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Review

Proton-Activated Chloride Channel: Physiology and Disease

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Abstract

The maintenance of intracellular and extracellular pH relies on multiple ion transporters/channels. Proton-activated chloride channel (PAC) precisely regulates extracellular and early/late endosomal pH by transporting chloride ion (Cl^-) across membranes and has been shown to be implicated in pH imbalance under hypoxic conditions, such as the acidic microenvironments of cancer and ischemia. In this article, the phenotypic characteristics, molecular mechanisms, physiology of PAC and its role in cancer, ischemic stroke and hypoxia will be discussed in order to provide some clues for developing potential therapeutic strategies.

Keywords: proton-activated chloride channel; endosomes; tissue acidosis; hypothermia

1. Introduction

The acid-base equilibrium is automatically adjusted in physiological conditions. The pH of extracellular fluid, including the blood plasma, is tightly set between 7.32 and 7.42 by chemical buffers, the respiratory system, and the renal system [1]. pH not only directly alters protein structure but also significantly influences cell activity by modifying pH-sensitive enzymes/channels [2–4]. Under pathological conditions, such as cancer, ischemic stroke, and hypoxia, the local pH is usually acidic (~5.5–7.0) [5–7]. Several acid-sensitive channels/exchangers (including acid-sensing ion channels (ASICs), Na^+/H^+ exchanger (NHE), and the recently identified Proton-activated chloride channel (PAC)) can regulate pH by modulating ion transport in local acidosis [8–10]. It is proposed that acid-sensitive ion channels are potential therapeutic targets for these diseases where low pH is a hallmark.

Recently, the structure, molecular mechanism and function of PAC have been characterized [11–13]. PAC exquisitely tunes the pH of extracellular milieu and early/late endosomes by adjusting the chloride ion (Cl^-) concentration, which acts as a counter ion to cations [13]. Cl^- is the most abundant anion in mammalian cells and a key factor in driving water molecules across the plasma membrane because of its synergistic coupling with other cations. Some physiological processes, such as cell volume regulation, membrane potential and intracellular/extracellular pH regulation, are closely related to Cl^- [14–18].

2. Characterization of PAC

2.1 Molecular Properties of PAC

The electrophysiological activity of PAC, also referred to acid-sensitive outwardly rectifying anion channel (ASOR) [19] or proton-activated outwardly rectifying anion channel (PAORAC) [20], was first observed in rat Sertoli cells [21]. The exact molecular identity and mechanism of PAC in maintaining acidic pH homeostasis remained ambiguous in last couple of decades. Three hypotheses were proposed, namely "PAC is a volume-regulated anion channel (VRAC)", "PAC is chloride channel (CLC)-3" and "PAC is CLC-7" [22–26]. Until recently, the gene PACC1 encoding PAC channel was identified by cell-based fluorescence reporter assay and an unbiased RNA interference screening [2]. Meanwhile, it was confirmed that PAC is identical to transmembrane protein 206 (TMEM206) with the similar unbiased method.

PAC belongs to a unique and highly conserved family of ion channels that are probably highly conserved in all vertebrate species and shares no significant sequence with any other families of membrane proteins, such as cystic fibrosis transmembrane regulator (CFTR), CLCs and NHE [2,27]. PAC has been identified in 27 kinds of tissues in human including brain, kidney, testis, lymph node and bone marrow [12]. PAC is activated at acidic pH and displays a strong outwardly rectifying current-voltage (I-V) relationship. At the positive membrane potential, PAC activation is time-dependent, and the order of ion permeability is $SCN^- > I^- > NO_3^- > Br^- > Cl^-$. In addition to pH, PAC is also sensitive to temperature. At room temperature and 37 °C, it can be activated by pHs below 5.5 and 6.0, respectively

[28].

