressing only occasionally the unique, but no less important, systems that have been developed by epithelial and other highly specialized cells.

Buffering power: rapid but finite

Because of its paramount importance, the pH of the cell is defended at multiple levels. Cellular compartments are protected from rapid, localized pH swings by their inherent pH buffering capacity (β). The total buffer capacity (β_{total}) consists of two components: $\beta_{intrinsic}$ and β_{HCO3-}. $\beta_{intrinsic}$ is provided by various intracellular weak acids and bases, including phosphate groups and side chains of amino acids[2]. Remarkably, the pK_a values of most ionizable groups in the cell are considerably above or below neutrality. As a result, $\beta_{intrinsic}$ is comparatively low at physiological pH (10–20 mM at pH 7.2), increasing at more extreme values (for example, 40 mM at pH 6.4)[3]. This apparent shortcoming is alleviated by the second component of the total buffering capacity, β_{HCO3-}. Mammalian cells are continuously exposed to CO_2, which is uncharged and readily traverses most biological membranes[4]. The hydration of CO_2 and the subsequent deprotonation of carbonic acid generate HCO_3^-, an effective proton buffer (BOX 1). At the prevailing CO_2 pressure (P_{CO2}; ~37 mm Hg) and using the equation in BOX 1, β_{HCO3-} can be calculated to contribute 29 mM to the total buffering capacity at pH 7.1.

Together, $\beta_{intrinsic}$ and β_{HCO3-} reduce the impact of acute challenges on the intracellular pH, but are insufficient to counteract sustained stress. In the absence of other regulatory processes, the continuous generation of metabolic acid equivalents, together with the ongoing transport of ions that alter the pH (for example, H^+, OH^- and HCO_3^-), would promptly consume the passive (physicochemical) buffers, which are finite. Therefore other, more dynamic and sustained mechanisms are required to ensure long-term pH homeostasis. As discussed in detail below, various different, often redundant, mechanisms have evolved to regulate pH in individual compartments.

Cytosolic pH

Under physiological conditions, the extracellular pH is slightly alkaline (~7.3–7.4). However, even when bathed in large volumes of this heavily buffered, alkaline medium, the cytosolic pH (pH_c) is slightly more acidic (FIG. 1) and, in fact, the cells have to guard against further acidification. Two reasons account for the tendency of the cytosol to acidify. First, the electrical potential across the membrane, which is negative inside, drives the uptake of positively charged protons and the efflux of negatively charged

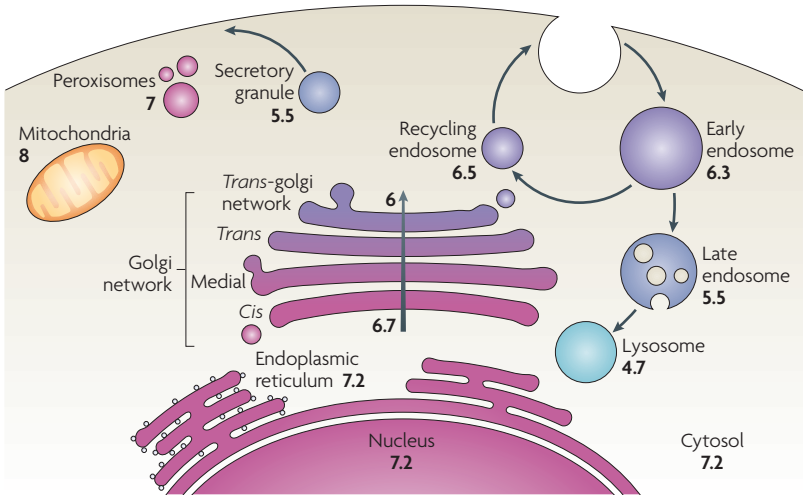


Figure 1 | **pH of the different subcellular compartments.** The pH of individual cellular organelles and compartments in a prototypical mammalian cell. The values were collected from various sources. The mitochondrial pH refers to the matrix, that is, the space contained by the inner mitochondrial membrane. Early endosomes refer to the sorting endosomal compartment. The pH of the multivesicular late endosome refers to the bulk luminal fluid; the pH of the fluid contained by the internal vesicles might differ.

bases, such as HCO_3^-, through conductive pathways. Second, net acid equivalents are generated by various metabolic reactions (for example, ATP production in the cytoplasm by glycolysis and in mitochondria by oxidative phosphorylation; see FIG. 2), a situation that is exacerbated during bursts of activity, such as in muscle contraction or on activation of leukocytes by pathogens[5].

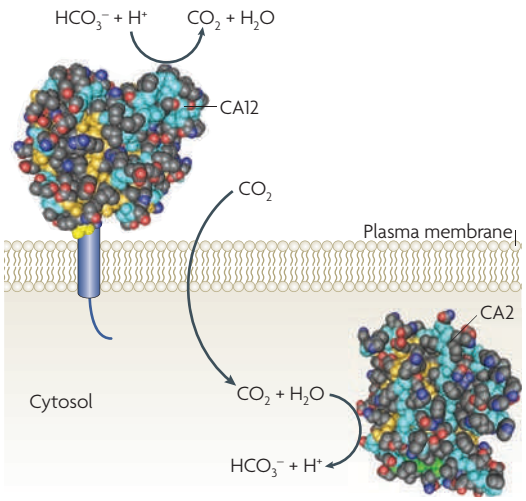
To prevent their gradual accumulation, protons must be continuously and actively extruded from the cytosol across the plasma membrane. The energy for proton extrusion can be provided directly or indirectly by ATP. Proton-pumping ATPases exist in the plasma membrane of some epithelial cells and in osteoclasts. These cells express on their surface vacuolar-type (V) electrogenic H^+-ATP hydrolases (V-ATPases) — energy-driven active proton pumps that are similar to those found in endomembranes (see below) and are characteristically sensitive to macrolide antibiotics such as bafilomycin[6]. The acid-secreting parietal cells of the stomach and distal tubule cells of the kidney additionally express a different type of ATPase, which exchanges K^+ for H^+ in an electroneutral manner[7]. Although these ATPases effectively extrude protons from the cytosol, their primary role is not the regulation of intracellular pH. Osteoclasts secrete acid into lacunae, where bone is resorbed, whereas the gastric and distal tubular ATPases, which are restricted to the apical surface of the cells, are intended for transepithelial delivery of protons to the lumen of the stomach and the renal tubules, respectively[7,8]. Thus, proton-pumping ATPases are not important participants in the regulation of pH_c in most mammalian cells. Instead, protons are extruded from the cells against their electrochemical gradient by coupling to other substrates through exchangers (antiporters) or co-transporters (symporters) (FIG. 2).

Alkali cation–H^+ exchangers. Alkali cation–H^+ exchangers (FIG. 2) are homodimeric complexes that directly couple the transfer of H^+ across biological membranes to the counter-transport of monovalent cations such as Na^+ or K^+, and are an evolutionarily conserved mechanism for protecting cells against excess acidification. Mammals possess at least eleven distinct orthologues: Na^+–H^+ exchanger 1 (NHE1; also known as SLC9A1)–NHE9 (also known as SLC9A9), Na^+–H^+ antiporter 1 (NHA1; also known as NHEDC1) and NHA2 (also known as NHEDC2). These are expressed in a ubiquitous or tissue-specific manner (see Supplementary information S1 (table))[9,10].

