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Roles of pH in control of cell proliferation

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Abbreviations

Akt, RAC-alpha serine/threonine-protein kinase; Amiloride, (3,5-diamino-6-chloropyrazinoyl-guanidine); AP-1, Activator protein-1; ASIC, Acid-sensing ion channel; $[Ca^{2+}]_I$, free intracellular Ca^{2+} concentration; CaM, Calmodulin; CaMK, Calmodulin kinase; Cariporide, N-(Diaminomethylene)-4-isopropyl-3-(methylsulfonyl)benzamide; CDK, Cyclin-dependent kinase; CREB, cAMP response element binding protein; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic

acid; DMA, 5-(N,N Dimethyl)amiloride; DMO, 5',5´-dimethyl oxazolidine 2,4-dione; EGFR, Epidermal growth factor receptor; EIPA, 5-(N-Ethyl-N-isopropyl)amiloride; EPPS, (iV-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid); ERK, Extracellular signal regulated kinase; GPCR, G-protein coupled receptor; haOC, human ascites-derived ovarian cancer cells; HEPES, (N-2 hydroxyethylpiperazine-N'-2-ethanesul- fonic acid); HSPD, Human sodium proton exchanger-deficient; HDAC, Histone deacetylase; HK, hexokinase; JNK, c-jun N-terminal kinase; KD, knockdown; KO, knockout; MAPK, Mitogen activated protein kinase; MCT(1/4), Monocarboxylate transporter (1/4); MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-Morpholino)propanesulfonic acid; MPF, mitosis promoting factor; mTORC1, Mammalian target of rapamycin complex 1; NBCn1, electroneutral Na^+/HCO_3^--cotransporter 1; NCX, Na^+/Ca^{2+}-exchanger; NFAT, Nuclear factor of activated T cells; NFκB,Nuclear factor κ-light-chain-enhancer of activated B cells; NHE1, Na^+/H^+-exchanger 1; p90RSK, Ribosomal S Kinase; PFK-1, Phosphofructokinase-1; pH_e, pericellular pH; pH_i, intracellular pH; pH_m, local pH at the membrane; $PI(4,5)P_2$, phosphatidylinositol 4,5-biphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-biphosphate; PI3PK, phosphatidylinositol-3-kinase; PIPES, (piperazine-N,N'-his 2-ethanesulfonic acid); RPTPγ, Receptor protein tyrosine phosphatase-γ; sAC, soluble adenylate cyclase; V-ATPase, Vacuolar type H^+-ATPase; V_m, membrane potential; VSMC, Vascular smooth muscle cells; Zoniporide, ([1-(quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl] guanidine))

Abstract

Precise spatiotemporal regulation of intracellular pH (pH_i) is a prerequisite for normal cell function, and changes in pH_i or pericellular pH (pH_e) exert important signaling functions. It is well established that proliferation of mammalian cells is dependent on a permissive pH_i in the slightly alkaline range (7.0-7.2). It is also clear that mitogen signaling in nominal absence of HCO_3^- is associated with an intracellular alkalinization (∼0.3 pH unit above steady state pH_i), which is secondary to activation of Na^+/H^+ exchange. However, it remains controversial whether this increase in pH_i is part of the mitogenic signal cascade leading to cell cycle entry and -progression, and whether it is relevant under physiological conditions. Furthermore, essentially all studies of pH_i in mammalian cell proliferation have focused on the mitogen-induced G0-G1 transition, and the regulation and roles of pH_i during the cell cycle remain poorly understood.

The aim of this review is to summarize and critically discuss the possible roles of pH_i and pH_e in cell cycle progression. While the focus is on the mammalian cell cycle, important insights from studies in lower eukaryotes are also discussed. We summarize current evidence of links between cell cycle progression and pH_i and discuss possible pH_i- and pH_e sensors and signaling pathways relevant to mammalian proliferation control. The possibility that changes in pH_i during cell cycle progression may be an integral part of the checkpoint control machinery is explored. Finally, we discuss the relevance of links between pH and proliferation in the context of the perturbed pH homeostasis and acidic microenvironment of solid tumors.

Intracellular pH (pH_i) is tightly regulated by the activity of multiple plasma membrane ion transport proteins, and is kept at a near-neutral range (7.0-7.2) in most normal cells. Because the function of numerous proteins is sensitive to pH in the physiological range, both pH_i and pericellular pH (pH_e) have a major impact on multiple cellular processes.[1] One example of this is cell proliferation, which has been shown to be pH_i dependent in a wide range of species. Cell cycle progression and ultimately cell division are initiated by mitogenic stimuli and very tightly regulated by the coordinated activity of cyclin-dependent kinases (CDKs), checkpoint controls, and DNA repair pathways.[2,3] As detailed below, multiple studies have shown that mitogen-induced cell proliferation is associated with, and in some cases dependent upon, an increase in pH_i. Furthermore, evidence is emerging that hyperproliferation in diseases such as cancer can be linked, at least in part, to dysregulation of pH.[4–6] However, mechanistic insight into how pH regulates cell cycle progression is still very sparse. Notably, much of the existing data was obtained in the nominal absence of HCO_3^-, excluding essential components of the physiological pH_i-regulatory machinery.

In this review, we present what we consider the most pertinent current knowledge about the possible roles of pH_i and pH_e in cell cycle progression and proliferation. We discuss how pH_i can impact proliferation via effects on G0-G1 transition, cell cycle progression, and protein synthesis, and we summarize existing evidence for pH_i changes during cell cycle progression. Based on this and on existing knowledge of the cellular pH-sensing machinery, we discuss possible models for how pH_i and pH_e may regulate cell cycle progression and hence proliferation, under normal conditions and in a cancer context. Finally, we point out essential directions for future research.