2.2 Structure of PAC

The structure of pufferfish PAC has been resolved by using single-particle cryo-electron microscopy (cryo-EM) [29]. Interestingly, PAC lacks obvious sequence homology with any known Cl^- channel, but the trimeric structure of PAC is highly like those of Na^+-selective channels like ASICs and epithelial Na^+ channels (ENaCs). For human PAC, significant conformational changes from the high-pH resting closed state to the low-pH proton-bound non-conducting state were observed while pH was dropped from 8.0 to 4.0 [30]. However, the limitation is that both structures at pH 8.0 and pH 4.0 are non-conductive and represent closed and pre-open/desensitizing states respectively, the activated structure of PAC is still unknown.

Recently, the structure of PAC in the resting state (pH 7.5), activated state (pH 4.5) and desensitized state (pH 4.0) was investigated [31]. In three conformational states, PAC were assembled from symmetric trimers of TMEM206 subunits, including a large extracellular domain (ECD) which would be the luminal structural domain in endosomes, a transmembrane domain (TMD) containing two TM helices per subunit (TM1 and TM2) and an intervening juxtamembrane interface (JMI) which connects ECD to TMD. Under acidic condition, acidic clusters in ECD act as primary proton sensors and lead to the helical reorganization of TMD, inducing metamorphosis of transmembrane helices to fashion an ion transport pathway unique to the activated conformation [32]. The similar conformational change also occurs in ASIC1a, where clusters of negatively charged amino acids in ECD sense extracellular acidification and trigger transient channel opening [33]. Similarities and differences in the structures of PAC and ASIC1a have been clearly described [31]. Although they transport different ions, the similar activation mechanism and conformational change indicate that PAC and ASIC1a may work synergistically under acidic conditions. Indeed, coactivation of PAC and ASIC currents has been observed in chondrocytes under simultaneous hypotonic and acidic stimulation [34]. The details of cooperation between both channels will be discussed later. Reportedly, human PAC can be converted into a cation-selective channel and display inward rectification by replacing positively charged Lys319 residue on TM2 with glutamate residues, which indicates that Lys319 is the determinant of anionic selectivity and strong outward rectification [30].

2.3 Functions of PAC

PAC was initially reported to be distributed in the plasma membrane. The swelling and death of HeLa cells and mouse cortical neurons induced by prolonged exposure to an acidic extracellular milieu were attenuated using non-specific blockers of chloride channels [19,28]. It was proposed that cell swelling induced by extracellular acidity is caused by the osmotic gradient generated by PAC-mediated Cl^- influx. Knockout of TMEM206 partially protects cells from cell death caused by extracellular acidity [27]. When exposed to extracellular pH 4.5, the cell volume gradually recovered their volume and even shrank after reaching the peak swelling. It was suggested that, although PAC mediates the swelling of acid exposed cells, it does not cause a sustained increase in cell volume. It must be pointed out that the parallel activation of PAC and acid-activated cation channels (like transient receptor potential melastatin subfamily member 7 (TRPM7) [35] and ASICs [8]) necessarily leads to cell swelling after the influx of cations with Cl^-. The observed cellular volume shrinkage may be attributed to the rapid inactivation of ASICs during acid exposure or due to the quenching error of fluorescence measurements.

Regarding its strong pH dependence, PAC may localize not only in the plasma membrane, but also in the luminal compartments of endocytic and secretory vesicles with lumen pH of ~4.5–6.5, such as lysosomes and late endosomes [20]. Actually, PAC is actively transferred from the cell surface to endosomes in a canonical YxxL motif-dependent manner, and mainly localizes in the early and late endosomal membranes, and a small amount locates in the plasma membrane [13]. By cooperating with vacuolar-ATPase (V-ATPase), CLCs and NHE, PAC regulates endosomal pH by precisely controlling the Cl^- concentration in endosomes (Fig. 1) [9,13,15]. The minimal or absent localization of PAC in lysosomes suggests that the lack of PAC may be one of the reasons for the increased Cl^- concentration and enhanced acidity of lysosomes [13,36]. Recently, it was reported that lysosomes rely on the efflux of luminal protons as an electric shunt to promote acidification [37]. PAC was also detected on macropinosomes. And the shrinkage of macrophage macropinosomes depends on TPC-mediated Na^+ efflux and PAC-mediated Cl^- exit [36]. These results indicate that PAC mainly plays conservative physiological functions in compartments of the endocytic pathway.