Box 1 | **CO_2, HCO_3^- and the regulation of pH**

Eukaryotic cells constantly produce CO_2, an end product of mitochondrial energy production. In the presence of water, CO_2 is effectively a conjugate acid by virtue of the following reactions: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$

Inter-conversion of CO_2 and HCO_3^- occurs spontaneously. Carbonic anhydrase (CA) enzymes (see the figure), however, greatly accelerate the reaction, with catalytic rates of up to 10^6 s^{-1} (REF. 117). The human genome encodes 11 different carbonic anhydrases[117]. Since they catalyse the production or consumption of H^+ (depending on the prevailing substrate concentrations), carbonic anhydrase isoforms exercise some degree of control on the kinetics and location of pH changes. Their function is likely to differ depending on their site of expression: some isoforms are located in the cytosol (CA1, CA2, CA3, CA7 and CA8), others are in mitochondria (CA5) or at the plasma membrane, where they can be anchored to the extracellular surface by a glycosylphosphatidylinositol anchor (CA4) or a transmembrane segment (CA9, CA12 and CA14). Other isoforms are secreted from the cell (CA6)[117].



Small, rapidly diffusible buffers facilitate the displacement of proton equivalents in cells. In this context, CO_2 (an uncharged acid equivalent) can diffuse without transiently binding to immobilized charged macromolecules and, therefore, plays an important part in dissipating cytosolic pH gradients. By accelerating the conversion of CO_2 to H_2CO_3, carbonic anhydrases speed up the dissipation of localized pH gradients[110].

pK_a
The acid dissociation constant. A quantitative measure of the tendency of an acid to dissociate in solution. It is calculated as $pK_a = -\log_{10}K_a$, where $K_a = [A^-][H^+]/[HA]$ and $[A^-]$, $[H^+]$ and $[HA]$ are the concentration of the dissociated acid, protons and the undissociated (protonated) acid, respectively.

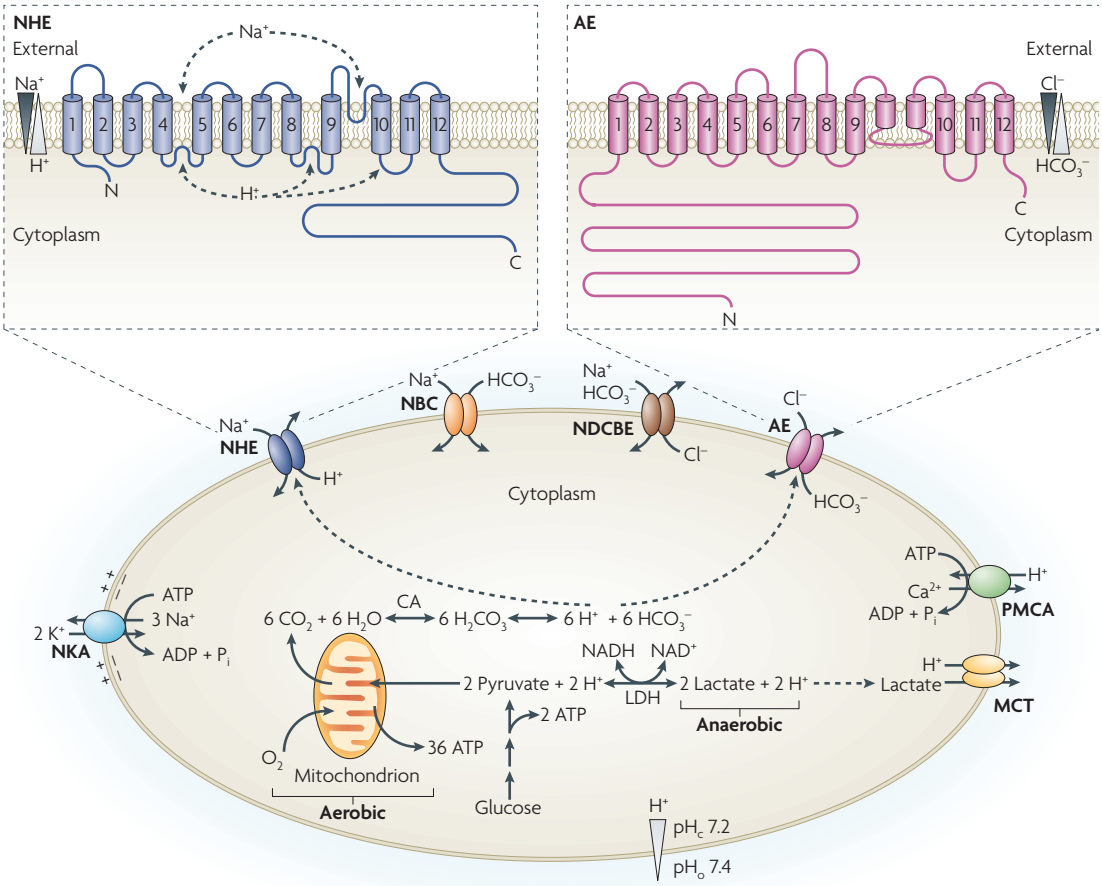


Figure 2 | **Ion carriers that regulate cytoplasmic pH.** The cytoplasm tends to acidify owing to the activities of various metabolic pathways, such as the ATP production in the cytoplasm by glycolysis that generates lactate (the anaerobic component in the figure) and the oxidative phosphorylation in mitochondria that produces CO_2 (the aerobic component shown in the figure). The predominant pH-regulatory transporters that are responsible for alkalinization are the plasma membrane Na^+–H^+ exchangers (NHEs) and Na^+–HCO_3^- co-transporters (NBCs). A limited number of cell types also alkalinize their cytosol through the actions of Na^+-dependent Cl^-–HCO_3^- exchangers (NDCBEs). These transporters use the energy stored in the inwardly directed electrochemical Na^+ gradient that is established by Na^+–K^+-ATPase pumps (NKAs) to drive solute transport. These alkalinizing mechanisms are counterbalanced by the actions of plasma membrane Cl^-–HCO_3^- or anion exchangers (AEs), which acidify the cell. Plasma membrane Ca^{2+}-ATPases (PMCAs), which exchange cytosolic Ca^{2+} for extracellular H^+, also acidify the cytosol in response to stimuli that elevate intracellular Ca^{2+}. In tissues undergoing anaerobic metabolism, alkalinization might be favoured by monocarboxylate–H^+ co-transporters (MCTs; for example, in muscle). The proposed transmembrane topology of NHE and AE proteins is shown in the insets. CA, carbonic anhydrase; LDH, lactate dehydrogenase; pH_c, cytosolic pH; pH_o, outside (or extracellular) pH.

In cells, these alkali cation–H^+ exchangers are differentially sorted not only to specific subdomains of the plasma membrane (for example, lamellipodia of fibroblasts[11], apical or basolateral surfaces of epithelia[12,13] and intercalated discs and T-tubules of cardiac myocytes[14]), but also to organelles along the secretory and endocytic pathways, in which they participate in a myriad of cellular and/or systemic processes.