Effectors of intracellular pH homeostasis

At the values of pH_i, pH_e, and membrane potential (V_m) in most cells, the driving force for H^+ flux is inward-directed. In conjunction with the continuous cellular metabolic acid production, this means that active acid extrusion is required to sustain normal cell function. As will be discussed in the last part of this review, this is even more critical for cancer cells in a solid tumor, which produce more acid and reside in a very acidic microenvironment. Widely expressed regulators of pH_i in eukaryotic cells (Fig. 1, left side) include the plasma membrane Na^+/H^+-exchangers (NHEs), of which the most ubiquitous is NHE1 (SLC9A1)[7] and the net acid extruding Na^+,HCO_3^--co-transporters such as the widely expressed NBCn1 (SLC4A7).[7,8] Vacuolar type H^+-ATPases (V-ATPases),[9] H^+/K^+-ATPases,[10] and other H^+ transporters such as the monocarboxylate transporters MCT1 and MCT4[11,12] also contribute to net acid extrusion in some cell types. Cytosolic and membrane-bound carbonic anhydrases (not shown in Fig. 1) are also important regulators of net acid extrusion rates, by facilitating the venting and cellular transport of H^+ and HCO_3^-.[13]

Early studies of the roles of pH in proliferation and cell cycle regulation

Table 1 lists central studies of the role of pH regulation in cell cycle progression in both lower eukaryotes and mammalian cells.

Studies in lower eukaryotes

One of the earliest studies suggesting a role for pH_i in cell division investigated the activation of sea urchin eggs following fertilization.[14] This activation occurs in two main phases, a rise in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) leading to cortical granule release, followed by a phase of increased protein synthesis, DNA synthesis, and division of the fertilized egg. Johnson et al.[14] demonstrated that the second phase was initiated by NHE activation, leading to a 0.3 unit increase in pH_i (from 6.5 to 6.8) 1 to 4 min after fertilization.[14] Two subsequent studies provided evidence that the increased pH_i upon sea urchin egg fertilization correlated with acceleration of protein synthesis, and that an increase in $[Ca^{2+}]_i$ could further amplify this effect.[15,16] Importantly, they showed that protein synthesis was completely inhibited when pH_i was experimentally set to the unfertilized level.[15,16] These studies established an increase in pH_i as a requirement for activation of protein synthesis upon fertilization.

The role of pH_i in regulation of cell cycle progression *per se* was first investigated in the slime mold *P. polycephalum*.[17] In this species (which has naturally synchronous, mitotic divisions approximately every 8 h) pH_i increased by 0.6 pH units at the point of mitosis, with an average pH_i of 6.3 during a cycle. A similar behavior of pH_i in this species was reported by Morisawa and Steinhardt,[18] although the absolute pH_i values reported in this study were higher, ranging between 7.0 during mid-interphase and 7.5 at mitosis. They further showed that artificially reducing pH_i to 7.0 delayed mitotic entry, and that pH_i must be >7.2 at least 1 h prior to mitosis to ensure timely entry into mitosis, but once initiatied, mitosis can ensue at lower pH values.[18] It should be noted that the cell cycle of *P. polycephalum* differs from that of mammalian cells in that G1 is very short or completely absent, meaning that S phase follows immediately after mitosis.[19] It is thus possible that the requirement for an increase in pH_i (>7.2) might be linked to a role in DNA synthesis. Such a relation was later shown in *D.*

discoideum, in which pH_i closely corresponded with the DNA replication cycle, oscillating 0.25 pH units with a peak of 7.45 during S phase and mitosis (Fig. 2a).[20] Inhibition of DNA- or protein synthesis had no effect on the pH_i oscillations. In contrast, when pH_i was raised from 7.3 to 7.4, dramatic increases in DNA- and protein synthesis (~3.5 and ~4.8 fold, respectively) were observed, whereas these processes were abolished at pH_i values below 7.3.[20] Taken together, this work established pH_i as important for regulation of the timing of the mitotic cycle. Similar observations have been made in *S. cerevisiae*, where increases in pH_i is associated with cell cycle progression and control of growth rate.[21–24] On the other hand, a study in *S. pombe* found no evidence for cell cycle-related changes in pH_i. pH_i was, however, associated with overall growth state, with starvation giving rise to intracellular acidification (<7.3) and metabolic quiescence.[25]. Together, the results of these studies thus indicate that the pattern of pH_i during cell cycle progression may depend on the specific cell type and conditions.

Studies in mammalian cells

From the early 1980s to the mid-1990s, a number of studies investigated the possible roles of pH_i in regulation of mammalian cell proliferation. An insightful discussion of these studies is provided by Grinstein, Rotin and Mason.[26] At that time, HCO_3^- dependent net acid extruding transporters were essentially unstudied. The focus was therefore logically on NHE, and to a limited extent, H^+ ATPases, and studies were frequently carried out in nominally HCO_3^- free conditions, essentially precluding contributions from HCO_3^- dependent transporters (see Table 1 for the conditions used in each study). The implications of this will be discussed later in this review. Additionally, some of the early studies cited below employed the first developed NHE inhibitor amiloride, which has later been shown to also inhibit the ENaC/Degenerin family of epithelial Na^+ channels,[27] the Na^+/Ca^{2+}-exchanger (NCX), and the acid-sensing cation channel-1 (ASIC-1).[28] Additionally, it has been shown to have cytotoxic effects, such as inhibition of protein synthesis,[29] protein kinase activity,[30] and

inhibition of the epidermal growth factor receptor (EGFR).[31] Later inhibitors with higher specificity for NHE1 and negligible cytotoxic effects were developed based on amiloride or benzoylguanidine derivatives, such as 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), 5-(N,N Dimethyl)amiloride (DMA), or Cariporide (HOE642), and so the choice of NHE inhibitor should be kept in mind when interpreting the studies discussed below.

An early indication of a link between proliferation and pH_i in mammalian cells was the demonstration that mitogen stimulation of murine spleen lymphocytes led to a biphasic pH_i response, with two alkaline peaks from 7.18 to 7.4 within 6 hours after stimulation, and from 7.2 to 7.45 after 12-48 h.[32,33] The second rise in pH_i correlated with initiation of DNA synthesis 48 h after activation. Accordingly, DNA synthesis was greatly dependent on pH_i, with the maximum rate reached at alkaline pH_i (7.32).[32,33] An increase in pH_i without mitogen stimulation was, however, not sufficient to activate DNA synthesis. The same year, mitogenic stimulation of mammalian fibroblasts was reported to lead to NHE activation, cytoplasmic alkalinization (from ~7.2 to ~7.4), and subsequent phosphorylation of the ribosomal S6 protein.[34,35,36], collectively pointing to a role for NHE-mediated alkalinization (>7.2) in mitogenic signaling. Such a role was directly demonstrated when it was found that fibroblasts lacking NHE activity, in contrast to their wild-type counterparts, were growth-arrested at neutral and acidic pH_e (<7.2) under HCO_3^- -free conditions.[37] Growth of the NHE-deficient cells could be restored either at alkaline pH_e (8-8.3), or by addition of HCO_3^-.[37] This was substantiated by the discovery of a tight correlation between NHE activity and the percentage of quiescent cells re-entering S phase and initiating DNA synthesis upon mitogenic activation,[38] and the demonstration that quiescent fibroblast mutants lacking NHE activity were unable to increase pH_i (to 7.2) and reinitiate DNA synthesis at neutral and acidic pH_e (<7.2) (Fig. 2b).[39] Restoration of pH_i to 7.2 in the NHE-depleted cells led to recovery of mitogen-induced DNA synthesis (Fig. 2b).[39]