3. PAC and Diseases

Under physiological conditions, the pH of extracellular fluid is maintained at ~7.32–7.42 and PAC cannot be activated because of its acidic threshold. However, acidic microenvironment is often found in most tissues in pathological situations including: (1) The extracellular pH at the inflammatory site can be as low as 5.5 due to the production of protons by activated neutrophils and macrophages [38]. (2) Even in presence of sufficient oxygen, cancers tend to produce energy by anaerobic oxidation instead of oxidative phosphorylation, the so-called Warburg effect [39], resulting in the extracellular pH even below 6.0 [40]. (3) When the blood supply to the brain is compromised, protons accumulate locally with available ATP being consumed, which makes the brain tissue more susceptible to ischemic injury. During ischemia, seizure and hyperglycemia, the extracellular pH can be reduced to less than 6.0 [6,41].

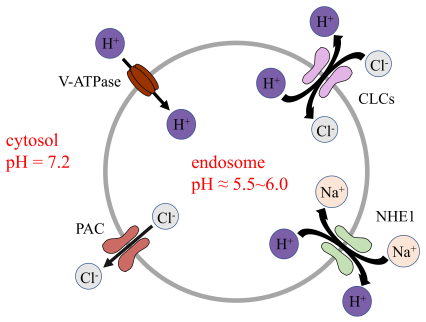

Fig. 1. Channels to maintain pH in endosomes. V-ATPase maintains luminal pH by inducing the influx of proton. CLC $2Cl^-/H^+$ exchangers (especially CLC-3-CLC-6) facilitate endosomal acidification by increasing luminal Cl^- level and providing the electric shunt for proton pumping by the V-ATPase. Intracellular domains of NHE1 regulate endosomal pH by protons efflux in exchange for Na^+ and K^+. Once the endosomal pH reaches the activation threshold of PAC, PAC activates and prevents endosomes from over-acidification by mediating the efflux of Cl^-. The above channels/exchangers cooperate and precisely regulate the homeostasis of endosomal pH.

3.1 PAC and Cancer

The intracellular and extracellular pH of cancer cells are out of balance, cancer cells operate at alkaline cytoplasmic pH higher than 7.4 and acidic extracellular pH around 6.7–7.1 [42]. It has been demonstrated that the acid-base equilibrium of the cancer microenvironment involves in the occurrence of numerous abnormalities of cancer cells. This reversed pH gradient facilitates tumorigenesis and development including proliferation, metabolic homeostasis, migration, and invasion [5,9,43–45]. On the other hand, more attentions have been directed to pH homeostasis within intracellular secretory or endo-lysosomal compartments regarding to cancer growth, metastasis, and drug resistance [46–48]. Genomic data indicate that endosomal pH regulation plays critical role in cancer survival, prognosis, and resistance to chemoradiation therapy. Mechanistically, growth factors, cytokines and other ligands initiate the genesis and development of cancer cells by binding to receptors on the plasma membrane. The ligands are separated from their receptors in the acidic pH of the endosomal compartments and then delivered to lysosomes for degradation to complete signal termination. Alternatively, the receptor-ligand complex can be removed from early endosomes for rapid return to the plasma membrane or be sorted into perinuclear recycling compartments for slow delivery to the plasma membrane. Therefore, as an important hub of cancer cell signaling, endosomes can determine the fate of cancer cells by terminating or prolonging oncogenic signals [10,49,50]. Because abnormal pH is a common finding in cancers, interventions aimed to manipulate the acidic extracellular microenvironment and endosomal pH of cancer cells may provide new therapeutic perspective. Thus, key channels or transporters (such as V-ATPase [51], CLCs [52], NHE [53] and PAC) to regulate extracellular/endosomal pH may be therapeutic targets for cancers treatment.