The NHE1 isoform has been studied extensively and is a useful paradigm for understanding this class of transporters. It is present at the cell surface of most cells (with some exceptions, such as renal α- and β-intercalated cells and macula densa cells [13,15]) and is the main isoform responsible for pH_c homeostasis. NHE1, along with other plasma membrane-type NHEs, are Na^+-selective and use the inwardly directed electrochemical Na^+ gradient generated by Na^+–K^+-ATPases to export H^+ in an electroneutral manner, although the precise Na^+/H^+ stoichiometry (1/1 or 2/2) is a subject of debate[16,17]. The magnitude of this transmembrane gradient (the extracellular Na^+ concentration is typically ~10-fold greater than the cytosolic Na^+ concentration) should, in principle, provide a driving force that is sufficient for NHE1 to alkalinize the cytosol at least one pH unit above the extracellular pH. However, resting pH_c is usually lower than the extracellular pH because the transporter becomes largely quiescent at $pH_c \geq 7.2$ by virtue of an internal pH-sensing regulatory site, known as the H^+ modifier, or H^+ sensor (described in more detail below). This regulatory property has two important functions: it minimizes dissipation of the transmembrane Na^+ gradient, which is required for the activities of other Na^+-coupled ion and nutrient transporters,

Na^+–K^+-ATPase
A ubiquitous plasmalemmal enzyme that uses ATP to extrude 3 Na^+ ions in exchange for 2 K^+ ions. Also known as the Na^+–K^+-pump or simply the Na-pump.

and it curtails undue alkalinization, thereby equilibrating pH_c near the level that is optimal for stable protein activities and interactions. Conversely, as the H^+ concentration of the cytosol rises, the activity of the transporter increases sharply and achieves near-maximal velocity in approximately one pH unit (Hill coefficient > 1), thereby minimizing exposure of the cytoplasm to excess acidification[16,18]. This complex kinetic behaviour has been widely attributed to the existence of not only a H^+ transport site but, as mentioned above, to an additional intracellular H^+-binding site, the modifier site, that senses pH_c and allosterically regulates transport activity[16,19].

Mechanistically, it has been proposed that the pH dependence of activation involves protonation or deprotonation of one or more of the ionizable side chains of the transporter that are exposed to the cytoplasmic milieu. Such changes could conceivably modulate ion pairs or salt bridges involved in conformational transitions that facilitate access of H^+ to the transport site. This postulate is supported by detailed structural and functional studies of the bacterial Na^+–H^+ antiporter A (NhaA), in which an amino acid cluster, identified as the H^+ sensor, at the entry of the cytoplasmic funnel is coupled electrostatically to conformational alterations that expose ionizable residues in another cluster involved in ion translocation[20–22]. Similarly, mutations of conserved charged residues located in intracellular loops of NHE1 alter its sensitivity to pH_c; these residues might constitute elements of the putative regulatory site [23–25]. However, the notion that the allosteric behaviour of NHE1 is explained by the presence of a discrete H^+ sensor has been questioned[26]. Instead, it has been proposed that the kinetic data best fit a Monod–Wyman–Changeux model[27], in which the transporter exists as a symmetrical dimer in thermodynamic equilibrium between two conformational states that are distinguished by their affinity (low or high) for H^+ at the transport site. At resting pH_c, the bulk of the transporters reside in the low-affinity, catalytically inactive state. As the cytosolic concentration of H^+ increases, however, the high-affinity form binds H^+ and becomes activated. This, in turn, shifts the balance between the two conformational states and promotes the transition of the low-affinity form to a high-affinity, transport-competent form. These two mechanistic models are not mutually exclusive and further experimentation will determine which one most accurately accounts for the exchanger's allosteric sensitivity to H^+.

In addition to its intrinsic sensitivity to cytosolic acidification, the internal pH sensitivity of NHE1 can be altered by various signals (for example, hormones, mitogens and physical stimuli such as mechanical stretch and hyperosmolarity) that modulate its state of phosphorylation[28–33] and its association with ancillary factors[34–43]. These associations are believed to elicit a change in the conformation of the cytoplasmic carboxy-terminal regulatory domain of NHE1, enhancing its affinity for H^+. Although the precise mechanism is uncertain, this produces an acute rise in pH_c that is thought to provide a permissive environment for the progression of diverse cellular processes, including changes in cell shape[36], adhesion[44], migration[45,46], chemotaxis[47,48] and proliferation[49–51].

Lactate–H^+ co-transporters. Monocarboxylate transporters (MCTs) mediate the co-transport of monocarboxylic acids (predominantly lactate) with protons[52]. Cytosolic accumulation of lactate occurs in tissues undergoing anaerobic metabolism (FIG. 2). In such tissues, the outward gradient of lactate provides the energy to ferry protons to the extracellular space by MCTs. In muscle, for example, MCT-mediated H^+–lactate efflux accounts for up to 40% of the pH_c recovery from ischemic acidosis — the accumulation of proton equivalents that results from oxygen deprivation[53]. However, because substantive lactate accumulation is limited to some types of skeletal muscle and solid tumours, MCT is not a widespread pH regulatory mechanism.

Bicarbonate transporters as acid extruders. Mammalian tissues are bathed in a milieu that typically contains about 25 mM HCO_3^-, and cells have developed a means to take up extracellular HCO_3^- to alkalinize their cytosol. In the process, carbonic acid is formed and subsequently converted by carbonic anhydrases into water and CO_2, which can exit the cells to regenerate HCO_3^- extracellularly (BOX 1). CO_2 is small and electroneutral and, therefore, membrane-permeant. Whether CO_2 merely dissolves into the lipid bilayer or additionally permeates aquaporin water channels or the Rhesus-associated glycoprotein (RHAG) of erythrocytes remains the subject of controversy[4,54,55]. It is clear, however, that the larger, anionic HCO_3^- requires transport proteins to facilitate its passage across the plasma membrane.

Predominant among the acid-extruding HCO_3^- transporters are the six mammalian Na^+-coupled HCO_3^- transporters (NBCs)[56] (FIG. 2; see Supplementary information S2 (table)). These plasma membrane transporters translocate Na^+ and HCO_3^- together in the same direction but, remarkably, the coupling stoichiometry varies among members of this family. The stoichiometry is 1 Na^+/1 HCO_3^- for NBC electroneutral isoform 1 (NBCn1; also known as solute carrier family 4 member 7 (SLC4A7)), NBCn2 (also known as SLC4A10) and SLC4A9, which are therefore electroneutral[56], but can be 1 Na^+/2 HCO_3^- or 1 Na^+/3 HCO_3^- for NBC electrogenic isoform 1 (NBCe1; also known as SLC4A4) and NBCe2 (also known as SLC4A5), which are consequently electrogenic[57]. The electroneutral isoforms are unaffected by the transmembrane potential and the magnitude and directionality of transport are dictated solely by the combined chemical gradients of Na^+ and HCO_3^-. In this case, the large Na^+ gradient (the extracellular Na^+ concentration is greater than the cytosolic Na^+ concentration) favours the net inward co-transport of HCO_3^-, which is equivalent to acid extrusion from the cells. For NBCe1 and NBCe2, the driving force is compounded by the electrical potential, which is normally negative inside the cell. When the electrical component outstrips the driving force of the concentration gradients, net HCO_3^- extrusion results, causing cytosolic acidification. Such is the case for NBCe1, which carries Na^+ and HCO_3^- into the blood across the basolateral surface of renal proximal tubule cells[58].

Hill coefficient
A measure of the cooperativity of a binding process. It is calculated by applying the Hill equation, which relates the fraction of filled ligand-binding sites to the ligand concentration.

