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# Functional analysis of acid-activated $Cl^-$ channels: Properties and mechanisms of regulation

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ABSTRACT

$Cl^-$ channels activated by acidic extracellular pH have been observed in various mammalian cells but their molecular identity and mechanisms of regulation are unknown. The aim of this study was to analyse the acid-activated $Cl^-$ current ($I_{Cl(H)}$) by elucidating its functional properties and mechanisms of regulation in three different cell types: primary human bronchial epithelial (HBE) cells, neuroblastoma SK-N-MC cells and HEK-293 cells. We found that outward rectification, sensitivity to acidic pH (50% activation at pH 5.15), permeability sequence ($SCN^- > I^- > Br^- > Cl^- >$ gluconate), voltage dependence and sensitivity to blockers of $I_{Cl(H)}$ were identical in all cells. These findings suggest a common molecular basis for $I_{Cl(H)}$. We analysed the possible relationship of $I_{Cl(H)}$ with members of ClC and TMEM16 protein families. By gene silencing, validated using RT-PCR, we found that $I_{Cl(H)}$ is unrelated to ClC-3, ClC-7, TMEM16A, TMEM16D, TMEM16F, TMEM16H and TMEM16K. Analysis of possible mechanisms of regulation indicate that $Ca^{2+}$, ATP and phosphorylation by PKA or PKC do not seem to be implicated in channel activation. Instead, the inhibition of $I_{Cl(H)}$ by genistein and wortmannin suggest regulation by other kinases, possibly a tyrosine kinase and a phosphatidylinositol-3-kinase. Moreover, by using dynasore, the dynamin inhibitor, we found indications that exo/endocytosis is a mechanism responsible for $I_{Cl(H)}$ regulation. Our results provide the first evidence about acid-activated $Cl^-$ channel regulation and, thus, could open the way for a better understanding of the channel function and for the molecular identification of the underlying protein.



## 1. Introduction

$Cl^-$ channels are widely expressed in various eukaryotic cells, where they fulfil different physiological functions [1]. Mutations in several of these channels lead to human diseases, such as cystic fibrosis, myotonias, ataxia, epilepsia, osteopetrosis, Dent disease and Bartter syndrome [2]. An anionic current activated by lowering the extracellular pH has been described in various mammalian cell types, i.e., HEK-293 cells [3,4], Sertoli cells [5], human monocytes [6], murine cardiac myocytes [7], human vein endothelial cells [8] and HeLa cells [9]. This acid-activated $Cl^-$ current ($I_{Cl(H)}$) is activated in the presence of low pH values and the current is characterized by outward rectification of the current–voltage relationship.

Although some biophysical characteristics of the channel have been assessed [4], almost no information exists about the mechanisms involved in the activation and regulation of $I_{Cl(H)}$. The presence of the $I_{Cl(H)}$ in different cell types seems to indicate that they may be of fundamental importance for cell housekeeping functions. However, the molecular identity of this channel is still unknown, and it is also unknown whether the associated currents have homogeneous properties among different cell types. It was first proposed that $I_{Cl(H)}$ and swelling-activated $Cl^-$ currents might be different manifestations of the same protein that changes biophysical properties upon protonation [3]. However, this does not seem to be the case [4]. Some other candidates have been put forward, such as ClC-3 and ClC-7, two members of the ClC voltage-gated $Cl^-$ channel/transporter family [10,11]. Matsuda and collaborators found that over-expression of ClC-3 in HEK-293 resulted in an increase of $Cl^-$ currents activated at pH 4 [10]. Given that the higher acid-activated $Cl^-$ currents could not be attributed to increased activity of the ClC-3 antiporter because $H^+$ exit is necessary to keep the $Cl^-/H^+$ exchange function at positive voltages, the authors proposed that at low pH the typical ClC-3 antiporter activity is uncoupled, thus resulting in pure $Cl^-$ conductance. In contrast, Ohgi et al. [11] performed whole-cell experiments in osteoclast cells in the presence, in the pipette solution, of a polyclonal antibody raised against ClC-7. In this condition, these authors found that the currents activated by acidic extracellular solution (pH 5) were significantly lower than in controls. Thus, available data do not explain in an incontrovertible