Shortly thereafter, it was shown that intracellular acidification (<7.3) inhibited protein synthesis and G0/G1 progression, whereas progression through S phase was unaltered.[40] S6 phosphorylation and the absolute rate of protein synthesis were extremely sensitive to changes in pH_i, with a lower threshold value of 7.3 (Fig. 2c),[40] i.e. very similar to that for re-initiation of

DNA synthesis. Also in this study, cytoplasmic alkalinization (7.3) alone was found to be insufficient in triggering S6 phosphorylation and DNA synthesis.[40] Finally, Lucas et al.[41] showed that preventing the mitogen-induced increase in pH_i (>7.0) inhibited DNA synthesis and proliferation in BALB/c-3T3 fibroblasts. Interestingly, alkaline pH_i (>7.0) was required for initiation of DNA synthesis, yet once initiated, DNA synthesis was pH-insensitive.[41] This led to the suggestion that the role of pH_i in DNA synthesis and hence proliferation might be secondary to a role for alkaline pH_i in protein synthesis.

Collectively, these studies provided the first insights into how mitogenic stimulation of mammalian cells might be controlled by NHE-mediated regulation of pH_i; furthermore, they point to a very prominent role of pH-dependent regulation of protein synthesis in the impact of pH_i on proliferation.

Signaling mechanisms linking pH to cell proliferation

Alkaline pH_i as permissive factor or alkalinization as a mitogen-driven signaling event?
The evidence outlined in the previous section raises the question of whether intracellular alkalinization (~0.3 pH unit above steady state pH_i) can act as a *bona fide* signal driving cell cycle initiation or -progression, or a certain minimal pH_i is simply permissive for this process. Obviously, if an increase in pH_i would be the only driver of proliferation, it should be sufficient to trigger proliferation in the absence of other stimuli. As discussed above, the available evidence suggests that this is not generally the case in mammalian cells, although it may be true under some conditions[37]. This, however, does not preclude a signaling role for pH_i in conjunction with other signals. We suggest that such a role is indicated if: (i) pH_i is elevated in response to mitogenic stimuli; (ii) preventing the increase in pH_i should inhibit proliferation; and (iii) a cellular sensor(s) of pH_i is involved.

As noted above, essentially all early studies were conducted under nominally HCO_3^- free conditions, in which contributions from HCO_3^-- transporters are precluded. Notably, the aberrant re-initiation of DNA synthesis in NHE-deficient fibroblasts was abolished upon culture in CO_2/HCO_3^--buffered media.[37,39] Furthermore, addition of HCO_3^- raised pH_i to growth-permissive values (>7.2) when the NHE-deficient cells were cultured in neutral or slightly acidic pH_e (6.6-7.2).[42] In congruence with this, addition of HCO_3^- increased steady-state pH_i of wild type fibroblasts (from ~7.0 to 7.2), and prevented further mitogen-induced alkalinization.[43] Importantly, mitogen-stimulated NHE and the ensuing cytoplasmic alkalinization (>7.2) were not required for proliferation initiation in lymphocytes,[44,45] suggesting that whether growth can be driven by an increase in pH_i may at least in part be cell type specific. Also arguing against a global driver role for pH_i, the NHE-dependence of mitogen-induced protein synthesis[34,40] was shown to be abolished when fibroblasts were grown in HCO_3^--buffered media, in which the NHE-dependent increase in pH_i was strongly attenuated.[46]

On the other hand, a recent study showed that proliferation of vascular smooth muscle cells (VSMCs) was reduced in HCO_3^--free medium due to the reduced pH_i under these conditions (~7.0 vs. ~7.4 in the presence of HCO_3^-).[47] Additionally, pH_i was shown to regulate yeast cell growth in response to glucose by modulating the activity of V-ATPase and subsequent downstream signaling via the Ras/PKA pathway.[48,49] These studies thus find a direct correlation between glucose availability, pH_i and growth, suggesting that pH_i might be a conserved regulator of cell growth.[49] In congruence with this notion, transfection of NIH3T3 fibroblasts with yeast V-ATPase (eliciting increased pH_i by 0.2 pH units compared to WT, even in the presence of HCO_3^-) was sufficient to endow these cells with tumorigenic characteristics such as

serum-independent growth under physiological conditions (Fig. 2d).[50] Thus, at least in this setting, an increase in pH_i can circumvent mitogenic signaling and stimulate growth directly. It is interesting to note that there appears to be a difference in the relation between NHE, HCO_3^- and proliferation *in vivo* vs *in vitro*: NHE-expressing human bladder carcinoma cells grew well both in absence and presence of HCO_3^-, and their growth was only precluded at very low medium pH (<6.6).[51] In contrast, *in vitro* growth of an NHE-deficient clone of these cells was attenuated by decreasing pH_i (<7.0) in the absence of HCO_3^-, whereas in its presence, growth was essentially unaffected by pH_i changes between 6.6 and 8,[51] (recapitulating findings from studies of NHE1-expressing and NHE1-deficient fibroblasts.[37,39,42]). Notably, however, *in vivo* growth of these cells as flank xenografts was strongly reduced by NHE depletion despite the fact that HCO_3^- is obviously present in the *in vivo* setting.[51] While the dependence on NHE1 in the presence of HCO_3^- *in vivo* but not *in vitro* could reflect the acidic microenvironment (and hence lower extracellular $[HCO_3^-]$) in growing tumors (see the last section of this review), growth rates of wild type and NHE-deficient xenografts appeared to differ already from shortly after injection,[51] indicating a more complex scenario. One hypothesis consistent with this would be a transport-independent role of NHE1 important for xenograft growth in the 3-dimensional/*in vivo* setting but not during in *in vitro* monolayer culture. This could include pH-*in*dependent roles of NHE1 in cell-cell adhesion or attachment to the extracellular matrix. Supporting this notion, NHE1-dependent, ion transport-independent regulation of the actin cytoskeleton has been demonstrated in monolayer culture.[52]