Aquaporin water channel
One of a family of proteins that facilitate the passage of water across biological membranes.

Acid-loading transporters. Although the ongoing net acid generation by metabolic reactions requires continued proton extrusion, cells have also developed acid-importing transport mechanisms. The most common acid-loading process is the exchange of Cl^- for HCO_3^-. Two distinct protein families are responsible for Cl^-–HCO_3^- exchange: anion exchangers (AEs; comprised of AE1 (also known as SLC4A1)–AE3 (also known as SLC4A3)) and SLC26A proteins (a group of five Cl^-–HCO_3^- exchangers, some of which are electrogenic and others electroneutral; see Supplementary information S2 (table))[56]. The inward Cl^- gradient (the cytosolic Cl^- concentration is typically several-fold lower than the extracellular Cl^- concentration) provides the driving force for net HCO_3^- efflux through these anion exchangers. Cellular acid loading by Cl^-–HCO_3^- exchange has four principal functions: the control of pH_c, the secretion of acids, the secretion of bases and NaCl transport.

The simultaneous operation of alkalinizing and acidifying systems working counter to each other enables the fine control of pH_c. In the event of over-alkalinization by regulatory acid extruders, acid-loading transporters can rapidly restore pH_c. Overcompensation is prevented by the intrinsic properties of the acid importers: transport activity of AE2 (also known as SLC4A2) decreases by 80–90% as pH_c drops from 7.3 to 6.8. The sensor for pH_c cannot be discretely localized to one or even a few residues of AE2 (FIG. 2). Instead, a region of the transmembrane domain and residues in the amino-terminal cytoplasmic domain all seem to contribute to inhibit AE2 activity at an acidic pH_c[59]. The combined action of AE2 (which is silenced at acidic pH) and NHE1 (which is activated by acid but becomes quiescent at alkaline pH) results in stringent control of pH_c near 7.2 (REFS 60,61).

Cells specialized in acid secretion, such as osteoclasts and gastric parietal cells, are susceptible to cytosolic alkalosis. In these cases, Cl^-–HCO_3^- exchangers such as AE2 provide a cytosolic acid reservoir that supports sustained acid secretion while preventing pH_c from reaching threateningly alkaline levels[62]. Other cells, such as those of the exocrine pancreas, are specialized in base secretion. Several systems cooperate to attain the high concentrations of HCO_3^- that are found in the fluid secreted by the pancreas (up to 140 mM). These include the Cl^-–HCO_3^- exchangers SLC26A3 and SLC26A6 (REFS 63,64) and the channel defective in cystic fibrosis (the cystic fibrosis transmembrane conductance regulator (CFTR)). Although best known as a Cl^- channel, CFTR is about one third as permeable to HCO_3^- as it is to Cl^-, and contributes significantly to pancreatic base secretion by using the negative membrane potential to drive HCO_3^- out of the cells[63].

Seemingly futile coupling of acid loading and acid extrusion occurs commonly in cells to achieve NaCl transport. Operation of Cl^-–HCO_3^- exchange in parallel with Na^+–H^+ exchange results in no net change of pH_c, but effectively loads a cell with NaCl. Cells take advantage of net NaCl uptake to regulate their volume, or for trans-epithelial secretion or resorption of salt and water. In lymphocytes, for instance, co-activation of AE2 with NHE1 restores cell volume following hyper-osmotic challenge, as water enters cells osmotically following the gain of NaCl[65]. In the lumen of the colon, NaCl is reabsorbed by the concerted action of SLC26A3 and NHE3 (also known as SLC9A3)[66]. The importance of acid loading in colonic NaCl–water uptake is evident in individuals with mutations in SLC26A3, who suffer chronic diarrhea[67].

The nuclear pH

Direct, reliable determinations of the pH of the nucleus are scarce, perhaps because most authors assume that the nuclear pH equals that of the cytosol. The nuclear envelope has an abundance of pores that are permeable to molecules 4–9 nm in diameter, the size of a small protein[68]. As such, the nuclear membrane presents only a weak diffusive barrier to H^+ and the pH of the nucleus is probably identical to that of the surrounding cytosol (FIG. 1). It is therefore assumed that the nuclear pH is indirectly controlled by the same mechanisms that safeguard the pH_c. This assumption, however, requires experimental validation.

The pH of the secretory pathway

Accurate delivery of newly synthesized glycoproteins and lipids to their intended destination is crucial for cell survival. Cells have therefore developed sophisticated means to properly identify and target biosynthetic products along the secretory pathway. One strategy is to control the interaction between cargo molecules and their receptors by controlling their state of protonation. Hence, stringent regulation of the pH of the secretory pathway is fundamental for proper protein sorting and processing.

Remarkably, the luminal pH is not homogeneous throughout the secretory pathway[69] (FIG. 1): whereas the pH of the endoplasmic reticulum (ER) is near neutral, similar to that of the cytosol, downstream compartments become progressively more acidic. The *cis*-Golgi is significantly more acidic (pH ~ 6.7) than the ER and the acidification becomes more marked in subsequent cisternae of the Golgi complex, reaching pH ~ 6.0 in the *trans*-Golgi network (TGN). The pH of secretory granules has been reported to be as low as 5.2.

Proton-pumping V-ATPases are the primary means of delivering protons to the organellar lumen. V-ATPases are multisubunit complexes composed of two distinct domains: V_0, an integral membrane complex consisting of six different types of subunits, and V_1, a peripheral complex made up of eight subunit types (FIG. 3). V_1 converts the chemical energy stored in ATP into the mechanical force required for proton displacement and V_0 provides the path for proton translocation across the membrane. Macrolide antibiotics such as the bafilomycins and concanamycins, which are specific inhibitors of the V-ATPases, effectively dissipate the pH gradient across all compartments of the secretory pathway[6].

If the pH gradient across all secretory organelles is generated by ostensibly similar V-ATPases, what accounts for the differences in pH of the individual compartments? The existence of alternative isoforms of some subunits of the V-ATPase could, in principle, explain the varying pH. The presence of multiple isoforms is, however, often associated

with the unique needs of specialized tissues, such as acid-secreting osteoclasts and epithelia[8]. Therefore, other factors are likely to account for the gradation of pH along the secretory pathway. These might include variation in the density of V-ATPases or in their rate of transport, and differences in counter-ion conductance and/or in the magnitude of the dissipative proton 'leak'. In some systems, disassembly of the V_1 and V_0 subunits has been shown[70] and this mechanism might lead to varying rates of pumping. The RAVE complex (regulator of the H^+-ATPase of the vacuolar and endosomal membranes) has been reported to regulate pump assembly. Lastly, the pumping rate also depends on the redox state of the enzyme[71].

V-ATPases translocate 'naked' H^+, unaccompanied by other ions. As a result, pumping generates a voltage (inside positive) across the membrane of the organelle, which antagonizes further inward transport of proton equivalents while promoting their outward leak. Net translocation of a substantial number of H^+ ions (that is, the development of luminal acidification) requires the parallel movement of neutralizing counter-ions. Entry of anions (probably Cl^-) or efflux of cations (such as K^+) could equally counteract the build up of an electrical potential. Several anion-conductive pathways have been detected in endomembranes, including Mid1-related chloride channel (MCLC; also known as CLCC1), a large anion-conductance channel called the Golgi anion channel (GOLAC), and members of the heterogeneous Cl^- channel and transporter ClC family. In addition, CFTR has been proposed to act in secretory membranes, but this is controversial[72,73]. By comparison, little is known about the cation channels and transporters that operate in the secretory pathway. Cations such as K^+, Na^+, Mg^{2+} and Ca^{2+} are all present in the lumen of the secretory pathway and might act as counter-ions, thereby contributing to pH homeostasis.