TMEM206 has significant modulating effects on cancer: (1) The silencing of TMEM206 can downregulate Wnt/β-catenin signaling and inhibit the proliferation and invasion of Osteosarcoma (OS) cells *in vitro* and *in vivo* [54]. This finding provides direct evidence for the inhibitory effect of TMEM206 silencing in OS, suggesting that TMEM206 is a promising new therapeutic target for the diagnosis and treatment of OS. (2) The expression level of TMEM206 in colorectal cancer (CRC) tissues was higher than that in normal adjacent tissues. TMEM206 upregulates the levels of phosphorylated AKT, downstream signaling pathway components and phosphorylated extracellular signal-regulated kinases to promote CRC cell proliferation and increase cell invasion and migration (especially promoting the interaction between the AKT and ERK signaling pathways) [55]. (3) The expression level of TMEM206 was significantly increased in colorectal, breast, liver cancer and lymphoma, and decreased in kidney cancer compared with the corresponding normal tissues through bioinformatics methods [11]. This study reported some genes co-expressed with TMEM206, suggesting that upregulation of TMEM206 is a potential prognostic biomarker for hepatocellular carcinoma. (4) Disruption of TMEM206 caused slow resolution and over-acidification of macropinosomes, increasing amino acid supply and albumin-dependent survival of cancer cells [36]. However, although these results are consistent with the expectation that TMEM206 may affect tumor proliferation and invasion by regulating the extracellular acidic microenvironment and endosomal pH gradient, the protective or adverse effects of TMEM206 in different types of cancer are uncertain, and it is necessary to further study the specific mechanism of TMEM206 in cancers to verify the results obtained by bioinformatics.

Targeted delivery to PAC via pH-sensitive nanosystems is a highly promising therapeutic strategy, the development of bio-responsive materials that undergo conformational or solubility changes in acidic environments offers great promise for the development of smart targeted drug delivery nano-systems [56]. However, at present, only some nonspecific drugs targeting chloride channels, such as 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), niflumic acid (NFA), phloretin and 5-nitro-2-(3phenylpropylamino) benzoic acid (NPPB) are available, while specific drugs targeting PAC in the endosomal pathway have not been reported [2,12]. More research is needed to establish the specific carcinogenic effect of endosomal pH in different types of cancer, which is crucial for the development of endosomal inhibitors and activators that are PAC-specific and have minimal off-target effects.

3.2 PAC and Ischemic Stroke

Ischemic acidosis is a sensitive metabolic indicator of cerebral ischemic injury progression in ischemic stroke. The pH of ischemic core tissue can be as low as pH 6.0, whereas the pH of penumbra tissue around infarction fluctuates between pH 6.5 and 6.9 [57]. TMEM206 has the highest expression in cerebral cortex [2], and three different approaches have demonstrated that PAC activity can be considered to play a major role in ischemic brain injury *in vivo*: (1) Acidosis-induced neuronal injury is largely prevented by pharmacological blockage of PAC using DIDS or phloretin in mouse cortical neurons [28], and ischemia-reperfusion induced neuronal injury is also alleviated by DIDS in hippocampal CA1 neurons [58]. (2) Hypothermia inhibited the PAC activity of mouse cortical neurons and reduced acidotoxic neuronal death [28]. (3) PAC knockout abolished the proton-activated Cl^- current in mouse neurons and reduced the acidosis-induced neuronal cell death and brain damage caused by ischemic stroke [59], and markedly reduced ischemic cerebral infarction by permanent middle cerebral artery occlusion (pMCAO) but not eliminated it [2].