Taken together, these studies demonstrate that an intracellular alkalinization (~0.3 pH unit above steady state pH_i) is at least in some cell types sufficient to stimulate proliferation, and that NHE loss renders proliferation more acid sensitive. Yet they also show that detectable increases in pH_i are not always required for mitogen-induced growth under physiological conditions. Collectively, this suggests that an increase in pH_i in general, or in NHE activity in particular, constitutes only part of the signal(s) driving proliferation. However, there are also results that point to more singular driver- or signaling roles of pH_i and/or NHE under physiological conditions. The next section addresses the possible mechanisms involved in such a scenario.

Cellular pH sensing

Activation of a net acid extruder such as an NHE will not only elicit an increase in pH_i, but also a corresponding decrease in pH_e.[53] In fact, activation of an acid-base transporter can cause multiple "sense-able" changes: a net acid/base flux across the membrane, a change in (local or global) pH_i, a concomitant pH_e change, and, if extracellular space is restricted or poorly perfused, also a more widespread change in microenvironmental pH; and finally parallel changes in intra- and extracellular $[HCO_3^-]$ and thus in CO_2/HCO_3^- buffering.

The molecular mechanisms through which H+ concentrations are sensed involve changes in the structure of titratable groups with a pKa in the physiological range (i.e. histidine, but also other groups may be relevant due to effects of the specific environment on the pKa values) and hence of the protein structure and properties [1]. *Extracellular* acid/base sensors (Fig. 1, right side) include *bona fide* acid/base receptors such as (i) H+-sensing G-protein coupled receptors (GPCRs),[54,55] which have been assigned a role in extracellular acid-induced increases in $[Ca^{2+}]_i$ and activation of extracellular signal regulated kinase (ERK),[56] and (ii) the receptor protein tyrosine phosphatase-γ (RPTPγ), a proposed alkali sensor, responding to extracellular CO_2/HCO_3^-.[57,58] Additionally, the affinity of multiple other receptors for their ligands is affected

by pH changes in the physiological range, rendering them indirect H^+ sensors. Well-studied examples include the prolactin receptor[59,60] and the EGFR,[61] both important regulators of cell proliferation. Numerous ion channels, not limited to the H^+-gated acid-sensing ion channels (ASICs),[62] are sensitive to pH_e in the physiological range,[54,63]. This establishes a link between pH_e and, e.g., V_m and $[Ca^{2+}]_i$ - important regulators of proliferation (see below). *Intracellular* acid/base sensors (Fig.1, right side) include ion channels regulated by pH_i,[64] the HCO_3^- sensor soluble adenylate cyclase sAC,[65] the non-receptor tyrosine kinase Pyk2,[66] the $G\alpha$-subunits of GPCRs,[67] and several other enzymes and signaling molecules (reviewed in[1]). More recently, histones,[68] (see below), as well as Smad5, whose nuclear-cytoplasmic translocation is regulated by direct proton binding,[69] have also been proposed to serve as cellular sensors of acid-base status, although additional work seems warranted before a functionally relevant role of these proteins in acid-base sensing can be established.

Several proteins pivotal to regulation of proliferation have been proposed to be regulated by pH, directly or indirectly, although the precise molecular mechanism involved is generally unknown. Pertinent examples will be summarized below.

Impact of acid-base transporters on cell cycle progression

pH sensitive cyclins and kinases. Using NHE1-deficient fibroblasts to exogenously overexpress NHE1 variants, Putney and Barber[70] found that cells expressing wild type NHE1 showed an alkaline peak in pH_i of ~0.3 pH units (~7.2 to ~7.5) from 0 h to 3 h after release from a double thymidine block (corresponding to G2/M phase), concomitant with a peak in Cdc2 activity at time 3 h. Both peaks were strongly attenuated in cells expressing a transport-deficient NHE1 (E266I), and the NHE1-E266I cells also exhibited delayed S phase progression, impaired G2/M entry, decreased Cyclin B1 expression and prolonged inhibitory phosphorylation of Cdc2 compared to cells expressing wild type NHE1.[70] Consistent with this, our recent work shows that knockdown of either NHE1 or NBCn1 in MCF-7 human breast cancer cells causes a prolonged S phase and a significant delay in G2/M entry,[71] hinting to possible non-redundant functions of different net acid extruding transport proteins.

The apparent importance of net acid extruding proteins for G2/M progression is consistent with the notion that the activity of these transporters may affect the mitosis promoting factor (MPF) complex of Cyclin B1 and Cdc2. Artificially alkalinizing the NHE1-E266I cells rescued G2/M entry as well as the activity and expression of the MPF proteins.[70] In a later study, the authors further found that a range of genes involved in DNA synthesis and cell cycle progression were significantly altered in NHE1-deficient cells compared to NHE1-WT cells.[72] Specifically, they found an upregulation of genes involved in G2/M arrest and DNA repair in NHE1-E266I cells, including increased expression of Wee-1 kinase (an inhibitory regulator of the MPF complex), as well as downregulation of Cdc25 (an activator of the MPF complex).[72] Interestingly, they found that the mRNA expression of Cyclin B1 was unaltered, which suggests that their previous finding of decreased Cyclin B1 protein expression in synchronized NHE1-deficient cells[70] may reflect its decreased protein stability upon loss of NHE1 activity. In congruence with this, extracellular alkalinization (pH_e 7.6 vs. pH_e 7.0) was shown to stimulate G1/S transition of murine hybridoma (7TD1) cells by upregulating the expression of c-Jun N-terminal Kinase 1 (JNK1) and Cyclin D2.[73] Although more studies are needed to establish

precisely how acid-base transporters may regulate cell cycle checkpoints, this work supports the notion that pH_i sensitivity (and/or other roles of these transporters than pH_i regulation, see the section "Other NHE1-dependent signaling pathways") of both the G2/M and G1/S checkpoints may contribute to control of cell cycle progression (Fig. 3).