When present, the counter-ion conductance dictates the rate, rather than the extent, to which an organelle can acidify. Given sufficient time, even a small neutralizing conductance should enable the pump to reach the theoretical maximal pH gradient that can be generated by the V-ATPases, which, based on thermodynamic considerations, can be calculated to exceed four units. Because the pH_c is near-neutral, the luminal pH can theoretically attain levels of ≤ 3.0. Such extreme values are never reached in secretory organelles. Two factors account for this. First, the secretory pathway is in a state of flux, such that the fluid in any one subcompartment is continuously delivered anterogradely towards the membrane and ultimately the extracellular space. For this reason, the time available for luminal acidification is limited, and the degree of acidification reached is influenced by the organellar flow, the rate of H^+ pumping and the counter-ion permeation. Second, and perhaps more importantly, H^+ ions (or equivalents) leak back to the cytosol, offsetting the ability of the pump to acidify the lumen. A steady state is attained when the rate of leakage (which increases with acidification) matches the rate of pumping (which decreases with acidification).

The precise mechanism or mechanisms responsible for the leak of H^+ equivalents have not been fully characterized. A Zn^{2+}-inhibitable, voltage-sensitive H^+ conductance

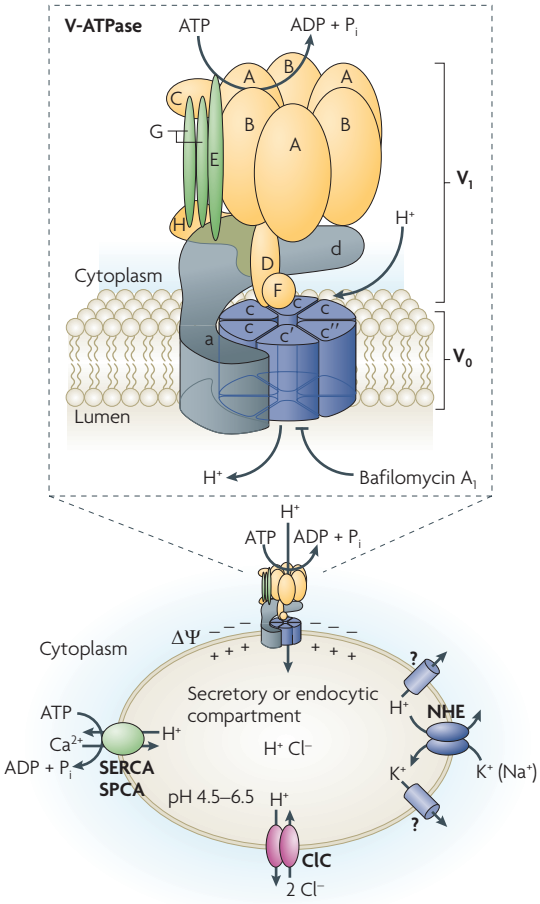


Figure 3 | **pH regulation in secretory and endocytic compartments.** Acidification of compartments along the secretory and endocytic pathways involves an intricate balance between H^+ influx and efflux pathways, as well as counter-ion (anion and cation) conductances. Active accumulation of H^+ in compartments is largely achieved by vacuolar-type H^+-translocating ATP-hydrolases (V_1V_0- or V-ATPases). These are multimeric complexes comprised of 14 distinct types of subunit (the V_1 domain comprises A_3, B_3, C_1, D_1, E_2, F_1, G_2 and $H_{1–2}$ subunits and the V_0 comprises a_1, d_1, e_n, $c_{4–5}$, c'_1, c''_1 subunits; the subscript number denotes the stoichiometry), which are shown in the inset. V-ATPases are electrogenic, generating a transmembrane voltage ($\Delta\psi$). Electrogenic 2 Cl^-/1 H^+ exchangers belonging to the Cl^- channel and transporter ClC family also make a large contribution to optimal luminal acidification by providing anions that tend to neutralize the build up of positive charge in the lumen, which would otherwise decrease V-ATPase activity. Moreover, this Cl^-–proton exchange mechanism accounts in part for the rapid (that is, in minutes) dissipation of the transmembrane H^+ gradient that occurs upon inhibition of the V-ATPase by macrolide antibiotics such as bafilomycin A_1. Other H^+ efflux pathways include sarcoplasmic, or endoplasmic, reticulum Ca^{2+}-ATPases (SERCAs) and secretory pathway Ca^{2+}-ATPases (SPCAs), the non-selective alkali cation–H^+ exchangers Na^+–H^+ exchanger 6 (NHE6)–NHE9 and a Zn^{2+}-inhibitable, voltage-sensitive H^+ conductance, although the precise molecular identity of this ion carrier is yet to be established. Cations such as K^+ are also present in the lumen of secretory and endocytic compartments and might act as counter-ions by extrusion through ill-defined carriers, thereby contributing to pH homeostasis.

seems to contribute to the H^+ leak from the Golgi complex[74], although the precise molecular identity of this ion carrier is yet to be established (FIG. 3). NHE7 (also known as SLC9A7) has been localized to the *trans*-Golgi network (TGN)[75] and transports not only Na^+ but also K^+, the major intracellular alkali cation, in exchange for H^+ (FIG.3; see Supplementary information S1 (table)). However, its relative contribution to pH regulation of the TGN remains to be confirmed. Similarly, anion exchangers are active as they transit the secretory pathway[76], but no Golgi-resident anion exchanger has been identified and direct evidence that exchange of Cl^- for OH^- or bicarbonate influences organellar pH homeostasis is lacking. Finally, Ca^{2+} accumulates in the lumen of several secretory organelles, possibly as a result of Ca^{2+}–H^+ exchange by sarcoplasmic, or endoplasmic, reticulum Ca^{2+}-ATPase (SERCA) and secretory pathway Ca^{2+}-ATPase (SPCA) pumps[77] (FIG. 3).

Even if the molecular entities responsible for counter-ion and leak permeabilities have not been fully defined, the determinants of the pH of the secretory pathway can be estimated. This task was undertaken by Machen and colleagues[78], who systematically analysed the absolute magnitude of the luminal pH of each subcompartment, as well as the rates of proton pumping and leakage and the permeability to counter-ions. The progressively more acidic luminal pH along the secretory path was attributed to two factors. First, proton-pumping activity (presumably a reflection of the density of active V-ATPases) increases progressively from the ER to the Golgi and thereafter. Secondly, the leak decreases progressively, favouring the retention of the protons pumped by the V-ATPase. These effects are synergistic and, as a result, the late components of the secretory pathway (for example, secretory granules) are much more acidic than the early ones (for example, the ER). Of note, Machen *et al.*[78] concluded that the magnitude of the counter-ion permeability is unlikely to have much influence on the development of the pH gradient.