For cerebral acidosis caused by ischemic stroke, a model describing the synergistic action of multiple channels can be proposed. Sustained extracellular acidification activates both ASIC1a and TRPM7, leading to Na^+ and Mg^{2+} influx and depolarization, driving PAC-mediated Cl^- entry, which is also activated by extracellular acidification [8,35,60]. Neuronal ASIC1a enables non-voltage-gated Ca^{2+} influx in response to acidosis [60,61], and NMDA receptor signaling augments ASIC1a-mediated Ca^{2+} current in ischemia [62]. Sustained extracellular acidification induces massive H^+ influx, causing intracellular acidification that stimulates the operation of NHE1, resulting in Na^+ entry into cells in exchange for H^+ [63]. Meanwhile, intracellular acidification inhibits the activity of volume-sensitive outwardly rectifying anion channel (VSOR)/volume-regulated anion channel (VRAC), thus compromising the VSOR-mediated cell volume reduction mechanism [64,65]. The cooperation of these channels increases water content of neurons, which in turn causes cytotoxic edema. Besides, intracellular Na^+ accumulation activates the Na^+/Ca^{2+} exchanger (NCX) and reverses the Na^+/Ca^{2+} exchange mechanism, thereby expelling excessive Na^+ while increasing intracellular Ca^{2+} concentration (Fig. 2) [66], which triggers a cell death cascade [67,68]. According to these studies, blocking ASIC1a, NCX, NHE1 and PAC may serve therapeutic opportunities for ischemic neuroprotection to limit acidosis-related neuronal injury. It should be emphasized that it is more efficient and applicable experimentally or clinically to block the single unique anion channel (PAC) which is widely expressed in human tissues than simultaneously block three different cation channels/exchangers (ASIC1a, NHE1, and NCX).

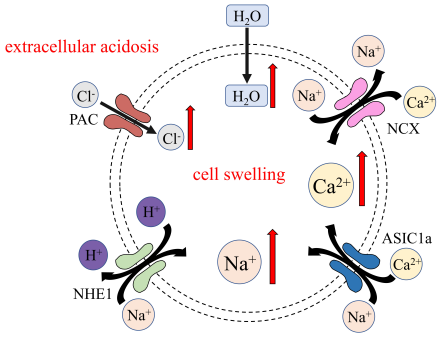

Fig. 2. Ion channels pertaining to cell edema caused by acidosis. Extracellular acidosis activates PAC, causing Cl^- influx, and simultaneously activates ASIC1a, causing Na^+ and Ca^{2+} influx. Low intracellular pH facilitates Na^+/H^+ exchange, thereby eliminating excessive intracellular Na^+ and leading to Ca^{2+} accumulation through Na^+/Ca^{2+} exchange. The entry of Na^+ and Cl^- leads to the increase of water content and cytotoxic edema of neurons. Intracellular Ca^{2+} accumulation leads to cell death cascade.

Based on the characteristics and mechanisms of PAC, there are two proposing therapeutic strategies. First, the application of pH-driven and bio-responsive nanomaterials in solid tumor therapy has inspired the application of nanotechnology in the treatment of ischemic stroke [69,70]. Tissue acidosis in ischemic stroke can act as a trigger to selectively deliver PAC-specific blockers to the ischemic penumbra via pH-responsive smart nano-systems, promising treatment at the site of injury without adverse off-target effects [71]. Second, since 1987, therapeutic hypothermia has been recognized as one of the most effective neuroprotective strategies for protecting the brain from ischemia, excitotoxicity, or traumatic brain injury [72–74]. Numerous preclinical studies have shown that hypothermia exerts neuroprotective effects mainly by reducing the cerebral metabolic rate [75], but it is also involved in cell death, inflammatory response, and white matter integrity [76], and PAC's temperature sensitivity may be one of the underlying mechanisms [59]. Precise cooling of acidotic brain tissue in ischemic stroke by therapeutic hypothermia can increase the opening threshold of PAC, thereby shutting the channel, reducing Cl^- influx, improving cytotoxic edema, and achieving neuroprotection [12,28]. It was reported that VSOR consisting of leucine-rich repeat containing 8 (LRRC8) family proteins can protect cells from necrotic death induced by hypotonia, hypoxia and hypothermia [77]. Clarifying the function and mechanism of PAC and VSOR in low temperature may be the key to the neuroprotective effect of hypothermia.