pH-dependent regulation of V_m. V_m has been shown to vary during cell cycle progression in a wide variety of cell types, generally depolarizing during G2/M and hyperpolarizing at the entry of S phase.[74–77] Furthermore, forced hyperpolarization of V_m prevents DNA synthesis and mitosis.[75,77–79] V_m changes during cell cycle progression in large part reflect changes in K^+ channel expression and activity, observed for several classes of K^+ channels.[75,80–83] Notably, these channels are often highly sensitive to both pH_i and pH_e.[54,63,84] It is therefore tempting to speculate that acid/base transport proteins may exert their effect on cell cycle progression by altering K^+-channel activity and thus V_m (Fig. 3). Interestingly, V_m has been shown to play a significant role in organization of lipid microdomains,[85,86] which in turn would affect signaling events related to such domains. Specifically, hyperpolarization of V_m was shown to induce clustering of phosphatidylinositol 4,5-biphosphate (PI(4,5)P$_2$) within the membrane. This in turn increased mitogen-activated protein kinase (MAPK) activity[87–90] and regulated, among many other processes, cell division.[91–93] Thus, in this scenario, PI(4,5)P$_2$ - MAPK signaling could be driven by acid/base transport-induced changes in local pH at the membrane (pH_m) (Fig. 3). Conversely, NHE1 and many K^+ channels are directly regulated by PI(4,5)P$_2$,[94–96] suggesting a complex regulatory ion transport-signaling interplay that contributes to the fine-tuning of cell cycle progression.

pH-dependent regulation of $[Ca^{2+}]_i$. Ca^{2+}-mediated signaling plays an important role during cell cycle progression, with oscillating concentrations during G1/S (accompanying DNA replication [97,98]), and G2/M transition, further proposed to regulate centrosome duplication and segregation, respectively.[99–101] The activation of Calmodulin (CaM), CaM kinases (CaMKs), MAPKs, Calcineurin and protein kinase C (PKC) by $[Ca^{2+}]_i$ in turn activates several transcription factors (e.g. nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), cAMP response element binding protein (CREB), and c-Myc), regulating the expression of cyclins and CDKS driving cell cycle progression.[98,100–102] NHE-dependent intracellular alkalinization (a 0.2-0.3 unit increase in pH_i compared to steady state pH_i of ~7.1) can increase $[Ca^{2+}]_i$ under HCO_3^--free conditions,[103] and alkalinization of pH_i (~0.4 pH unit above resting pH_i) can stimulate Ca^{2+} release from intracellular stores.[104–107] Furthermore, in membrane micro-domains containing both transporters[108] or in conditions with low Na^+/K^+-ATPase activity,[109] an NHE1-dependent increase in $[Na^+]_i$ can stimulate Ca^{2+} entry through NCX. Underscoring the importance of pH in Ca^{2+} signaling, binding of Ca^{2+} to CaM (which also interacts directly with and stimulates NHE1[110,111]) is highly pH sensitive under physiological conditions.[107] Furthermore, nuclear translocation of NFAT, which is driven by the Ca^{2+}-CaM-dependent phosphatase Calcineurin, is not only highly dependent on pH, but specifically on Calcineurin binding to NHE1.[112] Thus, an NHE1-Ca^{2+}-CaM-Calcineurin complex could also link NHE1, pH_i, and proliferation (Fig. 3). With respect to pH_e, ASICs and acid-sensing GPCRs[1,54,113] link pH_e acidification (<7.0) to increases in intracellular cAMP and $[Ca^{2+}]_i$,[54,56,64] and have been implicated in the dysregulated Ca^{2+}

homeostasis of cancer cells.[54,113,114] Finally, the above-mentioned pH sensitivity of K^+-channels and hence V_m will of course also regulate Ca^{2+}-mediated signaling.

In summary, numerous lines of evidence link pH_i and $[Ca^{2+}]_i$. Given the central role of Ca^{2+} in cell cycle regulation, this is likely to constitute a link between acid-base transporters and cell cycle progression (Fig. 3).

pH-dependent regulation of metabolism. Metabolic processes are the main source of cellular acid production, and metabolism is in turn highly sensitive to pH_i.[84,115] For instance, phosphofructokinase-1 (PFK-1),[116–120] hexokinase (HK),[121] and mammalian target of rapamycin complex 1 (mTORC1) activity are dependent on an alkaline pH_i (>7.0).[49,122] It seems likely, therefore, that pH_i changes during cell cycle progression play a role both in the production of ATP for catabolic processes and in ensuring ample supply of biosynthetic building blocks during cell growth and proliferation. Not only cancer cells, but also non-transformed, proliferating cells exhibit increased glycolytic flux.[123] Interestingly, genetic ablation of NHE1 is associated with increased expression of genes favoring ATP production by oxidative phosphorylation,[72] suggesting that a lack of NHE1 may cause a shift toward oxidative phosphorylation.

Other pH_i-dependent mechanisms. Another process relevant to cell division and recently proposed to be intricately coupled to pH_i is histone acetylation.[68] As pH_i was decreased by reducing pH_e, either by varying medium $[HCO_3^-]$ or by changing pH_e in a HEPES-buffered medium, the acetylation of histones H3 and H4 was substantially decreased, by more than 50% if pH_e was decreased to around 6.4.[68] Histone deacetylation was observed in multiple cancer and non-cancer cell types, and was validated using immunoblotting, immunofluorescence analysis, and chromatin immunoprecipitation, and could be inhibited by class I and II histone deacetylase (HDAC) inhibitors. The histone deacetylation was accompanied by free acetate anions being co-exported with H^+ by MCTs, thereby resisting further reduction in pH_i.[68] Conversely, as pH_i was increased, the flux of acetate and H^+ was in the uptake direction, resulting in global histone hyperacetylation.[68] While additional studies are required to fully assess the physiological relevance of this phenomenon, this raises the interesting possibility, as stated by the authors, that chromatin acetylation functions as a "pH_i rheostat". [68]

Other NHE1-dependent signaling pathways

While we focus here on pH-dependent processes, it is important to keep in mind that NHE1 also regulates cell function via processes, which are not, or not solely, dependent on pH_i changes, and which could contribute to its role in regulation of cell proliferation. A few such examples are given below.