The pH of the endocytic pathway

Cargo and fluid-phase contents taken up by cells experience an increasingly acidic environment as they progress through the endocytic pathway (FIG. 1). Endocytic vesicles are the least acidic, whereas lysosomes can reach pH values as low as 4.5–4.7. Progressive luminal acidification seems to be key to various aspects of endocytosis and phagocytosis. Uncoupling of ligands from receptors, activation of proteases, protonation of microbicidal factors and other essential reactions depend on the changing pH of the endosomal compartment. Indeed, even the normal traffic of membranes between endocytic subcompartments seems to be controlled by luminal acidification[79].

The principles governing the gradual acidification of the endocytic pathway are the same as those described for the secretory pathway. Proton accumulation is driven by V-ATPases, while counter-ion and leak permeabilities dictate the steady-state pH of individual organelles (FIG. 3). Members of the ClC family, Cl^- channel 3 (CLCN3)–CLCN7, have all been suggested to mediate conductive entry of Cl^- into endosomes and lysosomes[80]. Of note, at least some of the ClC family members provide a neutralizing charge by operating as electrogenic 2 Cl^-/1 H^+ exchangers, and not as channels as originally suspected. Additionally, CFTR has been proposed to function in lysosomes and thereby contribute to microbial killing in phagosomes[81]. Finally, cation-conductive pathways (for example, K^+ channels) might also dissipate the voltage generated by the V-ATPases.

The existence of H^+ (or equivalent) leakage pathways in endocytic organelles can be most readily determined by acutely arresting the V-ATPase. The addition of macrolide inhibitors unmasks the presence of a leak, manifested as an alkalinization that is rapid in endosomes but considerably slower in lysosomes. The rate of alkalinization is governed by the activity of leak pathways, as well as by the buffering power and surface-to-volume ratio of the individual organelles. As in the secretory pathway, little is known about the molecular identity of the leak, but NHE6 (also known as SLC9A6) and NHE9 are present in endocytic organelles (see Supplementary information S1 (table)) and might contribute to the dissipation of the H^+ gradient.

Mitochondrial pH

Unlike the lumen of compartments along the secretory and endocytic pathways, the mitochondrial matrix is markedly alkaline (pH ~8.0) owing to H^+ extrusion across the inner membrane by components of the electron transport chain[82,83] (FIG. 4). Together with the electrical potential (inside negative) generated by the electrogenic proton-extrusion process, the transmembrane pH gradient constitutes a proton-motive force (ψ_{H+}) that is harnessed by the inner membrane H^+-ATP synthase (F_1F_0-ATPase) to generate ATP from ADP and inorganic phosphate. The electrochemical H^+ gradient further acts to regulate Na^+, K^+ and Ca^+ homeostasis through the concerted actions of alkali cation–H^+ exchange, Na^+–Ca^{2+} exchange and Ca^{2+} uniport pathways[84–86] (FIG. 4). Matrix Ca^{2+}, in turn, modulates various dehydrogenases of the tricarboxylic acid cycle, which is essential for mitochondrial energy generation[87,88], the adenine nucleotide translocase[89] and the F_1F_0-ATPase[90], and has been proposed as a means of synchronizing energy production with cellular needs[87,88,91]. Thus, the matrix pH has a central role in the basal regulation of mitochondrial metabolism.

Alterations in mitochondrial pH homeostasis have been implicated as an important early event in mitochondrial-dependent apoptosis. Pro-apoptotic factors and stimuli, such as the endogenous apoptosis-inducing protein BAX, the kinase inhibitor staurosporine and ultraviolet radiation, induce mitochondrial–matrix alkalinization in parallel with cytosolic acidification. Matrix alkalinization seemingly requires F_1F_0-ATPase activity, suggesting that, contrary to its normal mode of operation as a synthase, during apoptosis the enzyme consumes ATP to pump protons from the lumen into the intermembrane space and ultimately into the cytosol[92,93]. However, this proposed mechanism has been challenged based on technical and thermodynamic considerations[94]. Alternatively, intracellular acidification associated with mitochondrial-dependent

Phagosome
A vacuole that forms inside cells following the engulfment of large (≥ 0.5 μm) particles by a receptor-mediated, actin-driven process.

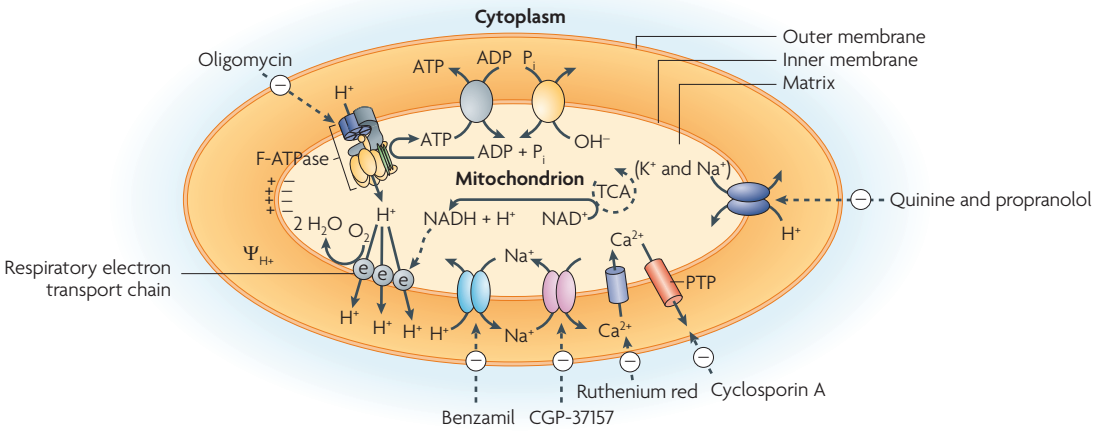


Figure 4 | **Mitochondrial pH regulation.** In mitochondria, the respiratory electron transport (e) chain moves H^+ out of the matrix into the inter-membrane space, whereas the F-ATPase allows the re-entry of H^+, a process that is coupled to ATP production. Intra-mitochondrial pH homeostasis is established by the interplay between the respiratory chain, F-ATPase and the transport of proton equivalents that are directly or indirectly coupled to other inorganic cations. Ca^{2+} tends to accumulate in the mitochondrial matrix through a ruthenium red-inhibitable Ca^{2+} uniport or channel, driven by the electrical potential generated by the respiratory chain. Excessive accumulation is averted by Na^+-coupled Ca^{2+} extrusion through an exchanger. Ca^{2+} can also exit the matrix on transient opening of the permeability transition pore (PTP). Na^+ taken up by Na^+–Ca^{2+} exchange is, in turn, prevented from accumulating by extrusion in exchange for H^+. The molecular activities of the exchangers and uniporters responsible for the cycling of H^+, Na^+, K^+ and Ca^{2+} across the inner membrane of mitochondria have primarily been distinguished by their sensitivities to various pharmacological antagonists (such as oligomycin, benzamil, CGP-37157, ruthenium red, cyclosporin A, quinine and propanolol), but their precise molecular identities are yet to be established. Recent immunological analyses have detected the presence of the mammalian Na^+–H^+ antiporter 2 (NHA2) isoform, although this finding is complicated by other studies reporting the localization of this transporter at the cell surface (see references 25–27 in Supplementary information S1 (table)). These findings require validation of the functional activity of NHA2. TCA, tricarboxylic acid cycle.

apoptosis has been attributed to the inhibition of plasma membrane NHE activity[95,96]. Although the precise mechanism remains obscure and is likely to be multifaceted and cell-type dependent, it is clear that the cytosol becomes acidified, which enhances the activation of the proteolytic activity of caspases by cytochrome C release from the mitochondria. That being said, it is worth noting that cell death associated with intracellular acidification elicited by blocking NHE activity is not necessarily linked to caspase-dependent apoptosis, but instead can resemble paraptosis — an alternative form of programmed cell death that is typified by cytoplasmic vacuolation and mitochondrial swelling[97].