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way which protein is responsible for $I_{Cl(H)}$. The outward rectification of the current–voltage relationship, the low pH threshold for activation and the slow activation kinetics found in some descriptions of acid-activated anion currents might suggest that the response to acidic pH is carried by a single type of $Cl^-$ channel. However, there are also differences in other current characteristics measured in the different studies. For instance, the anion permeation sequence is found to be $I^- > Br^- > Cl^-$ in HEK-293 cells [3,4], $Br^- > I^- > Cl^-$ in cardiac myocytes [7] and $Cl^- > Br^- > I^-$ in Sertoli cells and THP-1 monocytes [5,12], suggesting that the currents might be carried by different proteins.

Another point that has yet to be elucidated is the function of this channel. The threshold for activation of $I_{Cl(H)}$ is too acidic to be found in physiological conditions. Acidosis is common in patients suffering from different illness and has deep effects on the host. For instance, the interstitial fluid of tumours and abscesses has shown pH values of less than 6.0, [13]. Also in ischaemic stroke, and during the focal acidity levels that accompany epileptic seizures, brain pH can decrease to as low as 6.0, contributing to the occurrence of pain and brain damage [14]. Severe acidosis is also induced during myocardial ischaemia and might be the event that leads to arrhythmias [15]. A characteristic of inflammation is local acidosis, which is attributed to the increase of lactic-acid production by the anaerobic, glycolytic activity of infiltrated neutrophils and to the presence of fatty acid by-products of bacterial metabolism [16]. However, pH values hardly decrease below 5.5 in these pathological conditions. An explanation to link a putative function for $I_{Cl(H)}$ and acidosis may come from the recent observation that the pH dependence for activation of $I_{Cl(H)}$ is tightly modulated by the temperature, with a shift towards less acidic pH values at temperatures near 37 °C [17]. The physiological role of the inward current carried by $I_{Cl(H)}$ channels could be that of charge compensation as protons enter the cells. In this case, $Cl^-$ flux would help to maintain the membrane potential and the intracellular pH within physiological values. However, it is clear that many aspects related to $I_{Cl(H)}$ channels are still largely unexplored.

The aim of this study is to get further insight into the characteristics of $I_{Cl(H)}$ and to explore the mechanisms involved in the regulation of $I_{Cl(H)}$. We have analysed the functional expression of $I_{Cl(H)}$ in many cell types and compared the properties of these currents in primary human bronchial epithelial (HBE) cells, neuroblastoma SK-N-MC cells and HEK-293 cells. In the first part of the study, we examined the biophysical properties of $I_{Cl(H)}$, finding that outward rectification, permeability sequence, voltage dependence and sensitivity to blockers are common for the three cell lines. In addition, we have established that a PI3K and a tyrosine kinase are involved in $I_{Cl(H)}$ activation, and that exo/endocytosis is a mechanism that regulates $I_{Cl(H)}$ function, possibly by modulating the incorporation of vesicles containing the channel into the plasma membrane.

## 2. Materials and methods

### 2.1. Cell culture

HEK-293, CFPAC and SK-N-MC cells were cultured in DMEM/Ham's F12 (1/1) medium. CHO cells were cultured in Ham's F12. CFBE41o- cells were instead grown in MEM medium. Fisher Rat Thyroid (FRT) cells were cultured in Coon's modified Ham's F12 medium. RAW264.7 and H441 cells were grown in RPMI 1640 medium. All media were supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary human bronchial epithelial cells, obtained from lung resections or lung transplant and approved by the ethical committee, and CuFi-1 cells, homozygous for mutation F508del on the CFTR gene, were cultured in a serum-free culture mixture of LHC9 and RPMI 1640 (1:1).