Cell volume changes. In addition to its role in pH_i regulation, NHE1 is a major regulator of cell volume, acting in conjunction with Cl^-/HCO_3^- exchange to mediate net uptake of NaCl and hence osmotically obliged water.[124] While the precise mechanisms involved remain incompletely understood, cell swelling often stimulates, and cell shrinkage inhibits, cell cycle progression.[124–128] Cell volume varies during cell cycle progression, and this may in part be NHE1-dependent. Interestingly, in HeLa cells, NHE inhibition led to a 53% decrease in mitotic rounding pressure due to decreased intracellular osmolarity.[129] This correlates well with the proposed role of NHE1 in G2/M transition,[70] but indicates that the requirement for NHE1 could relate to cell volume rather than, or in addition to, pH_i (Fig. 3). Accordingly, an increase in $[Na^+]_i$ (favoring osmotic cell swelling) has been assigned a role as an early and necessary event in mitogenic signaling,[130] and a critical cell size threshold has been proposed to regulate G1/S transition.[131–133] Specifically, a size threshold in G1 was proposed to ensure balance between growth and proliferation rates by adjusting the length of the ensuing cell cycle.[133,134]

ERK and Akt signaling. NHE1 activity is tightly linked to that of the Ser/Thr kinases ERK and RAC-alpha serine/threonine-protein kinase (Akt), which play central roles in regulation of growth. Activation of NHE1 by mitogens involves ERK1/2, which phosphorylates NHE1 both directly and via activation of Ribosomal S Kinase (p90RSK), and Akt, which phosphorylates NHE1 on Ser648 of its C-terminal tail.[135–140] Conversely, NHE1 can regulate ERK[137,141–143] and Akt[144] activity, at least in part a result of scaffolding interactions.

Thus, although the clear pH_i sensitivity of proliferation and the equally unequivocal importance of NHE1 suggest that acid extrusion by NHE1 regulates proliferation, the impact of NHE1 on cell volume or pH_i-independent signaling could also contribute (Fig. 3). Substantial future work is, however, needed to understand the precise mechanisms involved.

Pathophysiological relevance

Altered pH homeostasis has major pathological consequences. Net acid extruding transporters, including NBCn1 and NHE1, are frequently upregulated in breast cancer and exhibit increased activity,[145–148] resulting in reversed pH gradients with highly acidic pH_e values (6-6.8) and slightly alkaline pH_i values (7.3-7.6).[5,84,149,150] This profoundly altered pH homeostasis affects all levels of tumor progression, including stimulating serum- and anchorage-independent growth.[5,8,151] Roles of NHE1- and NBCn1 in tumor development have been demonstrated both *in vitro and in vivo* and have been shown to be related to their

role in regulation of proliferation.[148,152–155] Conversely, in pathophysiological conditions with a decreased pH_i, such as neurodegenerative diseases[156], impaired proliferation could be expected to at least in part reflect this condition.

Conclusion and perspectives

The work summarized and discussed in this review leaves little doubt that cell proliferation is strongly affected by pH_i. However, the molecular mechanisms involved remain incompletely understood and numerous questions remain to be experimentally addressed. A central controversy is whether pH_i alone can play a driver/signaling role in driving cell proliferation. Other important open questions include the possible links between pH, V_m, $[Ca^{2+}]_i$ and mechanisms of pH-dependent regulation of the key cell cycle regulators.

Conflict of Interest:
No conflicts of interest.

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Tables

Species	CO_2/HCO_3^-	pH_i increase?	Role of pH_i	pH manipulation	Ref.
Sea urchin eggs	-	Yes (~0.3 after fertilization)	Alkaline pH_i correlated with initiation of protein- and DNA synthesis after fertilization	Inhibition of Na^+/H^+ exchange with amiloride	[14]
Sea urchin eggs/early embryos	-	N/A	pH_i controlled the rate of protein synthesis upon fertilization	pH_i manipulation by weak acid/base (CH_3COONa/ NH_4Cl)	[15]
Sea urchin eggs/early embryos	-	N/A	pH_i controlled the rate of protein synthesis in conjunction with Ca^{2+}	Manipulation of pH by omission and remission of Na^+ in artificial seawater, as well as addition of NH_4Cl	[16]
P. polycephalum	-	Yes (~0.6 prior to mitotic entry)	pH_i correlated with the mitotic cell cycle		[17]
P. polycephalum	-	Yes (~0.5 prior to mitotic entry)	pH_i correlated with the mitotic cell cycle, and acidic pHi (<7.2) delayed mitotic entry	Manipulation of pH_e with CH_3COONa	[18]
D. discoideum	-	Yes (~0.25 during S phase and mitosis)	Alkalinization of pH_i correlated with the DNA replication cycle and protein synthesis	pH_i manipulation by weak acid/base (DMO/NH_4Cl)	[20]

S. cerevisiae	-	Yes (~0.3 after mitogen stimulation)	Alkalinization of pH$_i$ was associated with "start" progression of the yeast cell cycle		[21]
S. cerevisiae	-	Yes (~0.2-0.4 after arrest recovery)	Alkalinization of pH$_i$ was associated with "start" progression of the yeast cell cycle		[22]
S. cerevisiae	-	N/A	pH$_i$ controlled growth rate	Investigated 39 mutants with altered pH$_i$ (out of 4,740 total) from the yeast deletion mutant array[157]	[24]
S. cerevisiae	-	Yes (~1.0 after mitogen stimulation)	pH$_i$ regulated cell growth in response to glucose via V-ATPase and the Ras/PKA pathway	48; Inhibition of V-ATPase with Concanamycin A. 49; Doxycycline-mediated repression of the yeast P-ATPase (*tet07-PMA1*)	[48,49]
S. pombe	+/-	N/A	pH$_i$ was associated with growth rate, but did not correlate with cell cycle progression		[25]
Murine lymphocytes	-	Yes (~0.25 6 and 12 h after mitogen stimulation)	Alkaline pH$_i$ correlated with initiation of DNA synthesis	pH manipulation using DMO	[32,33]
Chinese hamster lung fibroblasts (CCL39)	+/-	Yes (~0.2 after mitogen stimulation)	NHE associated alkalinization of pH$_i$ led to phosphorylation of S6	Inhibition of Na$^+$/H$^+$ exchange with amiloride	[34]
Swiss 3T3 fibroblasts	+	Yes (~0.2 after mitogen stimulation)	Mitogenic stimulation of quiescent cells caused NHE-dependent alkalinization of pH$_i$	Inhibition of Na$^+$/H$^+$ exchange with amiloride	[35]