Peroxisomal pH

Peroxisomes emanate from the ER as nascent vesicles and undergo a dynamic maturation process to form active organelles responsible for several crucial metabolic processes[98,99]. These include the breakdown of long- and branched-chain fatty acids by β-oxidation, the formation of cholesterol, bile acids and ether phospholipids, the catabolism of purines and polyamines, the metabolism of amino acids and glyoxylate, and the detoxification of reactive oxygen species[100]. Despite considerable understanding of their biogenesis and biochemical functions, knowledge of their luminal ionic composition — an important determinant of enzymatic activity — is sparse and conflicting. Earlier measurements in mammalian cells using the pH-sensitive organic probe carboxy-seminaphthofluorescein conjugated to a peroxisome-targeting sequence indicated that the luminal pH of peroxisomes was considerably alkaline[101]. However, the accuracy of these measurements is problematic owing to the limited dynamic range of the fluorophore (~10% change in fluorescence per pH unit)[102].

By contrast, subsequent analyses by others[102,103] using a different targeted fluorophore displaying broader pH sensitivity, the pH-sensitive green fluorescent protein variant pHluorin, were consistently unable to detect much of a H^+ gradient across the peroxisomal membrane. Steady-state peroxisomal pH (pH_p) was found to be near neutral and perturbations that altered pH_c were closely mirrored by parallel changes in pH_p[102]. These observations suggested that the peroxisomal membrane is highly permeable to H^+ (or acid equivalents). Assuming that pHluorin is a more reliable indicator of pH_p, this would indicate that peroxisomes, like the ER, lack an intrinsic pH regulatory system and instead use acid and base transporters at the plasma membrane and the buffering capacity of the cytoplasm to indirectly maintain pH_p homeostasis.

pH — homeostasis or signalling?

Numerous proteins that have crucial roles in membrane excitability, intercellular communication, signal transduction, cytoskeletal dynamics and vesicle trafficking have been reported to be highly sensitive to minute

Box 2 | **Diseases associated with dysregulation of intracellular pH**

Disturbances in cytoplasmic and organellar pH homeostasis, arising from either metabolic or genetic perturbations, are associated with the progression of distinct pathophysiologic states, as exemplified below.

Cytoplasmic pH. Reductions in blood flow (that is, ischemia) decrease the supply of oxygen required to maintain tissue ATP levels, especially in excitable organs such as the heart and brain that have a high demand for energy. As ATP stores are depleted, lactate, pyruvate and protons accumulate owing to anaerobic metabolism of glycogen stores. The accompanying cytoplasmic acidification causes hyperactivation of plasma membrane Na^+–H^+ exchanger 1 (NHE1) and the consequent accumulation of intracellular Na^+. The Na^+ overload reverses the mode of operation of Na^+–Ca^{2+} exchange, driving excess Ca^{2+} into the cell. The resulting elevation in intracellular Ca^{+2} concentration precipitates a cascade of deleterious effects, including altered membrane excitability and contractility, generation of toxic free radicals, cellular hypertrophy, apoptosis and necrosis — events that can culminate in cardiac arrhythmias or failure and stroke (for further details, see REFS 118, 119).

Organellar pH. Mutations of the electrogenic 2 Cl^-/1 H^+ exchanger Cl^- channel 5 (CLCN5) impair luminal acidification and biogenesis of apical early endosomes in renal proximal tubule epithelia, causing proteinuria, hypercalciuria, nephrolithiasis and ultimately renal failure (Dent disease)[120,121]. Mutations in other chloride carriers that are present in late endosomes and lysosomes, such as CLCN6 and CLCN7, can also give rise to distinct pathophysiologic states, such as osteopetrosis[122] and lysosomal storage disease[123,124]. Surprisingly, however, mutations to these proteins do not seem to perturb organellar pH, raising questions about the underlying mechanistic basis for the phenotypes.

Finally, mutations to the a2 subunit of the V-type H^+–ATPase, which resides in endosomes and in a compartment overlapping with the *trans*-Golgi network, lead to cutis laxa or wrinkly skin syndrome[125]. These mutations are associated with abnormal glycosylation and delays in the retrograde trafficking of Golgi membranes to the endoplasmic reticulum. Although it is assumed that these defects are a consequence of altered Golgi pH, this is yet to be proven.

changes in the surrounding pH. Salient examples include various neurotransmitter-gated receptors, cation channels, connexins, H^+-sensing G protein-coupled receptors, actin-binding proteins (cofilin, villin and gelsolin; reviewed in REF. 104) and the V-ATPase a2-isoform (see Supplementary information S3 (table) for other examples and references). Perhaps not unexpectedly, extreme metabolic disturbances or genetic perturbations that disrupt pH_c or organellar pH homeostasis can lead to the development of distinct disease states (BOX 2).

The exquisite sensitivity of these and other proteins to the concentration of H^+ might not be fortuitous and is suggestive of a role of pH in signalling. Accordingly, changes in pH_c have been proposed to initiate cellular proliferation[105], to initiate and direct cell migration[44,45] and to trigger apoptosis[106]. Although attractive, these hypotheses remain the subject of some controversy. The notion that alkalosis mediated by Na^+–H^+ exchange is essential for the initiation of cell proliferation was based in part on experiments in which NHE activity was inhibited for extended periods either pharmacologically or by omission of Na^+. Others found, however, that the impairment of NHE activity did not affect proliferation if bicarbonate was present in the medium[107]. These observations suggest that pH homeostasis is compromised when both Na^+–H^+ exchange and bicarbonate-dependent mechanisms are inactivated, and that failure to proliferate may result from pleiotropic effects caused by dysregulation of pH_c. In this context, pH can be regarded as permissive rather than as a signal for proliferation. A similar conclusion was reached more recently regarding the role of Na^+–H^+ exchange in cell migration[47], though most authors attribute a more direct signalling role for pH in cell spreading and motility[44–46].

Whereas global changes in cellular pH seem dangerous and unlikely to act as signals, transient, localized changes might have such a role. Intuitively, the small size of protons or hydronium ions — and hence their rapid diffusion — might be expected to rule out the generation of discontinuities in the profile of pH_c. However, the diffusion rate of protons in the cytosol (diffusion constant = 0.4–2.2 $\mu m^2\ s^{-1}$) is surprisingly low, two orders of magnitude slower than proton diffusion in water[108,109]. The slow rate is attributed to the delay caused by the transient and reversible association of protons with ionizable groups on cytosolic macromolecules (mainly proteins), many of which are fixed or only slowly mobile. Repeated association and dissociation events effectively reduce the free concentration of protons, lengthening the time taken for them to traverse the cytoplasm of an average cell[108,110]. The presence of rapidly diffusing H^+ buffers mitigates this effect (BOX 1), but formation of intracellular pH gradients is nevertheless possible. Accordingly, when operating at high rates, pH regulatory transporters and solute carriers coupled to H^+ transport (for example, H^+–dipeptide co-transporters) will change the pH in their immediate environment. Not only can pH gradients form in the submembranous layers of the cytosol, but in cases where the expression of the transporters is polarized (for example, in epithelial cells, which are asymmetric), gradients can form from one end of the cell to the other[110,111]. In this manner, the activation of Na^+–H^+ exchange at the leading edge of migrating cells might indeed generate a local pH gradient[112], which has been suggested to induce or at least support cytoskeletal rearrangement (FIG. 5a). Similarly, non-canonical Wnt–Frizzled signalling that controls planar epithelial polarization in the *Drosophila melanogaster* eye is highly dependent on Na^+–H^+ exchange, which, in its absence, can be replaced by manipulations that alkalinize the cell[113].