3.3 PAC and Hypoxia

Iron is essential to produce hemoglobin, the oxygen carrier in erythrocytes. Transferrin delivers iron into

cells through receptor-mediated endocytosis, so transferrin receptor-mediated endocytosis is important in erythropoiesis through its iron uptake function [78,79]. After iron binds to transferrin, it binds to cell surface receptors, forming transferrin-receptor complex into the endocytosis pathway. The endosomal pH is less than 6.0, which is conducive to the dissociation of iron from transferrin. Iron-free transferrin is then recycled back to the cell surface, where it rapidly dissociates from its receptor at neutral pH and enters the subsequent iron transport cycle [80]. Endosomal alkalization inhibits the dissociation of iron from transferrin, causing the undissociated iron-transferrin conjugate to return to the cell surface and bind closely to the receptor, reducing the binding rate of transferrin and iron [81]. PAC affects the efficiency of iron dissociation from transferrin by regulating pH in the endosomal lumen [13]. Knockout of PAC results in endosomal hyper-acidification, which improves the dissociation efficiency and cell surface receptor accessibility, and thus increases uptake of transferrin.

In addition, the role of PAC is conserved in long-term hypoxia adaptation. The results of exome sequencing in Tibetan highland population showed that PACC1 was one of the genomic regions with the highest frequency variation compared with ethnic Han Chinese population [82]. Natural selection for PACC1 has also been observed in pigs at high altitudes, allowing them to adapt to hypoxia [83]. The regulatory role of PAC after transferrin enters the endocytosis pathway provides a potential mechanism for these phenomena. However, the possibility of direct regulation of transferrin-receptor-mediated endocytosis by endosomal Cl^- concentration cannot be ruled out. Further research is needed to clarify whether and how PAC plays a physiological role in iron uptake and hypoxia induced erythropoiesis *in vivo*. Meanwhile, the regulation of transferrin by PAC implies that PAC may also regulate the transport and recycling of other membrane receptors related to endosome pH and thus participate in their respective physiological processes.

4. Conclusions

PAC is widely expressed in various cells and tissues, and highly conserved in mammals. The molecular properties, structures and functions of PAC were briefly reviewed. And emerging evidence has suggested that PAC may play critical roles in cancer, ischemic stroke and hypoxia by mediating the Cl^- transport to regulate extracellular/endosomal pH. There are three main points: (1) PAC can inhibit the proliferation and invasion of cancer by regulating extracellular pH, and terminate or prolong oncogenic signals by regulating the pH gradient and ion concentration in the secretory pathway and endo-lysosomal system in certain cancers. However, PAC-related experiments have currently only been performed in several cancers. The different or even opposite effects of PAC on some cancers are questionable. (2) Tissue acidosis caused by ischemic stroke involves the cooperation of PAC with ASIC1a, NCX, NHE1 and TRPM7. Targeted drug delivery and hypothermia can reduce PAC activity and help neurons resist toxic edema and cell death. (3) The regulation of PAC on endosomal acidification and receptor-mediated transferrin endocytosis provides a potential mechanism for hypoxia. Although there have been great advances in the understanding of PAC in recent years, the detailed molecular mechanisms of PAC, especially its function on the endosomal membrane, have not been clearly elucidated. There are still some problems to be solved:

(1) Further studies are needed to determine the molecular mechanisms responsible for both the endocytosis and recycling of PAC back to the plasma membrane.

(2) More research is required to determine the potential roles of PAC in other acidic intracellular organelles in the secretory and endocytic pathways.

(3) The protective or adverse effects of PAC in different types of cancer are uncertain, and further research is needed on the specific mechanism of PAC in cancer.

(4) The development of PAC-specific targeting drugs bears great prospects for the treatment of cancer and ischemic stroke.

(5) It would be interesting to investigate whether PAC affects other cargoes entering the endocytosis pathway, as is the case for iron uptake by erythrocytes via transferrin.

Author Contributions

Conceptualization—FP, YW, XD and PH; writing - original draft preparation—FP; writing - review and editing—XD and PH. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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