Human foreskin (HF) fibroblasts	-	Yes (~0.2 after mitogen stimulation)	Mitogenic stimulation of quiescent cells caused NHE-dependent alkalinization of pH_i	Inhibition of Na^+/H^+ exchange with amiloride	[36]
Chinese hamster lung fibroblast (CCL39)	+/-	N/A	Alkaline pH_e (8-8.3) or addition of HCO_3^- restored growth of NHE-deficient cells	Inhibition of Na^+/H^+ exchange with DMA. Manipulation of pH_e by PIPES, MOPS or HEPES	[37]
Chinese hamster lung fibroblasts (CCL39)	-	N/A	NHE inhibition abolished mitogen-induced re-initiation of DNA synthesis and S phase progression of quiescent cells	Inhibition of Na^+/H^+ exchange with 28 analogs of amiloride	[38]
Chinese hamster lung fibroblast (CCL39)	+/-	N/A	NHE-deficient cells failed to increase pH_i and reinitiate DNA synthesis after mitogen stimulation. Mitogen-induced DNA synthesis was recovered by addition of HCO_3^-, or by raising pH_i to 7.2	Mutation of NHE1, abrogating ion translocation activity; omission and remission of HCO_3^-. Manipulation of pH_e by MES or MOPS	[39]
Chinese hamster lung fibroblast (CCL39)	-	N/A	Acidic pH_i inhibited protein synthesis and progression through G0/G1, with a lower pH_i threshold level of 7.3	Mutation of NHE1, abrogating ion translocation activity. Manipulation of pH_e by MES, PIPES, MOPS, HEPES and EPPS	[40]
BALB/c-3T3 fibroblasts	+	Yes (~0.3 after mitogen stimulation)	Alkaline pH_i was required for initiation of DNA synthesis and proliferation	pH manipulation using DMO	[41]

Chinese hamster lung fibroblasts (CCL39)	+/-	N/A	Na^+-dependent HCO_3^-/Cl^- exchange protected against cytoplasmic acidification and established a steady-state pH_i permissive for growth at neutral or acidic pH_e	Inhibition of anion exchange by DIDS	[42]
Mouse fibroblasts (MES-1)	+	No	Addition of HCO_3^- to the media raised steady state pH_i by ~0.25 as compared to HCO_3^--free conditions, and prevented further mitogen-induced alkalinization unless pH_i was artificially lowered	Inhibition of anion exchange by DIDS and manipulation of pH_e by lowering [HCO_3^-] and CO_2	[43]
Rat thymic lymphocytes	-	N/A	Mitogen stimulated NHE was neither sufficient nor necessary for the initiation of cellular proliferation	Inhibition of Na^+/H^+-exchange with EIPA	[44]
Murine T-cells	-	Yes (~0.1 after mitogen stimulation)	Alkalinization of pH_i and mitogen-stimulated NHE were not required for IL2-induced proliferation initiation in lymphocytes	pH manipulation using DMO	[45]
Swiss 3T3 fibroblasts	+	No	NHE-dependent mitogen-induced alkalinizationi was attenuated in HCO_3^--buffered media and was not required for the activation of S6 phosphorylation	Inhibition of Na^+/H^+-exchange with DMA	[46]
Murine vascular smooth muscle cells (VSMCs)	+	Yes (~7.4 vs. 7.0 in presence of HCO_3^-)	pH_i correlated with proliferation rate of VSMCs	KO and inhibition of NBCn1 with S0859, and CO_2/HCO_3^- omission and remission	[47]
Balb-c/3T3 and NIH 3T3 fibroblasts	+	Yes (~0.2 pH units higher than WT)	Transfection of NIH 3T3 cells with yeast V-ATPase led to alkalinization of steady state pH_i and serum-independent growth	Overexpression of V-ATPase in NIH 3T3 cells, or expression of mutated V-ATPase with decreased ATPase-, and H^+ translocation activity	[50]

HSPD cells derived from human bladder carcinoma cells (MGH-U1)	+/-	N/A	HSPD cells were able to grow in the absence of bicarbonate *in vitro*, but were unable to grow and form tumors *in vivo*.	Na^+/H^+-exchange-deficient cells (HSPDs)	[51]
Ltk$^-$ mouse muscle fibroblasts	+	Yes (~0.3 pH units at G2/M entry)	NHE1-dependent alkaline pH_i timed G2/M entry and regulated expression and activity of the MPF complex	Mutation (NHE1^{E266I}) or inhibition of NHE1 with EIPA, abrogating ion translocation activity. NHE1-independent manipulation of pH_i by incubation with 50 mM $NaHCO_3$ at 15% CO_2	[70]
Ltk$^-$ mouse muscle fibroblast	+	N/A	Acidic pH_i induced by impaired NHE1 activity, differentially regulated genes involved in regulation of metabolism and cell cycle progression	Mutation of NHE1 (NHE1^{E266I}), abrogating ion translocation activity	[72]
Murine hybridoma (7TD1) cells	+	N/A	Alkalinization of pH_e (from 7.0 to 7.6) stimulated G1/S transition by upregulation of JNK1 and Cyclin D2 expression	Manipulation of pH_e by addition of triethylamine and inhibition of NHE1 by EIPA	[73]
NBCn1 KO C57BL/6J mice	+/-	N/A	KO of NBCn1 reduced *in vivo* tumor growth rate of chemically induced breast tumors, related to reduced pH_i and reduced proliferation	NBCn1 KO	[148]
Drosophila melanogaster	+/-	Yes (~0.4 pH units higher than WT)	Overexpression of NHE1 caused pH_i alkalinization, induced *in vivo* dysplasia and potentiated growth and invasion with oncogenic Ras	Overexpression of *Dnhe2* (a NHE isoform), or expression of mutated Dnhe2^{E358L}, which abrogates H^+ efflux	[152]

Human ovarian epithelial cells (HOSE) and cancer cells (A2780 + haOC)	+	N/A	Acidic pH_i, induced by NHE1 KD/inhibition, reduced pH_i and proliferation	NHE1 KD and inhibition of NHE1 with Zoniporide	[153]
Human breast cancer cells, MCF-7 and MDA-MB-231	+	N/A	KD of NBCn1 attenuated MCF-7 spheroid growth, but not MDA spheroid growth, whereas NHE1 KO reduced MDA spheroid growth	KD of NBCn1, MCT1, or MCT4. KO, KD or inhibition of NHE1 by Cariporide. Inhibition of NBC and MCT1 by S0859 and AR-C, respectively	[154]
Human breast cancer cells, MDA-MB-231	+	N/A	KD of NHE1, NBCn1, or MCT4 reduced *in vivo* primary tumor growth of MDA-MB-231 xenografts, via distinct, but overlapping mechanisms.	KD of NHE1, NBCn1, or MCT4	[155]

Table 1. Overview of studies of the role of pH in cell cycle progression.