### 2.2. Electrophysiology

Whole-cell membrane currents were recorded 2–6 days after plating the cells on 35 mm Petri dishes. Borosilicate glass pipettes were pulled on a vertical two steps puller to a final resistance of 1.5-2.5 MΩ, as measured in the working solution. For the measurements of acid-activated anionic currents, the internal solution contained the following (in mM): 130 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES and 1 ATP, pH adjusted to 7.4 with CsOH. The intracellular free $Ca^{2+}$ concentration was approximate 4.7 nM, as calculated with Patcher's Power tool (Dr. Francisco Mendez and Frank Würriehausen, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany). The bath solution contained the following (in mM): 140 NaCl, 10 CsCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$ and 10 glucose. The buffer was HEPES (10 mM) for solutions at pH 7.4 and MES (10 mM) for solutions at 6.1. The pH value was adjusted with CsOH. For solutions at lower pH values, we used 5 mM 3Na-citrate as buffer and the pH was adjusted at 5.4, 4.65 or 4.35 using citric acid. For anionic selectivity experiments, 125 mM $Cl^-$ was replaced with equimolar concentrations of $SCN^-$, $Br^-$, $I^-$ or gluconate. Given the calcium chelating characteristics of this anion, in gluconate-containing solutions, [$CaCl_2$] was increased to 10 mM. To minimized the liquid junction potential, the reference electrode was connected to the bath through an agar bridge filled with 1 M KCl. In any case, liquid junction potential was calculated and corrected offline. Experiments were done at room temperature (22-24 °C). The cell membrane was voltage-clamped using an EPC-7 patch-clamp amplifier (List Medical). Data were low-pass filtered at 1 kHz and digitized at 5 kHz using an Instrutech ITC-16 AD/DA interface and the PULSE software (Heka). Solutions were changed by a fast perfusion system controlled by valves (Fast step system, Warner) that allowed the tip of the inflow tube to be near the patched cell. Experiments were performed under continuous perfusion at a flow rate of about 2–3 μl/s. To follow the response of membrane currents to different pH values, the cell was clamped at −60 mV and alternatively depolarized to 100 mV and hyperpolarized to −100 mV. After stabilization of the current amplitude following a change of solution, this protocol was interrupted to record voltage pulses from −100 mV to 100 mV in 20 mV increments to generate current-to-voltage relationships. These were obtained by measuring the current at the end of 600 ms voltage pulse. The time interval between two pulses was 4 s.

#### 2.2.1. Data analysis

Experiments were analysed using IgorPro (Wavemetrics, Lake Oswego, OR) implemented with procedures created by Dr. Oscar Moran (Istituto di Biofisica, CNR, Italy). The currents measured at pH 7.4 were usually very small. However, we sometimes observed that application of solutions with acidic pH cause a reduction of the current at hyper-polarizing potentials while at depolarizing voltages the current first increased and then decreased to a slightly lower level. In some cases, the small current decrease at both hyperpolarizing and depolarizing potentials could be avoided by increasing the osmolarity of the bath solution with mannitol. This manoeuvre reduced the currents at pH 7.4. This suggests that the small currents at pH 7.4 depended in part on swelling-dependent $Cl^-$ currents and that they are inhibited by acidic pH with a slower rate than the development of $I_{Cl(H)}$. Therefore, the currents at pH 7.4 were not subtracted from those measured at acidic pH values. Current densities were calculated diving the currents by the cell capacitance. The pH dependence of $I_{Cl(H)}$ currents was analysed using Hill equation of the form

$$I/I_{max} = 1/\left(1 + (K_d/x)^{nH}\right) \quad (1)$$

where $K_d$ is the apparent dissociation constant of protons and $nH$ is the Hill coefficient. The relative anion permeability of $I_{Cl(H)}$ was calculated from the Goldman–Hodgkin–Katz zero-current expression after