Whereas examination of the vast majority of pH-sensitive intracellular proteins suggests that cytosolic H^+ ions act only as secondary regulators of their activities, extracellular H^+ can seemingly operate as a *bona fide* signal. A recent example was provided by observations of *Caenorhabditis elegans* intestinal cells that periodically extrude H^+ across their basolateral surface through the NHE PBO-4 (also known as NHX-7; the orthologue of mammalian NHE1)[114] (FIG. 5b). The resulting acidification of the extracellular space specifically activated H^+-gated cation channels (comprised of PBO-5 and PBO-6 subunits) located in the adjacent muscle cells, thereby inducing their contraction during the defecation cycle of the nematode. This cyclic release of H^+ is reminiscent of the temporal and spatial release of neurotransmitters in the central nervous system. This raises the distinct possibility

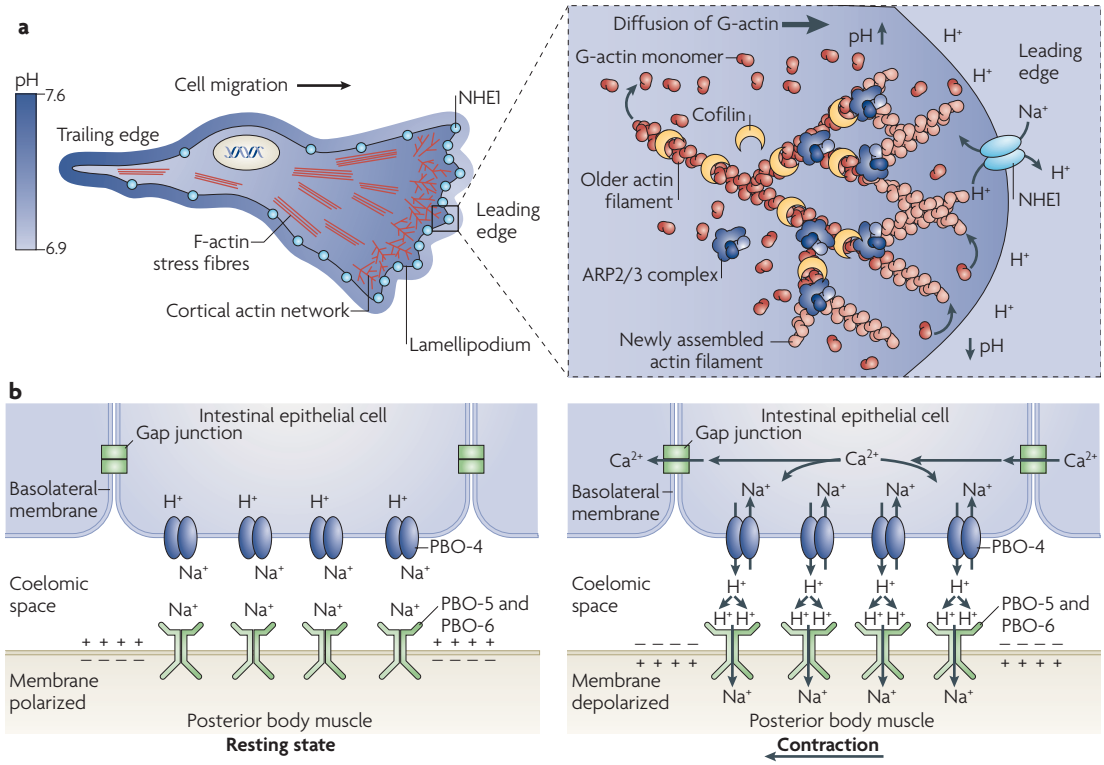


Figure 5 | **Role of protons in signal transduction. a** | Localized changes in intracellular pH have important roles in cell polarity and migration (reviewed in REFS 44–46). Na^+–H^+ exchanger 1 (NHE1) clusters at the leading edge of stimulated cells and establishes a more alkaline intracellular environment that facilitates the *de novo* assembly and organization of the underlying cortical branched actin filament network. Elevations in intracellular pH activate cofilin, an actin-binding protein that increases the recycling of actin monomers (globular actin (G-actin)) at the rear of 'older' actin filaments (filamentous actin (F-actin)). This promotes actin reorganization at the leading edge by severing established filaments, providing free barbed ends for further polymerization and nucleation by ARP2/3. Remodelling of the actin cytoskeleton, in turn, pushes the plasma membrane forwards to attain unidirectional cell movement. NHE1 activity at the leading edge also results in a more acidic external pH, which modulates integrin-mediated cell–matrix adhesion dynamics and, ultimately, cell migration. **b** | Protons as a signal transmitter in the defecation cycle of *Caenorhabditis elegans*. The rhythmic defecation cycle of *C. elegans* is initiated by inositol trisphosphate ($Ins(1,4,5)P_3$)-induced release of Ca^{2+} from intracellular stores in a pacemaking posterior intestinal epithelial cell. The Ca^{2+} spikes then travel anterogradely as a wave to neighbouring cells through gap junctions. Elevated intracellular Ca^{2+} leads to stimulation of the basolateral Na^+–H^+ exchanger PBO-4 (also known as NHX-7), which expels H^+ into the extracellular coelomic space. The secreted H^+ ions diffuse across the coelomic space and activate the H^+-gated cation channel PBO-5 and PBO-6, which is located in the facing posterior muscle cells, leading to cation influx, membrane depolarization and muscle contraction, which are required for defecation.

that protons that are secreted or released at synapses might also act as primary intercellular messengers in the modulation of pH-sensitive ion channels and neuronal function[115,116].

Conclusions

There is little doubt that the establishment and maintenance of an appropriate pH inside individual cellular compartments is of paramount importance to their normal physiology. Whereas acute departures from the resting state can be minimized or at least blunted by the buffering capacity, more durable, responsive and accurate means of controlling proton concentration had to evolve for long-term pH homeostasis. For the most part, homeostasis is accomplished by the interplay of multiple transporters that either extrude or import proton equivalents, and are tuned to the physiological state of the cell by coupling either physically or functionally to metabolic enzymes. The resulting 'metabolons' integrate proton production, conversion and transport to best regulate the intracellular pH.

Because metabolic demands vary upon stimulation and during the course of the cell cycle, proton transporters must adapt accordingly. For this reason, acid and base transporters are often the targets of signalling pathways that couple the effectors of pH homeostasis with the varying metabolic output of the cell. In turn, the pH changes associated with acute metabolic activity have been suggested to act as signals to readjust the functional activities of cells. Although the importance of pH changes as signals remains the subject of debate, it is clear that pH homeostasis and the maintenance of proton gradients across organellar membranes are key to cell survival, function and proliferation. A more profound understanding of the players and the rules governing pH regulation is therefore essential.

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