The table provides an overview of pertinent studies of the role(s) of pH and acid/base transporters in cell cycle progression and proliferation. The table is not exhaustive. For further details, see the text. Abbreviations: Amiloride, (3,5-diamino-6-chloropyrazinoyl-guanidine); Cariporide, N-(Diaminomethylene)-4-isopropyl-3-(methylsulfonyl)benzamide; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DMA, 5-(*N*,*N*-Dimethyl)amiloride; DMO, 5',5´-dimethyl oxazolidine 2,4-dione; EIPA, 5-(*N*-Ethyl-*N*-isopropyl)amiloride; EPPS, (iV-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid); haOC, human ascites-derived ovarian cancer cells; HEPES, (N-2 hydroxyethylpiperazine-N'-2-ethanesul- fonic acid); HSPD, Human sodium proton exchanger-deficient; KD, knockdown; KO, knockout; MCT(1/4), Monocarboxylate transporter (1/4); MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-Morpholino)propanesulfonic acid; MPF, mitosis promoting factor; NBC, Na^+/HCO_3^- cotransporter; NBCn1, electroneutral Na^+/HCO_3^--cotransporter 1; NHE1, Na^+/H^+-exchanger 1; pH_e, pericellular pH; pH_i, intracellular pH; PIPES, (piperazine-N,N'-his 2-ethanesulfonic acid); VSMCs, vascular smooth muscle cells; Zoniporide, ([1-(quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl] guanidine).

Figure legends

Figure 1. Effectors and sensors of cellular pH.

Na$^+$/H$^+$-exchanger 1 (NHE1), Na$^+$/HCO$_3^-$- cotransporter 1 (NBCn1), monocarboxylate transporter 1 and 4 (MCT1/4), H$^+$/K$^+$-ATPase (H$^+$/K$^+$-ATPase), vacuolar type H$^+$-ATPase (V-ATPase), H$^+$-sensing G-protein coupled receptor (GPCR), prolactin receptor (PRLR), epidermal growth factor receptor (EGFR), non-receptor tyrosine kinase (PYK2), proton gated acid-sensing ion channel (ASIC), receptor protein tyrosine phosphatase-g (PRLR-PRLR-g), HCO$_3^-$- sensing soluble adenylate cyclase (sAC). See text for details.

Figure 2. The importance of pH in cell cycle progression and proliferation.

(a) Correlation between pH$_i$, protein- and DNA synthesis, and cell number during three subsequent cell cycles of *D. discoideum*. From top to bottom: First, pH$_i$ as measured by the digitonin null point method in synchronized cells. Second, the fractional increase in total cellular protein. Third, the fractional increase in cellular DNA, and fourth, the relative increase in cell number.[20] **(b)** pH$_i$-dependence for reinitiation of DNA synthesis (right panel) and the relationship between pH$_i$ and pH$_e$ (left panel) in WT fibroblasts (Δ) and NHE-deficient fibroblasts (O) following growth factor stimulation of quiescent fibroblasts.[39] **(c)** pH$_e$-dependence of ribosomal protein S6 phosphorylation in quiescent cultures of WT fibroblasts (closed squares) and NHE-deficient fibroblasts (open squares). The pH indicated is the average pH$_o$ measured at the beginning and the end of incubation in different pH values for 1 h.[40] **(d)** Growth characteristics of RN1a (3T3 cells exogenously expressing the vacuolar H$^+$-ATPase), WT 3T3 cells, and their acid production. Top panel illustrates cell growth as a function of serum concentration, of cells plated at density indicated by dashed line and maintained for 72 hr at pH 7.2, after which cell mass was determined by crystal violet staining. Lower panel illustrates the H$^+$ production, which was determined by monitoring medium pH as a function of time, in the different cell lines.[50] See text for details. All panels reproduced with permission from the sources indicated.

Figure 3. Working hypothesis of possible mechanisms linking acid/base transporters and cell cycle progression.

We propose several mechanisms by which acid/base transporters may regulate cell cycle progression and proliferation. Thus NBCn1, NHE1 and/or other acid/base transporters might regulate cell cycle progression by i) raising pH_i to alkaline values (~0.3 pH units above resting pH_i) which affects the gene expression and/or protein stability of cell cycle checkpoint regulators, promoting G1/S and G2/M transition; by ii) increasing the rate of protein synthesis via alkaline pH_i and or Akt-dependent signaling; by iii) stimulating the expression and activity of K^+-channels followed by modulation of V_m, which regulates cell cycle progression, as well as affecting V_m-induced Ca^{2+} entry and V_m-induced clustering of lipids in microdomains ($PI(4,5)P_2$), which subsequently regulates cytokinesis and MAPK-induced stimulation of proliferation; by iv) pH-dependent and -independent regulation of Ca^{2+} and subsequent stimulation of CaM, CaMK, Calcineurin and induction of pro-proliferative transcription factors; by v) pH_i-independent regulation of cell volume-stimulated proliferation; by vi) pH_i-independent scaffolding functions of NHE1, regulating ERK and Akt-mediated signaling. See text for further discussion. Abbreviations: local pH at the membrane (pH_m), electroneutral Na^+/HCO_3^--cotransporter 1 (NBCn1), Na^+/H^+-exchanger 1 (NHE1), RAC-alpha serine/threonine-protein kinase (Akt), phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$), phosphatidylinositol 3,4,5-biphosphate ($PI(3,4,5)P_3$), phosphatidylinositol-3-kinase (PI3PK), mitogen activated protein kinase (MAPK), Calmodulin (CaM), CaM kinase (CaMK), extracellular signal regulated kinase (ERK).

(a)

(b)

(c)

(d)